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ASSOCIAÇÃO DOS NÍVEIS DE EXPRESSÃO DE PD-L1 COM DESFECHOS CLÍNICOS E CARACTERÍSTICAS CLINICO-PATOLÓGICAS EM PACIENTES COM NEOPLASIAS GENITO-URINÁRIAS DE BAIXA INCIDÊNCIA

ANDRÉ POISL FAY

PORTO ALEGRE, 04 JANEIRO DE 2016.

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Orientador: Carlos Eduardo Poli de Figueiredo

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Esta tese é dedicada a todos os pacientes com câncer e seus familiares.

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"Now this is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning."

Sir Winston Churchill[1]

[1] Churchill WS. The End of the Beginning. London: Cassell; 1943.

RESUMO

Objetivo: este estudo visa caracterizar a expressão de PD-L1 em amostras tumorais de pacientes com neoplasias genito-urinárias de baixa incidência e correlacionar seus níveis de expressão com características e desfechos clínicos. **Métodos:** blocos de parafina foram obtidos de pacientes com carcinoma de células renais (CCR) de células não claras e carcinoma do córtex da adrenal. A expressão de PD-L1 foi avaliada por imuno-histoquímica na membrana das células tumorais e nas células mononucleares infiltradas no tumor (CMIT). Comparações entre a expressão de PD-L1 e características clínico-patológicas foram analisadas utilizando teste t não pareado e teste exato de Fisher. Metodologia de Kaplan-Meier e teste de log-rank foram utilizados para avaliar a associação entre a expressão de PD-L1 e desfechos de sobrevida nas duas histologias. **Resultados:** Entre 101 pacientes com CCR de células não claras, 11 (10.9%) foram considerados PD-L1 positivo (+) na membrana das células tumorais: em 2/36 (5.6%) dos tumores de células cromófobas, 5/50 (10%) de tumores papilares, 3/10 (30%) dos tumores com translocação Xp11.2 e em 1/5 (20%) dos tumores do ducto coletor. Por outro lado, positividade de PD-L1 em CMIT foram observadas em 57 (56.4%) dos pacientes: em 13/36 (36.1%) dos tumores de células cromófobas, 30/50 (60%) de tumores papilares, 9/10 (90%) dos tumores com translocação de Xp11.2 e em 5/5 (100%) dos tumores do ducto coletor. PD-L1+ em ambos, membrana da células tumorais e CMIT, foram associados com um tempo mais curto até recorrência de doença em pacientes com CCR de células não claras (p=0.02 e p=0.03, respectivamente). Entre os 28 pacientes com tumores do córtex da glândula adrenal, 3 (10.7%) foram considerados positivos na membrana das células tumorais. Por outro lado, a expressão de PD-L1 em CMIT foram realizadas em 27 pacientes e PD-L1+ foi observado em 19 (70.4%) pacientes. Positividade para PD-L1 em ambos, membrana da células tumorais e CMIT, não foi associado com maior estágio clínico ao diagnóstico, alto grau tumoral, produção excessiva de hormônios ou sobrevida. **Conclusão:** Em suma, CCR de células não claras e carcinoma do córtex da adrenal expressam PD-L1 na membrana da célula tumoral e em infiltrados inflamatórios e isto pode representar um possível alvo para intervenções terapêuticas.

Palavras-chave: Carcinoma de Células Renais. Carcinoma Renal de Células Não Claras. Tumor Renal Benigno. Carcinoma Adrenocortical. PD-L1. Inibidores PD-1. Imunoterapia.

ABSTRACT

Objective: This study aims to characterize PD-L1 expression in tumor tissue from low incidence genitourinary malignancies and to correlate levels of PD-L1 expression with clinico-pathological features as well as survival outcomes. **Methods:** Formalin-fixed paraffin-embedded specimens were obtained from patients with non-clear cell renal cell carcinoma (non-ccRCC) and adrenocortical carcinoma (ACC). PD-L1 expression was evaluated by immunohistochemistry (IHC) in both tumor cell membrane and tumor infiltrating mononuclear cells (TIMC). Comparisons between PD-L1 expression and clinico-pathological features were evaluated using unpaired t-test and Fisher's exact test. Kaplan-Meier method and log-rank test were used to assess association between PD-L1 expression and survival outcome in both histologies. **Results:** Among 101 patients with non-ccRCC, 11 (10.9%) were considered PD-L1+ in tumor cells: 2/36 (5.6%) of chromophobe RCC, 5/50 (10%) of papillary RCC, 3/10 (30%) of Xp11.2 translocation RCC and 1/5 (20%) of collecting duct carcinoma. On the other hand, PD-L1 positivity by TIMC was observed in 57 (56.4%) patients: 13/36 (36.1%) of chromophobe RCC, 30/50 (60%) of papillary RCC, 9/10 (90%) of Xp11.2 translocation RCC and 5/5 (100%) of collecting duct carcinoma). PD-L1+ in both tumor cell membrane and TIMC cells were associated with shorter time to recurrence ($p=0.02$ and $p=0.03$, respectively). Among 28 patients with surgically treated ACC, 3 (10.7%) were considered PD-L1 positive on tumor cell membrane. On the other hand, PD-L1 expression in TIMC was performed in 27 specimens and PD-L1 positive staining was observed in 19 (70.4%) patients. PD-L1 positivity in either tumor cell membrane or TIMC was not significantly associated with higher stage at diagnosis, higher tumor grade, excessive hormone secretion, or survival. **Conclusion:** In summary, Non-ccRCC and ACC can express PD-L1 on both tumor cell membrane and immune cells and it may represent a potential target for therapeutic interventions.

Keywords: Renal Cell Carcinoma. Non clear-Cell Renal Cell Carcinoma. Benign Kidney Tumor. Adrenocortical Carcinoma. PD-L1. PD-1 Inhibitors. Immunotherapy.

SUMÁRIO

1 INTRODUÇÃO

Neoplasias malignas originadas no trato genito-urinário constituem uma importante causa de morbidade e mortalidade no Brasil e no mundo[1,2]. Para fins de estudos científicos, no campo da uro-oncologia, tais neoplasias são divididas em duas categorias de acordo com sua localização, incidência e estratégias terapêuticas: neoplasias da próstata e neoplasias não da próstata. Os principais representantes da segunda categoria são tumores de rim, bexiga, testículo, pênis e os tumores do córtex da glândula adrenal. À exceção dos tumores de próstata, a neoplasia mais frequente no sexo masculino[3], as neoplasias genito-urinárias não apresentam alta incidência, e alguns subtipos histológicos específicos são extremamente raros[4]. Entretanto, mesmo com baixas incidências, estas raras neoplasias podem apresentar um curso clínico bastante agressivo e os tratamentos disponíveis não proporcionam desfechos clínicos favoráveis, necessitando, portanto, serem desenvolvidos[5]. No presente estudo, dois subtipos histológicos de baixa incidência foram definidos como foco de investigação: carcinomas renais de células não claras, conjuntamente com seus subtipos histológicos, e carcinomas originados no córtex da glândula adrenal.

Aproximadamente 64.000 novos casos de câncer de rim são diagnosticados, anualmente, nos Estados Unidos da América e estima-se que 25-30% destes resultarão em óbito[6]. Recentes análises realizadas em bases populacionais identificam uma tendência no aumento da incidência em todo o mundo. Ao contrário dos países desenvolvidos, a mortalidade parece estar aumentando nos países em desenvolvimento, provavelmente por uma restrição ao acesso a novas terapias para a doença[7]. A cirurgia é o tratamento curativo nos estágios inicias. Entretanto, a recorrência da doença é comum e aproximadamente 20-30% dos pacientes irão desenvolver doença metastática após realizar um tratamento com intenção curativa[8]. O primeiro estudo avaliando o uso de terapias em caráter adjuvante teve seus resultados recentemente apresentados. Infelizmente, o uso de terapias sistêmicas não diminuiu o risco de recorrência em pacientes de alto risco [71].

O carcinoma renal de células claras (CCR) é o subtipo mais frequente, sendo responsável por aproximadamente 80% das neoplasias originadas no parênquima renal[9]. Embora menos frequentes, CCR de células não claras compreendem os subtipos histológicos: carcinoma papilar, carcinoma de células cromófobas, carcinoma de ductos coletores, e carcinoma associado a translocação do cromossomo Xp11.2[4]. Carcinomas papilares são

responsáveis por 10-15% dos CCR e tumores de células cromófobas por 5-10% desta neoplasia. O carcinoma associado a translocação cromossômica Xp11.2 é ainda mais raro, geralmente acomete jovens e está associado a um comportamento agressivo[10]. Carcinoma de ductos coletores corresponde a menos de 1% das neoplasias malignas renais.

Estudos epidemiológicos demostram que os CCR de células não claras tendem a ser diagnosticados em estágios inicias, conferindo um melhor prognóstico a esta situação quando submetidos a tratamento adequado[11]. Além disto, o risco de recorrência também é menor variando entre 2,9 e 14,9% quando comparados com o CCR de células claras que apresentam índices de recorrência oscilando entre 12 e 21.5%[12-14]. Entretanto, pacientes com diagnóstico de CCR de células não claras em estágio avançado ou que desenvolvem doença metastática após uma ressecção cirúrgica com intuito curativo apresentam uma doença de comportamento mais agressivo e o tratamento é desafiador[4]. Considerando a baixa incidência destes subtipos histológicos, estudos nesta população são difíceis de ser implementados fazendo com que muitas dúvidas permaneçam em relação ao melhor tratamento desta situação clínica.

Contudo, o prognóstico dos pacientes com neoplasias renais segue um curso heterogêneo e o risco de recorrência está estritamente associado com características clínico-patológicas, tais como, estadiamento TNM, "performance status" e grau de diferenciação tumoral, segundo Fuhrman[15]. Embora muitos biomarcadores moleculares tenham sido propostos para predizer risco de recorrência, estes marcadores ainda não estão validados e não são utilizados na pratica clinica[16].

Mutações germinativas no gene Von Hippel-Lindau (*VHL*) são responsáveis por uma síndrome hereditária que se caracteriza por hemangioblastomas de retina, cerebelo ou medula espinhal; cistos viscerais e tumores sólidos como carcinoma de células claras renais e feocromocitomas[17,18]. Da mesma forma, mutações somáticas no gene *VHL* e deleções no cromossomo 3p (locus do gene *VHL*) são encontrados na maioria das neoplasias renais de células claras esporádicos[19].

O gene *VHL* codifica a proteína VHL (pVHL). Esta proteína é um componente da via de ubiquitinização, interferindo diretamente na degradação de múltiplas proteínas intracelulares, incluindo os fatores induzíveis por hipóxia (HIFs, do inglês "Hypoxia-inducible factors")[20]. HIFs são responsáveis pela transcrição de genes que regulam o metabolismo e a angiogênese como o fator de crescimento vascular endotelial (VEGF, do

inglês "vascular endothelial growth factor") e fator de crescimento derivado de plaquetas (PDGF, do inglês "platelet-derived growth factor")[21-23]. Em suma, quando o gene *VHL* encontra-se inativado, existe uma hiperexpressão de HIFs, resultando na desregulação de vias de sinalização que influenciam no metabolismo, inflamação, angiogênese e morte celular[24-26].

A elucidação do *VHL* como um gene supressor tumoral e dos fatores que regulam a angiogênese, desencadeou importantes avanços no tratamento sistêmico do CCR de células claras metastático que se caracteriza fundamentalmente por esta biologia[27]. Agentes que ligam VEGF ou receptores tirosina quinase de VEGF, bem como drogas que atuam na via da alvo de rapamicina de mamíferos (mTOR, do inglês *"mammalian target of rapamycin"*) tem sido amplamente utilizados no tratamento do CCR avançado, resultando em aumento de sobrevida global e sobrevida livre de progressão nos pacientes portadores de doença metastática[28]. Desde 2005, diversos agentes foram aprovados para o tratamento da neoplasia renal metastática, tais como: Sunitinibe, Pazopanibe, Axitinibe, Bevacizumabe, Sorafenibe, Everolimo e Tensirolimo[29-34]. Tais medicações demonstraram desfechos clínicos superiores quando comparadas à imunoterapia tradicional com altas doses de Interleucina-2 (IL-2) ou Interferon-α (IFN), tornando-se o padrão ouro no tratamento desta patologia[29]. Hoje, com este arsenal terapêutico, a sobrevida média da doença metastática ultrapassa os 28 meses[35]. Entretanto, a maioria dos pacientes não apresentam respostas sustentadas com o uso de terapias anti-angiogênicas e em algum momento a doença irá progredir. Existem poucas comparações diretas entre tais medicações. Recentemente, um grande estudo de fase III, de não inferioridade, comparando Sunitinibe versus Pazopanibe mostrou que tais drogas possuem eficácia semelhante, porém com diferentes perfis de toxicidade[36]. Como na doença localizada, não existem biomarcadores disponíveis para o uso na prática clínica que possam determinar a melhor droga a ser utilizada em pacientes específicos ou predizer resposta a estes novos tratamentos[37,38]. Recentemente, Fay e colaboradores apresentaram os resultados de estudos investigando a presença de mutações genéticas como biomarcadores preditores de resposta à terapia anti-angiogênica (inibidores de receptores de tirosina quinase de VEGF e inibidores de mTOR)[39,40]. Estes estudos identificaram mutações específicas que são mais prevalentes em pacientes que apresentam uma resposta extraordinária a estas drogas (APÊNDICES M e O). Tais estudos precisam ser validados de forma prospectiva, porém caracterizam um importante passo na evolução da medicina individualizada e seleção de tratamentos no carcinoma renal avançado.

Paradoxalmente, todas as alterações descritas anteriormente são características do CCR de células claras, não sendo comuns às outras histologias do câncer renal[4]. Trabalhos recentes, reportaram a caracterização molecular de CCR de células não claras (carcinomas de células cromófobas e carcinomas papilares) identificando perfis moleculares distintos[41,42].

Carcinomas papilares são geneticamente caracterizados por trissomias dos cromossomos 3q, 7, 8, 12, 16 e 20. Dentre as alterações cromossômicas identificadas neste subtipo histológico, mutações no gene *c-MET* localizado no cromossomo 7, têm sido estudado como potencial alvo terapêutico[43]. Ademais, síndromes hereditárias associadas ao carcinoma papilar de células renais apresentam mutações no gene *FH.* Carcinomas de células cromófobas, por sua vez, se caracterizam por alterações nos cromossomos 1, 2, 6, 10, 13, 17, e 21. Desta forma, tais subtipos histológicos não se caracterizam pela inativação do gene *VHL,* característica dos CCR de células claras, previamente descrita. Assim, a ativação da angiogênese não ocorre da mesma forma que nos CCR de células claras e talvez não seja fator determinante na carcinogênese destes subtipos histológicos, o que pode impactar de forma significativa o tratamento destas patologias[4]. Da mesma forma que realizado no CCR, o "The Cancer Genome Atlas" - esforço colaborativo para a caracterização molecular de neoplasias malignas - permitiu uma caracterização molecular das neoplasias renais de baixa incidência e demonstrou diferenças importantes na biologia das mesmas.

Entretanto, estratégias de tratamento semelhantes às utilizadas no CCR de células claras, são aplicadas nos tumores de células não claras sem evidências consistentes em relação à eficácia[44]. As histologias não de células claras representam uma mínima parte da amostra nos estudos clínicos até hoje realizados e dados mostram que as respostas às terapias alvo neste subgrupo de pacientes tem resultados inferiores no que tange sobrevida global e taxas de resposta. A necessidade de se desenvolver novas terapias e biomarcadores de resposta às drogas pré-existentes é fator imperativo para a melhora dos desfechos clínicos neste grupo de pacientes.

Por sua vez, os carcinomas do córtex da glândula adrenal acometem 1-2 a cada 1 milhão de habitantes nos Estados Unidos da América anualmente[45]. Acredita-se que por falhas de registro esta estimativa deve estar subestimada. Curiosamente, tal neoplasia apresenta uma incidência até 18 vezes mais alta em crianças no sul do Brasil[46]. Especula-se que mutações germinativas no gene *TP53,* encontradas em até 90% dos pacientes, possam

responsáveis por tal diferença[47]. Estes achados reforçam um interesse local para o estudo e o entendimento da biologia desta patologia. Por não apresentar sintomas típicos em estágios iniciais, em torno de 70% dos pacientes diagnosticados com carcinoma do córtex da glândula adrenal apresentam doença em estágios avançados o que determina um prognóstico desfavorável e um curso clínico bastante agressivo[48].

O tratamento desta doença exige uma abordagem multidisciplinar, envolvendo endocrinologistas, cirurgiões, radio-oncologistas e oncologistas clínicos[49]. A ressecção cirúrgica completa ainda é a espinha dorsal do tratamento na doença localizada. Porém, mesmo após um tratamento cirúrgico com intenção curativa, até 80% dos pacientes apresentará recidiva da doença. A biologia desta doença é pouco compreendida[50]. O "The Cancer Genome Atlas" está estudando de forma cooperativa as alterações moleculares desta neoplasia(Ref:https://tcgadata.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=ACC&diseaseName=Adrenocortical carcinoma). Em breve, tal caracterização irá permitir um maior entendimento da biologia tumoral e o desenvolvimento de novos tratamentos para pacientes com doença avançada.

Até o momento, poucos estudos demonstraram o benefício da terapia sistêmica no tratamento desta rara neoplasia, onde o desenvolvimento de estudos clínicos se torna também bastante desafiador[51]. O primeiro estudo clínico de fase III realizado pra avaliar de forma comparativa tratamentos sistêmicos na doença avançada [FIRM-ACT *("first international randomized trial in locally advanced and metastatic adrenocortical carcinoma treatment")*] determinou que a combinação de Etoposide, Doxorrubicina e Cisplatina (EDP) em combinação com Mitotano (M) resulta em melhor desfecho clínico neste subgrupo de pacientes quando comparado ao tratamento com Estreptozocina e Mitotano[52]. É importante salientar que muitas terapias alvo específicas, que se mostraram eficazes em outras neoplasias genito-urinárias como CCR de células claras, não melhoraram o desfecho clínico de pacientes com doença metastática, reforçando a necessidade de uma melhor compreensão da biologia desta doença para o desenvolvimento de novos agentes[53-56].

Na última década, muitos estudos têm abordado o papel do sistema imune na fisiopatologia tumoral. As primeiras evidências desta associação são originárias dos experimentos do Dr. William Coley, no início do século 20, que acreditava que provocar infecções nas áreas tumorais poderiam desencadear uma resposta tumoral contra os mesmos.

Outras, foram publicadas na edição do "The Lancet" em 07 de janeiro de 1899[57]. Nesta publicação, Dr. William Bennett descreveu diferentes comportamentos das neoplasias, e questionou o papel da imunidade na progressão tumoral.

Muitos anos mais tarde, baseado nestas observações, estudos pré-clínicos mostraram que o uso de estimuladores imunológicos como interferon-γ, eram capazes de inibir o desenvolvimento de tumores em ratos imuno-comprometidos[58]. Surgem, então, princípios importantes para determinar a relação entre sistema imune e câncer: (1) imuno-vigilância, (2) equilíbrio e (3) escape[59].

O primeiro princípio determina que células do sistema imune são ativados na presença de qualquer célula tumoral, impedindo o desenvolvimento de neoplasias na sua origem. O segundo se refere ao controle exercido pela resposta imune na progressão de células tumorais: mesmo não conseguindo eliminar todas as células neoplásicas, o sistema imunológico conseguiria evitar a progressão das mesmas. Finalmente, o terceiro princípio defende a ideia que neoplasias clinicamente ativas são capazes de evadir ou atenuar respostas imunes, permitindo a progressão tumoral[59].

Carcinomas de células renais são classicamente tumores imunogênicos caracterizados por abundantes infiltrados linfocitários na maioria das vezes disfuncionais[60]. Muitos estudos sugerem que o CCR é capaz de produzir imunidade antitumoral, porém os mecanismos fisiopatológicos destas interações ainda não estão completamente definidos[61]. A doença avançada, historicamente foi tratada com imunoterapias (IL-2 em altas doses ou IFN), porém uma minoria destes tumores apresenta respostas clínicas prolongadas com esta abordagem[31].

A compreensão dos mecanismos de interação entre a célula tumoral e o sistema imunológico identificou diversos reguladores da imunidade. Estes reguladores, chamados de "imune-checkpoints" são capazes de ativar ou inativar a resposta inflamatória mediada por células T. A capacidade desenvolvida pelos tumores de expressar tais reguladores da imunidade foi descrita como sendo esta um "escudo molecular" contra a resposta imune. A intervenção sobre este complexo processo de regulação da resposta imunológica ganhou espaço como uma promissora estratégia para terapia anti-tumoral[62,63].

Inúmeros são os reguladores da imunidade que tem sido descritos como parte deste processo[64]. Receptores e ligantes presentes em linfócitos T, macrófagos e tecidos periféricos

constituem vias inibitórias e estimuladoras do sistema imune. Os principal mediador da via estimuladora é o receptor de CD28, enquanto os receptores CTLA-4 (do inglês "Cytotoxic T Lymphocyte Antigen-4") e PD-1 (do inglês, "Programmed Death-1") caracterizam a via inibitória[64]. Terapias utilizando como alvo estas duas vias de controle da resposta imune tem revolucionado o tratamento do câncer e respostas muito duradouras tem sido observadas em pacientes com melanoma, câncer de pulmão, CCR de células claras, câncer de bexiga entre outras neoplasias[72].

O receptor PD-1 encontra-se expresso em linfócitos T, B e "Natural Killers". Seus ligantes são PD-L1 (B7-H1) e PD-L2 (B7-DC). Estes ligantes apresentam uma ampla distribuição em tecidos periféricos, incluindo coração, pulmão, tecido muscular, pâncreas, placenta e tecido hematopoiético[65]. A interação entre ligante e receptor tem como objetivo limitar a atividade de células T no tecido periférico regulando a resposta inflamatória. Logo, a ligação entre PD-1 e PD-L1, fisiologicamente, inibe a resposta inflamatória[66]. Este é um mecanismo de proteção dos tecidos contra respostas inflamatórias exacerbadas.

A exposição crônica a determinados antígenos provoca um aumento nos níveis de expressão de PD-L1, como mecanismo de proteção daquele tecido frente a resposta inflamatória,. Tal fenômeno esta associado com um processo chamado de exaustão que limita a resposta imune. Este processo é descrito como uma das formas que as neoplasias malignas desenvolveram para escapar do controle exercido pelo sistema imune que tem como função não permitir o desenvolvimento tumoral. Entretanto, bloqueio da interação PD-L1/PD-1, visa reestabelecer o conceito de equilíbrio entre a resposta imune e a progressão tumoral impedindo sua evolução[67].

Recentemente, estudos demostraram que diversas neoplasias superexpressam PD-L1 tanto na membrana das células tumorais quanto nos infiltrados linfocitários intratumorais, e entre elas está o carcinoma renal de células claras[66]. Topalian e colaboradores, publicaram em 2012 importantes resultados de um estudo de fase I em que o bloqueio da via inibitória da resposta imune com anticorpos específicos anti-PD-1 resultou em importantes respostas clinicas objetivas e duradouras em diversas neoplasias solidas, incluindo CCR de células claras[68]. Dados preliminares deste estudo, sugeriram que a expressão de PD-L1 poderia apresentar associação com resposta clínica a esta estratégia de tratamento. Contudo, resultados subsequentes não confirmaram esta hipótese. Mesmo pacientes que não apresentam

expressão de PD-L1 na membrana de células tumorais ou em infiltrados linfocitários intratumorais podem apresentar respostas significativas a tais agentes. Por outro lado, em algumas neoplasias como carcinomas uroteliais de bexiga a hiperexpressão de tal marcador parece estar associado a melhores respostas a estes agentes[73].

Estes dados, desencadearam um retorno aos conceitos previamente publicados pelo Dr. Bennett e diversos estudos colaborativos tem sido realizados para elucidar o real papel do sistema imune no desenvolvimento de neoplasias malignas e como o bloqueio destas vias reguladoras da imunidade podem impactar no desfecho clínico dos pacientes acometidos por esta enfermidade.

Thompson e colaboradores reportaram dados da avaliação da expressão de PD-L1 em amostras tumorais de pacientes com CCR de células claras[61]. Notavelmente, altos níveis de expressão de PD-L1 se correlacionaram com fatores patológicos de alto riscos e desfechos clínicos desfavoráveis. Conjuntamente, estes dados suportaram a investigação do bloqueio da via PD1/PD-L1 no tratamento do CCR de células claras em estágios avançados. Recentemente, os resultados de um estudo de fase III comparando o uso de nivolumabe (um anticorpo monoclonal contra PD-1) versus uma terapia padrão utilizada em pacientes com CCR de células claras que progrediram a um tratamento prévio com uma terapia anti-angiogênica, evidenciou um aumento da sobrevida global com o uso deste novo agente imunoterápico, fazendo com que esta droga, provavelmente se torne o tratamento padrão neste cenário clínico em um curto espaço de tempo[74]. Este mesmo estudo avaliou a associação da expressão de PD-L1 na membrana das células tumorais com a eficácia da droga. O benefício do uso de nivolumabe não se correlacionou com a expressão de PD-L1 na membrana das células tumorais. Outros ensaios clínicos randomizados estão em andamento para responder confirmar este benefício e definir o papel da expressão de PD-L1 como biomarcador preditivo de resposta a esta estratégia terapêutica.

Recentemente, Choueiri e colaboradores avaliaram a expressão de PD-L1 em pacientes com CCR de células claras que receberam tratamento com sunitinibe ou pazopanibe como parte de um estudo clínico que incluiu mais de 1000 pacientes. Esta avaliação identificou que pacientes com hiperexpressão de PD-L1 apresentaram um pior prognóstico quando tratados com terapia antiangiogénica, sugerindo que eventualmente tais terapias devam ser utilizadas de forma mais precoce neste subgrupo de pacientes (APENDICE C).

Curiosamente, dentre as neoplasias genito-urinárias, os CCR de células não claras, bem como o carcinoma do córtex da glândula adrenal não estão sendo estudados em relação aos seus aspectos imunológicos. Talvez por uma incidência menor e pela ausência de estudos pré-clinicos neste subtipo histológico, os grandes estudos em câncer de rim estejam excluindo os pacientes com histologias raras[11].

A caracterização de reguladores imunológicos, como o PD-L1, neste subgrupo de neoplasias genito-urinárias com baixa incidência e sua correlação com variáveis clinico-patológicas, pode acarretar num avanço importante no entendimento da fisiopatologia das doenças, bem como no desenvolvimento de novas estratégias terapêuticas para estes pacientes, considerando que poucas opções estão disponíveis na prática clínica.

2 JUSTIFICATIVA

Na última década, esforços tem sido feitos para elucidar a biologia do CCR de células claras. O estabelecimento da inativação do gene de *VHL* como peça chave na carcinogênese, permitiu o desenvolvimento de terapias anti-angiogênicas que resultam em benefício clínico em até 80% dos pacientes com doença metastática[35]. Entretanto, a progressão da doença irá ocorrer após determinado período de tratamento em virtualmente todos os pacientes. Recentemente, imunoterapias tem mostrado respostas objetivas duradouras no tratamento de diversos tumores sólidos, incluindo CCR de células claras[68]. Apesar de apresentarem um melhor prognóstico quando diagnosticados precocemente, o tratamento do CCR de células não claras metastático é desafiador e poucas opções terapêuticas estão disponíveis. Pacientes portadores desta doença não apresentam respostas como as citadas anteriormente e a doença avançada possui prognóstico reservado.

As neoplasias renais, fundamentalmente o subtipo histológico de células claras, são classicamente imunogênicas e apresentam infiltrados leucocitários de forma abundante[60]. Contudo, o papel deste infiltrado linfocitário no prognóstico da doença ainda é pouco conhecido. A expressão de PD-L1, na membrana de células tumorais ou no infiltrado inflamatório intratumoral tem sido associada a fatores indicadores de pior prognóstico e desfechos clínicos adversos no CCR de células claras. Contudo, dados em CCR de células não claras ainda não foram investigados.

Os grandes estudos clínicos que norteiam o tratamento do CCR não incluem um número significativo de pacientes com esta histologia. Desta forma, dados consistentes para o estudo de imunoterapias no tratamento deste subtipo histológico são necessários.

Da mesma forma, o carcinoma do córtex da glândula adrenal é uma doença rara caracterizada por um curso clínico agressivo onde diversas estratégias terapêuticas tem falhado em demonstrar benefício clínico em pacientes com doença avançada. O papel do uso do sistema imunológico neste grupo de tumores não tem sido estudado e podem representar uma alternativa para futuros estudos clínicos neste cenário.

O estudo da expressão de PD-L1 neste subgrupo específico de tumores de baixa incidência visa elucidar aspectos relacionados a fisiopatologia das doenças, resultando em

potenciais estratégias terapêuticas baseadas na intervenção sobre as vias reguladoras da imunidade. A expressão de PD-L1 em tais tumores pode representar um importante racional teórico para a utilização de imunoterapias nestas neoplasias onde tais tratamento ainda não foram testados. Adicionalmente, a avaliação da expressão de PD-L1 como fator prognóstico em tais neoplasias pode auxiliar na tomada de decisões clínicas e desenvolvimento de estudos clínicos.

3 HIPÓTESE

O CCR não de células claras, bem como, o carcinoma do córtex da glândula adrenal apresentam expressão aberrante de PD-L1.

4 OBJETIVOS

4.1 OBJETIVO PRINCIPAL

O presente estudo visa caracterizar os níveis de expressão de PD-L1 em células tumorais e infiltrados leucocitários presentes em neoplasias genito-urinárias de baixa incidência (CCR de células não claras e carcinoma do córtex da glândula adrenal).

4.2 OBJETIVOS SECUNDÁRIOS

1. Correlacionar os níveis de expressão de PD-L1 com características clinico-patológicas dos pacientes portadores de tais neoplasias no momento do diagnóstico.
2. Correlacionar os níveis de expressão de PD-L1 com risco de recorrência da doença.
3. Correlacionar os níveis de expressão de PD-L1 com a sobrevida global dos pacientes portadores de CCR não de células claras ou carcinoma de adrenal.

5 MATERIAIS E MÉTODOS

5.1 DELINEAMENTO

Trata-se de um estudo de avaliação de biomarcadores baseado em uma coorte retrospectiva com análises exploratórias relacionadas às características clínico-patológicas, ao risco de recorrência e a dados de sobrevida global.

5.2 POPULAÇÃO E AMOSTRA

Foram incluídos no estudo 104 pacientes com o diagnóstico de CCR de células não claras submetidos a nefrectomia no Dana-Farber Cancer Institute/Harvard Medical School. Todos os pacientes desta amostra fazem parte de um estudo prospectivo que visa estudar biomarcadores associados a neoplasia renais. Desta forma, possuem consentimento informado livre e esclarecido para a participação do mesmo (vide item Considerações Éticas).

Da mesma forma, 28 pacientes, previamente consentidos, com carcinoma de adrenal tratados, na mesma instituição e com amostra tecidual disponível, oriunda de ressecções primárias ou biópsias foram incluídos no presente estudo. Não foram considerados, neste estudo, pacientes pediátricos ou portadores de síndromes genéticas conhecidas relacionadas a um risco aumentado para o desenvolvimento de carcinoma do córtex da adrenal.

5.3 MÉTODOS

Blocos de parafina (FFPE, do inglês “Formalin-Fixed Paraffin Embedded”) dos pacientes incluídos no estudo foram obtidos no Laboratório de Patologia da mesma instituição.

O diagnóstico foi revisado por patologista especializada em neoplasias genito-urinárias (Dra. Sabina Signoretti – Dana-Farber Cancer Institute, Brigham and Women’s Hospital, Harvard Medical School) para confirmar o diagnostico e delimitar área tumoral a ser avaliada para expressão de PD-L1 via imuno-histoquímica. A patologista não teve acesso as características clínicas dos pacientes.

Foram excluídos os pacientes que não possuíam tecido tumoral disponível para análise imuno-histoquímica.

Detalhamento metodológico específico é descrito nos respectivos manuscritos.

5.3.1 Avaliação Imuno-histoquímica

Lâminas foram preparadas conforme protocolos do laboratório de patologia do Dana-Farber Cancer Institute para avaliação imuno-histoquímica de PD-L1. Foi utilizado anticorpo anti-B7-H1 desenvolvido e validado pelo Dr. Gordan Freeman (Dana-Farber Cancer Institute/ Brigham Women's Hospital/ Harvar medical School). Tal anticorpo, evidenciou em análises prévias níveis apropriados de especificidade para identificação de B7-H1 em FFPE utilizando como controle citometria de fluxo. O anticorpo é incubado por 12 horas sob temperatura de 4° C, conforme previamente descrito na literatura[69].

Positividade para PD-L1 foi avaliada por dois patologistas especialistas em neoplasias genito-urinárias de forma independente. Foram consideradas positivas expressões de PD-L1 em 5% ou mais na membrana celular das células tumorais. Valores inferiores ao acima descrito foram considerados negativos. Discrepância entre os resultados foram esclarecidas pela revisão simultânea do material pelos dois patologistas para uma definição consensual. A contagem de células foi realizada com magnificação de 100 e 400 x em pelo menos 5 áreas tumorais selecionadas de forma randômica[70].

A expressão de PD-L1 em áreas de infiltrados linfocitários intra-tumorais foi descrita através de scores previamente descritos. A extensão de infiltrados tumorais foi caracterizada através da seguinte graduação: ausente (0), focalmente expresso (1), fracamente expresso (2), moderadamente expresso (3), fortemente expresso (4). A percentagem de PD-L1+ nos infiltrados linfocitários foi avaliada de forma independente por dois patologistas especialistas em neoplasias genito-urinárias de acordo com 3 categorias: 0%=0, <5%=1 e ≥5%=2. Um escore representativo da expressão de PD-L1 foi calculado multiplicando-se a extensão dos infiltrados linfocitários pela percentagem de positividade coradas nas células inflamatórias.

Alternativamente, para examinar se a expressão de PD-L1 está relacionada com áreas de infiltrados leucocitários tumorais, foi avaliada a expressão de CD45 no mesmo tecido

tumoral. Através de características morfológicas e histopatológicas similares, as áreas com hiperexpressão de PD-L1 foram identificadas nos slides corados para CD45. Foram avaliadas de forma sobreposta a expressão de CD45 em tais localizações. Dois patologista cegos para a expressão de PD-L1 revisaram as lâminas para definir a expressão de CD45[60,66].

5.3.2 Avaliação de Características Clínico-Patológicas

Dados demográficos e características clinico-patológicas foram extraídas de forma retrospectiva através de revisão de prontuário eletrônico de todos os pacientes incluídos no estudo. Os dados clínicos-patológicos a serem analisados nos pacientes com neoplasias renais incluem: sexo, raça, idade ao diagnóstico, data do diagnóstico, lateralidade do tumor primário, estadiamento clínico e patológico, subtipo histológico (papilar, células cromófobas ou associado a translocação X.p11.2), grau de diferenciação tumoral de Fuhrman (1, 2, 3 ou 4), presença de necrose. Para pacientes com carcinoma de adrenal as seguintes variáveis clínicas foram coletadas: estádio clínico ao diagnóstico, grau de diferenciação tumoral e tumores funcionais ao diagnóstico.

Foram registradas data do óbito, se ocorrido, ou data do último registro de seguimento para avaliações de sobrevida global. Da mesma forma, data da recorrência foi registrada caso aplicável.

Data do diagnóstico é definida como data da nefrectomia ou da biópsia precedente ao procedimento. Foram considerados contatos telefônicos ou via correio eletrônico para fins de avaliação da sobrevida.

Os dados clínicos Foram recuperados exclusivamente por um mesmo pesquisador (autor do estudo) previamente treinado para a correta extração dos dados e as informações foram registradas em planilhas de registro padronizadas.

5.4 ANÁLISE ESTATÍSTICA

Características clínico-patológicas são apresentadas de forma descritiva. A associação entre a expressão de PD-L1 e variáveis clinico-patológicas foi avaliada utilizando teste qui-quadrado e teste exato de Fisher de acordo com o tamanho da amostra. Valores de $p < 0,05$

(bi-caudados) indicarão significância estatística. Variáveis contínuas foram comparadas utilizando teste de Wilcoxon rank-sum.

Curvas de Kaplan-Meier foram utilizadas para estimar a distribuição de sobrevida global e tempo para recorrência entre os grupos que PD-L1 positivo versus PD-L1 negativo. Comparações entre os grupos, conforme a expressão de PD-L1 foram realizadas com o teste log-rank e modelos de Cox para definir associações entre sobrevida e fatores clínico patológicos.

Conforme previamente relatado nos estudos que avaliaram a expressão de PD-L1 em pacientes com CCR de células claras, foram consideradas positivas qualquer expressão de PD-L1 $\geq$ 5% na membrana da célula tumoral. Expressões inferiores a este ponto de corte são consideradas negativas. A expressão de PD-L1 em áreas de infiltrados linfocitários intra-tumorais foi considerada positiva quando escores $\geq$ 1.

6 CONSIDERAÇÕES SOBRE ESTA TESE

Durante o período de desenvolvimento deste projeto de pesquisa, foram possíveis diversas publicações na área de oncologia genito-urinária. Tais estudos envolvem principalmente pesquisa translacional na área de imunoterapia ou biomarcadores moleculares associados à terapia alvo. Optou-se, seguindo as normas do Programa de Pós-graduação, por centrar a discussão desta tese em dois artigos principais já publicados. Desta forma, os demais estudos desenvolvidos no mesmo período estão apresentados como apêndices da tese a fim de complementar os dados aqui apresentados e de documentar parte da produção científica e premiações recebidas durante o curso de doutorado.

7 CONSIDERAÇÕES ÉTICAS

Todos os pacientes selecionados para participar dos respectivos projetos de pesquisa aqui apresentados estudo apresentam termo de consentimento livre e esclarecido assinado referentes ao protocolo originalmente entitulado: “Collection of Specimen and Clinical Data from Patients with Renal Cell Carcinoma (DFCI Protocol No.: 01-130)”. Este estudo foi elaborado de acordo com as diretrizes e normas regulamentadoras de pesquisa envolvendo seres humanos vigentes neste país e nesta instituição, sendo registrado e aprovado pela comissão de ética local (IRB, do inglês “Institutional Review Board”) conforme legislações federais vigentes no país em questão. Este protocolo prevê o estudo de biomarcadores em células tumorais e a coleta de dados clínicos de paciente com câncer de rim, incluindo o biomarcador utilizado neste projeto.

Pacientes ou material biológico oriundos do Brasil não foram envolvidos na realização dos estudo. Os anticorpos utilizados para avaliação imunohistoquímica foram desenvolvidos pelo Dr. Gordon Freeman, dentro da mesma instituição, estando disponíveis para uso com autorização prévia.

Os projetos de pesquisa aqui descritos também receberam aprovação do Comitê Científico e do Comitê de Ética vinculados ao Programa de Pós-graduação da Pontifícia Universidade Católica do Rio Grande do Sul.

8 ARTIGOS

8.1 PD-L1 IN NON-CLEAR CELL RENAL CELL CARCINOMA

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PD-L1 expression in nonclear-cell renal cell carcinoma

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Background: Programmed death ligand-1 (PD-L1) expression in nonclear-cell RCC (non-ccRCC) and its association with clinical outcomes are unknown.
Methods: Formalin-fixed paraffin-embedded (FFPE) specimens were obtained from 101 patients with non-ccRCC. PD-L1 expression was evaluated by immunohistochemistry in both tumor cell membrane and tumor-infiltrating mononuclear cells (TIMC). PD-L1 tumor positivity was defined as ≥5% tumor cell membrane staining. For PD-L1 expression in TIMC, a combined score based on the extent of infiltrate and percentage of positive cells was used. Baseline clinico-pathological characteristics and outcome data [time to recurrence (TTR) and overall survival (OS)] were correlated with PD-L1 staining.
Results: Among 101 patients, 11 (10.9%) were considered PD-L1+ in tumor cells: 2/36 (5.6%) of chromophobe RCC, 5/50 (10%) of papillary RCC, 3/10 (30%) of Xp11.2 translocation RCC and 1/5 (20%) of collecting duct carcinoma. PD-L1 positivity (PD-L1+) in tumor cells was significantly associated with higher stage ($P = 0.01$) and grade ($P = 0.03$), as well as shorter OS ($P < 0.001$). On the other hand, PD-L1 positivity by TIMC was observed in 57 (56.4%) patients: 13/36 (36.1%) of chromophobe RCC, 30/50 (60%) of papillary RCC, 9/10 (90%) of Xp11.2 translocation RCC and 5/5 (100%) of collecting duct carcinoma. A trend toward shorter OS was observed in patients with PD-L1+ in TIMC ($P = 0.08$). PD-L1+ in both tumor cell membrane and TIMC cells were associated with shorter TTR ($P = 0.02$ and $P = 0.03$, respectively).
Conclusion: In non-ccRCC, patients with PD-L1+ tumors appear to have worse clinical outcomes, although only PD-L1 positivity in tumor cells is associated with higher tumor stage and grade.
Key words: renal cell carcinoma, nonclear-cell renal cell carcinoma, benign kidney tumors, PD-L1, PD-1 inhibitors, immunotherapy

introduction

Renal cell carcinoma (RCC) has been widely recognized as a heterogeneous disease encompassing different histological subtypes [1]. Clear-cell RCC (ccRCC) is the most common subtype and accounts for more than 80% of the tumors that arise from the renal epithelium [2]. The remaining renal epithelial malignancies, collectively named as nonclear-cell RCC (non-ccRCC), include several subtypes such as papillary RCC (10%–15%), chromophobe RCC (5%), and the more rare forms, which include as Xp11.2 translocation RCC, unclassified RCC, and collecting duct carcinoma, among others [3].

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In RCC, surgery can be curative for localized disease [4]. However, about 30% of patients treated with nephrectomy will still develop systemic metastases. The risk of recurrence has been associated with clinical and pathological factors such as tumor–node–metastasis (TNM) staging and Fuhrman nuclear grading [5]. Several reports suggested that localized non-ccRCC is more likely to have a favorable prognosis than ccRCC [6]. Paradoxically, some series showed that when metastatic, some types of non-ccRCC such as papillary and Xp11.2 translocation RCC [7, 8], may have an aggressive clinical course and a shorter overall survival (OS).

Immunotherapy strategies have been used for decades in patients with advanced RCC, with prolonged survival being seen in a very small proportion of patients treated with interferon-α or high-dose interleukin (IL)-2 therapy [9]. Based on the important role of angiogenesis in ccRCC, single-agent therapies blocking the vascular endothelial growth factor (VEGF) or its receptors, as well as the mammalian target of rapamycin (mTOR) produced significant clinical benefit in the majority of metastatic ccRCC, resulting in a median OS of 20–30 months, compared with ~13 months reported with traditional immunotherapy [10, 11]. Because of their relatively low prevalence and their distinct biology, patients with non-ccRCC have typically been excluded from the pivotal clinical trials of antiangiogenic and tumor targeted agents [12]. Although some series have suggested that these drugs may also have activity in patients with non-ccRCC, more effective therapies for this patient population are needed [6, 13–15].

The levels and clinical significance of PD-L1 expression in non-ccRCC subtypes is still unknown. In this study, we sought to examine PD-L1 expression and its association with clinical outcome in a large series of patients with non-ccRCC.

methods

patients and samples

One hundred one patients with non-ccRCC (chromophobe RCC, papillary RCC, collecting duct carcinoma and Xp.11.2 translocation RCC) treated surgically at two institutions: Brigham and Women's Hospital (BWH) and Mayo Clinic were indentified. For comparative purposes, 20 patients with oncocytoma or angiomyolipoma treated in the same institutions were also evaluated. Formalin-fixed paraffin-embedded (FFPE) blocks were retrieved and corresponding slides from all cases were re-reviewed by an expert genitourinary pathologist (SS) at BWH. Baseline clinico-pathological characteristics such as age, gender, tumor size, Fuhrman grade, pathological TNM stage at time of surgery and follow-up data were retrospectively collected for patients with non-ccRCC. Uniform data collection templates were used to ensure consistent data. Institutional Review Board approval was obtained before data acquisition and tumor staining.

immunohistochemistry

PD-L1 expression was evaluated by immunohistochemistry using a mouse monoclonal anti-PD-L1 antibody (405.9A11) developed in Dr Gordon Freeman's laboratory (Dana-Farber Cancer Institute, Boston, MA) (Figure 1) . The immunohistochemical assay was extensively validated using FFPE cell line controls known to be positive or negative for PD-L1

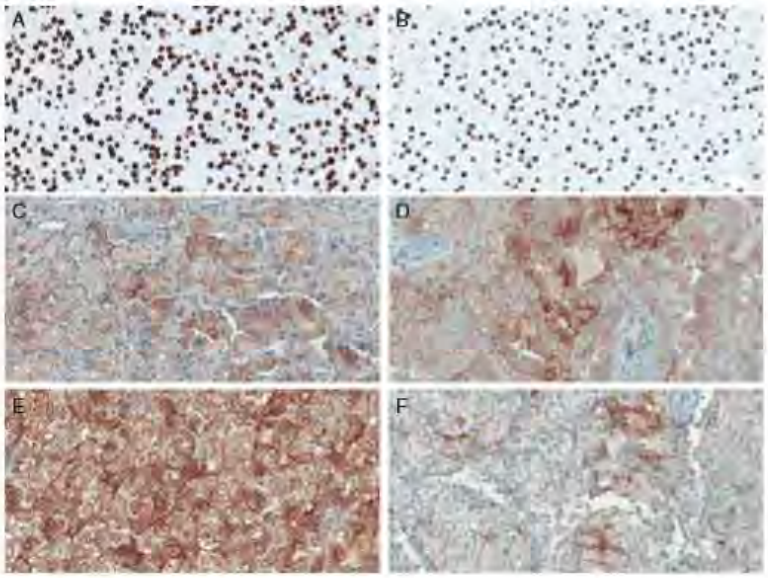

Figure 1. PD-L1 expression in FFPE samples stained with anti-PD-L1 antibody (clone 405.9A11). (A) Positive cell line control; (B) Negative cell line control; (C) Chromophobe RCC; (D) Papillary RCC. (E, F) Xp11.2 translocation RCC. Positive staining is present in tumor cells membrane in panels (C), (D) and (E). In panel (F), tumor cells are negative and tumor-infiltrating immune cells are positive for PD-L1.

expression by flow cytometry [16]. Four-micron-thick tumor sections were stained with an anti-PD-L1 antibody concentration of 3.25 μg/ml, on a Benchmark XT autostainer (Ventana Medical System, Tucson, AZ) with standard antigen retrieval (CC1 buffer, pH8.0, #950-124, Ventana). UltraView Universal DAB Detection kit (#760-500, Ventana) was used according to the manufacturer's instruction. Counterstaining was carried out as part of the automated staining protocol using hematoxylin (#760-2021, Ventana). After staining, slides were then washed in soap water and distilled water, dehydrated in graded alcohol and xylene, mounted and cover slipped.

quantification of PD-L1 expression on tumor cell membrane

Membranous expression in tumor cells was quantified semiquantitatively by two independent pathologists (SS and MC) blinded to clinical outcome. PD-L1 tumor positivity was defined as ≥5% tumor cell membrane staining.

quantification of PD-L1 expression in tumor-infiltrating mononuclear cells

The extent of tumor-infiltrating mononuclear cells (TIMC) (i.e. lymphocytes and macrophages) was assessed in hematoxylin and eosin-stained slides and recorded as absent (0), focal (1), mild (2), moderate (3) and marked (4). The percentage of PD-L1-positive TIMC was evaluated independently by two pathologists (SS and MC), according to three categories (0% = 0, <5% = 1, ≥5% = 2). An adjusted score representing PD-L1 expression was calculated multiplying the percentage of TIMC that stained positive for PD-L1 and the extent of mononuclear cell infiltration.

statistical analysis

The primary objective of this study was to characterize the PD-L1 expression in patients with non-ccRCC and to correlate the levels of expression with clinico-pathological features as well as disease outcomes. Two end points were analyzed: (i) TTR, defined as time from diagnosis to the date of development of metastatic disease; (ii) OS, defined as time from diagnosis to death. In the absence of an event, the end points were censored at last follow-up time. Patient and tumor characteristics were summarized descriptively. PD-L1 tumor positivity was defined as ≥5% tumor cell membrane staining. For PD-L1 expression in TIMCs, any score greater than zero was considered positive. Comparisons between PD-L1 expression and clinico-pathological features were evaluated using χ^2 or Fisher's exact test (when sample size was small) for categorical variables and Wilcoxon rank-sum test for continuous variables. Kaplan–Meier method estimated the distribution of TTR and OS by the PD-L1 positivity. Cox proportional regression assessed the associations with hazard ratio (HR) and 95% conference interval (CI). PD-L1 expression in patients with benign tumors was reported descriptively and correlations with clinico-pathological features as well as outcome variables were not carried out.

All statistical computations were carried out using SAS v.9.2 (SAS Institute, Inc., Cary, NC) and a P value (two-sided) <0.05 was considered statistically significant.

results

patients and tumor characteristics

Characteristics of patients with non-ccRCC are outlined in Table 1. The study cohort included a total of 101 patients with non-ccRCC. The histological subtypes included chromophobe RCC ($n = 36$), papillary RCC ($n = 50$) and Xp11.2 translocation RCC ($n = 10$) and collecting duct carcinoma ($n = 5$). The median follow-up time was 5 years [interquartile range (IQR): 3.5–6.2], and the median age was 59 years (range 24–81 years). For non-ccRCC, TNM clinical stages I, II, III and IV at diagnosis were identified in 54, 19, 18 and 9 patients, respectively. Additionally, 47 patients had high Fuhrman grade (III or IV) and 53 had low Fuhrman grade (I or II). In one tumor sample, the definition of tumor grade was not precisely possible and it was not reported. The median tumors' size was 4.7 cm (range 2.8–7.7 cm).

Table 1. Non-ccRCC patient characteristics

Characteristic	Total ($N = 101$)	
	No. of patients	%
Gender		
Male	55	54
Female	46	46
Stage		
1	54	53
2	19	19
3	18	18
4	9	9
Unknown	1	1
Fuhrman grade		
I/II	53	52.4
III	38	37.6
IV	9	9
Unknown	1	1
Histology		
Chromophobe	36	36
Papillary	50	49
Translocation	10	10
Collecting duct carcinomas	5	5
Metastatic disease		
No	78	77.2
Yes	23	22.8
PD-L1 expression in tumor cells membrane		
<5% (negative)	90	89.1
≥5% (positive)	11	10.9
PD-L1 expression in tumor-infiltrating mononuclear cells (TIMC)		
Score = 0 (negative)	44	43.6
Score > 0 (positive)	57	56.4
	Median	Min, max
Age at Dx (years)	59	24–81
Tumor size (cm)	4.7	0.6–30

For comparative purposes, 20 patients with benign kidney tumors were also evaluated for PD-L1 expression. The histological subtypes included oncocytoma ($n = 13$) and angiomyolipoma ($n = 7$). The median tumor's size was 3.2 cm (range 1.9–5.6 cm).

PD-L1 expression in tumor cells and clinico-pathological features

Among 101 patients with non-ccRCC, PD-L1 expression in tumor cell membrane was negative in 90 patients (89.1%) and positive in 11 patients (10.9%). Specifically, PD-L1 positivity in tumor cell membrane was detected in 2 of 36 (5%) chromophobe RCC, 5 of 50 (10%) papillary RCC, 3 of 10 (30%) Xp11.2 translocation RCC and 1 of 5 (20%) collecting duct carcinomas.

Table 2 Correlation of PD-L1 expression and clinico-pathological factors in non-ccRCC

Characteristic	% Positive tumor cell membrane			*P* value	TIMC			*P* value
	<5% (negative) (*N* = 90, 89.1%), *n* (%)	5% or more (positive) (*N* = 11, 10.9%), *n* (%)	Total (*N* = 101)		Score = 0 (negative) (*N* = 44, 43.6%), *n* (%)	Score >0 (positive) (*N* = 57, 56.4%), *n* (%)	Total (*N* = 101)	
Stage								
1	52 (58)	2 (20)	54 (53)	0.01	24 (55)	30 (54)	54 (53)	0.35
2	18 (20)	1 (10)	19 (19)		11 (25)	8 (14)	19 (19)	
3	14 (16)	4 (40)	18 (18)		7 (16)	11 (20)	18 (18)	
4	6 (7)	3 (30)	9 (9)		2 (5)	7 (12)	9 (9)	
Unknown	0	1 (1)	1 (1)		0	1 (1)	1 (1)	
Fuhrman grade								
I/II	51 (57)	2 (18)	53 (52.4)	0.03	23 (53)	30 (53)	53 (52.4)	0.11
III	31 (35)	7 (64)	38 (37.6)		19 (44)	19 (33)	38 (37.6)	
IV	7 (8)	2 (18)	9 (9)		1 (1)	8 (14)	9 (9)	
Unknown	1 (1)	0	1 (1)		1 (1)	0	1 (1)	
Histology								
Chromophobe	34 (94.4)	2 (5.6)	36 (36)	0.1	23 (63.9)	13 (36.1)	36 (36)	0.001
Collecting duct	4 (80)	1 (20)	5 (5)		0 (0)	5 (100)	5 (5)	
Papillary	45 (90)	5 (10)	50 (49)		20 (40)	30 (60)	50 (49)	
Translocation	7 (70)	3 (30)	10 (10)		1 (10)	9 (90)	10 (10)	

TIMC, tumor-infiltrating mononuclear cells.

PD-L1 positivity in tumor cell membrane was significantly associated with higher TNM stage ($P = 0.01$) and Fuhrman grade III/IV ($P = 0.03$) (Table 2). On the other hand, PD-L1 positivity was not correlated with gender, age at diagnosis or tumor size (data not shown).

PD-L1 expression in TIMCs and clinico-pathological features

Overall, the extent of TIMC infiltration was: absent in 11 patients, focal in 27 patients, mild in 31 patients, moderate in 20 patients and marked in 12 patients.

PD-L1 expression in TIMC was negative (score 0) in 44 patients (43.6%). Fifty-seven patients (56.4%) were considered PD-L1+ in the TIMC. Among the cases with PD-L1+ TIMC, 37 patients had expression in <5% of immune cells and 20 patients presented expression in more than 5% of immune cells. There was a significant association of histology subtype and PD-L1 expression levels in TIMC ($P = 0.001$). Specifically, among patients with PD-L1+, 13 of 36 (36%) had chromophobe RCC, 30 of 50 (60%) had papillary RCC, 9 of 10 (90%) Xp11.2 had translocation RCC and 5 of 5 (100%) had collecting duct carcinoma.

PD-L1 positivity in TIMC was not significantly associated with TNM stage ($P = 0.35$) or tumor grade ($P = 0.11$) (Table 2). In addition, PD-L1 positivity in TIMC did not correlate with gender, age at diagnosis or tumor size (data not shown).

PD-L1 expression and clinical outcome in non-ccRCC

The overall median follow-up of the cohort was 5 years, 17 patients died and 24 patients developed distant metastases. Patients with PD-L1+ in tumor cells were significantly associated with increased risk of death (HR = 6.41, 95% CI 2.17–18.88; $P < 0.001$) compared with patients with PD-L1 negative in tumor cells. A similar trend was observed when comparing PD-L1 expression in TIMC, but the result was not statistically significant (HR = 2.49, 95% CI 0.86–7.2; $P = 0.08$) (Figure 2A). In addition, PD-L1+ on tumor cell membrane and TIMC both were associated with lower TTR ($P = 0.02$ and $P = 0.03$, respectively) (Figure 2B).

PD-L1 expression in benign kidney tumors

PD-L1 expression in tumor cell membrane was positive in 4 of 13 (30.8%) oncocytomas and 0 of 7 (0%) angiomyolipomas. In addition, 7 of 13 (53.8%) of oncocytoma and 7 of 7 (100%) angiomyolipoma expressed PD-L1 in TIMC (score >0). Correlations with clinico-pathological features as well as outcome variables were not carried out.

discussion

Thompson et al. were among the first to describe the PD-L1 expression in ccRCC. In one study of 196 patients, PD-L1 expression was associated with aggressive features such as higher TNM stage, tumor size or Fuhrman grade and increased risk of cancer-specific mortality [17]. In another study of 306 patients, PD-L1+ was seen in 23% of cases. Similarly, PD-L1+ tumors were more likely to present adverse pathologic features including TNM stage III or IV, higher tumor size and Fuhrman grade III or IV ($P < 0.001$ for all), and higher risk of cancer-specific mortality (RR = 2.0 95% CI 1.27–3.15, $P < 0.003$) adjusting for TNM stage and grade [18]. Interestingly, the correlation between PD-L1 expression and adverse prognostic factors as well as OS was identified with PD-L1 expression in both tumor cell membrane and tumor-infiltrating lymphocytes (TILs). Based on these

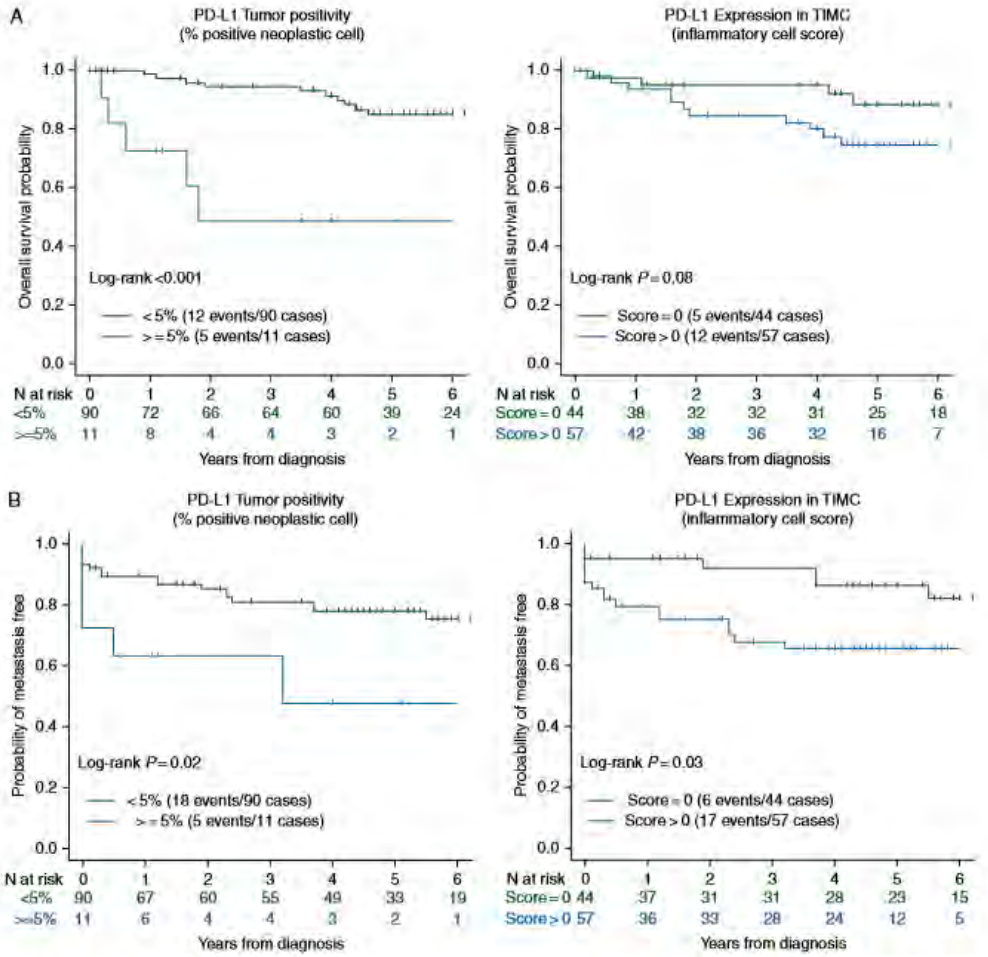

Figure 2. (A) Correlation of PD-L1 expression and OS (univariate analysis) in non-ccRCC; (B) Correlation of PD-L1 expression and TTR (univariate analysis) in non-ccRCC.

studies, PD-L1 expression may be considered as an independent predictor of poor prognosis in ccRCC [19].

Overcoming this adaptive mechanism of tolerance with therapies blocking the PD-1 or PD-L1 could restore the effectiveness of T-cell responses against tumor cells [20]. A phase I study evaluating the safety and efficacy of the anti-PD-1 monoclonal antibody (nivolumab) in patients with advanced cancer produced encouraging tumor responses in patients with RCC and other malignancies. Moreover, specimens from 42 patients, including 5 patients with RCC were analyzed for PD-L1 expression in tumor cells. Overall, 25 of 42 were considered PD-L1+. Among these 25 patients, 9 (36%) had objective response. On the other hand, none of the patients with PD-L1− expression achieved objective response ($P = 0.006$). These results supported the hypothesis that PD-L1 may be a promising predictive biomarker of response to agents that target the PD1/PD-L1 axis [21]. Since that landmark study, two other studies in RCC specifically showed that patients with PD-L1+ tumors have numerically higher response to agents that target the PD-L1/PD-1 axis than PD-L1 negative tumors, although it is important to note that responses were seen in PD-L1-negative tumors [22, 23].

To our knowledge, this is the first study to report PD-L1 expression in non-ccRCC and its correlation with clinical outcome. Consistent with previously published ccRCC studies, PD-L1 expression in tumor cell membrane was correlated with higher Fuhrman grade or TNM stage in patients with non-

ccRCC. In addition, on univariate analysis, patients with PD-L1 positivity in tumor cells were significantly more likely to have a shorter OS. Furthermore, a trend for shorter OS was also observed in patients with PD-L1+ TIMC and both PD-L1 positivity on tumor cell membrane and TIMC were associated with lower TTR. Our exploratory multivariate analyses suggest that tumor stage, Fuhrman grade and histology are significant effect modifiers for the association of PD-L1 positivity on clinical outcome (data not shown). Interestingly, we confirm that PD-L1 expression can exist in benign kidney tumors, as previously reported [24]. However, how it could affect the clinical course of this disease remains to be studied and addressed in other studies.

Infiltrating mononuclear cells in RCC release cytokines to either promote tumor growth or impair antitumor immune responses. In addition, high levels of TILs have been associated with an increased risk for cancer progression and death [25]. Similarly, higher expression of PD-L1 in TILs was also associated with aggressive features such as tumor grade and TNM stage in ccRCC [26]. Among non-ccRCC, we did not observe statistically significant association between PD-L1 expression in TIMC and clinico-pathological features or OS. Nonetheless, the percentage of patients who were considered PD-L1+ by this method was overall much higher than with the tumor membrane staining.

In this analysis, we showed that PD-L1 expression in non-ccRCC is heterogeneous and depends on histology. In 2004, the World Health Organization (WHO) classification of renal tumors recognized a new subtype of kidney cancer characterized by translocations involving the transcription factor E3 (TFE3) located on Xp11.2 gene [27]. These tumors share some morphological features with ccRCC and the real incidence of this subtype may be underestimated [8]. Aggressive clinical course in a younger adult population with a female predominance has been reported. Despite anti-VEGF drugs having some activity in these patients, there is no established treatment of patients with metastatic disease [28]. In our study, 3 of 10 patients who had Xp11.2 translocation RCC (30%) exhibited PD-L1+ in tumor cells and 9 of 10 (90%) harbored PD-L1+ TIMC. Collecting duct carcinomas are also a very aggressive disease and up to 40% of patients present with metastatic disease at diagnosis [14]. In our study, 1 of 5 patients expressed PD-L1 on tumor cells and all of them were considered positive in TIMC. Thus, we hypothesize that PD-L1 may play a key role in the biology of Xp11.2 translocation RCC and collecting duct carcinoma and could represent an important therapeutic target for these RCC subtypes for which few therapeutic options are currently available.

Our study has many limitations. First, the non-ccRCC is a very heterogeneous disease. In addition, considering the rarity of Xp11.2 translocation RCC and collecting duct carcinomas, even evaluating a large cohort, a small number of patients with these histologic subtypes have been represented limiting our conclusions. In addition, the majority of tumors did not have metastatic disease, and may underestimate the prevalence of PD-L1 staining in cytoreductive nephrectomies or distant metastases. Given the smaller group size for patients with PD-L1 positive and a small number of events (deaths), a multivariate analysis may not properly adjust the association of PD-L1 expression and clinical outcome for potential confounding factors. Moreover, the relatively short follow-up period may influence the correlation of PD-L1 expression and OS. Intratumor heterogeneity has been described in RCC. Although we have evaluated whole tissue sections, our results may not represent the PD-L1 expression in the entire tumor. Furthermore, the value of Fuhrman nuclear grading has been debated in non-ccRCC. However, it remains widely adopted in the clinical practice [29]. Finally, comparisons with other studies should be done with caution, since many different methodologies and antibodies have been applied to assess PD-L1 expression.

Notably, <10% of patients with nonclear-cell histologies were included on clinical trials of new investigational agents [30]. Our study suggests that patients with non-ccRCC, especially subsets with higher PD-L1 expression by either tumor or immune cells should not be automatically excluded from clinical trials of agents that target the PD-1/PD-L1 pathway.

In summary, PD-L1 expression in tumor and TIMC occurs in patients with non-ccRCC depending on histology subtype and tumor membrane versus immune cell scoring. In addition, PD-L1 positivity on tumors cell membrane was associated with aggressive clinico-pathological features. PD-L1 expression in Xp11.2 translocation RCC and collecting duct carcinomas as well as its correlation with clinical outcomes needs to be prospectively confirmed in larger series. Further evaluation of PD-L1 as a potential predictive biomarker to PD-1/PDL1 inhibitors in non-ccRCC is warranted.

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disclosure

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8.2 PROGREMMED DEATH-1 LIGAND IN ADRENOCORTICAL CARCINOMA: AN EXPLORATORY BIOMARKER STUDY

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RESEARCH ARTICLE Open Access

Programmed death ligand-1 expression in adrenocortical carcinoma: an exploratory biomarker study

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Abstract

Background: Adrenocortical carcinoma (ACC) is a rare tumor in which prognostic factors are still not well established. Programmed Death Ligand-1 (PD-L1) expression in ACC and its association with clinico-pathological features and survival outcomes are unknown.

Methods: Formalin-fixed paraffin-embedded (FFPE) specimens were obtained from 28 patients with ACC. PD-L1 expression was evaluated by immunohistochemistry (IHC) in both tumor cell membrane and tumor infiltrating mononuclear cells (TIMC). PD-L1 positivity on tumor cells was defined as ≥5% tumor cell membrane staining. TIMC were evaluated by IHC using a CD45 monoclonal antibody. For PD-L1 expression in TIMC, a combined score based on the extent of infiltrates and percentage of positive cells was developed. Any score greater that zero was considered PD-L1 positive. Baseline clinico-pathological characteristics and follow up data were retrospectively collected. Comparisons between PD-L1 expression and clinico-pathological features were evaluated using unpaired t-test and Fisher's exact test. Kaplan-Meier method and log-rank test were used to assess association between PD-L1 expression and 5-year overall survival (OS).

Results: Among 28 patients with surgically treated ACC, 3 (10.7%) were considered PD-L1 positive on tumor cell membrane. On the other hand, PD-L1 expression in TIMC was performed in 27 specimens and PD-L1 positive staining was observed in 19 (70.4%) patients. PD-L1 positivity in either tumor cell membrane or TIMC was not significantly associated with higher stage at diagnosis, higher tumor grade, excessive hormone secretion, or OS.

Conclusions: PD-L1 expression can exist in ACC in both tumor cell membrane and TIMC with no relationship to clinico-pathologic parameters or survival.

Keywords: Adrenocortical carcinoma, PD-L1, PD-1 inhibitors, Immunotherapy

Background

Adrenocortical carcinoma (ACC) is a rare and highly lethal malignancy arising from the adrenal cortex. In the United States, around 0.7-2 new cases per million are estimated every year [1,2]. Overall, ACC carries a poor prognosis with the most consistent prognostic factor being tumor stage at the time of diagnosis [3]. Unfortunately, retrospective studies have reported a 5-year survival rate of 24% for stage III and 0% for stage IV disease [4].

Complete surgical resection remains the only chance of cure for patients with early-stage disease (stage I and II). When feasible, resection is the mainstay of therapy, even for patients with locally advanced disease. Not without controversy, some studies suggest that adjuvant mitotane may improve clinical outcomes [5]. However, despite aggressive management, close to 80% of surgically treated patients will develop subsequent metastatic disease [6].

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The management of metastatic disease is challenging and disappointing results have been reported with the few available systemic therapeutic options [7]. Despite no statistically significant impact on overall survival (OS), results from the First International Randomized Trial in Locally Advanced and Metastatic ACC Treatment (FIRM-ACT) study have provided most consistent evidence for systemic treatment in advanced ACC [8]. This collaborative effort evaluated two different widely recommended regimens based on small phase II clinical trials [9,10]: mitotane plus a combination of etoposide, doxorubicin, and cisplatin (M/EDP) or mitotane plus streptozocin (SM). Patients who were treated with M/EDP had a significantly longer progression-free survival (PFS) compared with those who received SM (5.0 vs 2.1 months). Subsequent systemic options after progression on M/EDP or SM include gemcitabine plus capecitabine or metronomic 5-fluouracil which showed some clinical activity in a phase II clinical trial [11].

The biology underlying ACC is poorly understood [12]. So far, small studies have failed to demonstrate clinical benefit with targeted therapies blocking the epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF), mammalian target of rapamycin (mTOR), insulin-like growth factor 1 receptor (IGF-1R), or fibroblast growth factor receptor (FDFR) pathways in advanced disease, and no biomarkers have been established as predictors of survival or response to these agents [13-18]. The Tumor Cancer Genome Atlas is ongoing for ACC. This initiative will provide a comprehensive molecular characterization of this disease and may help to identify targets for drug development or a prognostic signature for risk stratification.

The current progress in understanding how the immune system can modulate tumor progression or effective responses against cancer is unfolding [19]. Immune checkpoints, like programmed death-1 (PD-1) and its ligand PD-L1, have been described as key regulators of T cell responses, and blocking the PD-1/PD-L1 axis using monoclonal antibodies has resulted in promising results in different malignancies [20]. Notably, in some series, levels of PD-L1 expression have correlated with clinical outcome [21,22]. However, the prognostic impact of PD-L1 expression still needs to be defined in many tumor types including ACC.

In this study, our goal is to characterize PD-L1 expression in ACC tissues and to correlate levels of PD-L1 expression with clinico-pathological features as well as survival outcomes.

Results

Patients and tumor characteristics

A total of 28 patients with ACC were included in this study. Patient characteristics are summarized in Table 1. Overall, the median age was 47.4 +/−13.2 years ranging from 19.8 to 73.7 years. Mean tumor size was 10.9 +/−4.4 cm ranging from 2.5 to 19 cm. The stage at diagnosis was defined pathologically according to International Union Against Cancer (UICC) and European Network for the Study of Adrenal Tumors (ENSAT) staging systems [23]. UICC stage I, II, III and IV were found in 1, 9, 3 and 11 patients, respectively, and ENSAT stage I, II, III, IV were found in 1, 9, 4 and 10 patients, respectively. Additionally, 19 patients developed metastasis during the follow up period and 14 patients presented with functional tumors at diagnosis.

In this cohort, 8 specimens were from metastasis and 20 from primary tumors. Among the metastatic specimens, only one had metastatic disease at diagnosis. Others had specimens collected at the time of relapse.

Correlation of PD-L1 expression and clinico-pathological features

Overall, PD-L1 expression on tumor cell membrane was considered positive in 3 of 28 patients (10.7%) (Figure 1). On univiariate analysis, PD-L1 expression was not correlated with stage (UICC and/or ENSAT), grade, or excessive secretion of hormones (Table 2).

A total of 27 patients were evaluated for PD-L1 expression in tumor infiltrating mononuclear cells (TIMC). The extent of TIMC were recorded as focal in 7 patients (26%), mild in 9 patients (33.3%), moderate in 9 patients (33.3%), and high in 2 patients (7.4%). For PD-L1 expression in TIMC, scores greater than zero were identified in 19 patients (70.4%). There was no significant correlation between PD-L1 expression in TIMC and stage (UICC and/or ENSAT), grade, or excessive hormone secretion (Table 3).

We further explored the effect of PD-L1 expression over other variables such as site of metastasis, number of mitosis per 10 high-power fields, age or tumor size. However, no association was found between any of these parameters and PD-L1 expression in either tumor cell membrane or TIMC (data not shown).

Correlation of PD-L1 expression and overall survival

Overall, 6 patients died during the follow up period. Positive PD-L1 expression in tumor cell membrane was not associated with 5-year survival (univariate analysis; two-sided $p = 0.65$) (Figure 2).

Discussion

Multiple retrospective analyses described the correlation between levels of PD-L1 expression and prognosis in several malignancies [20]. Some of these studies in renal cell carcinoma (RCC), breast cancer, and non-small cell lung cancer (NSCLC), demonstrated that higher levels of PD-L1 expression in tumor cells were associated with an

Table 1 Patient characteristics

Characteristics		Total (N = 28)	
		No. of patients	%
Sex	Male	13	46.4
	Female	15	53.6
Stage at Diagnosis (UICC)	I	1	4.2
	II	9	37.5
	III	3	12.5
	IV	11	45.8
	Missing	4	-
Stage at Diagnosis (ENSAT)	I	1	*4.2*
	II	9	*37.5*
	III	4	*16.6*
	IV	10	*41.7*
	Missing	4	-
Grade	Low	1	8.3
	High	11	91.7
	Missing	16	-
Metastatic disease	Yes	19	67.9
	No	9	32.1
Sites of Metastasis	Local Recurrence	4	14.3
	Lymph Node	8	28.6
	Lung	10	35.7
	Liver	8	28.6
	Bone	2	7.1
	Other	3	10.7
Functional Tumors at Diagnosis	Yes	14	50
	No	14	50
PD-L1 Expression on Tumor Cell Membrane	<5% (negative)	**25**	***89.3***
	≥5% (positive)	**3**	***10.7***
PD-L1 Expression in Tumor Infiltrating Mononuclear Cells (TIMC)*	Score = 0 (negative)	**8**	***29.6***
	Score > 0 (positive)	**19**	***70.4***
		Mean +/− SD	**Min, Max**
Age at Diagnosis (years)		47.4 +/−13.2	19.8 - 73.7
Tumor size (cm)		10.9 +/−4.4	2.5 -19
Mitosis/10HPF		13.7 +/−13.5	0.2-50

*In 1 patient, the PD-L1 staining in TIMC was not assessable.

unfavorable prognosis [24-26]. In contrast, higher PD-L1 expression on immune cells was associated with longer OS in melanoma and metastatic urothelial carcinoma [27,28]. To our knowledge, this is the first study to characterize PD-L1 expression and its clinical significance in ACC.

Tumor stage at diagnosis is the most important prognostic factor in ACC [29]. However, different clinical courses have been described among patients into the same tumor stage. Some retrospective analyses have reported that functional tumors may be associated with worse prognosis [30]. In addition, few studies have established histological or molecular markers, such as Ki67 index or *TP53* mutations, as predictors of poor prognosis and its value still needs to be confirmed [31]. From a clinician perspective, to investigate biomarkers that can predict response to treatments may be important in the decision-making process in the era of personalized medicine. In our analysis, PD-L1 positivity was observed in approximately 11% of ACC cases and did not correlate with stage at diagnosis (UICC or ENSAT), grade, and excessive secretion of hormones. Furthermore, no correlations were found between PD-L1 expression and survival at 5 years.

Some tumors are infiltrated by immune cells and it can dynamically influence the host immune response against tumor [32]. Interestingly, Willenberg and colleagues provided evidence of the involvement of immune cells and interleukin-2 (IL-2) cytokine stimulation in the formation of an adrenocortical tumor in a patient with Cushing's syndrome [33]. While little is known about the immune microenvironment in ACC, these findings may open new avenues on the understanding of tumor biology and development of new treatment strategies. The interaction between PD-1 and its ligand PD-L1 limits T cell activation in response to certain antigens in order to prevent immune-mediated damage in healthy tissue. Furthermore, chronic antigen exposure increases the levels of PD-L1 expression, resulting in T cell "exhaustion" and reduced immune control of tumor progression [34]. Tumor cells have the ability to express PD-L1 as an adaptive mechanism of resistance that can evade the immune system, resulting in tumor growth and more aggressive disease.

With the goal of restoring effective T cell responses, the inhibition of immune checkpoints such as PD-1 or PD-L1 has been considered attractive therapeutic targets using monoclonal antibodies. A set of well conducted clinical trials have reported encouraging clinical activity on PD-1/PD-L1 blockade across multiple tumor types. The first phase I clinical trial of nivolumab, an anti-PD-1 monoclonal antibody, showed significant clinical activity in RCC, melanoma, and NSCLC, leading to deeper investigations [35]. Other agents targeting this pathway have supported these early results [36]. In addition, combinations of immunomodulatory agents have been tested in different solid tumors and reported promising results [37].

No biomarkers have been established to precisely select patients for therapeutic strategies blocking the PD-1/PD-L1 axis. Moreover, while several studies have reported that

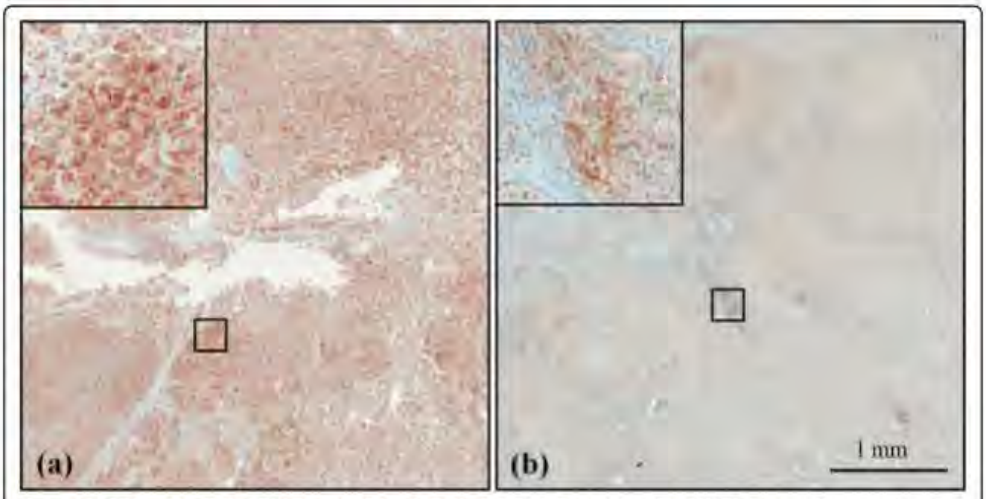

Figure 1 PD-L1 Expression in FFPE ACC Samples Stained with Anti-PD-L1 Antibody (clone 405.9A11). Positive membranous staining is present in tumor cells in panel **(a)**. In panel **(b)**, tumor cells are negative, and TIMC are positive for PD-L1.

PD-L1 expression in both tumor cell or tumor infiltrating immune cells is a potential predictor of response to immunomodulatory agents, the meaning and significance of PD-L1 expression in tumor cells or immune cells is still being investigated [20]. Preliminary results from a phase I study of an anti-PD-L1 inhibitor (MPDL3280A) in patients with advanced urothelial carcinoma showed response rates of 52% in patients with PD-L1 positive in immune cells vs. 14% in PD-L1 negative patients [38]. Interestingly, accumulating evidence shows that durable responses can also occur in patients who do not express PD-L1 on tumor cell membrane and/or tumor infiltrating immune cells [39]. This raises important considerations including tumor heterogeneity and tumor microenvironment alterations that need to be investigated in further studies.

Though this is the first study to date to evaluate the prevalence and prognostic significance of PD-L1 expression in ACC, there are several limitations. First, the retrospective nature of this analysis has led to missing data which may result in selection bias. In addition, considering that ACC incidence is low, it is difficult to perform studies with large and homogeneous cohorts. In our study, the sample size and the number of events in each group according to PD-L1 positivity were very small, limiting our ability to detect statistically significant

Table 2 Patient characteristics according to PD-L1 expression on tumor cell membrane

Characteristics (N = 28)	% Positive tumor cell membrane		
	<5% (negative) (n = 25, 89.3%) n(%)	5% or more (positive) (n = 3, 10.7%) n(%)	P-value*
UICC Stage**	I/II	9(90)	1(10)	–
	III/IV	13(92.8)	1(7.2)	
ENSAT Stage**	I/II	9(90)	1(10)	–
	III/IV	13(92.8)	1(7.2)	
Grade***	Low	1(100)	0(0)	–
	High	10(90.9)	1(9.1)	
Functional Tumors at Diagnosis	Yes	14(100)	0(0)	0.22
	No	11(78.6)	3(21.4)	

*Fisher's Exact Test.
**Missing: 4.
***Missing: 12.

Table 3 Patient characteristics according to PD-L1 expression on TIMC

Characteristics (n = 27)		Tumor infiltrating mononuclear cells		P-value*
		Score = 0 (negative) (n = 8, 29.6%) n(%)	Score > 0 (positive) (n = 19, 70.4%) n(%)	
UICC Stage**	I/II	1(11.1)	8(88.9)	0.34
	III/IV	5(35.7)	9(64.3)	
ENSAT Stage**	I/II	1(11.1)	8(88.9)	0.34
	III/IV	5(35.7)	9(64.3)	
Grade***	Low	0(0)	1(100)	-
	High	3(30)	7(70)	
Functional Tumors at Diagnosis	Yes	4(30.8)	9(69.2)	
	No	4(28.6)	10(71.4)	

*Fisher's Exact Test.
**Missing: 4.
***Missing: 16.

differences between groups. At the same time, there may be a missing-variable bias, since other clinico-pathological features such as Ki67 were not available for the majority of patients in this cohort. Furthermore, tumor heterogeneity is a confounding factor and differences between of primary and metastasis still need to be investigated. In 2 patients from our series, we had the opportunity to review primary and metastatic sites of disease. In one case, the primary tumor and one met were evaluated; in the other case the primary tumor plus 3 independent metastases were compared. Interestingly, in both cases the same results regarding PD-L1 expression (positive or negative) were observed in primary and metastatic samples stained for PD-L1. In this study, we tried to minimize the selection bias due to tumor heterogeneity by analyzing whole tissue sections from surgical resections and excluding needle biopsies. Finally, we focused on the clinical significance of PD-L1 expression in either, tumor cells and immune cells. However, the criteria defining PD-L1 positivity (cut-offs) as well as the antibodies used to stain for PD-L1 have varied among different studies. Therefore, comparisons with other studies should be done with caution and a more specific definition of immune cells subtypes should be focus of further studies. Although the same methodology used in this analysis has been applied to other studies, the methodology for PD-L1 staining should be standardized to allow for reliable evaluation of PD-L1 expression and comparison among differing cohorts [40].

Conclusions

In summary, ACC can express PD-L1 on both tumor cell membrane and immune cells and it may represent a potential target for therapeutic interventions. The current progress in cancer immunotherapy warrants prospective validation of our findings and further investigation of

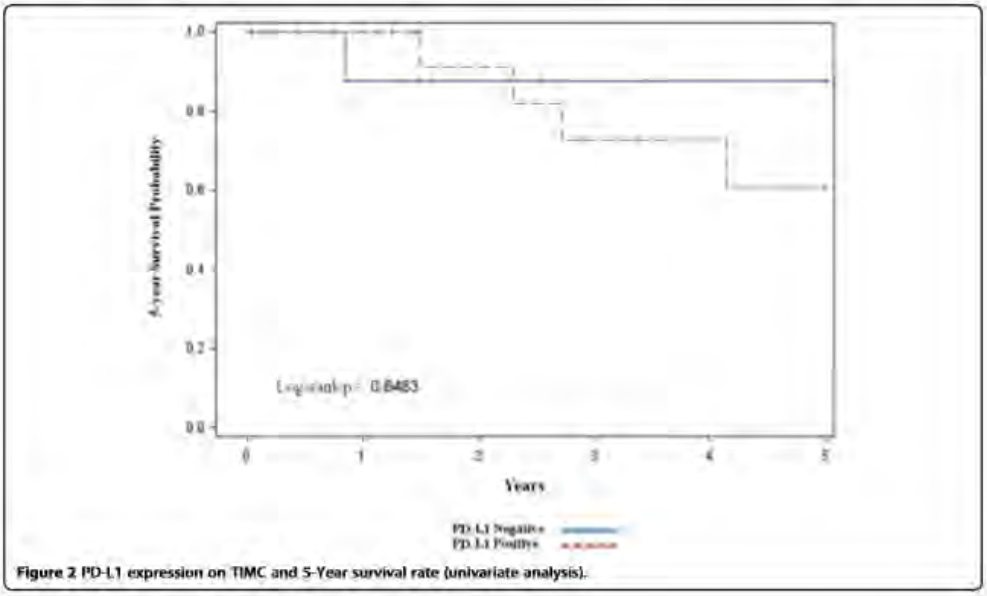

Figure 2 PD-L1 expression on TIMC and 5-Year survival rate (univariate analysis).

agents blocking the PD-1/PD-L1 pathway in this aggressive disease.

Methods

Patients and samples

Twenty-eight patients with ACC treated surgically at Dana-Farber Cancer Institute or Brigham and Women's Hospital were retrospectively selected. Formalin fixed paraffin-embedded (FFPE) blocks from primary tumors or metastases were retrieved, and for each patient one representative tumor block was selected for analysis by a genitourinary pathologist. Baseline clinico-pathological characteristics such as age, gender, tumor size, grade, stage using UICC and ENSAT staging systems, hormone-related symptoms at clinical presentation, as well as follow up data were retrospectively collected for all patients. Patients who presented with Cushing's syndrome, virilization, or feminilization were classified as functional tumors. This study received Institutional Review Board approval before tumor staining and data acquisition.

Immunohistochemistry

PD-L1 expression was evaluated by immunohistochemistry (IHC) using a mouse monoclonal anti-PD-L1 antibody (405.9A11) developed in Dr. Gordon Freeman's laboratory at Dana-Farber Cancer Institute. The immunohistochemical assay was validated using FFPE cell line controls known to be positive or negative for PD-L1 expression by flow cytometry [41]. Four micron-thick tumor sections were stained with an anti-PD-L1 antibody at a concentration of 3.25 ug/ml on a Benchmark XT autostainer (Ventana Medical System, Tucson, AZ) with standard antigen retrieval (CC1 buffer, pH8.0, #950-124, Ventana). UltraView Universal DAB Detection kit (#760-500, Ventana) was used according to the manufacturer's instruction. Counterstaining was performed as part of the automated staining protocol using hematoxylin (#760-2021, Ventana). After staining, slides were then washed in soap water and distilled water, dehydrated in graded alcohol and xylene, mounted, and cover slipped.

CD45 immunostaining was performed on adjacent four micron-thick tumor sections, which were initially deparaffinized, rehydrated and heated with a pressure cooker to 125°C for 30 seconds in citrate buffer for antigen retrieval and then incubated with peroxidase (Dako #S2003, Carpinteria, CA) and protein blocking reagents (Dako #X0909) each for 5 minutes. Sections were then incubated with anti-CD45 (1:100, Dako, clone 2B11 + PD7/26) antibody for 1 hour at room temperature followed by incubation with the Dako EnVision + System HRP labeled polymer anti-mouse (Dako #K4001) for 30 minutes. All sections were developed using the DAB chromogen kit (Dako K3468) for 2 minutes and then lightly counterstained with hematoxylin.

Scoring of PD-L1 expression on tumor cell membrane

For each sample, the percentage of tumor cells with PD-L1 expression on the cell membrane was estimated by two independent genitourinary pathologists who were blinded to clinical outcomes.

Scoring of PD-L1 expression in Tumor Infiltrating Mononuclear Cells (TIMC)

TIMC were identified on the basis of IHC positivity for CD45, a pan-leukocyte marker expressed in lymphocytes, macrophages and dendritic cells [42,43]. The extent of TIMC was recorded as absent (0), focal (1), mild (2), moderate (3) and marked (4). The percentage of PD-L1 expression in TIMC was evaluated semi-quantitatively according to three categories: 0% = 0, <5% = 1, and ≥5% = 2. An adjusted score was then calculated multiplying the percentage of TIMC that stained positive for PD-L1 and the extent of infiltrating immune cells, as previously reported [44]. Staining for PD-L1 was not performed in one patient in whom the available specimen was from a lymph node given that tumoral and non-tumoral immune cells could not be distinguished.

Statistical analysis

In this exploratory biomarker study, the pre-defined primary objective of this study was to characterize levels of PD-L1 expression on tumor cell membrane and TIMC in patients with ACC. Secondary endpoints were the correlation of PD-L1 expression with clinico-pathological features as well as 5-year survival rates. Five-year survival rate was defined as the time period between date of diagnosis and the date of death, or censored at 5 years after diagnosis. PD-L1 tumor positivity on tumor cells was defined as ≥5% tumor cell membrane staining. For PD-L1 expression in TIMC, any score greater than zero was considered positive.

Statistical analyses were performed using SAS (version 9.2; SAS Institute Inc., Cary, NC, USA). Descriptive data are presented as mean and standard deviation (SD), or percentage. Comparisons between PD-L1 expression and clinico-pathological features were evaluated using Fisher's exact test for categorical variables and unpaired t-test for continuous variables. Kaplan-Meier method estimated the distribution of 5-year survival rates by PD-L1 positivity. The association of 5-year survival rates with PD-L1 expression (negative vs. positive) was assessed by log-rank test and univariate Cox proportional regression analysis. Multivariate analysis were to be performed only if p-values were <0.05, considered statistically significant.

Abbreviations

ACC: Adrenocortical carcinoma; EGFR: Epidermal growth factor receptor; ENSAT: European network for the study of adrenal tumors; FDFR: Fibroblast growth factor receptor; FFPE: Formalin fixed paraffin-embedded; FIRM-ACT: First international randomized trial in locally advanced and metastatic ACC

treatment; IGF-1R: Insulin-like growth factor 1 receptor; IHC: Immunohistochemistry; IL-2: Interleukin-2; M/EDP: Mitotane, etoposide, doxorubicin, and cisplatin; mTOR: Mammalian target of rapamycin; NSCLC: Non-small cell lung cancer; OS: Overall survival; PD-1: Programmed death-1; PD-L1: Programmed death ligand-1; PFS: Progression-free survival; RCC: Renal cell carcinoma; SD: Standard deviation; SM: Mitotane and streptozocin; TIMC: Tumor infiltrating mononuclear cells; UICC: International Union Against Cancer; VEGF: Vascular endothelial growth factor.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MC and SS carried out the pathological studies and immunoassays. GF developed the antibody used in immunoassays. ML JS, IC and AF carried out the data acquisition. AF, AE, SS, TC, conceived of the study, and participated in its design and coordination and helped to draft the manuscript. GT and AF participated in the design of the study and performed the statistical analysis. All authors participated of analysis and interpretation of data. AF drafted the manuscript. All authors helped to draft the final version of the manuscript. All authors read and approved the final manuscript.

Authors' information
André P. Fay is a medical oncologist and a PhD student at PUCRS, Brazil. Since 2013, Dr. Fay is working with clinical and translational research in the Lank Center of Genitourinary Oncology at Dana-Farber Cancer Institute/ Harvard Medical School under Dr. Toni Choueiri's supervision. Recently, he was selected to receive the MERIT Award of American Society of Clinical Oncology for two times (GU ASCO Symposium 2014 and 2015). One of the research projects awarded was: PD-L1 expression in non-clear cell renal cell carcinoma. Dr. Fay and the authors involved in this work have published important contributions highlighting the role of immunotherapy in genitourinary malignancies. Toni Choueiri is the clinical director and Kidney Cancer Center director of the Lank Center of Genitourinary oncology and has contributions as a researcher, clinician and mentor of many oncology trainees. His research interests include the development of novel agents and biomarkers in genitourinary cancers, with a particular focus in renal cell carcinoma. Currently, Dr. Choueiri is the overall primary investigator in several Phase I, II and III studies of novel compounds and combinations using both clinical and correlative tissue-based endpoints.

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9 CONSIDERAÇÕES FINAIS

Os estudos apresentados acima caracterizaram de forma inédita na literatura médica a expressão de PD-L1 em células tumorais e infiltrados leucocitários em neoplasias genito-urinárias de baixa incidência (CCR de células não claras e carcinomas do córtex da glândula adrenal). Estes estudos mostram que tais neoplasias são capazes de expressar PD-L1 sugerindo que o sistema imunológico pode fazer parte da fisiopatologia da doença e de sua progressão. Entretanto, são vários os fatores que influenciam na expressão destes marcadores e estes devem ser avaliados com cautela na interpretação dos dados apresentados. O valor prognóstico deste marcador não pode ser estabelecido de forma definitiva nestes estudos. Contudo, a expressão deste marcador parece estar associada a um pior prognóstico naqueles pacientes com CCR de células não claras.

É importante ressaltar, conforme previamente descrito nos respectivos manuscritos, que estes estudos possuem limitações: a associação dos achados apresentados com os diversos outros fatores que são parte da resposta imunológica estão fora do escopo destes projetos de pesquisa e devem ser abordados em estudos futuros. Da mesma forma, a correlação destes achados com as demais vias de ativação molecular que participam do processo do ciclo celular e progressão tumoral, também não podem ser respondidas e devem ser foco de estudo. Além disso, a metodologia utilizada para avaliar a expressão imuno-histoquímica desta proteína é controversa em relação a sua validade externa. Diferentes anticorpos e métodos de quantificação tem sido utilizados para caracterização deste marcador em diferentes neoplasias sólidas. Desta forma, comparações entre estas metodologias tornam-se difíceis e a padronização dos métodos deve ser prioridade.

Estudos recentes mostram que a utilização do sistema imunológico no combate ao câncer aumenta a sobrevida global de pacientes com histologias específicas, produzindo resposta duradouras em um subgrupo de pacientes respondedores ao tratamento. Tal conhecimento caracteriza uma mudança de paradigmas no tratamento do câncer; porém muitas questões relacionadas a este tema ainda precisam ser respondidas.

Em suma, os estudos apresentados nesta tese estudos respaldam a hipótese de que inibidores da via do PD-L1 podem ser drogas ativas no tratamento de neoplasias genito-urinárias de baixa incidência em estágio avançado onde poucos tratamentos estão disponíveis

e o prognóstico é reservado. Estes estudos justificam a inclusão de pacientes portadores desta patologia em estudos clínicos prospectivos avaliando tais drogas. Estudos futuros, já planejados em grandes centros, poderão validar os dados aqui apresentados e abordar as demais associações e correlações não abordadas neste estudo.

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APÊNDICE A - Association of PD-L1 expression on tumor-infiltrating mononuclear cells and overall survival in patients with urothelial carcinoma

original articles

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Association of PD-L1 expression on tumor-infiltrating mononuclear cells and overall survival in patients with urothelial carcinoma

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Background: Programmed death-1 (PD-1) receptor/PD-1 ligand (PD-L1) pathway negatively regulates T-cell-mediated responses. The prognostic impact of PD-L1 expression needs to be defined in urothelial carcinoma (UC).

Patients and methods: Formalin-fixed paraffin-embedded tumor samples from 160 patients with UC were retrieved. PD-L1 expression was evaluated by immunohistochemistry using a mouse monoclonal anti-PD-L1 antibody (405.9A11). PD-L1 positivity on tumor cell membrane was defined as ≥5% of tumor cell membrane staining. The extent of tumor-infiltrating mononuclear cells (TIMCs) as well as PD-L1 expression on TIMCs was scored from 0 to 4. A score of 2, 3, or 4 was considered PD-L1-positive. Clinico-pathological variables were documented. The Cox regression model was used to assess the association of PD-L1 expression with overall survival (OS) in patients who developed metastases.

Results: TIMCs were present in 143 of the 160 patient samples. Out of 160 samples, 32 (20%) had positive PD-L1 expression in tumor cell membrane. Out of 143 samples with TIMCs, 58 (40%) had positive PD-L1 expression in TIMCs. Smoking history, prior BCG use and chromosome 9 loss did not correlate with PD-L1 expression in either tumor cell membrane or TIMCs. PD-L1 positivity was not different between non-invasive or invasive UC. In patients who developed metastases (M1) and were treated with systemic therapy ($n = 100$), PD-L1 positivity on tumor cell membrane was seen in 14% of patients and did not correlate with OS ($P = 0.45$). Out of 89 M1 patients who had evaluable PD-L1 on TIMCs, PD-L1 expression was seen in 33% of patients and was significantly associated with longer OS on multivariate analysis ($P = 0.0007$).

Conclusion: PD-L1 is widely expressed in tumor cell membrane and TIMCs in UC. PD-L1 in tumor cells was not predictive of OS. However, positive PD-L1 expression in TIMCs was significantly associated with longer survival in those patients who developed metastases.

Key words: urothelial carcinoma, PD-1, PD-L1, immunotherapy, prognosis, overall survival

introduction

Metastatic urothelial carcinoma (UC) remains largely incurable and the mortality rates have not changed substantially over the past two decades [1] with the median overall survival (OS) of 14–15 months seen with the use of cisplatin-based chemotherapy. Targeted therapies have produced limited clinical activity and when responses occur, they are usually transient.

In the metastatic setting, clinical factors, such as performance status, visceral metastases, hemoglobin level, or liver metastases, have been used to predict clinical outcome in both first- and second-line [2]. Although The Cancer Genome Atlas (TCGA) has provided insights on the genomic profile of urothelial tumors, potentially opening new avenues for prognosis and therapy [3], its clinical application is still premature.

Non-muscle invasive UC (NMIUC) has been historically recognized as an immunogenic tumor [4]. Tumor-infiltrating mononuclear cells (TIMCs) appear to be involved in the local anti-tumor responses [5] when immunotherapy with Bacillus Calmette–Guerin (BCG) is used, preventing local recurrences and tumor progression in High-grade/ Carcinoma *in situ* (CIS) non-invasive disease [6].

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Recently, blocking immune checkpoint molecules with monoclonal antibodies has emerged as a promising strategy in advanced urothelial cancer treatment [7]. The interaction of programmed cell death-1 (PD-1) on T-cells with its ligand PD-L1 (B7-H1) on tumor cells and immune cells limits T-cell-mediated immune responses [8]. Therefore, it is hypothesized that the PD-1/PD-L1 signaling pathway plays an important role in immune system escape by the tumor [9].

PD-L1 has been shown to be expressed in several malignancies, including UC [10–12]. In addition, it has been suggested that higher PD-L1 expression in tumor cell membrane or tumor-infiltrating immune cells is associated with different clinico-pathological features and clinical outcome in multiple different tumor types [13]. However, the prognostic impact of this biomarker has not been established across different tumor types.

In this study, we sought to characterize PD-L1 expression and its correlation with clinico-pathological features as well as OS in a large series of patients with UC as well as OS including patients who developed metastatic disease and were subsequently treated with platinum-based chemotherapy (M1).

methods

patients and samples

A total of 160 patients with UC from two institutions, Dana-Farber Cancer Institute, Boston, USA, and Hospital del Mar, Barcelona, Spain, were identified. Formalin-fixed paraffin-embedded (FFPE) blocks from radical cystectomy or transurethral resection of bladder tumors (TURB) were retrieved from the Department of Pathology. Baseline clinico-pathological characteristics including smoking history, prior BCG treatment, TNM stage at diagnosis, copy number variation (CNV) at chromosome 9, prognostic factors in patients with metastatic disease, and clinical follow-up data were retrospectively collected from our database. Institutional Review Board approval was obtained at both institutions before data acquisition and tumor staining.

immunohistochemistry

PD-L1 expression was evaluated in a tissue micro array (TMA) by immunohistochemistry using a mouse monoclonal anti-PD-L1 antibody (405.9A11) developed in Dr Gordon Freeman's laboratory (Figure 1). This antibody attaches to the PD-L1 ligand in the cytoplasmic domain, providing a clearer stain on the membrane of cells. The immunohistochemical assay is described in supplementary material S1, available at *Annals of Oncology* online.

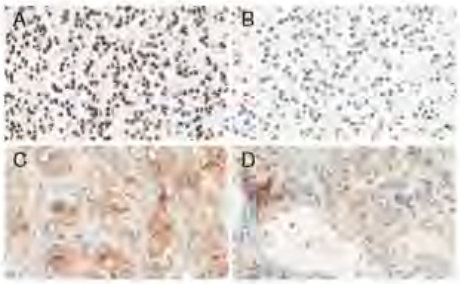

Figure 1. PD-L1 expression in FFPE samples stained with anti-PD-L1 antibody (405.9A11). Positive and negative controls for PD-L1 expression are presented in (A) and (B), respectively. Positive membranous staining is present in tumor cells in (C). In (D), tumor cells are negative, and tumor-infiltrating mononuclear cells are positive for PD-L1.

scoring of PD-L1 expression

For each sample, the TIMCs infiltrate and the membranous expression of PD-L1 in tumor cells or TIMCs were determined by two independent pathologists (SS and MC) blinded to clinical data.

PD-L1 tumor positivity was defined by the presence of ≥5% of tumor cells with membrane staining.

The extent of TIMCs was assessed in hematoxylin and eosin-stained slides and recorded as absent (0), focal (1), mild (2), moderate (3), and severe (4) with score 0 or 1 considered negative. The extent of PD-L1-positive TIMCs was also assessed using the same scoring scale (0–4) and samples with a score of 2–4 were considered PD-L1-positive. Seventeen samples were non-evaluable for TIMCs extent or PD-L1 staining in TIMCs.

recurrent copy number alterations

Array comparative genomic hybridization was carried out on 71 samples as previously described (supplementary material S2, available at *Annals of Oncology* online) [14].

statistical analysis

The primary objective of this study was to correlate the levels of PD-L1 expression with OS in patients with metastatic disease and who received first-line platinum-based chemotherapy. We also carried out an exploratory analysis to correlate PD-L1 expression and clinico-pathological features. Patient clinico-pathological characteristics were summarized descriptively. OS was defined as the time period between the date of the first chemotherapy application and the date of death, or censored on the date of last follow-up. Smoking history was only available for 74 patients from one institution. The time point for current smokers was at the time of cystectomy. Current and former smokers were combined into the smokers' category for analysis. Fisher's exact tests were used to assess the associations of smoking status, use of BCG with PD-L1 positivity in tumor cells, and TIMCs. The Cox regression model was used to assess the association of PD-L1 positivity and TIMCs with OS in both univariate and multivariate analysis adjusting for Eastern Cooperative Oncology Group (ECOG) performance status and whether patients had visceral disease or not. Hazard ratio and 95% confidence interval were also listed.

All statistical computations were carried out using SAS v.9.2 (SAS Institute Inc., Cary, NC) and a *P*-value (two-sided) of <0.05 was considered statistically significant.

results

Patient and tumor characteristics are described in Table 1. One hundred and sixty patients had tumor samples and adequate clinical data to be evaluated for PD-L1 expression in tumor cells. Among them, 143 had TIMCs in tumor samples and were evaluable for PD-L1 expression in TIMCs. Out of the 160 patients, 100 patients developed metastatic disease and received treatment (M1) (Figure 2). Of the 100 M1 patients, 89 had TIMCs in their tumor sample and were evaluable for PD-L1 expression in TIMCs ($M1^{wTIMCs}$) (supplementary Figure S3, available at *Annals of Oncology* online). Of note, patients were not treated with immunotherapy during the course of metastatic disease.

Table 1. Patient characteristics [patient characteristics at the time of initial diagnosis (Stage) and at the time of starting treatment for metastatic disease]

Clinico-pathological features	All cohort ($n = 160$), n (%)	Patients with metastatic disease ($n = 100$), n (%)
Staging		
Non-invasive tumors	23 (14.4)	
T2	60 (37.5)	
T3	57 (35.7)	
T4	16 (10)	
Not available	4 (2.5)	
Visceral disease		
Yes		47 (47%)
No		53 (53%)
ECOG PS		
0		35 (35%)
1		58 (58%)
2 or 3		7 (7%)
PD-L1 expression on tumor cell membrane		
Negative (<5%)	128 (80)	86 (86%)
Positive (≥5%)	32 (20)	14 (14%)
Extent of TIMCs		
Absent	3 (1.9)	2 (2%)
Focal	43 (26.9)	32 (32%)
Mild	50 (31.2)	28 (28%)
Moderate	34 (21.2)	21 (21%)
Severe	13 (8.1)	6 (6%)
Not available	17 (10.6)	11 (11%)
PD-L1 expression in TIMC*		
Absent	34 (23.8)	26 (29.2%)
Focal	51 (35.6)	30 (33.7%)
Mild	42 (29.4)	23 (25.8%)
Moderate	13 (9.1)	8 (9%)
Severe	3 (2.1)	2 (2.3%)

*Patients with absent TIMCs were not stained for PD-L1 in TIMCs ($n = 143$ and $n = 89$ in the metastatic cohort).

PD-L1 expression on tumor cell membrane or TIMCs

Overall, PD-L1 expression in tumor cells was negative in 128 patients (80%) and positive in 32 patients (20%). In the M1 subset ($n = 100$), PD-L1 expression was negative in 86 (86%) and positive in 14 patients (14%) (Table 1).

Seventeen patients (10.6%) were not evaluable for TIMCs and were not included in the PD-L1 expression analysis. Out of the 143 patients with TIMCs present, PD-L1 expression in TIMCs was scored as absent (0) in 34 patients (23.8%), focal (1) in 51 patients (35.6%), mild (2) in 42 patients (29.3%), moderate (3) in 13 patients (9.1%), and severe (4) in 3 patients (2.1%). PD-L1 expression in TIMCs was considered negative (0 or 1) in 85 of 143 patients (63%) and positive (2–4) in 58 patients (37%).

Among the $M1^{+TIMCs}$ subset ($n = 89$), PD-L1 expression in TIMCs were scored as absent (0) in 25 patients (28.1%), focal (1) in 30 patients (33.7%), mild (2) in 23 patients (25.8%), moderate (3) in 8 patients (9.0%), and severe (4) in 2 patients (2.2%). PD-L1 in TIMCs expression was considered negative (0–1) in 56 of 89 patients (63%) and positive (2–4) in 33 of 89 patients (37.1%) (Table 1).

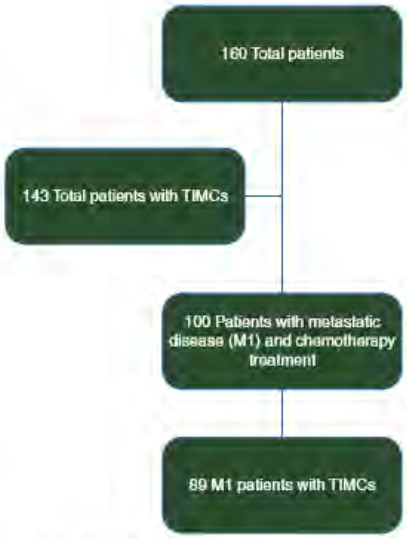

Figure 2. Study design.

association of PD-L1 expression and OS in patients with metastatic disease

The median time from cystectomy to the development of metastatic disease in these patients was 20 months. All received first-line treatment with platinum-based chemotherapy. The median follow-up was 25 months for M1 patients. In the $M1^{+TIMCs}$ subset, the presence (score of 2–4) versus the absence (score of 0–1) of TIMCs infiltrate was associated with longer OS (11 versus 18 months, $P = 0.02$). Positive PD-L1 expression (score of 2–4) in TIMCs was significantly associated with longer OS (12 versus 23 months) in both univariate ($P = 0.04$) and multivariable analysis ($P = 0.0007$) (adjusting for ECOG status and visceral disease) (Table 2; Figure 3). PD-L1 expression in tumor cell membrane was not associated with survival ($P = 0.45$).

association of PD-L1 expression and staging

Overall, 23 patients had NMIUC (T0 and T1) and 133 patients had high-grade muscle invasive UC (≥T2). Staging was not available in four patients. For muscle-invasive UC, TNM stages II, III, and IV at diagnosis were found in 60, 57, and 16 patients, respectively. There were no statistically significant differences in PD-L1 expression on TIMCs or on tumor cells between non-invasive or invasive bladder cancer (41.8% versus 30%; $P = 0.53$; 8.7% versus 21.8%; $P = 0.25$) (Table 3).

Table 2. Association of PD-L1 expression and OS in patients who develop metastatic disease

	n	Deaths	Median OS and 95% CI	HR and 95% CI (univariate)	*P*-value	HR and 95% CI (multivariable)	*P*-value
PD-L1 expression in TIMCs							
Absent, focal	56	37	12 (9, 16)	1.87 (1.02, 3.47)	0.04	3.19 (1.64, 6.22)	0.0007
Mild, moderate, severe	33	14	23 (12, not reached)	1 (reference)		1 (reference)	
PD-L1 expression in tumor cell membrane							
<5%	86	52	14 (11, 18)	1.42 (0.57, 3.55)	0.45	1.72 (0.67, 4.40)	0.26
≥5%	14	5	Not reached	1 (reference)		1 (reference)	

The extent of PD-L1-positive TIMCs was also assessed using a scale (0–4): absent (0), focal (1), mild (2), moderate (3), and severe (4) with score 0 or 1 considered negative. Samples with a score of 2–4 were considered PD-L1-positive. PD-L1 tumor positivity was defined by the presence of ≥5% of tumor cells (membrane staining).

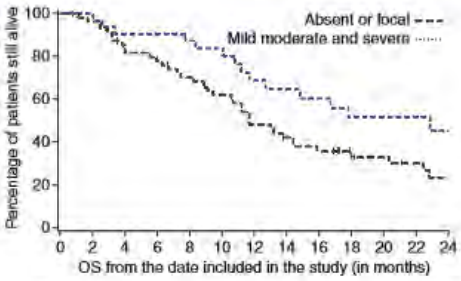

Figure 3. PD-L1 expression in TIMC and OS ($n = 89$). Correlation of PD-L1 expression (Absent or Focal vs. Mild, Moderate or Severe) and OS (months) in patients with metastatic disease.

association of PD-L1 expression and BCG treatment

Information regarding the prior use of BCG was available in a subset of 69 out of the total 160 patients (43.1%). Out of the 69 patients, 17 patients (23%) were treated with at least one BCG instillation and 52 (70%) did not receive any BCG therapy (supplementary Table S4, available at *Annals of Oncology* online). All patients who underwent BCG treatment had their last treatment within 1 year of cystectomy. There was no correlation with prior adjuvant BCG exposure and PD-L1 expression in tumor cell membrane or TIMCs ($P = 0.12$ and 0.99, respectively) (Table 3).

association of PD-L1 expression and smoking status

In a subset of 73 patients, smoking history was available: 9 (12%) were active smokers, 46 (62%) were former smokers, and 18 (24%) had never smoked. Smoking history was not associated with PD-L1 expression in tumor cell membrane or TIMCs ($P = 0.86$ and 0.99, respectively) (Table 3).

association of PD-L1 expression and CNV at chromosome 9

CNV data were available for 71 of the 100 M1 patients. CNV at the PD-L1 locus (9p24) was not significant in terms of standard GISTIC parameters. We also looked for the correlation of loss of all of chromosome 9 defined as having a loss in all four loci (9p11.2, 9p21.3, 9q34.3, and 9p23) that were shown to be significant based on GISTIC cut-offs. Chromosome 9 loss was identified in five patients. In our analysis, loss of chromosome 9 did not correlate with PD-L1 expression in tumor cell membrane nor TIMCs ($P > 0.99$).

discussion

Higher PD-L1 expression in tumor cells has been correlated with both favorable and unfavorable outcome in several malignancies [15–17]. In UC, PD-L1 expression on tumor cells has been associated with high grade, stage, and worse outcome in some reports. However, the overall impact of PD-L1 expression on prognosis remains controversial in UC [18]. To our knowledge, this is the first study to demonstrate that PD-L1 expression in TIMCs is correlated with improved OS in patients with UC who developed metastatic disease and were homogeneously treated with platinum-based chemotherapy. PD-L1 expression can occur on the tumor cell or on TIMCs. When T-cells recognize antigen and become activated, they express cytokines such as interferon-γ which in turn can induce PD-L1 expression on surrounding immune and tumor cells. The expression of PD-L1 on TIMCs is consistent with the idea that these intratumoral lymphocytes are tumor antigen-specific and responding to the tumor.

The correlation between PD-L1 expression in tumors cells and worse clinical outcome (higher risk of recurrence and shorter OS) in 65 patients with UC was first reported by Nakanishi et al. [19]. In addition, levels of PD-L1 expression were found to be high in inflammatory cells in 13 randomly selected patients.

Recently, Boorjian et al. [20] reported that higher PD-L1 expression in tumor cells was associated with the presence of advanced disease in patients with UC and also correlated with shorter OS in patients with organ-confined UC after radical cystectomy. In another series, which evaluated 302 UC patients, PD-L1 expression in tumor cell membrane was not correlated with recurrence, cancer-specific, or OS. However, in patients with organ-confined UC, higher PD-L1 expression was associated with an increased risk of death ($P = 0.02$) [21].

Table 3. Association of PD-L1 expression and clinico-pathological features

Staging	PD-L1 expression in tumor cell membrane (*n*)		*P*-value	PD-L1 expression TIMCs (*n*)		*P*-value
	Negative	Positive		Negative	Positive	
Association of PD-L1 expression with staging at the time of radical cystectomy						
Non-invasive tumors	21	2	0.25	7	3	0.53
Muscle-invasive tumors	104	29		75	54	
Clinical features	PD-L1 expression in tumor cell (*n*)		*P*-value	D-L1 expression in TIMCs (n)		*P*-value
	<5%	≥5%		Positive	Negative	
Association of PD-L1 expression with BCG use or smoking history						
Prior BCG						
No	35	17	0.12	21	27	0.99
Yes	15	2		7	8	
Smoking history						
Active smokers	7	2	0.86	4	5	0.99
Former smokers	32	14		19	23	
Never smoked	14	4		7	9	

In the first part, the association between PD-L1 expression and staging (non-invasive tumors versus muscle invasive tumors) at the time of radical cystectomy is presented. The association between PD-L1 expression and BCG use or smoking history is presented in the second part.

Based on the potential predictive role recently described for PD-L1 expression on immune cells in patients receiving checkpoint inhibitors in UC, attention has now switched toward the analysis of PD-L1 expression in immune cells instead of tumors cells. In our study, no association between tumor cell PD-L1 expression and clinical outcome was found. However, in addition to seeing a correlation with the presence of TIMCs and survival (supplementary Table S5, available at *Annals of Oncology* online) as previously reported [22], higher PD-L1 expression in TIMCs was statistically correlated with longer OS in the multivariate analysis in patients who developed metastatic disease and subsequently received chemotherapy. Moreover, we found that patients with TIMCs were more likely to have higher PD-L1 expression on tumor cell membrane (supplementary Table S5, available at *Annals of Oncology* online). It is still an open question how these exploratory correlations will impact patient's prognosis. Therefore, further prospective studies are warranted.

Recently, preliminary results from a phase I study to evaluate the efficacy of MPDL3280A, an anti-PD-L1 mAb, in patients with advanced UC were presented. The overall response rate in those patients who express PD-L1 (score 2/3) in immune cells was 52% versus 14% in those who were considered PD-L1 negative (score 0/1) [23]. Interestingly, most of the responses were ongoing at the cut-off time of analysis. These results support the rationale of PD-L1 expression in immune cells as a potential predictive biomarker for immunotherapies in UC. The fact that PD-L1-negative patients had response to anti-PD-L1 therapy highlights the need for better biomarkers for response to agents targeting this pathway.

The success of BCG in NMIUC has highlighted UC as an immune sensitive disease and the role of immune checkpoints like PD-1/PD-L1 in patients failing BCG is under evaluation [24]. Inman et al. evaluated PD-L1 expression in tumor cells in 280 UC of the bladder. In this study, they reported that PD-L1 expression was associated with high-grade tumors and tumor infiltration by mononuclear cells ($P = 0.009$ and 0.004, respectively). Interestingly, higher PD-L1 expression was seen in 11 out of 12 patients who had BCG-induced pathological inflammatory changes and failed BCG treatment suggesting that tumor cells might be protected from attack by immune cells through immune checkpoints, like PD-L1 [12]. Notably, in our analysis, PD-L1 expression was not correlated with prior use of BCG.

Smoking history is the most important risk factor for bladder cancer and highly complex mutational profiles have been described for both, smokers and non-smokers [25]. Responses to agents targeting PD-1/PD-L1 pathway have been described to be more robust in smokers than non-smokers in patients with lung cancer [26]. In our study, no correlation between PD-L1 expression and smoking history was observed in an exploratory analysis.

PD-L1 gene is located on chromosome 9p24. Green et al. [27] demonstrated that *PD-L1* amplification was associated with significantly higher PD-L1 expression Hodgkin's lymphomas cells. UC is associated with multiple somatic CNVs, including frequent chromosome 9 loss [28]. Therefore, we speculated that CNV on chromosome 9 may correlate with PD-L1 expression in UC. No correlation was found between copy number changes and PD-L1 expression.

Our study has limitations. First, although we have analyzed a large cohort, this is a retrospective analysis and there is a potential for selection bias. Secondly, clinical data were limited for some of our patients, resulting in lack of power for the analyses of smoking status and BCG due to the small sample size. Thirdly, whether the presence of TIMCs correlates with PD-L1 expression, as shown in our study, should be a focus of further prospective investigations. In addition, it is difficult to compare the results from our study with previous studies due to different methodologies used to evaluate PD-L1 expression in tumor cell membrane and TIMCs. Finally, even though we tried to minimize tumor

heterogeneity using three different cores for each patient in the TMAs, heterogeneity of PD-L1 expression within the tumor may limit the ability for an adequate assessment.

In conclusion, PD-L1 is widely expressed in tumor cell membrane and TIMCs in UC. No significant correlation was found with prior BCG treatment, smoking history, staging, or chromosome 9 copy number changes. However, PD-L1 positivity in TIMCs and not in tumor cells was significantly associated with better OS in those patients who subsequently developed metastatic disease and received platinum-based chemotherapy. Further prospective studies should be carried out in order to define the role of PD-L1 expression in immune cells as a predictive and prognostic biomarker in UC.

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disclosure

APF, MC, LW, SAM, JJL, and SS declare no conflict of interest for this study. GJF: significant financial interest from DFCI administered patent royalties from BMS, Merck, Roche/Genentech, EMD-Serono, Amplimmune, Boehringer-Mannheim, CoStim. Scientific founder and scientific board member of CoStim. TKC: consultancy: Pfizer, Novartis; advisory board: Pfizer, Novartis, Aveo, GlaxoSmithKline, Exelixis; research: Pfizer; No Speakers bureau. FSH: advisory board: Genentech. JB: advisory board: Merck, Genentech, OncoGenex.

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APÊNDICE B - Differential expression of PD-L1 between primary and metastatic sites in clear cell renal cell carcinoma

Differential expression of PD-L1 between primary and metastatic sites in clear cell Renal Cell Carcinoma

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Abstract: 245 /250 word limit

Main text: 3276/5000 word limit

Number of tables and figures: 7/7 (4 tables, 2 figures, 2 supplemental tables)

Abstract

PD-L1 expression in primary clear cell renal cell carcinoma (ccRCC) increases the likelihood of response to anti-PD-1 inhibition, but fails to identify all responders. We hypothesized that PD-L1 levels assessed in randomly selected areas of the primary tumors may not accurately reflect expression levels in metastatic lesions, which are the target of systemic therapy. Therefore, we compared PD-L1 expression in a series of primary ccRCC and their metastases. Tissue blocks from 53 primary ccRCCs and 76 corresponding metastases were retrieved. Areas with predominant and highest nuclear grade were selected. Slides were immunostained with a validated anti-PD-L1 antibody (405.9A11). Membranous expression in tumor cells was quantified using H-score. Expression in tumor-infiltrating mononuclear cells (TIMC) was quantified using a combined score. Discordant tumor cell PD-L1 staining between primary tumors and metastases was observed in 11/53 cases (20.8%). Overall, tumor cell PD-L1 levels were not different in primary tumors and metastases ($p=0.51$). Tumor cell PD-L1 positivity was associated with higher T stage ($p=0.03$) and higher Fuhrman Nuclear Grade (FNG) ($p<0.01$). Within individual lesions, PD-L1 positivity was heterogeneous and almost exclusively detected in high nuclear grade areas ($p<0.001$). No difference was found in PD-L1 levels in TIMCs between primary tumors and metastases ($p=0.82$).

Heterogeneity of PD-L1 expression in ccRCC suggests that its assessment as predictive biomarker for PD-1 blockade may require analysis of metastatic lesions. Notably, since PD-L1 expression was mostly detected in high nuclear grade areas, to avoid false negative results, these areas should be specifically selected for assessment.

Keywords: PD-L1, PD-1/PD-L1 inhibitors, renal cell carcinoma, clear cell, metastases, predictive biomarker, immunotherapy

Introduction

The most common type of renal cell carcinoma (RCC) is clear cell RCC (ccRCC), which represents >80% of cases, and accounts for 2–3% of all adult malignant neoplasms (1). Median survival for patients with metastatic disease with approved targeted therapies remains poor and ranges from 8 to 30 months according to prognostic risk groups (2), Therefore, more effective systemic therapies for the treatment of advanced RCC are needed (3). For more than two decades, ccRCC has been recognized as an immunogenic tumor and cytokine-based immunotherapy can produce durable responses in a small subset of patients (4–7).

Recent studies have demonstrated the role of the Programmed Death-1 (PD-1) T-cell co-receptor and its ligand PD-L1 (also known as B7-H1) in maintaining an immunosuppressive tumor microenvironment (8). The PD-1/PD-L1 pathway is known to be activated in many tumor types, including lung, ovarian, colorectal, breast, liver, head and neck, kidney, and bladder cancers and melanoma (9) . PD-1 is mainly expressed on tumor-infiltrating lymphocytes, whereas its ligand PD-L1 is expressed on both hematopoietic cells (B, T, myeloid and dendritic cells) and tumor cells (10). There is evidence that similar to epithelial and stromal cells in normal tissues, tumor cells can express PD-L1 on the cell membrane in response to interferon gamma production by activated T cells. Thus, many tumors co-opt the natural physiology of the PD-1 pathway for tissue protection in the face of inflammation, to protect themselves from an antitumor immune response. In line with this hypothesis, it has been shown that tumors expressing PD-L1 are able to inhibit antitumoral T-cell immunity by binding PD-1 on T-cells (11).

It has been reported that PD-L1 is aberrantly expressed in human ccRCC and that patients with PD-L1-positive tumors display a higher risk of cancer-specific mortality (12–15). Currently, anti-PD-1 and anti-PD-L1 antibodies are actively being investigated in clinical development for metastatic ccRCC (8,10) and several datasets suggest that primary ccRCC tumors with PD-L1 positivity either on tumor cell membranes or inflammatory cells achieve better response to PD-1/PD-L1 targeting therapies (16–19). Although PD-L1 expression in primary ccRCC tissue increases the likelihood of response to PD-1 pathway inhibition, it fails to identify all responders. Moreover, many patients with PD-L1-positive tumors do not respond to this therapy. Developing

biomarkers that reliably predict response will be essential for narrowing the application of PD-1 blockade to those patients most likely to benefit.

Clear cell RCC is characterized by intratumoral heterogeneity (20). We hypothesized that PD-L1 expression may vary significantly throughout the primary tumors (e.g. high nuclear grade versus low nuclear grade) and/or in the primary tumor versus the metastases and potentially constrain the predictive value of this biomarker. This knowledge is important to determine whether the development of optimal predictive models for PD-1/PD-L1 blockade can be conducted on primary tumor tissue or whether tissue from metastatic sites is likely to be more informative. For this reason, we performed an extensive analysis of PD-L1 expression in a series of primary ccRCCs and corresponding metastases (surgical resections). We assessed PD-L1 expression in both tumor cells and tumor-infiltrating immune cells.

Materials and Methods

Patients and samples

A cohort of 53 primary ccRCC tumors and 76 corresponding metastases from 53 patients, who had undergone surgical tumor resections, were selected from two institutions: Brigham and Women's Hospital and Beth Israel Deaconess Medical Center. Formalin-fixed paraffin-embedded (FFPE) tissue blocks from primary tumor and corresponding lymph node or distant metastases were retrieved. For each nephrectomy or metastasectomy specimen, all hematoxylin and eosin-stained slides containing tumor were reviewed by expert genitourinary pathologists (SS, EMG, MG). To address intratumoral morphologic heterogeneity, the nuclear grade was assessed in all slides using the criteria established by Fuhrman (21). For each specimen, both areas of highest nuclear grade, also known as Fuhrman nuclear grade (FNG), and areas of predominant nuclear grade were selected for analysis.

Immunohistochemistry

PD-L1 expression was evaluated by immunohistochemistry (IHC) using a mouse monoclonal anti-PD-L1 antibody (405.9A11) developed by Dr. Gordon Freeman (Boston, MA). The assay was validated using FFPE cell line controls known to be either positive or negative for PD-L1 expression by flow cytometry (22).

Four micron-thick tumor sections were stained with the anti-PD-L1 antibody (final concentration of 3.25ug/ml), on a Benchmark XT autostainer (Ventana Medical System, Tucson, AZ) with standard antigen retrieval (CC1 buffer, pH8.0, #950-124, Ventana). UltraView Universal DAB Detection kit (#760-500, Ventana) was used according to the manufacturer's instruction. Counterstaining was performed as part of the automated staining protocol using hematoxylin (#760-2021, Ventana). After staining, slides were then washed in soapy water and distilled water, dehydrated in graded alcohol and xylene, mounted and cover slipped.

CD45 immunostaining was performed on adjacent four micron-thick tumor sections. Sections were initially deparaffinized, rehydrated and heated with a pressure cooker to 125°C for 30 seconds in citrate buffer for antigen retrieval and then incubated with peroxidase (Dako #S2003, Carpinteria, CA) and protein blocking reagents (Dako #X0909) each for 5 minutes. Sections were then incubated with anti-CD45 (1:100, Dako, clone 2B11+PD7/26) antibody for 1 hour at room temperature followed by incubation with the Dako EnVision+ System HRP-labeled polymer anti-mouse (Dako #K4001) for 30 minutes. All sections were developed using the DAB chromogen kit (Dako K3468) for 2 minutes and then lightly counterstained with hematoxylin.

Quantification of PD-L1 expression in tumor cell membranes and tumor-infiltrating mononuclear cells

Evaluation of PD-L1 expression in neoplastic cells and tumor-infiltrating mononuclear cells (TIMC) was independently performed by three pathologists (SS, MG and MC), blinded to clinical data.

Membranous PD-L1 expression in tumor cells was quantified using an H-score (23), which takes into consideration the percentage of positive tumor cells within each staining category (0 = negative, 1= weak, 2 = moderate, 3 = strong). In cases with focal positivity (<1%, positive tumor cells), the H score was calculated considering the positive tumor cell percentage equal to 1. A case was considered positive when any tumor cell membrane positivity was detected. In addition, in cases with any positivity in either the primary tumor or in the metastases, we recorded PD-L1 status (positive versus negative) in each nuclear grade component (1-4) present in the lesion.

The extent of TIMCs was evaluated on the basis of the immunoreactivity for CD45, a pan-leukocyte marker (24,25) and recorded as absent (0), focal (1), mild (2), moderate (3) or marked (4). The percentage of PD-L1-positive TIMCs was determined according to six categories (0%= 0, ≤5%=1, 6-25%=2, 26-50%=3, 51-75%=4 and >75%=5). PD-L1 expression in TIMCs was then quantified using an Immune Cells Adjusted Score, calculated by multiplying the extent of TIMCs by the "percentage of positive cells" category (26). Any score greater than zero was considered positive.

Statistical Analysis

The primary objective of this study was to characterize PD-L1 expression in primary ccRCC and their corresponding metastases, and to correlate the levels of expression with clinico-pathologic features. Patient and tumor characteristics were summarized descriptively. When several samples were available within one primary or multiple metastatic sites, an average was calculated for each case (similar results were obtained when considering a maximum or median value). Proportions of positive PD-L1 expression in matching primary and metastases from an individual case were compared with the exact McNemar test. Median H-score and Median Immune Cells Adjusted Score in matching primary and metastatic case were compared with the exact Wilcoxon signed rank test. Comparisons between PD-L1 expression and clinico-pathologic features were evaluated using Fisher's exact test. All statistical computations were performed using Stata v.13.1 (StataCorp, College Station, TX, USA) and a p value (two-sided) <0.05 was considered statistically significant.

Results

Patient population and tumor tissue selection

We collected tissue samples from 53 primary clear cell RCCs and 76 matching metastases. In all cases, the metastatic lesions had been removed by surgical excision, providing sufficient and representative tumor tissue for analysis. ccRCCs are characterized by considerable Intratumoral morphologic heterogeneity with areas of low nuclear grade frequently intermixed with areas of high nuclear grade. In order to address the impact of this heterogeneity, for each primary or metastatic lesion, tumor tissue blocks containing both areas of highest nuclear grade, also known as Fuhrman nuclear grade (FNG), and areas of predominant nuclear grade were selected for analysis.

Metastatic sites included lung (n=20), bone (n=12), lymph node (n=11), soft tissues (n=9) adrenal gland (n=8), pleura (n=3), brain (n=2), thyroid (n=2) and others (n=9). While most primary tumors had only one matching metastasis, in 14 cases (26%), two or more metastatic lesions could be retrieved.

Patient characteristics are summarized in **Table 1**. Median age was 58 years (range 40-85). Pathologic T stages at diagnosis were T1/T2 in 18 patients and T3/T4 in 32 patients and unknown in 3 cases. No FNG I or II were identified in the cohort; 35 patients had FNG III and 18 had FNG IV.

Extent of discordant PD-L1 expression in primary tumors and metastases

Of the 53 cases analyzed, 17 cases (32%) presented PD-L1 tumor cell membrane positivity in the primary tumor and 12 cases (23%) presented PD-L1 tumor cell membrane positivity in the metastases (**Table 2** and **Figure 1A, B)**. The percentage of positive tumor cells ranged between [0-40%] in primary tumors, and [0-70%] in the metastases.

Discordant tumor cells PD-L1 staining between primary tumors and metastases was detected in 11 of 53 cases (20.8%, 95% CI: 10.8% -34.1%). Of the 36 cases with primary tumors that did not express PD-L1, 33 cases were also PD-L1-negative in the metastases. Of the 17 cases with primary tumors that expressed PD-L1 only 9 cases also expressed PD-L1 in the

metastases (**Table 3** and **Figure 1C-F**). Among the 11 discordant cases, 6 cases had less than 3-month time interval between the resection of primary tumor and the resection of the metastasis (**Supplemental Table 1**).

It should be noted that several samples were characterized by low percentage (<5%) of PD-L1-positive tumor cells and only 6 cases (11%) showed ≥5% positive tumor cells in the primary tumor. Similarly, only 8 (15%) cases showed ≥ 5% positive tumor cells in the metastatic sites. Using the 5% cutoff, we observed discordant tumor cell PD-L1 staining between primary tumors and metastases in 6 of 53 cases (11.3%, 95% CI: % 4.3%-23.0%).

In the 20 cases with positive PD-L1 expression in the primary tumors and/or metastases, tumor cell PD-L1 levels (determined by an H-score) were not significantly different in primary tumors compared to the metastatic sites (median H-score: 1.3 [0, 85] versus 1.5 [0, 170], p=0.25) (**Table 2**).

All but one primary tumor and all metastases displayed PD-L1-positive TIMCs (range: 5% to 75%). PD-L1 expression levels in TIMCs assessed by median Immune Cells Adjusted Score was not significantly different in primary tumors and metastases (4 versus 3, p=0.82) (**Table 2**).

PD-L1 expression in multiple metastases from the same primary tumor

Among the 14 cases in which more than one metastatic lesion was analyzed, only one case (7%) was discordant for tumor cell PD-L1 positivity across the different metastases. Specifically, PD-L1 positivity was observed in a lung lesion but not in a pancreatic lesion. This case also did not present PD-L1 expression on the primary tumor. In the remaining 13 cases, all metastases were PD-L1 negative.

PD-L1 positivity is associated with poor pathologic features and is mostly restricted to high nuclear grade areas

We correlated PD-L1 expression with pathologic features within the cohort of 53 primary tumors (**Table 4**). We observed that tumor cell PD-L1 positivity was detected in 2 of 18 cases

(11.1%) with T stage 1/2 compared to 14 of 32 cases (43.8%) with T stage 3/4, p=0.03. Furthermore, tumor cell PD-L1 positivity was more frequently detected in primary tumors with FNG IV (n=12) versus tumors with FNG III (n=5), p<0.01.

Pathologic evaluation revealed that in both primary tumors and metastases, PD-L1 positivity was heterogeneous and only present in a subset of tumor cells. Since our analysis was purposely conducted on multiple morphologically different tumor areas that included both the predominant and the highest nuclear grade (i.e. FNG), we further correlated PD-L1 expression with the distinct nuclear grade components detected within each primary or metastatic lesion. We found that PD-L1 expression was strongly associated with areas of nuclear grade 3 or 4 (i.e. high grade) (p<0.001) while areas of nuclear grade 1 or 2 (i.e. low grade) were negative in all but one lesion (**Figure 2** and **Supplemental Table 2**). It should be noted that within the subset of PD-L1-positive cases, the coexistence of low nuclear grade (mostly PD-L1 negative) and high nuclear grade (PD-L1 positive) areas was observed in 18/20 (90%) primary tumors but only in 9/21 (43%) metastases. The vast majority of the remaining lesions (2 of 2 primaries and 10 of 12 metastases) were exclusively composed of high grade tumor cells. Therefore, intratumoral heterogeneity for PD-L1 expression was extensive in primary tumors but more limited in metastases (**Supplemental Table 2**).

Discussion

While systemic therapies targeting the vascular endothelial growth factor (VEGF)/VEGF receptor (VEGFR) axis and the mammalian target of rapamycin (mTOR) pathway represent major advances in the treatment of patients with mRCC, a plateau has been reached in terms of their impact on progression-free survival and overall survival (3). Very encouraging results have been obtained recently with new immunotherapy modalities that target immune checkpoints, including agents blocking the PD-1/PD-L1 pathway. It has been established that interaction of PD-1 with its ligands (PD-L1 and PD-L2) limits T-cell activation and there is evidence that chronic antigen exposure increases PD-L1 levels in immune cells within the tumor microenvironment, resulting in T-cell "exhaustion" and reduced immune control of tumor progression. Of note, cancer cells can also express PD-L1 and directly contribute to the inhibition of an antitumor immune attack. In this regard, PD-L1 expression has been investigated in several tumor types as both a prognostic biomarker and a potential predictive factor of response to therapeutic antibodies that block the PD-1/PD-L1 axis.

Studies from Thompson and colleagues first demonstrated that PD-L1 expression in RCC is associated with aggressive features such as higher TNM stage, tumor size or FNG and increased risk of cancer-specific mortality (12–15). In these reports, the expression of PD-L1 in either tumor cells or tumor-infiltrating immune cells was found to be an indicator of poor prognosis.

Initial clinical investigations of PD-1- and PD-L1-targeting antibodies in mRCC have raised high expectations and suggested that PD-L1 expression might be a useful biomarker of response to PD-1/PD-L1 inhibition. To date, several distinct clinical trials have shown that responses to PD-L1/PD-1 inhibition are more frequently observed among ccRCC patients whose tumors are positive for PD-L1 expression (16–19,27,28). However, it has become increasingly clear that IHC staining for PD-L1 in nephrectomy specimens fails to identify all responders to PD-1/PD-L1 blockade. Indeed, up to 18% of patients with PD-L1 negative tumors have been found to respond to the treatment (19), while many patients with PD-L1 positive tumors fail to respond (18). While there are several potential explanations for these results, it is possible that the predictive value of PD-L1

expression is negatively impacted by tumor heterogeneity. Predictive tissue biomarker research is usually conducted by analyzing the primary tumor because it is easier to obtain. However, given the significant tumor heterogeneity in ccRCC, nephrectomy specimens may not accurately reflect the biology of the metastatic tumors that are the target of the systemic therapy. In line with this hypothesis, we found discordant tumor cell PD-L1 staining between primary tumors and corresponding metastases in a high proportion of cases (~20%). In contrast, multiple metastases from the same patient presented limited discordance in PD-L1 expression (7%) in the relatively small number of samples that we analyzed (14 cases). Taken together, these data suggest that robust predictive models that include the assessment of PD-L1 expression in ccRCC tumor cells might require the analysis of tissue from metastatic lesions. This possibility should be tested in prospective clinical trials.

Our study also highlights the considerable intratumor heterogeneity of PD-L1 expression in ccRCC. We demonstrate for the first time that PD-L1-positive tumors (especially primary lesions) present considerably morphologic heterogeneity and harbor tumor areas of both low and high nuclear grade, with PD-L1 protein almost exclusively expressed in high grade areas. These findings have important implications for future predictive biomarker studies and imply that the random selection of tumor blocks for PD-L1 analysis might lead to false negative results. To avoid this possible bias, we recommend that in resected lesions characterized by morphologic heterogeneity, high nuclear grade areas should be specifically selected for assessment.

A recent study by Jilaveanu and colleagues explored PD-L1 expression in a cohort of 34 matched pairs of nephrectomy and metastatic tissue samples (29). The authors used an Automated Quantitative Analysis (AQUA) method on a tissue micro-array (TMA) consisting of four tissue cores per specimen. Similarly to our current work, they found that the correlation between tumor cell PD-L1 expression in matched primary and metastatic specimens was weak and the study highlighted PD-L1 staining heterogeneity within one specimen. In contrast to our results, however, the median AQUA score was higher in metastatic sites compared to primary specimens. Since our extensive analysis of whole tissue sections from both primary and metastatic tumors

reveals that PD-L1 expression is highly heterogeneous and largely restricted to areas with aggressive pathologic features (i.e. high nuclear grade), it is possible that the analysis of only four tissue cores per lesion in a TMA is impacted by considerable selection bias.

Several papers have described PD-L1 expression in primary ccRCC, and the reported rate of positivity is highly variable and ranges from 15% to 66% (13–19,28–30). In the present study, we report a membranous tumor cell PD-L1-positivity rate of 32%, which is higher than the 23.9% rate previously reported by Thompson and colleagues (14). This difference can be ascribed to several factors, including the use of a different anti-PD-L1 antibody, the analysis of a metastatic patient population, the evaluation of multiple tumor blocks per primary tumor, and the fact that in our study a case was considered positive when any tumor cell positivity was detected, while in Thompson's study cases with <5% tumor staining were considered negative. In this study, we decided to utilize any positivity as the cut-off for the following reasons: (i) the correlation between PD-L1 levels and inhibition of anticancer immunity is currently unknown and any level of PD-L1 protein detected by IHC might have significant biologic consequences; (ii) the optimal cut-off for PD-L1 expression as a biomarker of response to PD-1/PD-L1 inhibitors still needs to be established and recent clinical results show that responses can be achieved in patients whose tumors were considered negative using a 5% cut-off; (iii) pathologist-based evaluation is semi-quantitative and subjective, and the reproducibility of discerning 1% versus 5% PD-L1-positive tumor staining is questionable.

One major limitation of the PD-L1 staining reports published to date, including ours, is the variability in staining methodologies that utilize antibodies that are not commercially available, and thus prevent a direct comparison of their performance. Standardization of both staining procedures and scoring methods is warranted before PD-L1 can be widely used as predictive biomarker in the clinic.

Conclusions

Targeting the PD-1/PD-L1 interaction to reinvigorate the immune system is showing promising clinical efficacy in metastatic ccRCC and the ability to select patients that are more likely to benefit from this therapeutic approach relies on the development of predictive biomarkers such as PD-L1 expression. We report that discordant expression of PD-L1 between primary tumors and their metastases is detected in approximately 20% of cases suggesting that accurate assessment of PD-L1 as predictive biomarkers for PD-1 blockade in ccRCC may require the analysis of metastatic lesions. Moreover, we found that PD-L1 staining is almost exclusively observed in the high grade component of a tumor. This finding should guide pathologists to select appropriate tumor areas for PD-L1 immunohistochemical analysis.

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Table 1. Patient characteristics

Characteristics		Total (n=53)	
		No. of Patients	%
Gender	Male	33	62.3
	Female	20	37.7
Median age at primary surgery, years (range)	58 (40-85)		
T Stage	T1	4	7.5
	T2	14	26.4
	T3	28	52.8
	T4	4	7.5
	Tx	3	5.7
N Stage	N0	16	30.2
	N1	14	26.4
	Nx	23	43.4
Fuhrman Nuclear Grade	III	35	66
	IV	18	34
Number of metastatic sites analyzed per case	1	39	73.6
	2	10	18.9
	3-6	4	7.5

Table 2. PD-L1 expression levels in primary tumors and metastases

PD-L1 expression		PRIMARY	METASTASIS	P value
Tumor Cells Membrane	Staining>0%: n (%)	17 (32%)	12 (23%)	p=0.23
	H-score: Median (range)	1.3 (0, 85)	1.5 (0, 170)	p=0.25
Tumor Infiltrating Immune Cells	Immune Cells Adjusted Score: Median (range)	4 (0, 9)	3 (1, 16)	p=0.82

Immune Cells Adjusted Score [0-20] = inflammatory extent*x percentage of positive immune cells **
*inflammatory extent (absent= 0 ; focal= 1 ; mild=2; moderate= 3 ; marked= 4)
**percentage of positive immune cells (0% = 0; ≤5% = 1; 6-25%= 2; 26-50%=3; 51-75%=4; >75%=5)

Table 3. PD-L1 expression in primary tumors versus corresponding metastases

		Metastases		Total
		PD-L1-	PD-L1+	
Primary Tumors	PD-L1-	33	3	36
	PD-L1+	8	9	17
Total		41	12	53

Table 4: Primary tumor characteristics associated with PD-L1 positivity

Characteristic		n	PD-L1+	P-value
T Stage	1/2	18	2 (11.1%)	0.03[1]
	3/4	32	14 (43.8%)	
	unknown	3	1 (33.3%)	
Fuhrman Nuclear Grade	III	35	5 (14.3%)	<0.01
	IV	18	12 (66.7%)	

[1] Comparing T3/T4 to T1/T2

Figures legends

Figure 1. FFPE samples immunostained with anti-PD-L1 antibody (clone 9A11). Representative images of three primary ccRCC tumors (A, C, E) and their corresponding metastases (B, D, F) immunostained for PD-L1. A, B. Membranous expression of PD-L1 in tumor cells is detected in both the primary tumor and the metastasis. C, D. Membranous expression of PD-L1 in tumor cells is only detected in the metastasis. E, F. Membranous expression of PD-L1 in tumor cells is only detected in the primary tumor. Scale bar: 50 µm

Figure 2. PD-L1 positivity is detected in high-grade tumor areas. a. Representative images of a primary ccRCC tumor with heterogeneous PD-L1 expression. Membranous expression of PD-L1 in tumor cells is negative in low nuclear grade areas (A) but present in high nuclear grade areas (B). Scale bar: 50 µm**. b**. Graphic representation of PD-L1 status in distinct nuclear grade areas within primary and metastatic lesions from PD-L1-positive cases. The height of each bar indicates the number of lesions that contain a given tumor grade component (i.e. Grade 1, Grade 2, Grade 3, Grade 4). PD-L1 positivity is indicated in red and PD-L1 negativity is indicated in blue.

Figure 1

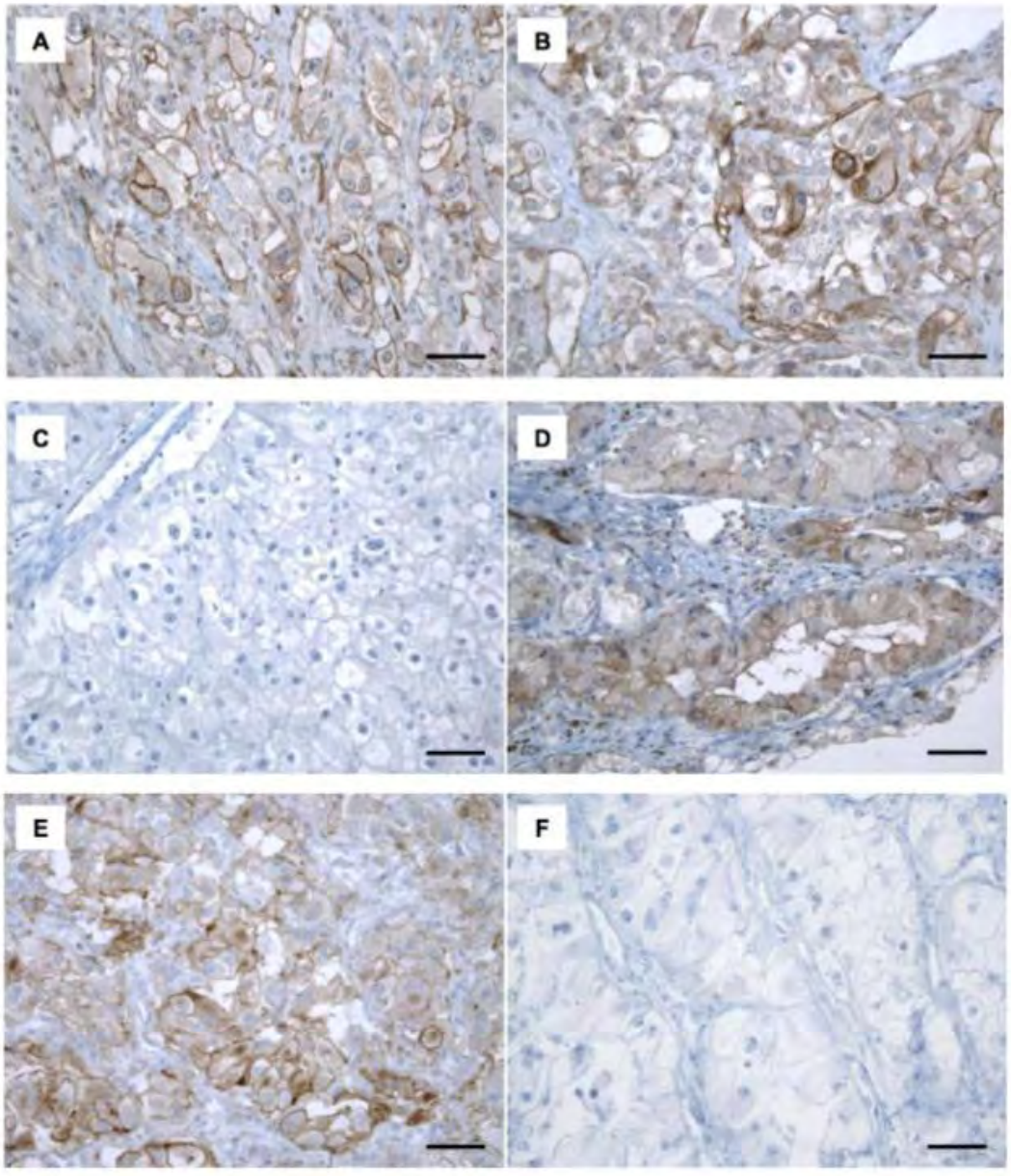
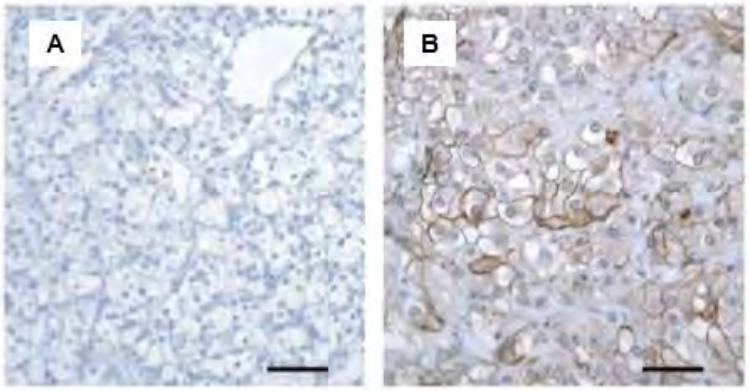


Figure 2

a.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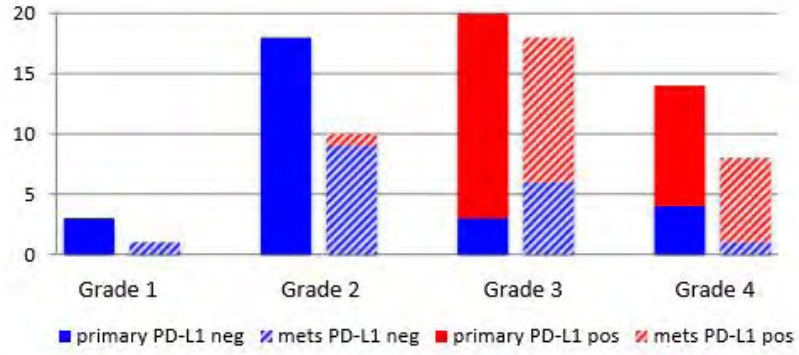
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APÊNDICE C - Correlation of PD-L1 tumor expression and treatment outcomes in patients with renal cell carcinoma receiving sunitinib or pazopanib: results from COMPARZ, a randomized controlled trial

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Personalized Medicine and Imaging

Clinical Cancer Research

Correlation of PD-L1 Tumor Expression and Treatment Outcomes in Patients with Renal Cell Carcinoma Receiving Sunitinib or Pazopanib: Results from COMPARZ, a Randomized Controlled Trial

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Abstract

Purpose: The interaction of programmed death-1 ligand (PD-L1) with its receptor (PD-1) on T cells inactivates antitumor immune responses. PD-L1 expression has been associated with poor outcomes in renal cell carcinoma (RCC) but has not been investigated in advanced RCC patients receiving VEGF-targeted therapy.

Experimental Design: Formalin-fixed paraffin-embedded specimens were collected at baseline from patients in the COMPARZ trial. Tumor cell PD-L1 expression by IHC was evaluated using H-score (HS). Dual PD-L1/CD68 staining was used to differentiate PD-L1 tumor expression from tumor-associated macrophages. Intratumor CD8-positive T cells were quantified morphometrically. Associations between biomarkers and survival were investigated using the log rank test.

Results: HS data were available from 453 of 1,110 patients. Sixty four percent of patients had negative PD-L1 expression (HS = 0). Patients with HS > 55 ($n = 59$, 13%) had significantly shorter overall survival (OS) than those with HS ≤ 55 in both pazopanib and sunitinib arms (median 15.1 vs. 35.6 and 15.3 vs. 27.8 months, respectively, $P = 0.03$). In both arms, median OS was shortest in patients with HS > 55 and intratumor CD8-positive T-cell counts > 300 (9.6 and 11.9 months with pazopanib and sunitinib, respectively). Median OS in patients with HS ≤ 55 and CD8-positive T-cell counts ≤ 300 was 36.8 and 28.0 months with pazopanib and sunitinib, respectively. Progression-free survival results were similar to OS results.

Conclusions: Increased tumor cell PD-L1, or PD-L1 plus tumor CD8-positive T-cell counts, were associated with shorter survival in patients with metastatic RCC receiving VEGF-targeted agents. These findings may have implications for future design of randomized clinical trials in advanced RCC. *Clin Cancer Res; 21(5); 1071–7. ©2014 AACR.*

Introduction

Kidney cancer accounts for at least 3% of malignant diseases (1). The incidence and mortality of renal cell carcinoma (RCC) seem to be rising, and approximately 65,000 new cases are diagnosed every year in the United States (2), resulting in more than 13,000 deaths, usually from metastatic disease.

Clear cell RCC (ccRCC), the most common type of RCC, is characterized by a dysregulation of hypoxia-inducible transcription factors resulting in the activation of several genes that regulate angiogenesis, such as *VEGF* (3). Detailed investigation of these genetic pathways has identified multiple targets for therapeutic intervention; in the last decade, agents targeting the VEGF ligand and its receptors (VEGFR-1, -2, and -3) have become the standard of care for patients with advanced disease (3).

Sunitinib and pazopanib, as compared with IFN or placebo, respectively, have significantly improved progression-free survival (PFS) benefit in patients with advanced disease, and are widely established as first-line therapies in this setting (4, 5). Recently, a large phase III, randomized trial (COMPARZ) compared the efficacy and safety of pazopanib versus sunitinib as first-line systemic treatment of patients with metastatic RCC (6). This noninferiority study showed that pazopanib and sunitinib have similar efficacy, but different safety and quality-of-life profiles (7). Although a number of potential biomarkers to predict response to targeted therapy have been investigated in RCC, none have entered clinical practice (8).

The understanding of how tumor cells evade antitumor immune response has provided a rationale for new therapeutic

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Immune checkpoint molecules such as programmed death-1 (PD-1) and its ligand (PD-L1) are negative regulators of T-cell–mediated antitumor response. PD-L1 is aberrantly expressed in several malignancies, including clear cell renal cell carcinoma (ccRCC), and this may be associated with an unfavorable prognosis and adverse clinicopathologic features. However, the prognostic impact of tumoral PD-L1 overexpression remains unclear in patients with ccRCC treated with VEGF-targeted agents. By evaluating the association of PD-L1 expression with clinical outcomes in patients who received sunitinib or pazopanib in COMPARZ, the largest randomized trial of targeted agents in ccRCC, we show that PD-L1 expression is associated with shorter survival in patients with metastatic RCC. Our results may help predict response to available targeted therapies and may assist in the design and patient selection strategies of future clinical trials of therapies that target the PD-1 axis.

strategies (9). Immune checkpoint molecules such as programmed death-1 (PD-1) and the PD-1 ligand (PD-L1) are key regulators of T-cell–mediated response. The interaction of PD-1 with its ligand (PD-L1 or B7-H1) negatively regulates T-cell activation (10). Therefore, by overcoming this adaptive mechanism with therapies that inhibit the PD-1/PD-L1 pathway, the effectiveness of T-cell responses against tumor cells can be restored (11).

PD-L1 is aberrantly expressed in ccRCC, and this is often associated with worse prognosis and adverse clinicopathologic features (12–18). Preliminary data for monoclonal antibodies that block the interaction of PD-1 and its ligand have shown encouraging results in patients with RCC, as well as other tumors such as melanoma and non–small cell lung cancer (19). In addition, preliminary data on a limited number of patients with RCC showed that PD-L1 expression may be a potential biomarker of response to PD-1 inhibitors (19–21).

In this study, we evaluate the correlation between the expression of PD-L1 on tumor cell membrane and clinical outcomes in a large cohort of patients with metastatic RCC who received pazopanib or sunitinib as part of the COMPARZ trial (NCT00720941).

Materials and Methods

Patients and samples

We analyzed data from patients who were enrolled in the COMPARZ clinical trial, which was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent for participation in the clinical trial.

Between August 2008 and September 2011, this phase III study enrolled 1,110 patients with metastatic ccRCC to randomly receive pazopanib ($n = 557$) or sunitinib ($n = 553$) at standard dosages. The primary endpoint was PFS, and the study was designed to evaluate the noninferiority of pazopanib versus sunitinib. Secondary endpoints included overall survival (OS), safety, and quality of life (6). Formalin-fixed paraffin-embedded tumor blocks were available from 453 patients who provided consent for tissue analysis: 221 of 557 in the pazopanib arm and 232 of 553 in the sunitinib arm. Archival tumor tissue samples were collected at baseline from these 453 patients.

IHC

PD-L1 expression was retrospectively evaluated by IHC using the monoclonal anti-PD-L1 mouse IgG1 antibody (clone 5H1) on the Leica automated IHC platform (MEDTOX Laboratories) as previously described (22). All cases were also stained for CD8 using a commercially available monoclonal mouse antibody (4B11) on the Leica Bond platform using recommended antigen retrieval conditions and an alkaline phosphatase red detection system. Formalin-fixed paraffin-embedded tonsil tissue was used as positive and negative control material for each staining run.

PD-L1 expression on tumor cell membrane was determined semiquantitatively on a 0+ to 3+ scale: 0+, no appreciable staining above background; 1+, any degree of cytoplasmic or membranous staining above background, but less than 2+ or 3+; 2+, moderately to intensely positive membranous staining in single or small groups of cells, or moderate cytoplasmic staining; 3+, intensely positive membranous staining matching or exceeding control material, in more than single or small groups of cells (Fig. 1A). H-scores [HS; HS = (% cells 3+) × 3 + (% cells 2+) × 2 + (% cells 1+)] were evaluated, and a case was considered positive when any tumor cell positivity was detected (HS > 0; ref. 23).

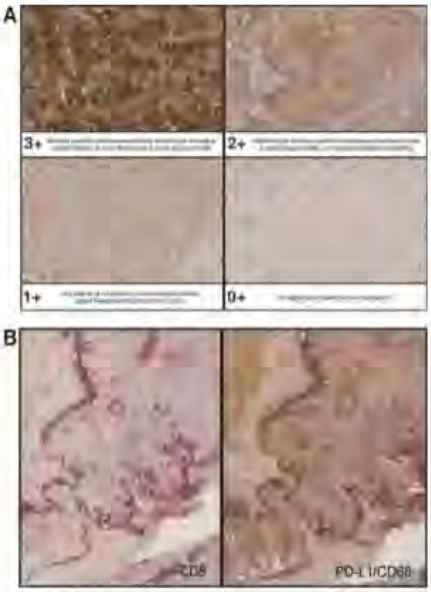

Figure 1.
Ad hoc semiquantitative scoring scheme for PD-L1 expression by tumor cells in RCC (A) and example of prominent peripheral inflammatory response and corresponding PD-L1 expression (B).

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In all cases showing any possible staining for PD-L1, a dual-color PD-L1/CD68 stain was performed on adjacent sections using the Leica Bond automated IHC platform to differentiate PD-L1 expression by tumor cells from that by tumor-associated macrophages (TAM). Staining was carried out sequentially, first for PD-L1 and then for CD68 (clone 514H12), using a horseradish peroxidase linker antibody conjugate with DAB and alkaline phosphatase red detection system, respectively. The number of TAMs expressing PD-L1 was noted separately and semiquantitatively graded as absent, rare, moderate, or numerous. The TAM PD-L1 staining was not included in the final PD-L1 HS. For all patients, intratumor CD8-positive ($CD8^+$) cells were quantified morphometrically (number of $CD8^+$ cells/mm^2 of tumor tissue) using a proprietary digital image analysis and counting program (BioImagene; Ventana/Roche Medical Systems) on CD8-stained slides scanned at ×20 on an automated whole slide imaging system (iScan BioImagene; Ventana/Roche Medical Systems). The intensity of the inflammatory response at the periphery of the tumor and its interface with surrounding stroma was graded using a semiquantitative scale (Supplementary Fig. S1).

Statistical analysis

The objectives of this study were to investigate the association between PD-L1 expression on tumor cells and treatment outcome; the primary objective was correlation with OS and the secondary objective was the correlation with PFS. Other objectives included the correlation between PD-L1 expression on tumor cells and the corresponding TAMs, and the association of intratumoral peripheral $CD8^+$ T-cell counts with OS and PFS. A test of the combined association between CD8 counts/PD-L1 HS and clinical outcome was also performed. OS was defined as the time period between initiation of targeted therapy and the date of death or censoring on the day of the last follow-up visit. Patient and tumor characteristics were summarized descriptively. PFS was defined as the time period from initiation of targeted therapy to disease progression, death, or censoring at the last follow-up visit; patients who discontinued treatment before progression continued disease assessments until progression or initiation of another cancer therapy. Those initiating another therapy were censored at the time of the last disease assessment before initiating the other therapy.

The association between PD-L1 HS and treatment outcomes (OS and PFS) was explored by the log-rank test across a sliding window of HS. The HS threshold was the minimum HS with log-rank $P < 0.05$. Multivariate analysis (Cox proportional hazards regression) was adjusted by individual adverse risk factors: Karnofsky Performance Score (KPS), lactate dehydrogenase (LDH), and number of metastatic sites. In Cox analysis of OS, data from pazopanib and sunitinib patients were combined. The association of rate of response (complete response or partial response vs. stable disease or progressive disease) for patients with PD-L1 levels above and below the threshold was assessed using logistic regression.

All statistical analyses were *post hoc*; computations were performed using SAS v.9.2 (SAS Institute Inc.), and a P value (two-sided) <0.05 was considered statistically significant.

Results

Patient and tumor characteristics

Patient and tumor characteristics are described in Table 1. The KPS was 90 or 100 for 164 patients (74%) in the pazopanib arm and 169 patients (74%) in the sunitinib arm. In addition, the Memorial Sloan-Kettering Cancer Center (MSKCC; New York, NY) prognostic risk scores were considered favorable, intermediate, and poor for 64 (29%), 127 (57%), and 26 (12%) patients in the pazopanib arm, respectively, and 55 (24%), 142 (61%), and 24 (10%) patients in the sunitinib arm, respectively. The OS and PFS in the subset of patients who were included in the PD-L1 analysis ($n = 453$) were comparable with the outcomes reported in the COMPARZ trial (Supplementary Table S1).

PD-L1 expression on tumor cells and immune cells

Formalin-fixed paraffin-embedded specimens were available from 453 of 1,110 patients. Overall, membranous PD-L1 expression in tumor cells was detected (HS > 0) in 163 of 453 patients (36%); HS ranged from 0 to 290 (Table 2). A total of 85 patient samples (18.8%) showed a robust PD-L1 staining (2+ or 3+; Tables 3 and 4). Interestingly, a robust PD-L1 signal was seen in fewer core biopsies than in tissue samples from surgical resections, although the numbers are too small to make definitive comparisons (Table 3).

Overall, the dual staining with PD-L1 and CD68 identified 157 samples with moderate to numerous PD-L1–positive ($PD\text{-}L1^+$) macrophages (Fig. 1B). In some of the cases, PD-L1 expression was determined to be exclusively on macrophages and no tumor expression was noted; these cases were excluded from the analysis. The correlation of PD-L1 expression on tumor cells and macrophages is summarized in Table 4.

In addition, the inflammatory response as represented by the presence of peripheral $CD8^+$ T cells in the invasive margin surrounding the tumor was graded as very strong in 36 cases, strong in 31 cases, moderate in 161 cases, weak in 132 cases, and minimal in 38 cases. In 88 cases, no invasive tumor/stromal margin was present in the tissue block analyzed.

Correlation of PD-L1 expression on tumor cells with treatment outcomes

An HS of >55 was found to be the threshold at which log-rank analysis demonstrated statistically significant association

Table 1. Patient characteristics

Characteristic	Pazopanib ($N = 221$)	Sunitinib ($N = 232$)
Median age, y (range)	62 (30–86)	62 (33–86)
Sex, *n* (%)		
Male	158 (71)	178 (77)
Female	63 (29)	54 (23)
Prior nephrectomy, *n* (%)	191 (86)	205 (88)
KPS, *n* (%)		
70 or 80	57 (26)	60 (26)
90 or 100	164 (74)	169 (74)
LDH, *n* (%)		
>1.5× ULN	17 (8)	11 (5)
≤1.5× ULN	204 (92)	214 (95)
Metastatic sites at baseline, *n* (%)		
≤2	151 (68)	152 (65)
>2	70 (32)	80 (34)
MSKCC risk category, *n* (%)		
Favorable	64 (29)	55 (24)
Intermediate	127 (57)	142 (61)
Poor	26 (12)	24 (10)
Unknown	4 (2)	11 (5)

Abbreviation: ULN, upper limit of the normal range.

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Table 2. PD-L1 expression levels in available samples

	HS, *n* (%)						
Treatment	**0**	**1–5**	**6–10**	**11–25**	**26–50**	**>50**	**Total**
Pazopanib	142 (64)	17 (7)	12 (5)	9 (4)	14 (6)	27 (12)	221
Sunitinib	148 (64)	15 (6)	7 (3)	16 (7)	12 (5)	34 (15)	232

between HS and OS ($P = 0.0302$, Fig. 2). In the pazopanib arm, 25 of 221 patients (11.3%) had HS > 55 (median OS, 15.1 months) and 196 had HS ≤ 55 (median OS, 35.6 months). In the sunitinib arm, 34 of 232 patients (14.7%) had HS > 55 (median OS, 15.3 months) and 198 patients had HS ≤ 55 (median OS, 27.8 months). At higher HS cutoff values, patients had a shorter OS. For example, in patients with HS > 125, median OS was 5.1 and 8.9 months in the pazopanib ($n = 8$) and sunitinib ($n = 7$) arms, respectively (Supplementary Fig. S2). Similarly, patients with HS > 125 had significantly shorter PFS in both the pazopanib (3.1 vs. 10.2 months) and sunitinib (4.0 vs. 8.4 months) arms ($P = 0.017$).

A covariate analysis was performed to adjust the association of PD-L1 expression and OS for potential confounding factors. In a multivariate analysis ($N = 450$), a model that includes number of metastatic sites and KPS, PD-L1 expression (HS > 55 vs. HS ≤ 55) was an independent prognostic indicator of poor OS (HR = 1.43, $P = 0.028$). The number of metastatic sites [> 2 vs. ≤ 2 (HR = 1.52, $P < 0.0001$)] and KPS [70–80 vs. 90–100 (HR = 1.55, $P = 0.0005$)] were also indicators of poor OS.

In addition, using an HS threshold of 55, as we did for the PFS and OS endpoints, we did not find statistically significant differences in the rates of response for patients with PD-L1 levels above versus below the threshold (sunitinib $P = 0.6$; pazopanib $P = 0.7$).

Combined effect of PD-L1 H-Score and CD8 level on OS

A combination of higher PD-L1 tumor expression and higher intratumor $CD8^+$ cell counts correlated with shorter OS. In both arms, patients with both HS > 55 and intratumoral $CD8^+$ T-cell counts > 300 had the shortest OS (11.9 months for sunitinib and 9.6 months for pazopanib).

Discussion

Several studies have been conducted to determine the predictive and/or prognostic value of PD-L1 expression in pretreatment specimens (24). To our knowledge, this is the largest series in a randomized clinical trial to correlate higher PD-L1 expression on tumor cells with worse clinical outcomes in patients with metastatic RCC (and solid tumors) receiving standard first-line VEGF-targeted therapy.

Thompson and colleagues reported that PD-L1 expression was associated with aggressive features such as higher tumor–node–metastasis (TNM) stage, tumor size, or Fuhrman grade, and increased risk of cancer-specific mortality in 196 patients with RCC (25). In another study of 306 patients with ccRCC, 23% of patients were deemed PD-L1 positive and were more likely to present higher risk of cancer-specific mortality (risk ratio: 2.0; 95% confidence interval, 1.27–3.15; $P = 0.003$) adjusting for TNM stage and grade (12). Interestingly, the correlation between PD-L1 expression and adverse prognostic features as well as OS was identified with PD-L1 expression in both tumor cell membrane and tumor-infiltrating lymphocytes (22). In our analysis, we showed that higher PD-L1 tumor expression was an independent prognostic marker for OS in patients treated with pazopanib or sunitinib.

High levels of tumor-infiltrating immune cells, particularly $CD8^+$ T cells, have been associated with adverse clinical outcomes in RCC, possibly due to an impairment of antitumor immune responses (26). Similarly, higher expression of PD-L1 in these cells has been also correlated with more aggressive features in RCC (22). In our study, higher numbers of infiltrating macrophages were correlated with PD-L1 tumor expression.

Recently, we investigated the correlation between PD-L1 tumor expression and clinical outcome in patients with metastatic RCC who were enrolled in an older and smaller phase III placebo-controlled clinical trial of pazopanib (VEG105192; NCT00334282; ref. 27). Using a similar HS methodology for scoring, patients in the pazopanib arm with HS > 3 (23/113, 20%) had a trend toward shorter OS (7.3 vs. 11 months; $P = 0.14$) and a shorter PFS (2.3 vs. 5.5 months; $P = 0.02$). Interestingly, a much lower level of PD-L1 expression was observed in this clinical trial when compared with patients enrolled in the COMPARZ trial. It is important to note that although patients with PD-$L1^+$ tumors have shorter PFS/OS on pazopanib treatment, the data from the VEG105192 trial showed that patients with PD-$L1^+$ tumors continue to benefit from pazopanib (27), suggesting that tumor PD-L1 expression is a prognostic marker.

In a phase I study of an anti-PD-1 monoclonal antibody (nivolumab) in metastatic RCC, melanoma, and non–small cell lung cancer, therapeutic blockade of the PD-1/PD-L1 pathway produced encouraging responses in patients with RCC. For PD-$L1^+$ tumors, an objective response rate of 36% (9/25) was observed compared with no response in the PD-L1–negative tumors ($P = 0.006$; ref. 28), suggesting that PD-L1 expression in tumor cells may be a promising biomarker for agents that block the PD-1/PD-L1 pathway. More recent data with nivolumab suggest that although PD-$L1^+$ tumors have numerically higher response rates (22%) than PD-L1–negative tumors (8%), responses can be seen in PD-L1–negative tumors (20). Clinical trials have reported encouraging results with combinations of agents blocking the PD-1/PD-L1 axis, either with other immune checkpoint blockers or VEGF-targeted therapies (29, 30). The correlation between tumor PD-L1

Table 3. Comparison of PD-L1 expression between full tissue sections and core biopsies

	PD-L1 semiquantitative score						
Specimen	**0**	**1+**	**2+**	**3+**	**Total *N***	**1+ to 3+**	**2+ or 3+**
All, *n* (%)	289 (63.8)	79 (17.4)	51 (11.3)	34 (7.5)	453	163 (36.2)	85 (18.8)
Full tissue, *n* (%)	252 (63.5)	66 (16.6)	50 (12.6)	29 (7.3)	397	145 (36.5)	79 (19.9)
Core biopsy, *n* (%)	37 (66.1)	13 (23.2)	1 (1.8)	5 (8.9)	56	19 (33.9)	6 (10.7)

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Table 4. Correlation of PD-L1 expression between tumor and macrophages

	PD-L1$^+$ macrophages				
IHC score of tumor sample	**Absent**	**Rare**	**Moderate**	**Numerous**	**Total**
0/1+	188	93	67	20	368
2+/3+	3	12	29	41	85
Total	191	105	96	61	453

expression and prognosis in patients with RCC receiving VEGF-targeted therapies supports the hypothesis that this molecule may also serve as a predictive biomarker for agents targeting PD-1 or PD-L1.

In addition to PD-L1 expression on tumor membranes, PD-L1 expression in immune cells may correlate with treatment response. Preliminary data from a phase I expansion cohort of patients with RCC (as part of a larger cohort of patients with solid tumors) treated with an anti-PD-L1 antibody (MPDL3280A) revealed an overall response rate of 20% in PD-L1$^+$ patients compared with 10% in patients with negative PD-L1 tumor expression (21, 31). Interestingly, we showed that increased baseline PD-L1 expression or increased PD-L1 expression plus intratumor CD8$^+$ T-cell counts >300 at baseline was associated with shorter OS in patients treated with sunitinib or pazopanib, suggesting that these patients may be ideal candidates for a therapeutic strategy that targets the PD-1/PD-L1 axis.

The tumor microenvironment is recognized to encompass important factors supporting tumor growth and progression (32). Similarly, mechanisms of resistance may be driven by interactions between stromal and tumor cells that can modulate response to targeted therapies (33). The immune system can also play an important role in treatment response. For example, activated intratumor lymphocytes can induce PD-L1 expression on tumor cells or surrounding immune cells by releasing several cytokines (33). A recent study of biomarker expression in patients with metastatic ccRCC found that VEGF-targeted therapy caused a significant reduction in vessel density (CD31) and PD-L1 expression, but no correlation between PD-L1 expression and clinical outcome was reported (34). In addition, exposure to sunitinib, but not pazopanib, resulted in reduced expression of the immune cell markers CD45 and CD3 (34). The questions of how different VEGF-targeted therapies may impact the expression of regulatory T-cell molecules and other biomarkers and how that could be associated with treatment outcome in patients with metastatic RCC still need to be addressed.

Although we have evaluated a large cohort, our study has limitations. First, there is potential selection bias in any retrospective analysis. However, there was no statistical difference between the PFS and OS of the PD-L1 study population when compared with the overall COMPARZ population. Second, the impact of PD-L1 expression on response to VEGF-targeted therapies remains undefined. In this analysis, we defined as primary endpoints the correlation between PD-L1 expression and survival outcomes (OS or PFS). Therefore, future studies, especially those based on current trials combining VEGF-targeted therapies with anti-PD-1 therapies, should address that question. In addition, several methodologies with different PD-L1 IHC protocols are used to assess PD-L1 in other studies; direct comparisons of our results with those of other investigations should be done with caution. We evaluated baseline PD-L1 expression, but the question of how different VEGF-targeted therapies may influence the expression of this biomarker in posttreatment biopsies still needs to be investigated. Finally, although we evaluated patients who were part of a clinical trial, we found that information was missing

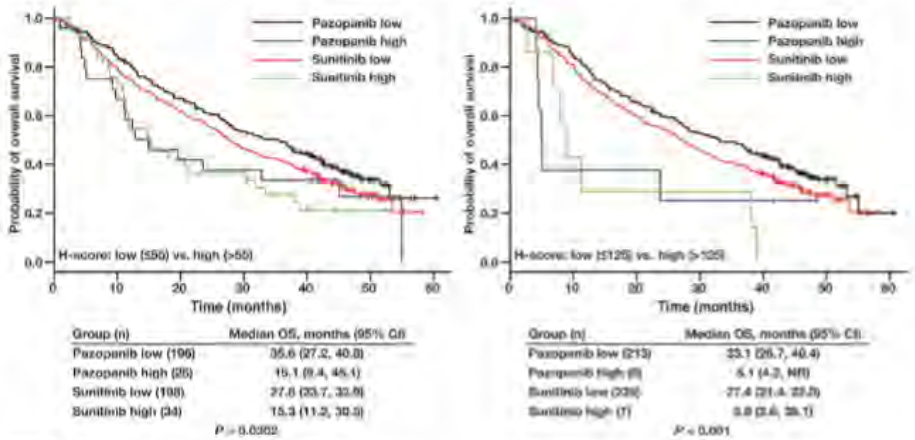

Figure 2.
Association of OS with PD-L1 expression status on tumor cell membrane.

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which could classify patients according to prognostic risk score groups. Therefore, in the multivariate analysis, we include only known (i.e., data not missing) single variables that impact the prognosis of RCC. The strengths of our study include the large number of patients who were part of a well-conducted clinical trial and the adjustment of the analysis for the prognostic risk factors previously associated with worse prognosis.

In conclusion, our study shows that PD-L1 expression is associated with treatment outcome in patients with metastatic RCC treated with VEGF-targeted therapies. Increased levels of PD-L1, or increased PD-L1 plus tumor $CD8^+$ T-cell counts, were independently associated with shorter survival. The role of PD-L1 as a predictor of survival on VEGF-targeted therapy needs to be validated in prospective clinical trials; a phase I trial of pazopanib plus the PD-1 inhibitor MK-3475 is under way (NCT02014636). Results from this and other trials may have major implications for the design of future trials that include PD-1/PD-L1 inhibitors.

Disclosure of Potential Conflicts of Interest

T.K. Choueiri reports receiving a commercial research grant from Pfizer and is a consultant/advisory board member for Bayer, GlaxoSmithKline, Novartis, and Pfizer. Y. Liu and I. Pandite are employees of and have ownership interest (including patents) in GlaxoSmithKline. R. Gagnon is an employee of GlaxoSmithKline. K. Deen and C. Carpenter have ownership interest (including patents) in GlaxoSmithKline. P. Benson reports receiving other research grants from GlaxoSmithKline. P. de Souza is a consultant/advisory board member for GlaxoSmithKline Australia, Janssen Australia, and Pfizer Australia. T. Powles reports receiving speakers bureau honoraria from and is a consultant/advisory board member for GlaxoSmithKline. R.J. Motzer reports receiving a commercial research grant from BristolMyers-Squibb, Genentech, GlaxoSmithKline, and Pfizer and is a consultant/advisory board member for Pfizer. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.J. Figueroa, Y. Liu, P. Benson, P. de Souza, R.J. Motzer

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): T.K. Choueiri, D.J. Figueroa, A.P. Fay, Y. Liu, R. Gagnon, K. Deen, T.H. Ho, P. de Souza, T. Powles, R.J. Motzer

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Study supervision: T.K. Choueiri, Y. Liu, I. Pandite, R.J. Motzer

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Clinical Cancer Research

Correlation of PD-L1 Tumor Expression and Treatment Outcomes in Patients with Renal Cell Carcinoma Receiving Sunitinib or Pazopanib: Results from COMPARZ, a Randomized Controlled Trial

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APÊNDICE D - Characteristics of long-term and short-term survivors of metastatic renal cell carcinoma treated with targeted therapies: results from the International mRCC Database Consortium

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Original Study

Characteristics of Long-Term and Short-Term Survivors of Metastatic Renal Cell Carcinoma Treated With Targeted Therapies: Results From the International mRCC Database Consortium

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Abstract

Patients with mRCC may have variable clinical courses when treated with targeted therapy. The two extremes of the survival spectrum need to be characterized. Analyzing data from a large database (International Metastatic Renal Cell Carcinoma Database Consortium - IMDC) we found that baseline prognostic criteria and absence of PD after first- and second-line targeted therapy may discriminate long-term survival.

Background: Targeted therapies improve survival in metastatic renal cell carcinoma (mRCC). However, survival patterns can be divergent, and patients at the 2 extremes of the survival spectrum need to be characterized. **Patients and Methods:** Data from 2161 patients included in the International mRCC Database Consortium (IMDC) were analyzed. We identified patients on the basis of their duration of survival. Long-term survival (LTS) was defined as overall survival (OS) of $\geq$ 4 years, and short-term survival (STS) was defined as OS of $\leq$ 6 months from the start of targeted therapy. Baseline characteristics, including demographic, clinicopathologic, and laboratory data, were compared between LTS and STS. Treatment response by the RECIST criteria was summarized for the 2 survival groups. **Results:** A total of 152 patients experienced LTS and 218 experienced STS. Adverse clinical and laboratory prognostic factors previously described in the IMDC prognostic model were significantly more frequent in the STS group ($P < .0001$). In the LTS group, 138 patients (91%) had nonprogressive disease (non-PD) as best response to first-line targeted therapy, and 56 (60%) of 94 patients who received second-line therapy had non-PD. In the STS group, only 51 patients (23%) had non-PD on first-line therapy. None of 21 the patients who received second-line therapy had non-PD as best response. In LTS, the median duration of therapy was 23.6 months (range 0.4 to

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81.8+ months) for first-line therapy and 11.5 months (range 0.6 to 45.7 months) for second-line therapy, compared to 2.0 and 0.8 months for the STS group, respectively. **Conclusion:** Baseline prognostic criteria and absence of PD after first and second-line targeted therapy may characterize long-term survival.

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Introduction

The incidence and mortality of renal cell carcinoma (RCC) appears to be rising in the United States, with approximately 65,000 new cases of RCC diagnosed every year.[1,2] Complete surgical resection remains the only curative treatment for localized disease.[3] However, about 30% of patients will develop metastatic disease after nephrectomy, which is generally associated with poor long-term survival.[4]

In the last decade, major advances have contributed to the understanding of the biology underlying RCC. The knowledge that this disease is driven by dysregulation of angiogenesis provided a rationale for the development of new targeted agents.[5] Drugs targeting vascular endothelial growth factor (VEGF) or mammalian target of rapamycin (mTOR) produce objective response or disease stabilization in the majority of patients, resulting in a median survival approaching 30 months with metastatic RCC (mRCC).[6–10] However, variable outcomes in terms of duration of survival and degree of response to targeted therapies have been reported.

Several clinical and laboratory factors have been identified as important prognostic factors and have been integrated into prognostic models to estimate survival in the metastatic setting.[11,12] Patients are stratified into poor-, intermediate-, and favorable-risk categories.[11] Recently, conditional survival defined as the probability of surviving an additional period of time after the patient has already survived a specific time period was shown to provide relevant individual prognostic information.[13]

However, patients with similar survival estimates at baseline may have very divergent clinical outcomes.[14] For this reason, characterization of patients at the 2 extremes of the survival spectrum may provide insights into the biology of RCC. We analyzed data from our large international multi-institutional database, the International Metastatic Renal Cell Carcinoma Database Consortium (IMDC), to provide information about treatment response and clinical features of patients with overall survival (OS) of greater than 4 years or less than 6 months.

Patients and Methods

The IMDC database includes 20 centers from North America (Canada, United States), Europe (Denmark, Greece), and Asia (Singapore, Japan, South Korea). As of January 2012, the database contained data of 2161 patients with mRCC who had received targeted therapy. Patients who survived at least 4 years and patients who survived 6 months or less over the same period (2004-2007) were identified. Long-term survival (LTS) was defined as an OS of $\geq$ 4 years after initiating therapy, and short-term survival (STS) was defined as an OS of $\leq$ 6 months after initiating therapy (Figure 1). Survival cutoffs were defined arbitrarily based on median OS reported in clinical trials with targeted agents.[7] Baseline patient characteristics, including demographic, clinicopathologic, and laboratory data, as well as outcomes data were collected from medical chart reviews and electronic records, as previously outlined.[13] Laboratory values were standardized against institutional upper limit of normal (ULN) and lower limit of normal (LLN) values when appropriate. Response to targeted therapy was assessed by Response Evaluation Criteria in Solid Tumors (RECIST v.1.0).[15] Uniform data collection templates were used to ensure consistent data. Most of the patients had been treated with standardly available agents, but a subset may have been participants in clinical trials. All patients were collected in consecutive series to avoid selection bias. All IMDC centers obtained local institutional review board approvals before data acquisition.

The primary objective of this study was to evaluate patient and tumor characteristics as well as tumor response to the targeted therapy according to the 2 divergent survival outcomes (LTS vs. STS). OS was defined as the time period between targeted therapy initiation and the date of death, or censored on the day of the last follow-up visit. Duration of targeted therapy was defined as the time period between treatment from targeted therapy initiation to progression, drug cessation, death, or censored at the last follow-up visit. Radiographic criteria with RECIST v.1.0 grouped patients into 4 categories according to their best response during treatment: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). Non-PD was defined as CR, PR, or SD and clinically implies the absence of primary refractory disease to targeted therapy. Patient and tumor characteristics outlined in Table 1 were compared between LTS and STS by Fisher's exact test for categorical variables. The percentage of patients who experienced

Figure 1 Study Design

Table 1 Patient and Disease Characteristics at Initiation of Targeted Therapy

Characteristic	Short-Term Survival (≤6 months, n = 218)	Long-Term Survival (≥4 years, n = 152)	P
Age			NS
<60 years	97 (44%)	69 (45%)	
≥60 years	121 (56%)	83 (55%)	
Gender			NS
Female	61 (28%)	30 (20%)	
Male	157 (72%)	122 (80%)	
No. of Metastases			.019
1	36 (17%)	41 (27%)	
>1	181 (83%)	111 (73%)	
Brain Metastasis			NS
No	196 (90%)	143 (94%)	
Yes	21 (10%)	9 (6%)	
Histology			NS
Clear cell histology	171 (85%)	127 (90%)	
Non–clear-cell histology	31 (15%)	14 (10%)	
Sarcomatoid Pathology			.0001
No	163 (84%)	129 (97%)	
Yes	31 (16%)	4 (3%)	
Previous Nephrectomy			<.0001
No	76 (35%)	8 (5%)	
Yes	142 (65%)	144 (95%)	
Previous Immunotherapy			NS
No	138 (63%)	96 (63%)	
Yes	80 (37%)	56 (37%)	
LDH >1.5 ULN			.03
Yes	50 (23%)	20 (13%)	
No	168 (77%)	132 (87%)	
IMDC Prognostic Risk Factor			
KPS			<.0001
≥80	99 (47%)	136 (93%)	
<80	111 (53%)	11 (7%)	
Diagnosis to TKI Therapy <1 Year			<.0001
No	85 (39%)	110 (72%)	
Yes	131 (61%)	42 (28%)	
Low Hemoglobin Level (<LLN)			<.0001
No	41 (19%)	96 (65%)	
Yes	170 (81%)	52 (35%)	
Hypercalcemia			<.0001
No	155 (75%)	142 (97%)	
Yes	53 (25%)	4 (3%)	
Neutrophilia (>ULN)			<.0001
No	137 (66%)	142 (98%)	
Yes	70 (34%)	3 (2%)	
Thrombophilia (>ULN)			<.0001
No	123 (58%)	142 (96%)	
Yes	87 (42%)	6 (4%)	

Table 1 Continued

Characteristic	Short-Term Survival (≤6 months, n = 218)	Long-Term Survival (≥4 years, n = 152)	P
IMDC Risk Group			<.0001
Favorable, 0 RF	5 (2%)	64 (44%)	
Intermediate, 1 RF	21 (10%)	55 (38%)	
Intermediate, 2 RFs	54 (25%)	20 (14%)	
Poor, 3-6 RFs	130 (62%)	5 (4%)	

Abbreviations: IMDC = International mRCC Database Consortium; KPS = Karnofsky performance status; LDH = lactate dehydrogenase; LLN = lower limit of normal; NS = not significant; RF = risk factor; TKI = tyrosine kinase inhibitor; ULN = upper limit of normal.

non-PD was summarized by the 2 survival extremes as well as by the IMDC prognostic risk groups. Duration of therapy was estimated by the Kaplan-Meier methodology.

All statistical computations were performed by SAS v.9.2 (SAS Institute, Cary, NC, USA), and a P value (2-sided) of $< .05$ was considered statistically significant.

Results

Patient and Tumor Characteristics

Patient characteristics at initiation of targeted therapy are outlined in Table 1. The study cohorts included a total of 370 patients out of 2161 patients registered in the IMDC database. There were 152 patients who survived at least 4 years (LTS) and 218 patients who survived < 6 months (STS) since therapy initiation. All first-line targeted therapies were administered between 2004 and 2007. The majority of patients received a first-line VEGF targeted therapy of sunitinib (72%), sorafenib (22%), and bevacizumab (5%) in the STS group; the same distribution was 55%, 34%, and 11% in the LTS group. Thirty-seven percent of patients in both groups had been treated with previous immunotherapy.

IMDC Prognostic Risk Criteria in LTS and STS Groups

Karnofsky performance status of < 80% was observed in 53% of patients in the STS group and only 7% in the LTS group. STS were more likely to have anemia (81% vs. 35%) and time from diagnosis to targeted therapy of < 1 year (61% vs. 28%). Presence of other IMDC laboratory risk factors, including neutrophilia, thrombocytosis, and hypercalcemia, were extremely low (less than 5%) in the LTS group. Overall, the presence of the IMDC adverse prognostic risk factors was significantly associated with STS ($P < .0001$, Table 1).

By the IMDC prognostic risk group criteria, the majority of patients (82%) in the LTS group had favorable risk (44%) or intermediate risk with 1 risk factor (38%). By contrast, most of patients (87%) in the STS group had poor (3 to 6 risk factors, 62%) or intermediate risk with 2 risk factors (25%).

Response to Targeted Therapy in LTS and STS Groups

In the LTS group, the majority of patients (91%, 95% confidence interval 85-95) had non-PD as best response to first-line targeted therapy, including CR (4%), PR (34%), and SD (53%).

Table 2 Nonprogressive Disease by Prognostic Group

Characteristic	Short-Term Survival (≤6 months, n = 218)		Long-Term Survival (≥48 months, n = 152)	
	First-Line Therapy	Second-Line Therapy	First-Line Therapy	Second-Line Therapy
All	51/218 (23%)	0/21	138/152 (91%)	56/94 (60%)
IMDC Risk Group*				
Favorable	3/5 (60%)	0/1	59/64 (92%)	21/43 (49%)
Intermediate	23/75 (31%)	0/9	68/75 (91%)	32/45 (71%)
Poor	25/130 (19%)	0/11	5/5 (100%)	2/3 (67%)

*Excludes 16 patients without prognostic data.

In addition, 59 (92%) of 64 patients who had favorable prognostic risk score, 68 (91%) of 75 patients with intermediate risk score, and all the 5 patients in the poor prognostic risk score category had non-PD as best response to first-line targeted therapy.

Ninety-four (62%) of 152 LTS received second-line sequential therapy. In this setting, 21 (49%) of 43 patients with favorable prognostic risk score, 32 (71%) of 45 patients with intermediate risk score, and 2 (67%) of 3 with poor risk score had non-PD as best response (Table 2). Overall, 56 (60%) of all 94 patients had non-PD on second-line therapy.

In the STS group, PD was the best response to first-line targeted therapy in 93 patients (43%). Only 42 patients (19%) had stable disease, and in 74 (34%) of 218 patients, the best response was not available in the database (Table 1). Three (60%) of 5 patients who had favorable prognostic risk scores, 23 (31%) of 75 patients who had intermediate risk scores, and 25 (19%) of 130 who had poor prognostic risk scores had non-PD as best response to first-line targeted therapy. Overall, 51 patients (23%) had non-PD on first-line therapy.

Only 21 of 218 patients in the STS group received second-line targeted therapy; all had PD as best response, independent of risk score category (Table 2).

The median duration of receipt of first-line therapy was 23.6 months (range 0.4 to 81.8+ months) for LTS (Table 3). In addition, for patients who received second-line targeted therapy, the median treatment duration was 11.5 months (range 0.6 to 45.7 months). In the STS group, the median duration of first-line and second-line therapy was 2.0 (range 0.2 to 5.9 months) and 0.8 months (range 0.2 to 4.1 months), respectively. The duration of therapy according to response to first-line therapy is outlined in Table 3.

In the LTS group, 57 patients (38%) who had CR or PR as best response to first-line targeted therapy had a median duration of therapy of 35.9 months; 32 of these 57 patients also received second-line therapy for a median of 5.0 months. The median duration of therapy for the 5 patients (3%) who had PD while receiving first-line therapy was 2.9 months. All these patients received second-line treatment, and if they had non-PD as best response, the median duration of therapy was 20.1 months.

Among STS, 9 patients (4%) who had CR or PR to first-line treatment achieved a median duration of receipt of therapy of 3.7 months. In patients who had PD (n = 93, 43%) as best response, the median duration of treatment was 1.6 months. As previously reported, response data from 74 patients (34%) were not available

Table 3 Response and Duration of First- and Second-Line Therapy

Characteristic	First-Line Therapy		Second-Line Therapy		Non-PD While Receiving Second-Line Therapy, n (%)
	n (%)	Median Duration of Therapy (months)	n (%)	Median Duration of Therapy (months)	
Short-Term Survival					
CR + PR	9 (4%)	3.7	—	—	—
SD	42 (19%)	2.8	4	—	—
PD	93 (43%)	1.6	14	—	—
Unknown	74 (34%)	1.7	3	—	—
All	218	2.0	21	0.8	0
Long-Term Survival					
CR + PR	57 (38%)	35.9	32	5.0	13 (41%)
SD	81 (53%)	17.0	53	12.7	36 (68%)
PD	5 (3%)	2.9	5	20.1	4 (80%)
Unknown	9 (6%)	28.5	4	7.6	3 (75%)
All	152	23.6	94	11.5	56 (60%)

Abbreviations: CR = complete response; IMDC = International mRCC Database Consortium; PD = progressive disease; PR = partial response; SD = stable disease.

for analysis; the median duration of treatment in this subgroup was 1.7 months.

Discussion

Data from large randomized clinical trials and prognostic models have provided survival estimates for patients with metastatic RCC.[11,12] The Memorial Sloan-Kettering Cancer Center (MSKCC) system was developed based on patients previously treated with interferon α. The study identified 5 adverse prognostic factors associated with different clinical outcome: (1) an interval from diagnosis to treatment < 1 year; (2) Karnofsky performance status < 80%; (3) serum lactate dehydrogenase > 1.5 times the ULN; (4) corrected serum calcium greater than ULN; and (5) serum hemoglobin less than the LLN.[12] In the era of targeted therapies, the IMDC has validated 4 of these 5 factors and incorporated 2 new markers of poor prognosis: neutrophil and platelet counts greater than ULN.[11]

As expected, our data demonstrate that the presence of adverse clinical and laboratory prognostic factors previously described by the IMDC were significantly more frequent in the STS group. These findings confirm the importance of the baseline prognostic criteria to aid in the discrimination between long- and short-term survival. Intermediate risk score is defined as 1 or 2 adverse prognostic factors.[11] Interestingly, 54 (72%) of 75 patients in the STS group who had intermediate risk prognostic scores had 2 risk factors. On the other hand, the majority of subjects with LTS in the same category (n = 112; 74%) had only 1 risk factor. These findings suggest that the presence of 1 additional adverse prognostic feature may be related to distinct clinical outcomes.

As previously reported, our analysis showed that tumor histology with sarcomatoid features, more than 1 site of metastasis, and absence of nephrectomy were significantly more prevalent in STS.[16] In addition, metastatic RCC with non–clear-cell histologies has been associated with poor prognosis.[17,18] In this series, although a small number of patients presented this pathologic covariate, the presence of this feature was not significantly associated with duration of survival (Table 1).

A retrospective analysis of 1059 patients with mRCC treated with sunitinib on clinical trials demonstrated that patients who had objective response to this agent achieved significantly longer survival. The median OS was 48.8 months for those with disease that responded to therapy and 14.5 months for those whose disease did not respond to therapy ($P < .001$). The survival duration was not associated with time to tumor response, and those whose disease responded to therapy also had significantly better performance status and more favorable MSKCC prognostic factors.[19] It is important to note that this analysis was not designed to compare LTS and STS. Another study reported that tumor reduction within 60 days of treatment initiation was associated with 74% decreased risk of death and early response was considered an independent predictor of better OS (hazard ratio 0.26; $P = .031$).[20] In addition, Grünwald and colleagues[21] presented at the 2013 European Cancer Congress the results from a study that explored the impact of tumor shrinkage in the clinical outcome of 2749 patients with mRCC who were treated as part of clinical trials. In this analysis, the tumor shrinkage was an independent predictor of OS. In our study, 57 patients (38%) in the LTS group had CR or PR to first-line therapy compared with 9 patients (4%) in the STS group. In addition, 81 patients (53%) in the first group had stable disease compared with 42 patients (19%) in the other. Over 90% of patients in the LTS achieved disease control (non-PD), independent of the prognostic risk category. Interestingly, although rare, even patients with poor prognostic risk scores experienced LTS if their disease responded to targeted therapies. Alternatively, response to therapy does not ensure long-term survival, as 9 subjects in the STS group with objective responses had a median duration of therapy of only 3.7 months.

To our knowledge, this is the largest series characterizing patients in the 2 extremes of survival spectrum. Among the 2161 patients registered in the IMDC database, we identified 152 patients (7%) who have survived more than 4 years. This subgroup of patients experienced higher response rates, longer treatment durations, and exposure to second-line agents compared to patients in the STS group, although we recognize that patients in the LTS group had more favorable baseline prognostic risk factors. A better characterization of these 2 extreme phenotypes of survival may help in planning clinical decisions. Predictions are clinically useful for the following: (1) selecting therapy (eg, define best subsequent therapy or good candidates for new therapeutic strategies, such as immunotherapy); (2) prognostication; and (3) planning end-of-life clinical care. However, they should be used carefully; patients with similar survival estimates may present different clinical outcomes. In addition, known and emerging pathologic and genetic biomarkers in these 2 groups of patients might be informative.

Although we have evaluated a large database, our study has several limitations. There is potential selection bias in any retrospective analysis. Despite standard data collection templates and inclusion of consecutive patients from each center in the database, data were missing for several patients, especially in the STS group. The short time of receipt of therapy for patients missing response data (1.7 months) is possibly due to the fact that these patients had rapid clinical progression and were taken off therapy, sometimes without detailed imaging. Furthermore, patients included in this analysis were treated with different targeted agents (although only VEGF and mTOR inhibitors); how it could distinctly impact clinical outcome needs to addressed in future studies. Unfortunately, tumor specimens and blood samples were not available, and molecular characterization of these 2 groups was not an objective of this work. However, the role of tumor biology in the survival duration should be addressed in future studies. Finally, our analysis was descriptive in nature, and no formal comparison was made for treatment outcomes, given that we included 2 extremes of survival spectrum by the study design. The strengths of our study include the large number of patients and the adjustment for the validated IMDC prognostic risk criteria.

In summary, baseline prognostic criteria used in the targeted therapy era may predict 2 extreme survival patterns. Our analysis supports response to targeted therapies and the absence of PD as important elements related to long-term survival.

Clinical Practice Points

- Patients with metastatic RCC have variable clinical courses in terms of survival and response to targeted therapy.
- Prognostic models have been widely used to predict patient outcomes. However, patients at the two extremes of the survival spectrum need to be characterized.

- In this analysis of patients from a large international database (IMDC), baseline prognostic criteria were able to discriminate between long- and short- term survivors.
- In addition, patients with poor prognostic factors may be able to achieve long-term survival if they could respond to targeted therapy.

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Disclosure

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APÊNDICE E - Adrenocortical carcinoma: The management of metastatic disease

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Adrenocortical carcinoma: The management of metastatic disease

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Contents

Abstract

Adrenocortical cancer is a rare malignancy. While surgery is the cornerstone of the management of localized disease, metastatic disease is hard to treat. Cytotoxic chemotherapy and mitotane have been utilized with a variable degree of benefit and few long-term responses. A growing understanding of the molecular pathogenesis of this malignancy as well as multidisciplinary and multi-institutional collaborative efforts will result in better defined targets and subsequently, effective novel therapies.

Keywords: Adrenocortical carcinoma; Therapeutics; Targeted therapies; Metastatic disease

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1. Introduction

Adrenocortical carcinoma (ACC) is a rare and aggressive malignancy of the adrenal cortex with an annual US incidence around 1–2 cases per million population [1,2]. Notably, given reliance of incidence data on NCI surveys from the 1970s as well as the challenge in proper histopathologic diagnosis, the true incidence may be underestimated.

ACC can occur at any age, but there is a bimodal distribution with a first peak at childhood (1–6 years old) and the second peak in the fourth to fifth decade of life [3]. The Surveillance Epidemiology End Results (SEER) data reports an incidence of 0.3 per million in children younger than 15 years. Notably, there is up to 18-fold higher incidence of cases in children in southern Brazil due to environmental and genetic risk factors which have been identified [4]. In this population, germline mutations of the *TP53* tumor suppressor gene (R337H) have been detected in 34% of the patients [5]. In addition to the above demographics, women have a higher incidence compared to men of about 2:1 with studies showing proliferative effects of estrogen on ACC cells, although not establishing this as a clear cause for the higher female incidence [6,7].

Sporadic ACC is a heterogeneous neoplasm with a poorly understood molecular pathogenesis [8]. The relationship between ACC tumorigenesis and familial hereditary syndromes has provided some insights into the molecular biology of this disease (Table 1) [9]. Chromosome imbalances (losses and gains) in specific loci of DNA have been reported with impact on several genes such as *TP53*, insulin-like growth factor type II (*IGF-2*), steroidogenic factor 1(*SF1*) and β-catenin. Given their roles, these genes have been identified as potential candidates for targeted therapies [10–12].

Overall, ACC carries a poor prognosis with the most important prognostic factors being the tumor stage at time of diagnosis. Unfortunately, with the absence of specific cancer-related early symptoms about 70% of patients are diagnosed with stage III or IV disease. In a European series of patients, the 5-year survival rates were 60% for stage I, 58% for stage II, 24% for stage III, and 0% for stage IV. Importantly, the median survival for metastatic disease (stage IV) at the time of diagnosis is less than a year [13].

ACC management often requires a multidisciplinary approach, frequently involving a medical oncologist, an endocrine surgeon, an endocrinologist and several other disciplines. Surgical resection remains the cornerstone of the treatment and represents the only curative option for patients with early stage ACC. However, around 80% of these patients will present local or distant recurrence after a complete resection [14]. With regard to recurrent or advanced disease, ACC is modestly responsive to standard cytotoxic chemotherapies, although various combinations have shown clear palliative benefit. Radiation and ablative techniques have been utilized with variable benefit depending on the clinical scenario.

The above realities highlight the fact that effective systemic treatments for advanced disease are lacking. The pipeline for novel drug development and testing in clinical trials has been limited. The goal of this manuscript is to review the advances in the therapy of advanced ACC. With the recent evolution of new technologies producing genetic data and the molecular characterization of multiple solid tumors described by The Cancer Genome Atlas, we will also focus on the potential of targeted signaling pathways and personalized therapies.

2. Evidence acquisition

A systematic review of the MEDLINE databases was performed on September 2013. The search was conducted using the keywords "general surgery", "therapeutics", "mitotane", "radiotherapy", "biological markers", "oncogenes", "tumor suppressor genes", "drug therapy" and "adrenocortical carcinoma". In total, 266 abstracts were identified. Articles about advanced disease were manually selected. Full text of potentially relevant studies (97 articles) were carefully examined by the authors and considered for analysis. Review articles were also analyzed. We searched abstracts and

Table 1
Hereditary ACC-related hereditary syndromes.

Hereditary syndrome	Chromosome alterations	Gene	Comments
Li–Fraumeni syndrome [10]	17p13	*TP53* *hCHK2*	Mutations in TP53 are present in about 25% of sporadic ACC
Beckwith–Wiedemann syndrome [10]	11p15	*IGF-2* *CDKN1C* *H19*	Macroglossia, macrosomia, Wilms' tumor, ACC
Multiple Endocrine Neoplasia I (MEN I) [10]	11q13	*MEN1*	Adrenal adenomas did not present alterations in this locus
Carney syndrome [10]	17q22-24	*PRKAR1A*	53% of sporadic ACC
Lynch syndrome (LS) [9]	2p16 2q31 3p21 2p16	*MSH2* *PMS1* *MLH1* *MSH6*	The prevalence of LS among patients with ACC is 3.2%

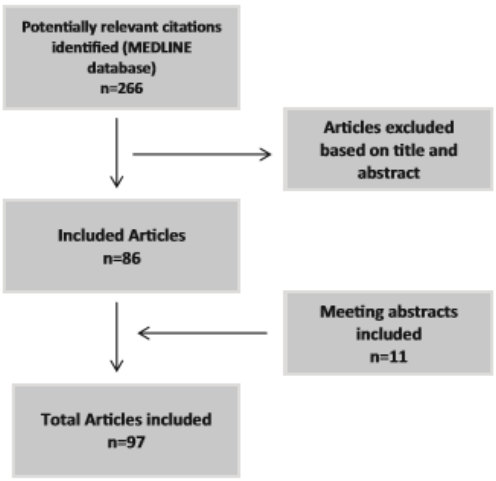

Fig. 1. Flow diagram.

virtual meeting presentations from the American Society of Clinical Oncology (ASCO) (http://www.asco.org/ASCO) and Endocrine Society conferences from 2004 to 2013 Pertinent material was retrieved and critically evaluated (Fig. 1).

3. Evidence synthesis

3.1. Surgery

In patients with advanced disease, surgery remains an important consideration when complete resection of the primary tumor and all metastases is feasible. In contrast, cytoreductive debulking surgery should be carried out in selected patients with severe hormone excess that is refractory to medical management [15,16].

Despite aggressive surgical intervention, local recurrences are frequent. Salvage resections may be considered especially if more than 12 months have been elapsed from the initial surgical treatment and single site of metastasis [17–20]. However, data on the effectiveness of surgery in the management of recurrent disease are still lacking. Erdogan and colleagues suggested two major predictors for clinical outcome after first recurrence, namely time to first recurrence over 12 months and resectability of tumor lesions (R0-resection) [21].

A recently published study reported that, in selected patients with ACC and liver metastases, major liver metastactomy was associated with long-term survival, with a 5 year-survival of 39%. However, cure is generally not achievable [22].

Based on data from the adjuvant setting, we suggest the application of "adjuvant" treatment concepts as soon as complete surgical resection of metastatic disease has been performed.

3.2. Radiation therapy

Radiation therapy has been labeled as ineffective in adjuvant or primary treatments for ACC [23]. However, increasing evidence supports the rational that ACC is not a radiotherapy-resistant tumor and palliative radiotherapy has been successfully used to treat symptomatic metastatic lesions [24,25].

We identified 9 articles reporting palliative radiation therapy for advanced ACC with administered radiation doses ranging from 10 Gy to 60 Gy [26–28]. Response criteria included mainly pain relief, and were obtained in about 72% of the cases [20,27–30]. In addition, radiotherapy is effective for improving neurologic symptoms from brain metastasis [31]. It remains an individual decision whether a patient with widespread metastasis and a very limited life expectancy should receive radiotherapy. Non specific recommendation exists for cerebral metastasis, vena cava obstruction and bone metastasis with spinal cord compression [31].

Interestingly, pre-clinical models suggest that mitotane may function as a radiosensitizer [32]. This is an important question which requires further investigation given the implications across a number of clinical scenarios like the adjuvant setting, which are beyond the scope of this paper.

3.3. Other interventional therapies

Local therapies as chemoembolization or radiofrequency ablation have also shown to play a palliative role in metastatic disease. Percutaneous radiofrequency ablation may provide short-term local control in unresectable primary tumors, particularly for those with less than 5 cm in diameter [33,34]. In addition, transcatheter arterial chemoembolization may be considered as part of the therapeutic arsenal to treat liver metastasis from ACC [35], especially in cases in which the diameter of the target metastasis is 3 cm or smaller [36]. Similar to considerations for metastatectomy, an individualized decision is required for each patient based on both disease- and patient-specific characteristics.

3.4. Systemic therapies

The most frequently used systemic drugs in the advanced disease include mitotane, cisplatin and etoposide used alone or in combination with other agents [37]. Fareau and coworkers described retrospectively the experience of one institution over 20 years with different first line chemotherapies including mitotane, cisplatin, etoposide, streptozocin, doxorubicin, paclitaxel, gemcitabine, cyclophosphamide and ifosphamide. No overall survival (OS) advantage was observed for any single agent or combination over others [38]. Systemic chemotherapy will be reviewed in more details later.

3.4.1. Adrenolytics

Mitotane is an adrenal-specfic cytotoxic agent, which can also lead to necrosis of the adrenal gland. It plays an important role in palliation of effects of hypercortisolism from hormonally active ACC. Evidence for use of mitotane as the only approved drug for advanced ACC is based on retropective series of data as opposed to any prospective, randomized controlled trials. Mitotane is considered one of the most active agents in ACC with response rates ranging from 13 to 31% [39]. However, complete responses rarely occur [15]. In terms of overall survival, four studies concluded that mitotane treatment does not increase the survival rate whereas five found an increase in the survival rate [7,20,39–45]. It is important to note that mitotane therapy may prolong recurrence-free survival when used as an adjuvant treatment for patients who have undergone radical resection of their primary tumor [46].

The optimal dosing regimens of mitotane are not largely known. Among the studies in which survival influence of mitotane was addressed, three studies reported superior clinical benefits in patients with plasma levels above 14 mg/l. Plasma levels of 14 mg/l or greater have produced a significant response rate whereas only occasional responses have been described when plasma mitotane levels remain below this threshold [39,47,48]. In addition, significant toxicities have been reported when plasma levels exceed 20 mg/l [49,50]. Therefore, we recommend to start mitotane at 0.5 g daily and increase 0.5 g weekly according to tolerance targeting mitotane blood level between 14 and 20 mg/l [51,52]. The narrow therapeutic window can be difficult to achieve, and severe side effects are generally dose limiting during the treatment. It is important to note that mitotane itself is not active, rather must be metabolized to be transformed into therapeutic metabolites that are capable of adrenolysis [53]. Because of this unique characteristic, measuring circulating mitotane levels serve as a surrogate for the active metabolites. Some individuals are clearly capable of metabolizing mitotane into its active constituents more readily than others, as evidenced by therapeutic and toxic effects occurring in some individuals at very low mitotane doses, while much higher doses in others.

Although mitotane is metabolized and cleared primarily by the liver, one of its most profound and dynamic influences is accelerating CYP3A4 activity. Since the vast majority of medications are metabolized or altered by hepatic CYP3A4, mitotane therefore has a significant impact on drug metabolism and drug-drug interactions. A few notable examples of mitotane-induced hepatic CYP3A4 effects include: (1) glucocorticoid insufficiency; (2) hyperlipidemia and HMG-CoA inhibitor metabolism; (3) and increased macrolide metabolism.

Almost all patients on mitotane agent will develop some form of glucocorticoid (and occasional mineralocorticoid) insufficiency, and therefore require adrenal supplementation. This is the result of dual mitotane effects: direct adrenolysis and accelerated hepatic metabolism of adrenal steroids by CYP3A4. In addition, mitotane increase hepatic production of globulins (such as cortisol binding globulin [CBG] and thyroid binding globulin [TBG]), thereby further lowering the free concentrations of circulating cortisol.

Patients on mitotane should be empirically treated with low dose hydrocortisone to prevent adrenal crises, and treating physicians must be aware that patients may require increasing doses of glucocorticoids (well into the supraphysiologic range) to achieve normal adrenal replacement. Patients on full dose mitotane therapy often need 50 mg of hydrocortisone daily, or more, to treat the vague and diffuse symptoms of cortisol insufficiency. Our usual approach is to start by hydrocortisone 20 mg in the morning and 10 mg in the afternoon when patients achieve a daily mitotane intake of 2 g. Measuring ACTH, 24 h urine free cortisol, and urinary cortisol, and plasma renin activity, in addition to detailed history, can help guide adrenal steroid replacement.

Mitotane induces hypercholesterolemia (primary high LDL and triglycerides) via unclear mechanisms. Mitotane also accelerates the clearance of simvastatin and atorvastatin which are metabolized by CYP3A4; therefore, when statins are used, pravastatin or rosuvastatin are preferred for maximal effectiveness. Lastly, mitotane-induced CYP3A4 activity prevents adequate levels of macrolide antibiotics; therefore, when antibiotics of this class are indicated, consideration for using levofloxacin or ciprofloxacin should be made.

Other toxicities experienced with mitotane can vary and need active medical monitoring and intervention. Local gastrointestinal discomfort (i.e. nausea, vomting or diarrhea), fatigue, and central nervous system toxicities (memory loss, ataxia, dizziness) are common. Mitotane can cause a central hypothyroidism and inhibits the conversion of testosterone to dihydrotestosterone [54]; both of these effects can further compound fatigue and systemic symptoms [55,56].

Patients on mitotane should iniatially undergo laboratory evaluation every 4–8 weeks to monitor: electrolytes, kidney and liver function tests. Free thyroxine levels, testosterone in men, and lipid profile should be assessed every 3–4 months. Plasma renin activity, ACTH, and a 24 h urine collection for free cortisol can help assess the adequacy of adrenal steroid replacement, and can be considered at every visit or if suggestive symptoms.

3.4.2. Cytotoxic chemotherapy

3.4.2.1. First-line. Chemotherapy with single agents has produced disappointing results and low response rates [57]. With the exception of one large randomized clinical trial, combination of cytotoxic agents to attack ACC was exclusively investigated in retrospective series or small phase II studies (Table 2).

Platinum-based chemotherapies achieve responses ranging from 11 to 48% and the best results may be explained by patient selection [58]. A small phase II study conducted by the Southwest Oncology Group Study (SWOG) enrolled 47 patients with advanced disease to evaluate first-line treatment with cisplatin and etoposide followed by mitotane at disease progression. This trial reported 11% of patients with

Table 2
Cytotoxic chemotherapy in ACC.

Drug	Study phase/*n*	Clinical benefit
Cisplatin + etopside followed by mitotane [59]	II/*n* = 47	ORR:11% Median OS: 10 months
M/EDP [60]	II/*n* = 28	CR: 2 patients PR: 13 patients ORR 53.5%
SM [61]	II/*n* = 22	ORR: 36.4%
M/EDP vs SM FIRMACT trial [62]	III/*n* = 304	PFS: 5.0 vs 2.1 months ($p < 0.001$) OS: 14 vs 12 months ($p = 0.07$) ORR: 23.2 vs 9.2%, ($p < 0.001$)

M/EDP: mitotane + etoposide, doxorubicin, and cisplatin; SM: streptozotocin + mitotane.

objective response and confirmed some activity with this strategy [59].

The best outcomes in advanced ACC were achieved by etoposide, doxorubicin, and cisplatin (EDP) or streptozotocin, both in combination with mitotane. The first combination was studied in a phase II trial with 28 patients in which complete response (CR) was reported in two patients and partial response (PR) in 13, for an overall response rate of 53.5% [60] The second regimen was evaluated by Khan and colleagues in 22 patients with advanced ACC. In this phase II study, CR or PR were obtained in 36.4% of patients treated with streptozotocin combined with mitotane (SM) [61]. These results provided the rationale for an international initiative establishing the standard first-line treatment in advanced ACC.

The first international randomized trial in locally advanced and metastatic adrenocortical carcinoma treatment (FIRM-ACT) compared these two successful regimens in the first-line to establish a standard of care in this setting. In this large collaborative effort, 304 patients were enrolled to receive mitotane plus a combination of etoposide, doxorrubicin and cisplatin (M/EDP) or streptozocin plus mitotane (SM). The progression-free survival (PFS) was significantly longer in the M/EDP than SM groups (5.0 vs 2.1 months). The response rate was also higher in the M/EDP group (23.2% vs 9.2%) and the toxicity profile was similar. There was no significant difference in OS (14.8 vs 12.0 months). Importantly, the study design included cross-over at treatment failure and the M/EDP group included many patients who had first-line SM failures. These findings may explain the similar OS in the two groups. The quality of life and rate of serious adverse events were comparable between the two groups. Despite no differences in OS were found, this study provides the most robust evidence for the systemic treatment of advanced ACC [62].

Most recently, a phase II study enrolled 19 patients with advanced ACC to demonstrate the efficacy of cisplatin plus docetaxel. The median PFS was 3 months. Therefore, the combination of different agents, such as taxanes, did not appear to improve the clinical outcome when compared with other combinations usually used to treat this disease [63].

3.4.2.2. Second-line. Studies evaluating the impact of chemotherapies in the second-line are limited and have described disappointing results. A secondary endpoint of the FIRM-ACT trial was to evaluate the efficacy of both regimens in the second-line. The PFS was 2.2 months among the 84 patients who were treated with second-line SM. Among 101 patients who are treated with M/EDP in this setting the PFS was 5.6 months. These findings reassure the higher antitumor efficacy of M/EDP as both first- and second-line therapy [62].

A phase II trial addressed the role of gemcitabine plus metronomic 5-fluorouracil or capecitabine in heavily pretreated patients with advanced ACC. One of the 28 patients (3.5%) presented complete response and other one patient a partial regression. Notably, 11 patients (39.3%) achieved stable disease (SD). Therefore, this treatment scheme may potentially delay the disease progression in pretreated advanced ACC [58]. One explanation for these results is the concept of metronomic therapy. The administration of low doses of chemotherapy on a frequent or continuous schedule may have both antiangiogenic and immunomodulatory effects resulting in disease stabilization in tumors that had became resistant to conventional treatments [64]. This hypothesis should be addressed in future studies to define the role of metronomic chemotherapy in advanced ACC.

Recently, Germano and colleagues presented the efficacy of gemcitabine alone or in combination with mitotane in ACC cell lines. Interestingly, in mitotane-sensitive cells the combination of these two drugs showed an antagonistic effect. Paradoxically, in mitotane-insensitive cells the same drug combination was synergistic, except when mitotane was sequentially administered prior gemcitabine. One explanation for these findings may be a different interaction with the Ribonucleotide Reductase large Subunit 1 (RRM1) gene. Further investigations are needed to define the role of gemcitabine in ACC treatment [65].

3.4.3. Molecular pathways and targeted therapies

A better understanding of ACC biology has provided some rationale for the development of novel agents in this disease. Unfortunately, new agents have resulted and minimal

Table 3
Targeted therapies in ACC.

Drug	Target	Study phase/*n*	Clinical benefit
Sunitinib [71]	VEGF pathway	II/*n* = 35	5 patients with SD
Sorafenib + paclitaxel [72]	VEGF pathway + cytotoxic chemotherapy	II/*n* = 9	No activity
Bevacizumab + capecitabine [73]	VEGF pathway + cytotoxic chemotherapy	II/*n* = 10	No activity
Erlotinib + gemcitabine [77]	EGFR pathway	II/*n* = 10	1 patient with SD
Gefitinib [78]	EGFR pathway + cytotoxic chemotherapy	II/*n* = 19	No activity
Everolimus [89]	mTOR pathway	II/*n* = 4	No activity
Imatinib [94]	C-ABL, PDGFR and C-kit tyrosine kinase inhibitor	II/*n* = 4	No activity
Cixutumumab (IMC-A12) [84]	IGF-IR pathway	II/*n* = 10	1 patient with SD
Figitumumab [82]	IGF-IR pathway	I/*n* = 14	8 patients with SD
Cixutumumab + temsirolimus [88]	IGF-IR pathway + mTOR pathway	I/*n* = 10	4 patients with SD

VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; mTOR, mammalian target of rapamycin; IGF-IR, insulin growth factor 1 receptor; PDGFR, platelet-derived growth factor receptor.

or no activity and new investigations/clinical trials need to be encouraged to incorporate this strategy in the clinical practice. Here we summarize the existing evidence and potential pathways and targeted drugs that are being studied in ACC (Table 3).

3.4.3.1. Vascular endothelial growth factor (VEGF) pathway. Sunitinib is a small molecule tyrosine kinase inhibitor (TKI) involved in tumor proliferation and angiogenesis targeting platelet-derived growth factor receptor (PDGFR), vascular endothelial growth factor receptor 2 (VEGFR2), stem cell factor receptor (c-KIT) and fms-related tyrosine kinase 3 (FLT-3) pathways [66]. This drug produces important anti-tumor activity in different tumors and is widely used in renal cell carcinoma and gastrointestinal stromal tumor (GIST) [67]. Interestingly, ACC is characterized by higher levels of VEGF expression than others adrenal tumors. In preclinical models sunitinib induced adrenal hemorrhage, leading to adrenal insufficiency [68,69], and down-regulated *HSD3B2* gene blocking steroidogenesis in vitro [70].

Based on these pre-clinical data, a phase II, single arm, trial was conducted to evaluate the efficacy of sunitinib in 35 patients with refractory ACC who had progressed after mitotane and 1–3 previous chemotherapies. The median PFS was 2.8 months and the response rate was 15.4%. More than half of the patients enrolled in this study received mitotane concomitantly. Notably, a drug interaction between sunitinib and mitotane might result in reduced sunitinib levels by induction of cytochrome P450-3A4 activity. These findings may explain the modest antitumor activity of sunitinib reported in this study [71].

Sorafenib is another multi-targeted TKI inhibitor targeting VEGF and angiogenesis. This agent was evaluated in a phase II trial in combination with weekly paclitaxel. Nine patients were enrolled. However this trial had to be stopped prematurely because all patients progressed after 8 weeks of treatment [72].

Ten patients with advanced ACC who failed to prior cytotoxic agents were treated with bevacizumab and capecitabine combination on a compassionate use basis. None of these patients experienced any objective response or SD [73].

3.4.3.2. Epidermal growth factor receptor (EGFR) pathway. Inhibition of EGFR pathway results in clinical benefit in several malignancies like non small cell lung cancer (NSCLC) and pancreatic cancer [74,75]. Recently, Fassnatch and colleagues reported that 78% of ACCs express EGFR by immunohistochemistry, though none of the tumors harbored EGFR mutations in exons 19–21, [76]. In a small study of patients with ACC progressing after two to four systemic therapies, salvage chemotherapy using gemcitabine plus erlotinib had no benefit among 10 patients who received this treatment [77].

A small North American phase II clinical trial addressed the efficacy of gefitinib in 19 patients. No complete responses, partial responses or patients with SD were also observed [78].

3.4.3.3. Insulin-like growth factor 1 receptor (IGF-IR) pathway. The IGF-IR is a transmembrane receptor with tyrosine kinase activity and is overexpressed in several malignancies. This seems to be the most common molecular event in ACC, but the molecular mechanism involved in this process is still unknown [79]. Using DNA microarray Barlaskar and colleagues showed that the majority of ACC overexpressed *IGF* gene transcripts [8]. Two pathways are closely related to IGF-1R cascade: the phosphatidylinositol 3-kinase (PI3K)/AKT/mTOR and the RAS/MAPK pathways. The IGF-1R phosphorylation activates PI3K/AKT signaling pathway leading to cell survival. Similarly, it activates RAS and extracellular-signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway leading to tumor growth and proliferation [80].

Cixutumumab (IMC-A12) and figitumumab (CP-751,871) are IGF-IR-targeting antibodies currently in development to block the interaction between IGF-IR and its ligand [81]. Figitumumab was evaluated in a phase I study which enrolled 14 patients with advanced ACC. Eight of 14 patients (57%) had stable disease as their best response and none remains on therapy after 7 cycles due to progressive disease (PD) or toxicity [82]. Notably, 10 patients with ACC were treated with cixutumumab alone in a phase II study and no objective responses were reported. One patient achieved SD for 7 cycles [83]. In addition, a small-molecule TKI of the

IGF-IR (OSI-906) is being investigated. In a phase I study presented in the American Society of Clincial Oncology (ASCO) Annual Meeting in 2010, 1 patient with ACC had SD for more than 1 year. Results from this phase I trial led to the development of a randomized, placebo-controlled, phase III trial with this compound [84]. Results from this study that finished accrual are awaited (GALACCTIC – NCT00924989).

3.4.3.4. Mammalian target of rapamycin (mTOR) pathway. mTOR is a protein kinase of PI3K/AKT signaling pathway which regulates cell growth, metabolism, and proliferation [85]. Pre-clinical studies have shown the anti-proliferative effect of the dual PI3K/mTOR inhibition in adrenocortical cell lines and xenograft models [86,87]. These findings support the study of mTOR inhibitors in refractory ACC. Recently, a phase I study evaluated IMC-A12, a human IgG_1 monoclonal antibody to the IGF-IR, in combination with temsirolimus in patients with advanced cancer including 10 patients with ACC. In this study, 4 of 10 patients with ACC had SD as best response lasting 8 months. Among them, 2 patients presented tumor reduction without RECIST criteria of PR. PD was the best response in the other patients. Further investigations are needed to establish whether dual inhibition of the IGF-1R and mTOR pathways will impact in clinical outcomes of ACC patients [88]. Interestingly, targeting this pathway with everolimus alone resulted in no meaningful responses [89].

3.4.3.5. Wnt/β-catenin pathway. β-Catenin is important for the adrenal physiological maintenance and is a part of the Wnt signaling pathway which regulates the tissue self-renewal [90]. Mutations in *APC* and *CTNNB1*genes, which encode this protein, have been described in several malignancies [91]. Interestingly, this pathway is also upregulated in ACC and may be a new target for therapeutic intervention [92]. The rationale to test this drug as a potential new therapy came from an experiment in which a β-catenin antagonist inhibited proliferation in ACC cell lines [93]. Studies demonstrating the efficacy of this strategy in ACC treatment are not published up to the time this review was performed.

3.4.3.6. Other targeted agents and approaches.

3.4.3.6.1. Imatinib. Imatinib mesylate, is a small molecule selective inhibitor of the c-ABL, PDGFR and c-kit tyrosine kinases. Dramatic responses are seen in gastrointestinal stromal tumors (GIST) treated with Imatinib. Additionally, patients with other solid tumors expressing c-kit and/or PDGFR have also shown responses to this agent. A phase II trial evaluated the role of imatinib in a variety of solid tumors including 4 patients with ACC. No clinical benefit was reported in this subgroup of patients [94].

3.4.3.6.2. Dovitinib. Dovitinib is a multi-target TKI that inhibit the fibroblast growth factor receptor (FGFR). Pre-clinical studies have suggested that this pathway may play a role in ACC carcinogenesis. Recently, the first results from a phase II trial to evaluate the efficacy of this drug in 17 patients were presented. No objective response was observed. However, clinical benefit was achieved with SD for >6 months in 23% of the patients [95].

3.4.3.6.3. Polo-like kinase 1 (PLK1) inhibitors. Polo-like kinase 1 (*PLK1*) gene is involved in multiple aspects of cell progression and is highly expressed in several malignancies. Agents against *PLK1* have been investigated in advanced solid tumors. Results from a phase I study to evaluate a small interfering RNA (siRNA) inhibiting *PLK1* were presented in the last ASCO annual meeting. This study included 4 patients with ACC in the expansion cohort. Among the ACC patients, 3 presented SD and the longest duration of response was 6 months. In addition, 1 patient had tumor size reduction of 19.3% after 2 cycles on therapy [96]. Further investigation is warranted to define the role of PLK1 inhibition in patients with advanced ACC.

3.4.3.7. Combination of targeted therapies. The combination of targeted therapies to block synergistically different pathways at the same time is being investigated in solid tumors to overcome therapy resistance. As mentioned before, combinations using mTOR inhibitors may be an important therapeutic option considering its impact in cell metabolism and pathways interactions [88]. Moreover, single agent targeted therapies do not result in markedly responses.

A phase I study examined the safety and clinical effect of combination therapy targeting the VEGF pathway. In this study, bevacizumab plus sunitinib were evaluated in different advanced solid tumors, including 5 patients with ACC. This regimen showed some tumor activity across all tumor subtypes. 2 out of 5 patients with ACC presented some tumor reduction. However, higher toxicities levels were reported [97]. Another phase I study of sorafenib and bevacizumab in patients with refractory, metastatic or unresectable solid tumors is ongoing and may help us to answer this question. (NCT00098592). A combination of anti-IGF-IR and EGFR-targeted therapy (OSI-906 + Erlotinib) is under investigation and in ACC. However, what is the optimal combination to treat ACC remains an important question.

4. Future directions

Advanced ACC is a very aggressive disease and its management is challenging. During the past 10 years collaborative efforts have been made to improve the ACC treatment. Although multidisciplinary approaches may result in long-term disease control and survival, conventional chemotherapy is not curative and targeted therapies did not yield so far any noticeable results. The combination of mitotane plus EDP offers the best approach to extension of survival based on a rigorous and large study. However, effective treatments are lacking and these efforts have resulted in minimal progress in disease outcomes.

In the genomic era, the understanding of the ACC biology may lead to improvements in therapeutics. The Cancer

Genome Atlas (TCGA) has allowed the molecular characterization of a large number of tumors, and an initiative for ACC is ongoing (Ref: https://tcga-data.nci.nih.gov/tcga/tcgaCancerDetails.jsp?diseaseType=ACC&diseaseName=Adrenocortical carcinoma). It may help us identifying new targets for therapeutic interventions and biomarkers that can predict response to targeted therapies. In addition, the role of immune regulation in tumor progression and metastasis has been investigated and the interest in this subject is tremendously increasing. As an example, blocking programmed death-1 (PD-1), an inhibitory receptor expressed on activated T cells, results in significant antitumor activity in several malignancies. Efforts should be also done to clarify the potential of novel immunotherapies to treat ACC and at least to interrogate the expression of the PD-1 ligand on tumor cells.

Conflict of interest

Toni K. Choueiri: Consultancy: Pfizer, Novartis; Advisory board: Pfizer, Novartis, Aveo, GlaxoSmithKline, Exelixis; Research: Pfizer; No Speakers bureau. All remaining authors have declared no conflict of interest for this work.

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Biography

André P. Fay is a medical oncologist. Since July 2013, Andre is working with clinical and translational research in the Lank Center of Genitourinary Oncology at Dana-Farber Cancer Institute/Harvard Medical School under Dr. Toni Choueiri's mentoring. Recently, he was selected to receive the MERIT Award of American Society of Clinical Oncology (GU ASCO Symposium 2014) with the research project: PD-L1 expression in non-clear cell renal cell carcinoma. Toni Choueiri is the clinical director and Kidney cancer Center director of the Lank Center of Genitourinary oncology and has contributions as a researcher, clinician and mentor of many oncology trainees. His research interests include the development of novel agents and biomarkers in genitourinary cancers, with a particular focus in renal cell carcinoma. Dr. Choueiri is currently the overall primary investigator in several phase I, II and III studies of novel agents and combinations using both clinical and correlative tissue-based endpoints.

APÊNDICE F - MET as a Target in Papillary Renal Cell Carcinoma

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Clinical Cancer Research

CCR Translations
See related article by Albiges et al., p. 3411

MET as a Target in Papillary Renal Cell Carcinoma

André P. Fay[1], Sabina Signoretti[1,2,3], and Toni K. Choueiri[1,2,3]

The biology underlying papillary renal cell carcinoma (pRCC) is largely unknown, and no specific therapies have been developed for advanced disease. The elucidation of the *MET* pathway status in types I and II pRCC may help to select patients who are more likely to benefit from MET inhibitors. *Clin Cancer Res; 20(13); 3361–3. ©2014 AACR.*

In this issue of *Clinical Cancer Research*, Albiges and colleagues substantially contribute to the understanding of the biology of papillary renal cell carcinoma (pRCC) through a rigorous study of a large number of patients (1). RCC is widely recognized as a heterogeneous disease characterized by multiple histologic subtypes and distinct biologies as well as variable clinical courses. Clear-cell RCC (ccRCC) is the most common subtype of kidney cancer and accounts for more than 80% of cancers that arise from the renal epithelium. pRCC is the most common subtype of non-ccRCC, accounting for 10% to 15% of all RCCs. Two main types of pRCC with divergent pathologic and clinical features have been recently recognized: type I, which is characterized by low nuclear grade and usually (but not always) an indolent clinical course, and type II, which presents with higher nuclear grade and a more aggressive clinical behavior (2).

Drugs targeting angiogenesis and specifically vascular endothelial growth factor (VEGF) have dramatically improved the clinical outcome of patients with advanced ccRCC, in which the von Hippel–Lindau/hypoxia-inducible factors (VHL/HIF) axis plays an essential role. Because non–clear-cell tumors seem to have a different biology from their clear-cell counterparts and HIF/VEGF signaling is likely to play a pro-oncogenic role only in a subset of non–clear-cell cancers (3), it is not surprising that less impressive results from VEGF-targeted agents have been described for advanced non-ccRCC, including pRCC. In addition, some series have suggested that metastatic pRCC may even carry a worse prognosis than ccRCC, justifying an urgent need for novel drugs in this particular subtype (4).

The MET protein is a transmembrane receptor tyrosine kinase. The interaction with its only known ligand, hepatocyte growth factor (HGF)/scatter factor, regulates cell growth, migration, invasion, proliferation, and angiogenesis, promoting malignant transformation when inappropriately activated. HGF/MET signaling activates several downstream intracellular pathways, including focal adhesion kinase (FAK), Ras/Raf/MEK/ERK, and PI3K/Akt (5). The aberrant expression of elements of the MET pathway such as MET protein has been associated with poor prognosis and aggressive features in several malignancies, including RCC (6).

Trisomy of chromosome 7, in which *MET* is located, has been seen to be a common occurrence in pRCC (7). In addition, mutations in *MET* have been identified in an inherited syndrome of type I pRCC and in a few sporadic pRCC (8), justifying MET inhibitors as a therapeutic strategy in advanced pRCC. Choueiri and colleagues conducted a clinical trial to investigate the role of a dual MET/VEGF inhibitor (foretinib) in pRCC. In this phase II study, 74 patients were stratified on the basis of MET pathway activation, defined as the presence of a germline or somatic *MET* mutation, *MET* 7q31 amplification, or gain of chromosome 7 (9). The primary endpoint of an objective response rate of at least 25% was not met. However, the objective response rate of 13.5% and a median progression-free survival duration (PFS) of 9.3 months were noteworthy, because agents targeting angiogenesis have shown modest activity with PFS rates of 1.6 to 6.6 months and objective response rates ranging from 3% to 13% in pRCC (10). Interestingly, germline *MET* mutations (hereditary type I pRCC) were highly predictive of response, with 50% of patients with mutations having an objective response compared with 9% of patients without mutations (5/10 vs. 5/57, respectively; ref. 9). Notably, differences between the two pRCC subtypes were not assessed.

Albiges and colleagues (1) investigated the *MET* gene status in a large well-annotated cohort of 220 patients with pRCC. Each sample was independently reviewed by two specialized pathologists, both blinded to the clinical outcome. This robust dataset expands our knowledge about *MET* gene status for both type I and II pRCC subtypes by reporting on different mechanisms of MET activation: gene expression, copy-number alterations (CNA), mutational status, and potential coactivators of MET protein. As previously reported (6), MET expression was significantly higher in both type I and type II pRCC than in clear-cell histology. However, type I pRCC presented a higher expression of MET

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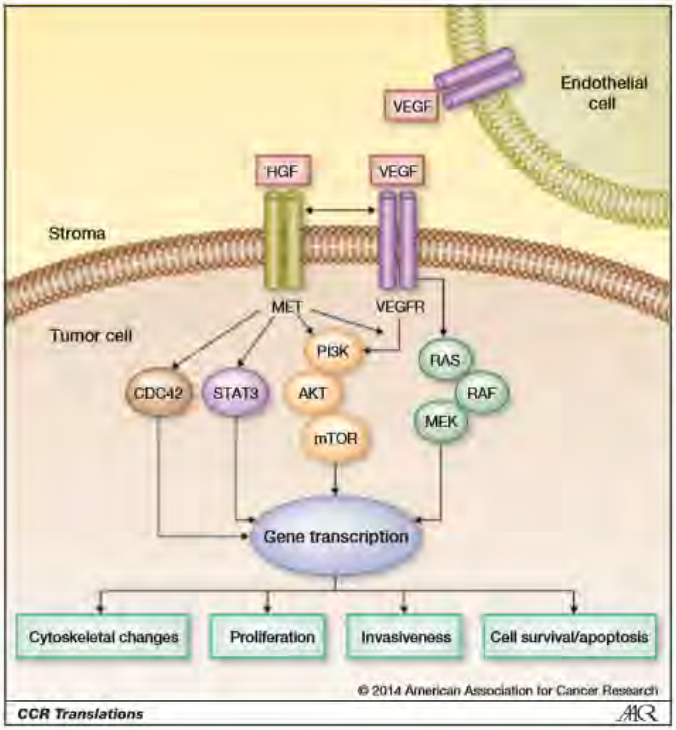

Figure 1. Binding of HGF to MET induces activation of the PI3K/AKT/mTOR, RAS/RAF/MEK, STAT3, and CDC42 downstream pathways. In a subset of pRCC, VEGF signaling in tumor cells may also contribute to the activation of the PI3K/AKT/mTOR and RAS/RAF/MEK pathways. VEGF signaling in endothelial cells may drive angiogenesis.

when compared with the type II subtype ($P < 0.0001$). CNAs of *MET* were identified in 46% of type II pRCC and in 81% of type I pRCC. The correlation of CNA and *MET* mRNA expression was significantly high ($P < 0.0001$), which may provide a biologic basis for enhanced MET signaling. Of note, 11 somatic mutations of the *MET* gene, including four new mutations, were identified in 51 type I pRCC (21.5%), whereas smaller series had previously reported a mutation rate around 13% in this setting. Importantly, the impact of CNA and mutations in *MET* on MET pathway downstream activation should be addressed in further studies.

Consistent with this framework, additional investigations are needed to translate these findings into clinical practice. The first issue raised from this study is how clinicopathologic features and clinical outcome correlate with the molecular findings, because the authors have evaluated a heterogeneous cohort. Second, assessment of MET protein expression by immunohistochemistry, which was not performed, may be helpful to select patients for further studies and clinical trials and would certainly be an important addition to the field. Third, the authors evaluated gene expression in both types of pRCC, but *MET* sequencing was arbitrarily performed only in type I pRCC. Identification of specific mutations in type II pRCC would need to be performed in future work.

Although the MET pathway seems to play an important role in pRCC, the inhibition of this pathway could be insufficient to control tumor growth. As the MAPK/ERK and PI3K/AKT pathways are known to be part of the MET cascade, questions still remain about how the cross-talk among distinct elements of these downstream pathways are involved in tumor progression. It is also very possible that a subset of pRCC depends on the VEGF axis signaling in tumor cells and/or endothelial cells. In fact, small tissue-based studies showed that overexpression of VEGF and VEGF receptors (by immunohistochemistry or qRT-PCR) in pRCC can be associated with worse prognostic features (11). The elucidation of these interactions could provide a rationale for combinatorial strategies in advanced pRCC (Fig. 1).

The deeper understanding of MET activation in pRCC may also help patient selection. A study enriching for

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patients whose tumors harbor genomic alteration in *MET* may be the ideal population for testing the efficacy of agents targeting MET in pRCC. The Cancer Genome Atlas has allowed the molecular characterization of a large number of solid tumors, and an initiative for pRCC is ongoing (12). This important initiative will help us to validate the findings from the study by Albiges and colleagues, and provide a global unbiased approach to understanding the genetic basis of pRCC, with the hope of providing more effective treatment strategies that are tailored to the genetic profile of each patient's cancer, thus advancing our ultimate goal toward precision medicine in RCC (13).

Disclosure of Potential Conflicts of Interest

T. Choueiri reports receiving a commercial research grant from Pfizer and is a consultant/advisory board member for Aveo, Exelexis, GlaxoSmithKline, Novartis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: A.P. Fay, T.K. Choueiri
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.P. Fay, T.K. Choueiri
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T.K. Choueiri
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MET as a Target in Papillary Renal Cell Carcinoma

André P. Fay, Sabina Signoretti and Toni K. Choueiri

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APÊNDICE G - Sequential Targeted Therapy After Pazopanib Therapy in Patients With Metastatic Renal Cell Cancer: Efficacy and Toxicity

Original Study

Sequential Targeted Therapy After Pazopanib Therapy in Patients With Metastatic Renal Cell Cancer: Efficacy and Toxicity

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Abstract

Sequential therapy benefits patients with metastatic RCC. However, the best sequence of drugs has not been established. We evaluated the efficacy and toxicity of subsequent therapies in 35 patients after pazopanib progression. On second-line, targeting VEGF was an effective strategy, although OS was not significantly different among patients treated with VEGF targeted therapies or mTOR inhibitors.

Introduction/Background: Patients with metastatic renal cell carcinoma (mRCC) in whom first-line therapies have failed might derive clinical benefit with sequential targeted agents. Limited data are available on the efficacy and toxicity of subsequent therapies after disease progression during pazopanib therapy. **Patients and Methods:** Patients with mRCC who received subsequent systemic treatment after pazopanib treatment failure were identified across 7 institutions. Pazopanib was given as first-line therapy in 28 patients and after cytokines therapy in 7 patients. Clinical outcome and toxicity analyses of 2 sequential treatment options (anti-vascular endothelial growth factor [VEGF] or mammalian target of rapamycin inhibitor [mTORi]) is presented. **Results:** Subsequent therapy was anti-VEGF in 22 patients and mTORi in 13. One patient who received bevacizumab and temsirolimus combination was excluded. VEGF-targeted therapies included sorafenib (n = 10), sunitinib (n = 3), bevacizumab (n = 2), cediranib (n = 4) and cabozantinib (n = 3). Patients treated with mTORi received everolimus. Median progression-free survival was 5.6 months from the start of subsequent therapy with anti-VEGF and 2.4 months with mTORi ($P = .009$). Overall survival (OS) was not significantly different ($P = .68$). Clinical benefit (including partial response and stable disease) on subsequent therapy was observed in 15 patients (64%) and 4 patients (31%) of anti–VEGF- and everolimus-treated patients, respectively ($P = .021$). **Conclusion:** In this retrospective study, targeting VEGF was an effective strategy after disease progression during pazopanib treatment, although OS was not different among patients treated with VEGF or mTORi.

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Keywords: mTOR inhibitor, Pazopanib, Renal cell carcinoma, Sequential therapy, VEGF-targeted therapy

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Introduction

Clear cell renal cell carcinoma (RCC) is the most common subtype of kidney cancer and accounts for approximately 80% of cancers that arise from the renal epithelium.[1] Although surgery is potentially curative at early stages, recurrences will occur in up to 30% of these patients.[2] In the advanced disease, there are now several treatments available that provide a substantial clinical benefit.[3]

Inactivation of von Hippel-Lindau gene in clear cell RCC upregulates hypoxia-inducible factor (HIF) expression.[3] The over-expression of HIFs promotes activation of important pathways

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that regulate angiogenesis such as vascular endothelial growth factor (VEGF) and mammalian target of rapamycin (mTOR).[5,6] This rationale has resulted in multiple targets for therapeutic intervention.

Over the past years, several drugs that target angiogenesis have been developed and have improved the clinical outcome of patients with metastatic RCC (mRCC).[3] VEGF-targeted therapies are the standard first-line treatment for most patients with this disease,[7] with sunitinib being the most widely used agent in this setting. A recent large noninferiority study of >1100 patients (COMPARZ)[8] demonstrated that the efficacy of pazopanib was not inferior to sunitinib in the first-line setting. In addition, a patient preference study showed that patients significantly preferred pazopanib over sunitinib.[9] These results added important information in the decision-making process and pazopanib might become widely used in the first-line setting.[10,11] Despite these advances in the RCC therapeutic armamentarium, the vast majority of patients fail to achieve durable responses,[12] and currently, there are no clinical factors or biomarkers that can predict the targeted therapies to which patients will respond.[13,14]

Although targeted therapies became the cornerstone of the treatment of mRCC, the best sequence of drugs has not been established.[15] Currently, 3 phase III randomized trials: RECORD-1,[16] AXIS,[17] and INTORSECT,[18] have addressed this question. However, there is a lack of data on the efficacy and safety profiles of subsequent therapy after pazopanib treatment failure.

In this multicenter retrospective study, we sought to evaluate the outcome of patients whose disease progressed after pazopanib therapy, analyzing the outcome and tolerability of the 2 sequential treatment options: anti-VEGF or mTOR inhibitors.

Patients and Methods

Patients and Characteristics

We retrospectively collected patient data from 35 patients identified in the databases of 7 institutions (Hospital del Mar, Barcelona, Spain; Dana-Farber Cancer Institute, Boston, MA; GU Center of Excellence Texas Oncology, Dallas, TX; St Bartholomew's Hospital, London, UK; San Matteo University Hospital Foundation, Pavia, Italy; Istituto Toscano Tumori, Arezzo, Italy; and San Camillo and Forlanini Hospitals, Rome, Italy) between 2009 and 2012. The characteristics and outcome of these patients were recorded using standard data collection templates. This study was approved by the local institutional review boards.

Inclusion criteria were the following: (1) patients with mRCC in whom pazopanib therapy had failed and subsequently received another targeted therapy; (2) patients in whom previous cytokine therapy had failed (n = 7) but must have received pazopanib as part of first-line VEGF targeted therapy; (3) patients who switched from sunitinib to pazopanib after receiving ≤ 1 cycle during first-line therapy for toxicity reasons (n = 2).

Further therapy included mTOR inhibitor (everolimus)- or further VEGF-targeted therapy (sorafenib, sunitinib, bevacizumab, cediranib, and cabozantinib). One patient who had data collected was not included in this analysis because of subsequent therapy with a combination of VEGF- and mTOR-targeted therapies as part of a clinical trial (bevacizumab with temsirolimus).

Statistical Analysis

The predefined primary end point of this study was to establish the progression-free survival (PFS) for subsequent therapy after exposure to pazopanib. Secondary end points included a comparison of PFS and OS with mTOR- and VEGF-targeted therapies after pazopanib treatment. PFS was defined as the period from targeted therapy initiation to progression, drug cessation, death, or censored at the last follow-up visit. OS was defined as the period between targeted therapy initiation and the date of death, or censored on the last follow-up visit. Toxicities were assessed according to Common Terminology Criteria for Adverse Events version 4.0 criteria.

Analysis was limited to identify factors that were likely to affect PFS, OS, and toxicity. Potential relationships between patient characteristics (Memorial Sloan-Kettering Cancer Center [MSKCC] and Heng risk scores, age, sex, Eastern Cooperative Oncology Group [ECOG] performance status [PS], and histology) and response were explored. Treatment breaks, dose reduction, and pazopanib taken as first- or second-line therapy were also explored.

We used Kaplan-Meier plots to obtain median PFS and OS estimates for pazopanib treatment and subsequent anti-VEGF and mTOR inhibitor therapies. Log rank tests enabled us to check that the results were statistically significant.

Results

Patient Characteristics

Patient characteristics are outlined in Table 1. Twenty-five percent of all patients had received previous systemic therapy before taking pazopanib including cytokines (n = 7) and sunitinib (n = 2). Both patients who took sunitinib as first-line therapy stopped the drug before completing the first cycle because of toxicity. Therefore, pazopanib was considered to be part of the first-line targeted therapy regimen in these 2 patients.

Outcome With First-Line Therapy

Most patients had a good/intermediate MSKCC score (91%) and Heng prognostic score (71%) at the time of starting pazopanib therapy. MSKCC risk score was not available for 2 patients and 1 patient had a poor MSKCC score. Heng score was not available for 8 patients (23%).

The overall PFS was 7.9 months (range, 0.4-34.8 months). The OS from the time of starting pazopanib was 23.1 months (range, 5.4-68.8 months). Median PFS for patients with good MSKCC prognostic score was 8.0 months compared with 7.5 months for patients with intermediate or poor risk scores, as shown in Table 2 and Figure 1A. OS for good MSKCC risk score patients has not yet been reached and for intermediate and poor risk scores the OS was 28.1 months (Figure 1B).

According to Response Evaluation Criteria In Solid Tumors 1.1, overall partial response (PR) to pazopanib was 40% (14/35 patients). Six of 9 patients with previous cytokine or sunitinib intolerance had a PR with the remainder having stable disease (SD). Eight of 26 patients (31%) given pure first-line pazopanib had a PR (Table 2).

Outcome After Failure of Pazopanib Therapy

Reasons for stopping therapy after first-line pazopanib were progressive disease in 29 patients and cumulative toxicity in 6 patients.

Table 1 Patient Characteristics

Characteristic	Anti-VEGF	mTOR Inhibitors	Total
Patient n	22	13	35
Sex			
Male	21 (95.5)	10 (76.9)	31 (88.6)
Female	1 (4.5)	3 (23.1)	4 (11.4)
Histology, Clear Cell Component	22 (100)	13 (100)	35 (100)
Treatment Before Pazopanib			
INFα	4 (18.2)	2 (15.4)	6 (17.1)
IL-2	1 (4.5)	0	1 (2.9)
Sunitinib	1 (4.5)	1 (7.7)	2 (5.7)
Characteristics at Start of Pazopanib Treatment			
Median age, years	61	63	61
MSKCC risk score			
Good	11 (50)	2 (15.4)	13 (37.1)
Intermediate	9 (40.9)	10 (76.9)	19 (54.3)
Poor	1 (4.5)	0	1 (2.9)
Heng risk score			
Good	10 (45.5)	2 (15.4)	12 (34.3)
Intermediate	5 (22.7)	8 (61.5)	13 (37.1)
Poor	2 (9.0)	0	2 (5.7)
Nephrectomy	20 (90.9)	13 (100)	33 (94.3)
Characteristics at Time of Disease Progression During Pazopanib Therapy			
Median age, years	61	63	62
ECOG PS			
0	9 (40.9)	7 (30.4)	16 (45.7)
1	9 (40.9)	5 (21.9)	14 (40)
2	3 (13.6)	0	3 (8.6)
3	1 (4.5)	0	1 (2.9)
Unknown	0	1 (4.3)	1 (2.9)
Nephrectomy	22 (100)	13 (100)	35 (100)

Data are presented as n (%) except where otherwise noted.
Abbreviations: ECOG PS = Eastern Cooperative Oncology Group performance status; IL = interleukin; INF = interferon; MSKCC = Memorial Sloan-Kettering Cancer Center; mTOR = mammalian target of rapamycin; VEGF = vascular endothelial growth factor.

All patients considered in this analysis had switched to taking either an mTOR inhibitor (n = 13) or further anti-VEGF therapy (n = 22). VEGF-targeted therapies included sorafenib (n = 10), sunitinib (n = 3), bevacizumab (n = 2), cediranib (n = 4), and cabozantinib (n = 3). All 13 patients who received mTOR therapy were treated with everolimus. ECOG PS was recorded at the time at which it was determined that pazopanib therapy had failed and the results are described in Table 1.

Table 2 Pazopanib Treatment

	Good MSKCC	Intermediate/Poor MSKCC
Median Treatment Duration (*P* = .37)	8.0 (5.3-11.0)	7.5 (5.7-13.8)
Overall Survival (*P* = .73)	NYR (15.3-NE)	28.1 (11.2-NE)
Best Response	**Anti-VEGF**	**mTOR Inhibitor**
CR	0	0
PR	8 (36.4)	6 (46.1)
SD	13 (59.1)	4 (30.8)
PD	1 (4.5)	1 (7.7)
Unknown	0	2 (15.4)

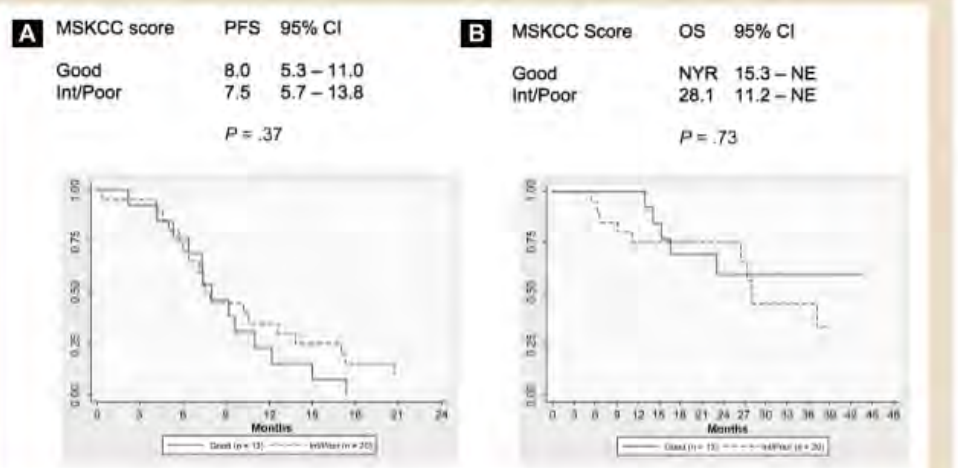
Abbreviations: MSKCC = Memorial Sloan-Kettering Cancer Center; mTOR = mammalian target of rapamycin; NYR = not yet reached; VEGF = vascular endothelial growth factor.

For anti–VEGF-treated patients, the PFS was 5.6 months (range, 0.9-31.4 months) and OS was 17.8 months. With mTOR inhibitor treatment, the PFS was 2.4 months (range, 0.6-12.2 months) and OS was 20.8 months. Only 1 patient (3%) had a PR to therapy, whose subsequent therapy was VEGF-targeted therapy. The patient receiving bevacizumab with temsirolimus was not included in the analysis but achieved a PR to this second-line therapy after has had SD as best response to first-line on pazopanib.

Comparison of VEGF and mTOR Inhibition After Pazopanib Therapy

On univariate analysis, ECOG PS at relapse inicated a significant difference in PFS, but not OS (Fig. 2) in terms of subsequent

Figure 1 (A) Progression-Free Survival and (B) OS With Pazopanib Therapy According to MSKCC Score

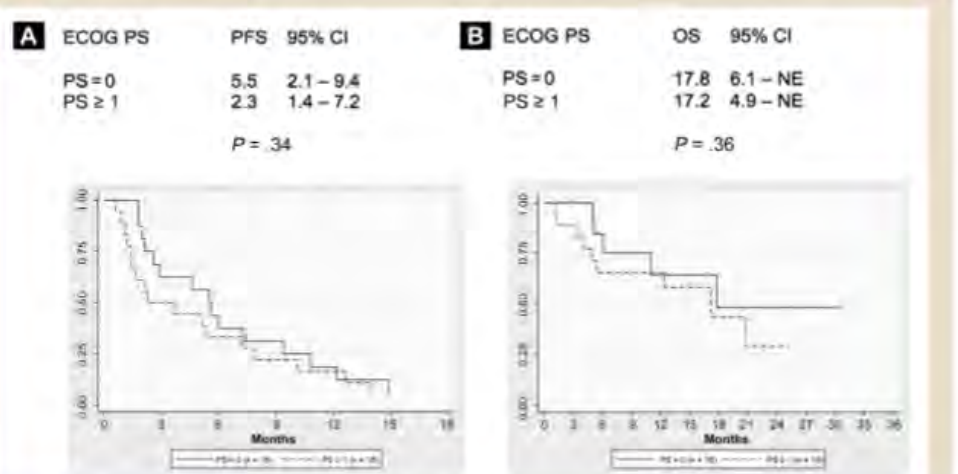


Abbreviations: Int. = intermediate; MSKCC = Memorial Sloan-Kettering Cancer Center; NE = non-estimable; NYR = not yet reached; OS = overall survival; PFS = progression-free survival.

therapy. The PFS and OS for the ECOG PS 0 or 1 groups were 5.5 months (range, 1.8-31.4 months) and 2.3 months (range, 0.6-12.6 months), respectively.

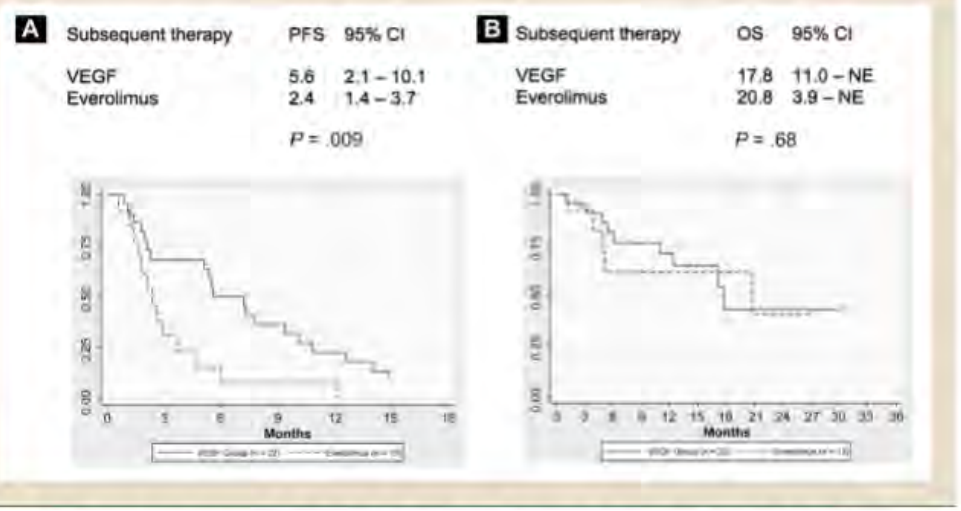
Overall, the median PFS was significantly longer for patients given anti-VEGF than for mTOR inhibitor therapy (5.6 months and 2.4 months, respectively; unadjusted $P = .009$; Fig. 3). There were slightly fewer patients with ECOG PS 0/1 who were given mTOR inhibitors than were given VEGF inhibitors, but also more patients with ECOG PS 2/3 who were given VEGF inhibitors than were given mTOR inhibitor therapy (Table 1).

Figure 2 (A) Progression-Free Survival After Pazopanib Therapy and (B) OS From the Start of Subsequent Therapy According to ECOG PS 0 and ECOG PS ≥1



Abbreviations: ECOG = Eastern Cooperative Oncology Group; NE = non-estimable; OS = overall survival; PFS = progression-free survival; PS = performance status.

Figure 3 (A) Progression-Free Survival After Pazopanib Therapy With VEGF- and mTOR-Targeted Therapies, and (B) OS From the Start of Subsequent Therapy



Abbreviations: mTOR = mammalian target of rapamycin; NE = non-estimable; OS = overall survival; PFS = progression-free survival; VEGF = vascular endothelial growth factor.

Clinical benefit (PR + SD) was observed in 15 patients (64%) and 4 patients (31%) of anti-VEGF and everolimus-treated patients, respectively (unadjusted $P = .021$). The median OS for anti-VEGF and mTOR inhibitors were 17.8 and 20.8 months, respectively, from the starting point of therapies (unadjusted $P = .68$) (Table 3).

Toxicity After Pazopanib Treatment: Anti-VEGF Versus mTOR Inhibitors

Overall, toxicities in both groups are summarized in Table 4. Treatment interruption for more than 7 days occurred in 30.8% of the mTOR group, compared with 21.7% of the VEGF-treated patients.

Discussion

Multiple retrospective analyses provided the first insights on the role of subsequent therapies in mRCC, showing benefits of second-line therapies with an acceptable toxicity profile.[15,19,20]

The sequential administration of sunitinib after sorafenib and vice versa has provided a rationale for the sequential administration of VEGF-targeted therapies. Interestingly, in 3 retrospective studies, PFS was longer in patients who were treated with the sequence sorafenib then sunitinib compared with sunitinib then sorafenib.[20-22] Based on these results, a phase III, randomized trial (SWITCH) was designed to validate these findings (NCT00732914). Another

Table 3 Response to Further Therapy After Pazopanib Therapy

Variable	Anti-VEGF	mTOR Inhibitors	Total
Median Second-Line Treatment Duration ($P = .009$)	5.6 (2.1-10.1)	2.4 (1.4-3.7)	—
Treatment Interruption			
Total	4 (18.2)	4 (30.8)	8 (22.9)
<7 Days	3 (13.6)	4 (30.8)	7 (20)
Dose Reduction	7 (31.8)	3 (23.1)	10 (28.6)
Subsequent Third-Line Treatment	10 (45.5)	9 (69.2)	19 (54.3)
Overall Survival From Second-Line Treatment Start ($P = .68$)	17.8	20.8	17.8
Best Response			
CR	0	0	0
PR	1 (4.5)	0	1 (2.9)
SD	14 (63.6)	4 (30.8)	18 (51.4)
PD	4 (18.2)	8 (61.5)	12 (34.3)

Abbreviations: mTOR = mammalian target of rapamycin; VEGF = vascular endothelial growth factor.

Table 4 Toxicity After Pazopanib Treatment

	Anti-VEGF		mTOR Inhibitors	
	G1-2	G3-4	G1-2	G3-4
Hand-foot Syndrome	6 (27.3)	2 (9.0)	0	0
Rash	5 (22.7)	0	0	0
Asthenia	11 (50)	0	4 (30.8)	0
Diarrhea	7 (31.8)	0	0	0
Hypertension	1 (4.5)	0	0	0
Stomatitis	3 (13.6)	0	0	1 (7.7)
Anemia	1 (4.5)	1 (4.5)	0	1 (7.7)
Nausea	3 (13.6)	0	0	0
Hepatotoxicity	0	0	1 (7.7)	0
Thrombocytopenia	1 (4.5)	0	0	1 (7.7)
Respiratory	2 (9.0)	0	3 (23.1)	0
Edema	1 (4.5)	0	1 (7.7)	0
Metabolic (Hyperglycemia, Hypercholesterolemia, Hypomagnesemia, Hypothyroidism)	5 (22.7)	0	2 (15.4)	0
Leucopenia	1 (4.5)	0	0	0
Fever	0	0	2 (15.4)	0
Other	13 (59.0)	0	3 (23.1)	0
Laboratory Findings (Creatine Kinase Increase, Hyperamylasemia, Creatinine Increase)	3 (13.6)	0	1 (7.7)	0

Abbreviations: G = Grade; mTOR = mammalian target of rapamycin; VEGF = vascular endothelial growth factor.

phase III trial, (SWITCH-II), is designed to evaluate the efficacy and safety of sorafenib followed by pazopanib versus pazopanib followed by sorafenib in the treatment of mRCC (NCT01613846).

Sorafenib was evaluated after bevacizumab or sunitinib progression and there was limited efficacy of this agent in a small number of patients who were refractory to VEGF-targeted therapies.[24] These findings were corroborated by another phase II trial that enrolled 52 patients to receive sorafenib as second-line therapy after disease progression with sunitinib therapy.[24]

Trying to answer the question of anti-VEGF treatment followed by another anti-VEGF treatment versus anti-VEGF followed by mTOR inhibitor treatment, data from the International Metastatic RCC Database Consortium reported the results from a large retrospective analysis of 216 mRCC patients in whom VEGF-targeted therapy had failed and then received second-line therapy. There was no significant difference in OS among patients treated with VEGF-targeted therapies or mTOR inhibitors, and targeting VEGF was an active strategy resulting in a longer time to treatment failure.[13]

Although the best sequence is not yet established, there is a rationale to switch a drug to another with a different mechanism of action.[25,26] To answer this question, the RECORD-1 trial evaluated the role of mTOR inhibitors after disease progression during anti-VEGF therapy. This phase III, randomized study showed a significantly better PFS in patients who received mTOR inhibitors compared with placebo after failure of VEGF-targeted therapy (sunitinib or sorafenib) in the first-line setting.[16] Another study (RECORD-3) compared the sequence, everolimus then sunitinib versus sunitinib then everolimus. Median OS was 22.4 months for everolimus then sunitinib therapy and 32.0 months for sunitinib then everolimus therapy.[27] Although the primary end point of this trial was PFS with sunitinib versus everolimus in the first-line setting, the analysis of the sequential therapy considering PFS on first-line and PFS on second-line (secondary end points) showed a trend for an OS benefit in patients who were treated with the sequence sunitinib then everolimus rather than everolimus then sunitinib. Results from the final OS analysis are awaited to confirm this difference.

Direct comparison between a second-line VEGF-targeted therapy and second-line mTOR inhibitor therapy have been reported in the INTORSECT trial. This trial compared temsirolimus versus sorafenib in patients in whom first-line sunitinib treatment had failed. Results showed no significant difference in PFS, which was defined as the primary end point. However, patients who received sorafenib experienced longer OS (16.6 vs. 12.3 months; $P = .01$).[18] These data raise questions about the role of PFS as a valid surrogate marker to evaluate outcome in this setting and the time required to achieve the best outcome using the same VEGF-targeted therapy.[28] These findings support the hypothesis that continuing blockade of the VEGF pathway might be a better option than mTOR inhibitor therapy and that the biology of mRCC might be altered by first-line therapy.

A phase III, randomized trial (METEOR) will evaluate the effect of cabozantinib, an anti-VEGF therapy, compared with everolimus on PFS and OS in mRCC patients who have progressed after previous VEGF-targeted therapy including pazopanib (NCT01865747). It is important to note that the anti mesenquymal epithelial transition factor activity of cabozantinib might influence the nature of response.

The prospective sequential use of a first-line VEGF-targeted therapy followed by 2 different anti-VEGF therapies has been studied in the AXIS trial. Rini and collegues conducted a trial comparing axitinib versus sorafenib in patients whose disease had

progressed after sunitinib or cytokine therapy. The PFS favored axitinib in both groups, albeit the benefit in patients who were treated with sunitinib in the first-line setting was modest (4.8 vs. 3.4 months; $P = .011$). These results led to approval of this drug by regulatory agencies and support the use of axitinib after disease progression during sunitinib therapy.[17]

An indirect comparison between axitinib and everolimus after sunitinib treatment failure using the results from the phase III trials suggest a similar efficacy (PFS 4.9 months for everolimus and 4.8 months for axitinib)[18,27] of both sequences.[18,26] These results support the hypothesis that the 2 drugs could be part of the sequential treatment in advanced RCC and the decision of which sequence to use needs to be based on other end points.

An important question is how to explain the benefit of sequential use of an anti-VEGF treatment followed by another anti-VEGF treatment. Distinct mechanisms of resistance of these agents have been proposed, based on their individual pharmacokinetics and different affinities for target kinases,[28] supporting the lack of cross-resistance in this class of drugs.[29] As an example, 1 patient who had SD with pazopanib treatment achieved a PR with sorafenib as a subsequent therapy (PFS = 13.8 months). In addition, it is not known if combination therapy targeting multiple pathways could compensate for resistance in 1 of these targets. Although the combination of antiangiogenic drugs did not increase PFS in the first-line setting,[30] the role of this strategy was being evaluated in the second-line setting in a phase III randomized trial which combined everolimus and bevacizumab versus everolimus alone (NCT01198158).[14] Unfortunately, this study closed for poor accrual.

As seen in previous reports,[15,31] our results showed a longer PFS with VEGF-targeted therapy compared with everolimus, with a good safety profile. This is provocative and needs to be interpreted with caution, because results could be explained by a selection bias. Imbalances in MSKCC risk score at pazopanib treatment initiation, differences in ECOG PS, and also specific reasons to receive a second-line mTOR inhibitor like cumulative toxicity to previous anti-VEGF therapy might also explain these different results. It is also noteworthy that OS was not significantly different in a comparison of these 2 groups of agents.

Our study has several limitations. First, the retrospective analysis could result in missing data or patient characteristics that were not collected which might lead to a selection bias. Although we have selected patients across 7 institutions, the sample size was small with only 35 patients included. In addition, the mTOR inhibitor-treated subgroup was significantly smaller than the VEGF inhibitor-treated group and differences for choosing second-line therapy were not controlled. Thus, a comparison between these 2 different cohorts limits the conclusion. Moreover, different types of VEGF-targeted therapies, including some agents that are not yet approved as standard treatment, were used as part of clinical trials and could certainly have an effect on our results.

This is, to our knowledge, the first retrospective analysis to address clinical outcomes of patients who receive therapies after pazopanib therapy. Our results suggest that targeting VEGF after pazopanib treatment is an effective and tolerable strategy. The PFS and OS from the time of starting second-line therapy are what one would expect after treatment failure with sunitinib.

Conclusion

Randomized data support the use of sequential therapy in mRCC, although the best sequence of agents is not well established. Our findings support the use of VEGF-targeted therapy and mTOR inhibitors in patients whose disease has progressed after first-line pazopanib therapy. Additional efforts must be done to identify predictive biomarkers for selected agents and results from ongoing clinical trials are awaited.

Clinical Practice Points

- Sequential therapy may benefit patients with metastatic RCC who failed to first-line. However, the best sequence of drugs has not been established.
- Limited data is available on the efficacy and toxicity of subsequent therapies after progression on pazopanib.
- In this retrospective study, we sought to evaluate subsequent therapies in 35 patients who received pazopanib on first-line.
- Targeting VEGF was an effective and tolerable strategy after pazopanib progression, although OS was not significantly different among patients treated with VEGF targeted therapies or mTOR inhibitors.
- Additional efforts must be done to identify predictive biomarkers for selected agents and to optimize the therapy in patients with metastatic RCC.

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Disclosure

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APÊNDICE H - HER2 as a target in invasive urothelial carcinoma

Cancer Medicine

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ORIGINAL RESEARCH

HER2 as a target in invasive urothelial carcinoma

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ERBB2, genomic alterations, HER2, prognosis, urothelial carcinomas

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Abstract

We evaluated primary tumors from two cohorts, Spain ($N = 111$) and Greece ($N = 102$), for patients who were treated with platinum-based chemotherapy. Patients were tested for HER2 status (IHC score of 3+ or FISH ratio of ≥2.2) by immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), DNA copy number, mRNA expression, and mutation status in patients with metastatic urothelial carcinoma (UC), and its impact on survival. *ERBB2* mutation was determined by hotspot sequencing. mRNA expression was assessed using NanoString counting. Association of overall survival (OS) and HER2 status was assessed by a Cox regression model. NIH-3T3 cells containing HER2 V777L were assessed for growth, invasion, and HER2 kinase activation. In all, 22% of Spanish and 4% of Greek cohorts had 3+ HER2 staining by IHC. FISH amplification was identified in 20% of Spanish and 4% of Greek cohorts. Kappa coefficient between FISH and IHC was 0.47. HER2 status was not associated with OS in univariate (Spanish $P = 0.34$; Greek $P = 0.11$) or multivariate (Spanish $P = 0.49$; Greek $P = 0.12$) analysis. HER2-positive tumors expressed higher levels of HER2 mRNA than HER2-negative tumors ($P < 0.001$). HER2 mutations (V777L and L755S) were identified in two (2%) patients. In vitro analysis of V777L results in transformation of NIH-3T3 cells, leading to increased growth, invasion on soft agar, and HER2 kinase constitutive activation. In summary, HER2 overexpression or amplification in the primary tumor did not predict OS in patients with metastatic UC. HER2 positivity rates can differ between different populations. Further trials in genomically screened patients are needed to assess HER2-targeted therapies in UC.

Introduction

Of all patients diagnosed with urothelial carcinoma (UC), roughly 20% will present with metastatic UC, and another 20% will progress to metastatic disease over time, which is nearly uniformly fatal [1]. Although untreated UC is frequently chemosensitive, nearly all tumors become resistant to standard platinum-based combination therapies. Unfortunately, the treatment of metastatic UC has not improved significantly in 20 years, in part, due to the lack of validated therapeutic targets beyond cytotoxic agents.

Human epidermal growth factor receptor 2 (HER2) overexpression (encoded by the *ERBB2* gene) has long been a prognostic marker and predictive tool in the treatment of breast cancer, and more recently in esophagogastric cancer [2, 3]. In breast cancer, overexpression and *ERBB2* DNA amplification are generally closely linked. For specimens with intermediate HER2 protein expression, fluorescence in situ hybridization (FISH) identifies patients which will benefit from HER2-targeted therapies [4].

In addition to amplification and overexpression, mutations in *ERBB2* have been reported in multiple cancer types, including UC, and are oncogenic in vitro [5–8]. Recently, mutations in the extracellular domain of *ERBB2* were found to be present in 40% of micropapillary UC [9]. Since extracellular domain mutations may confer sensitivity to *ERBB2* kinase inhibitors, these findings may result in new therapeutic opportunities in selected UC patients [7].

Similar to breast cancer, the mechanisms of UC HER2 overexpression include DNA amplification and/or protein overexpression. Reports of HER2 overexpression in UC have demonstrated frequencies of alteration ranging from 6% to 80% [10–18].

Several studies have shown a significantly higher incidence of HER2-positive tumors in advanced disease and metastases, suggesting HER2 may not only be a biomarker for more aggressive disease but also a potential therapeutic target [11, 13]. However, other studies have found no such association [1, 10, 11, 14–17, 19–21]. Furthermore, the association between HER2 status and overall survival (OS) in UC remains unclear with published studies providing conflicting results [22–24].

To address these issues, we undertook an analysis of HER2 in bladder cancer in patients who developed metastatic disease, by evaluating immunohistochemical (IHC) staining for HER2, FISH for *ERBB2* on two cohorts of patients, targeted *ERBB2* mutation hotspot sequencing, mRNA expression by NanoString, and *ERBB2* copy number by array-based comparative genomic hybridization (aCHG) in primary tumors from one of these cohorts. For selected hotspot mutations, in vitro evaluation of their oncogenic potential was undertaken in NIH-3T3 cells.

Methods

Patients

Patients from two cohorts were used for this analysis. One cohort of 111 patients was obtained from biospecimen banks from three Spanish hospitals (University Hospital del Mar in Barcelona, Hospital Parc Taulí in Sabadell, and Fundación Jimenez Diaz in Madrid). Each patient received platinum-based combination chemotherapy for metastatic disease. The other cohort of 102 consisted of patients treated on a phase III study of dose-dense gemcitabine and cisplatin or dose-dense MVAC (methotrexate/vinblastine/doxorubicin hydrochloride/cisplatin) for metastatic UC, as well as some patients treated with gemcitabine and carboplatin [25]. Formalin-fixed paraffin-embedded tissue (FFPE) was collected from prior transurethral resection or cystectomy. All the translational studies were performed using standard protocols in the Cytogenetics Laboratory and the Center for Molecular Oncologic Pathology (CMOP). All cases were collected under Institutional Review Board (IRB)-approved protocols at the different institutions, de-identified and approved for use by the Dana-Farber Cancer Institute IRB.

Tissue preparation

Slides from FFPE tissue blocks were evaluated by two genitourinary pathologists (D. M. B. and J. A. B.). Tumor-bearing areas were identified, and 0.6-mm cores were taken for tissue microarray (TMA) construction and DNA extraction. Each specimen was represented in triplicate in the TMA.

Immunohistochemistry

Detailed laboratory methods can be found in Data S1. Tumor samples on TMAs in triplicate were analyzed for HER2 expression. For all cases, the assessment of HER2 was performed using the 2010 USCO/CAP HER2 guidelines as established for breast cancer [26], which has been previously employed to assess HER2 expression in bladder cancer [16, 27]. HER2 staining and its categorization based on localization and intensity is depicted in Figure 1A. All cases were scored for HER2 IHC status by a single pathologist (E. S.). For any sample with a score of less than 3+, status was validated by FISH.

FISH

To assess the genetic status of *ERBB2*, FISH was performed on FFPE tissue from TMAs. Detailed laboratory methods can be found in Data S1. Assessments of ratios below 1.8 were considered negative and ratios more than 2.2 were considered positive for *ERBB2* gene amplification. Ratios varied between 1.8 and 2.2 were considered as equivocal. In these cases, 60 additional cells were analyzed by a second scorer to obtain a conclusive result. When the average number of chromosome 17 signal numbers exceeded 2.5 per cell, the case was considered

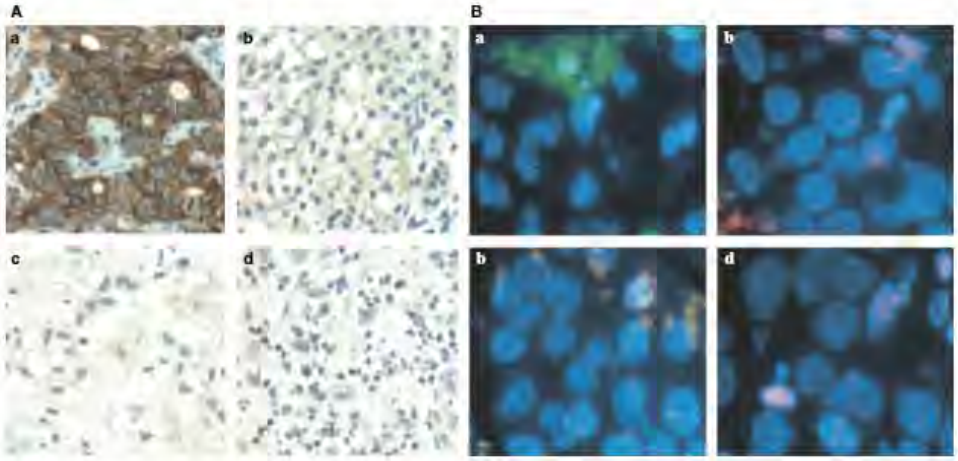

Figure 1. (A) HER2 expression in urothelial carcinoma. (a) HER2-positive staining scored as 3+, showing heavy membranous HER2 staining. (b) Moderate HER2 staining scored as 2+, demonstrating moderate membranous HER2 staining. (c) Weak HER2 staining, which demonstrates weak membranous staining which is scored as 1+. (d) HER2-negative urothelial cancer, showing no membranous HER2 expression, and scored as 0. (B) *ERBB2* status in urothelial carcinoma. (a) Normal, nonamplified (ratio = 1), (b) polysomic, nonamplified (ratio = 1), (c) amplified (ratio = 3.5), and (d) amplified (ratio = 5). HER2, human epidermal growth factor receptor 2.

polysomic. Representative examples of HER2 FISH are shown in Figure 1B.

Copy number analysis

Copy number variation (CNV) was evaluated only in the Spanish cohort by aCGH. Detailed laboratory methods can be found in Data S1. CGH Analytics software version 3.4 (Agilent Technologies, Santa Clara, CA, US) was used to analyze the aCGH data. *ERBB2* copy number gain was determined as specimens with a log base 2 ratio greater than 0.9.

mRNA analysis

Total RNA was extracted from tumor specimens following manufacturer's protocols (Ambion RecoverAll, Life Technologies, Grand Island, NY). mRNA transcript expression of HER2 was quantified using color-coded oligonucleotides, synthesized by NanoString Technologies, Seattle, Wa, US and hybridized to these transcripts. Transcripts were counted using the automated NanoString nCounter® system. Counts were normalized with the nSolver Analysis Software (version 1.0) in which mRNA expression was compared to internal NanoString Technologies, Seattle, Wa, US controls, several housekeeping genes (*ACTB*, *GAPDH*, *HPRT1*, *LDHA*, *PFKP*, *PGAM1*, *STAT1*, *TUBA4A*, *VIM*), and invariant genes (*ANGEL1*, *DDX19A*, *NAGA*, *RPS10*, *RPS16*, *RPS24*, *RPS29*) in UC. These invariant genes were identified by analyzing gene expression variances in several published datasets [28, 29]. Differential expression of HER2 status versus wild-type tumors was calculated with the edgeR package [30].

Mutation status

For each sample, 100 ng of tumor-derived genomic DNA was subjected to whole genome amplification. Next, regions containing loci of interest were amplified using polymerase chain reaction and then mass spectrometric genotyping using iPLEX: Sequenom, San Diego, Ca, US chemistries was performed. An automated mutation-calling algorithm was performed to identify candidate mutations. Putative mutations were further filtered by a manual review and selected for validation using multibase homogenous Mass-Extend (hME) chemistry. Only mutations found in iPLEX and confirmed by hME were considered validated mutations. *ERBB2* hotspot mutations sequenced are listed in Table S1.

Soft agar assays

NIH-3T3 cells (ATCC Cell Lines, Middlesex, UK) transfected with pBabe-puro constructs containing mutant *ERBB2* cDNAs were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Cellgro/Mediatech,

Manassas, Va, US) supplemented with 10% calf serum (Invitrogen, Life Technologies, Carlsbad, Ca, US). Soft agar assays were performed as described previously [31].

Statistical analysis

Fisher's exact tests were used to measure associations between patient clinical characteristics and HER2 IHC or *ERBB2* FISH amplification. Since there is no standard scoring for HER2 in bladder cancer, we followed the protocol used for breast cancer: all specimens that scored either 3+ by IHC or a ratio of greater than or equal to 2.2 by FISH were considered positive. OS was defined as the time from start of treatment for metastatic disease to death or last follow-up.

Kaplan–Meier method was used to summarize the median OS, and Cox proportional hazard models were used to assess the associations of HER2 positivity and OS.

Results

HER2 status

Table 1 summarizes baseline patient characteristics for all patients with clinical information (Spanish $N = 111$; Greek $N = 102$). The number of patients with available HER2 status is lower because of tissue fall-off during antigen retrieval and/or hybridization procedures, age-dependent decrease in DNA integrity, or a lack of neoplastic tissue within the TMA sample. Similarly, aCGH data were indeterminate for 17 patients in the Spanish cohort, most likely due to inefficient hybridization as a result of fragment and crosslink-dependent decrease in DNA integrity.

Table 1. Patient clinical characteristics and outcomes for any patient with HER2 or clinical data.

	Spanish ($N = 111$), N (%)	Greek ($N = 102$), N (%)
Eastern Clinical Oncology Group Performance Status (ECOG PS)		
0	40 (36)	57 (56)
1, 2	71 (64)	40 (39)
Missing	0	5 (5)
Visceral disease		
Yes	42 (38)	38 (37)
No	69 (62)	58 (57)
Missing	0	6 (6)
Complete response		
Yes	25 (23)	10 (10)
No	81 (73)	87 (85)
Unevaluable	5 (5)	5 (5)
Death		
Yes	57 (51)	66 (65)
No	54 (49)	33 (32)
Missing	0 (0)	3 (3)

HER2, human epidermal growth factor receptor 2

Table 2. Frequency of IHC 3+, FISH amplification, and aCGH gain.

	Spanish, N (%)	Greek, N (%)
FISH		
Normal	57 (80)	90 (96)
Amplified	14 (20)	4 (4)
IHC		
Negative (scored 0, 1+, 2+)	69 (78)	89 (96)
Positive (scored 3+)	19 (22)	4 (4)
aCGH		
Negative	80 (85)	
Positive	14 (15)	

IHC, immunohistochemistry; FISH, fluorescence in situ hybridization; aCGH, array-based comparative genomic hybridization.

ERBB2 amplification by FISH was more frequent in the Spanish cohort compared to the Greek cohort (14/71 vs. 4/94, $P = 0.006$; Table 2). Similarly, IHC 3+ was more common in the Spanish cohort (19/88 vs. 4/93, $P = 0.00058$). The concordance rate between FISH and IHC was relatively low, indicated by a Kappa statistic of 0.47 (Table S2). These results are visualized in Figure 2.

Survival analysis and association of HER2 status with clinical characteristics

Based on 3+ IHC and/or FISH ratio ≥2.2, 26 patients for Spanish and 5 patients for Greek cohorts were HER2-positive. Due to the differences between the rates of HER2 positivity, we analyzed the Spanish and Greek cohorts separately. We assessed the association of HER2 status and OS in both univariate and multivariate analysis and found no significant associations in either cohort

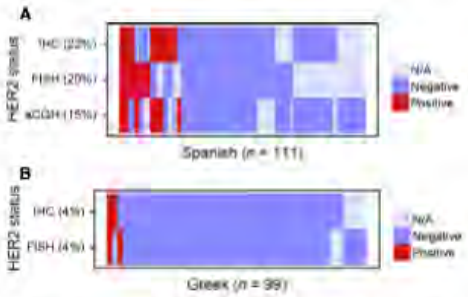

Figure 2. Heatmap of (A) Spanish cohort and (B) Greek cohort. Visualization of HER2 status based on different methodologies. N/A – data not available. HER2, human epidermal growth factor receptor 2.

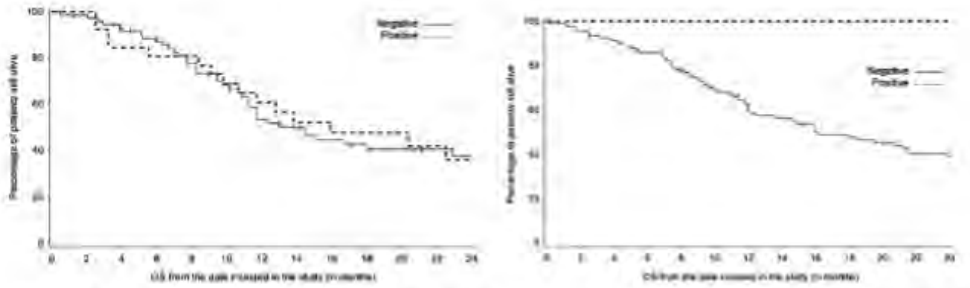

Figure 3. MOverall survival by HER2 status for Spanish and Greek cohorts. No difference in overall survival was observed in the two cohorts. For the Spanish cohort, the hazard ratio was 0.94 (95% CI = 0.52–1.70, $P = 0.83$) and for the Greek cohort, the hazard ratio was 0.2 (95% CI = 0.03–1.48, $P = 0.11$). Multivariable analysis incorporating known prognostic factors showed similar nonsignificant results (Tables S2 and S3). HER2, human epidermal growth factor receptor 2.

(Fig. 3). We also tested associations between HER2 status and clinical characteristics and found no significant associations between prognostic variables or response and HER2 status in either cohort. Detailed result tables can be found in Tables S3A and S3B.

Mutation hotspot sequencing

ERBB2 mutations were identified at amino acid 755 and 777 in two (2%) patients in the Spanish cohort. These mutations were L755S and V777L. The specimen containing mutation L755S was also HER2-positive by IHC and copy number by aCGH, but HER2-negative by FISH. No HER2 IHC or FISH data for the specimen with mutation V777L were available.

mRNA expression

HER2-positive tumors had increased levels of HER2 mRNA by NanoString in both the Spanish and Greek cohorts. The results are visualized in box plots in Figure 4.

Functional analysis of HER2 V777L

Wild-type and mutant HER2 were ectopically expressed in murine NIH-3T3 cells and tested for oncogenic activity by assessing anchorage-independent proliferation in soft agar. Although HER2 V777L supported soft agar colony formation, two other mutants reported in the COSMIC database, V777A and V777M, did not (Fig. S1). HER2 C334S, a highly oncogenic extracellular domain mutant [7], was used here as a positive control. All HER2 mutants were expressed to similar levels, with the V777L mutant also exhibiting an increase in C-terminal phosphorylation (Fig. S2). These data are consistent with previous reports [32].

Discussion

The impact of HER2 status on prognosis in metastatic UC has been controversial. To address the impact of

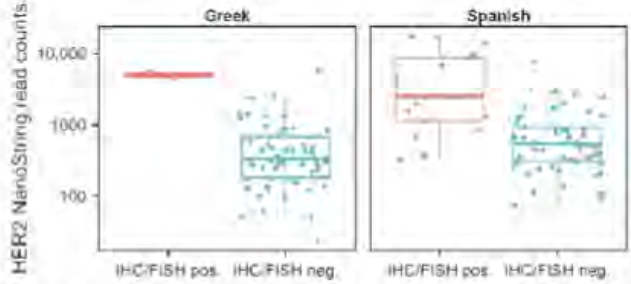

Figure 4. HER2 status and mRNA expression in Spanish and Greek cohorts.. The NanoString distributions of read counts HER2-positive and HER2-negative patients are visualized with box plots for both cohorts. HER2, human epidermal growth factor receptor 2.

HER2 status on survival in patients with metastatic UC, this study analyzed the primary tumors of two clinically characterized cohorts of 111 and 102 patients with UC that would later develop metastases using standard clinical tests, IHC and FISH. To further explore the pathway, we performed aCGH, although was only technically possible in one cohort. Between the two cohorts, we found that 16% of primary tumors demonstrated either IHC 3+ or FISH amplification. In addition, the concordance between FISH and IHC results was low, with many IHC-positive samples being FISH negative (Table S5). HER2-negative IHC staining demonstrated a high predictive value and specificity for negative *ERBB2* gene amplification, suggesting that it is a reasonable screening test for HER2 status in bladder cancer. IHC sensitivity for gene amplification is quite low (53%). We analyzed each cohort separately for clinical outcomes, and no significant associations between HER2 status and clinical outcomes were observed when controlling for known prognostic factors. While some clinical characteristics of the two cohorts were different, investigating both cohorts allows us to analyze the HER2 status across a large population of patients with metastatic UC.

The dependence of cancer cells on oncogenes for proliferation is well known. Whether HER2 is truly oncogenic in UC is not clear. However, we show that HER2 mRNA expression was increased in those tumors that were HER2-positive by IHC and FISH. These findings are similar to those found in HER2-positive breast cancer [33], suggesting that in fact the genomic and IHC findings in UC highlight an oncogenic dependence on the pathway in selected tumors.

HER2 copy number gains were also assessed by aCGH in the Spanish cohort as an exploratory analysis, and while there was significant overlap with the other modalities of assessing HER2, there were many specimens which were discordant (Fig. 3). Since aCGH integrates the results of all cells within a sample, it is not capable of distinguishing heterogeneity within a specimen, compared to FISH.

We expected to find similar frequencies of HER2 in both cohorts due to use of the same methodologies in the same laboratories and their similar clinical outcomes. Interestingly, there were significant differences between the Spanish and Greek cohorts, where 27% and 4% had HER2 overexpression and/or amplification, respectively. The large difference observed in these series suggests that HER2 status varies between populations, and raises the hypothesis that there is significant etiologic heterogeneity within bladder cancer that can lead to these differences. While we cannot rule out that subtle differences in fixation and storage could contribute to changes in HER2 antigenicity and *ERBB2* DNA, it is unlikely that these would affect DNA and proteins in the same manner.

Recently, Ross and colleagues reported the results of next-generation sequencing in 35 patients with UC. In this study, two (6%) patients presented genomic alterations in ERBB2: one patient with gene amplification and the other with mutation (S310F) [34]. In addition, The Cancer Genome Atlas Project (TCGA) performed an integrated analysis to characterize molecular alterations in 131 patients with high-grade UC. This study identified mutations in 32 genes. Mutations or amplifications in *ERRB2* were also identified in 9% of patients [35]. Interestingly, some of these molecular alterations are similar to those found in the TCGA for breast cancer, suggesting that these two tumors may share pathways for tumor progression. Interestingly, a high frequency (40%) of activating extracellular domain HER2 mutation has been detected in the infrequently found histological variant of micropapillary UC.

We identified a low frequency of *ERBB2* activating mutations in patients who developed metastatic UC. Not using next-generation sequencing might have overlooked the presence of some mutations. No patient in our series was described to have the micropapillary histological variant. Two mutations were identified by hotspot sequencing, both of which have been documented in other tumor types. HER2 L755S is a mutation identified in breast, gastric, colon, and lung cancers. In vitro testing indicates that this mutation confers resistance to lapatinib [32]. In addition, HER2 V777L has also been documented as an oncogenic mutation in gastric and breast cancer, and remains sensitive to lapatinib [32]. Although these molecular alterations have been identified in low frequencies, it may represent potential therapeutic targets in a specific subset of patients. In addition, future functional analysis of those mutations will be important to determine whether they represent targets for HER2-directed therapy.

While HER2 alterations do not lead to poorer outcomes in patients with advanced disease in this dataset, these findings do not exclude the utility of HER2 as a promising therapeutic target in UC. The presence of activating mutations in a small number of patients, as well as evidence of copy number gain and mRNA and protein overexpression, all suggest the importance of HER2 to the oncogenic phenotype of a subset of bladder cancers, and likely represents a therapeutic opportunity in a selected patients with locally advanced and metastatic UC.

Further work will be needed to ascertain the frequency of HER2 alterations in UC metastases and to examine the extent of HER2 concordance between primary and metastatic tissue, as there is some evidence that HER2 expression is increased in UC metastases [36]. The addition of trastuzumab to cytotoxic chemotherapy in patients with evidence of HER2 expression was tested in a phase II study, and showed high levels of activity, although the

study was designed to test safety and not efficacy [37]. Single agent lapatinib was tested in unselected second line patients, and did not show significant evidence of anticancer activity [38]. An ongoing randomized phase III study of maintenance lapatinib in UC patients with HER2 2 to 3+ IHC is ongoing (NCT00949455) and might shed additional light. Appropriately designed trials enriched for patients with HER2 alterations are needed to determine whether HER2-targeted therapies provide benefit.

The strengths of this study include the detailed clinical information available, the uniform treatment with platinum-based combinations (real-world use), the use of routine clinical tests to measure HER2 alterations (IHC and FISH), and findings of rare oncogenic mutations in bladder cancer. Limitations include the targeted rather than whole gene sequencing of *ERBB2*, which may underestimate the frequency of mutations and the relatively small sample size. In addition, we evaluated a selected group of patients which portend a poor prognosis and it can influence the association between HER2 status and survival outcomes in this population. Finally, we believe that exploratory analysis regarding clinical outcome or other clinical parameter will not produce reliable results. So, these correlations were not performed; thus, these results are hypothesis generating and require further external validation.

In summary, the impact of HER2 status on UC prognosis remains controversial. Our data suggest that neither IHC nor *ERBB2* gene amplification in the primary tumor play a role in prognosis after diagnosis of metastatic disease. However, the strong association between HER2 status and mRNA expression suggests that this pathway is active in selected cancers, and may yet represents a therapeutic target in UC.

Acknowledgments

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. NIH-3T3 soft agar assay. HER2 V777L leads to growth in soft agar compared to other V777 mutations. HER2 C334S was used as a positive control and the kinase-inactive mutant D845A was used as a negative control.

Figure S2. NIH-3T3 *ERBB2* western blot. HER2 V777L is associated with increased C-terminal phosphorylation.

Table S1. *ERBB2* mutation hotspots.

Table S2. Concordance between FISH and IHC.

Table S3. Association between HER2 status and OS from metastatic disease for (A) Spanish cohort and (B) Greek cohort.

Table S4. Association of HER2 status with prognostic variables and treatment response.

Table S5. Concordance IHC status versus FISH status.

Data S1. Supplementary Methods

APÊNDICE I - Perioperative Therapy for Muscle Invasive Bladder Cancer

Perioperative Therapy for Muscle Invasive Bladder Cancer

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KEYWORDS

• Urinary bladder neoplasms • Neoadjuvant therapy • Adjuvant therapy • Chemotherapy • Radiotherapy • Radical cystectomy • Chemotherapy

KEY POINTS

- Bladder cancer has a high incidence of local and distant recurrence, which may be the result of micrometastatic disease at the time of localized treatment.
- Eradicating deposits of micrometastases from bladder cancer is best achieved via perioperative systemic neoadjuvant or adjuvant therapy.
- Postcystectomy nomograms and risk stratification help to identify patients who may benefit from adjuvant therapy.
- Use of platinum-based combination chemotherapy in the neoadjuvant setting improves survival. Adjuvant chemotherapy is also beneficial, although the evidence is less robust.
- Investigation of molecular pathways underlying bladder cancer has led to the discovery of genomic alterations, which may lead to the development of patient-specific therapies.

INTRODUCTION

Urothelial carcinoma (UC) of the bladder is the fourth most commonly diagnosed malignancy in the United States. About 20% to 30% of patients present with muscle invasive (≥T2) bladder cancer (MIBC).[1] Initial treatment for most of these patients consists of localized therapy, including surgery or radiation; however, the risk of recurrence after localized therapy exceeds 50%,[2] and the 5-year mortality rate ranges from 33% to

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0889-8588/15/$ – see front matter

73%.[3] It is thought that the high incidence of local and distant recurrence is due to micrometastatic disease at the time of localized treatment. Therefore, perioperative systemic therapy is often used in the form of neoadjuvant or adjuvant therapy, with the goal of eradicating deposits of micrometastases.

Based on level I evidence (meta-analysis of randomized trials), the current gold standard for the treatment of MIBC is neoadjuvant cisplatin-based chemotherapy, followed by surgery, which shows an increased overall survival benefit of 5%.[4] Despite this evidence, recent studies have reported that this therapeutic strategy is still not widely used.[5]

Adjuvant treatment has increased survival in patients with different malignancies such as breast and colon cancer.[6,7] In MIBC, the role of adjuvant chemotherapy has been investigated throughout the last 3 decades, but the benefit still remains controversial. Most clinical trials evaluating the impact of adjuvant chemotherapy on MIBC have important methodological limitations, including small sample size, early termination owing to poor accrual, few events (deaths), and different chemotherapy regimens, leading to unequivocal results and few studies reporting a survival benefit.

This article discusses advantages and disadvantages of each therapeutic strategy, highlighting the most important studies supporting their use.

STRATIFICATION OF RISK AND PROGNOSTIC VARIABLES

Whereas there is strong evidence for the use of neoadjuvant chemotherapy in MIBC, there has been little information on the risk stratification of this group in the prelocalized treatment setting. In the meta-analysis of neoadjuvant trials performed in 2005, there was no specific risk stratification involving age, gender, clinical T or N stage, or performance status.[4]

The most widely used risk stratification in the precystectomy setting is staging. However, there is a large difference between clinical and pathologic staging at radical cystectomy (RC), with up to 54% of patients being upstaged[8] and 18% being downstaged at the time of surgery.[9] A nomogram designed to help predict pT3 or pT4 at RC was found to confer only a modest (4%) improvement over clinical staging alone.[10] Qureshi and colleagues[11] constructed an artificial neural network with 2 difference categories (Ta/T1 and T2–T4), using variables including genomic alterations, smoking status, gender, carcinoma in situ (CIS), metaplasia, architecture, and location of the tumor. This model predicted progression-free survival (PFS) and 1-year cancer-specific survival (CSS) at 80% and 82% accuracy, respectively. Catto and colleagues'[12] neuro–fuzzy models predicted recurrence-free survival (RFS) of Ta-T4 cases with 88% to 95% accuracy. The prediction model included p53, mismatch repair proteins, stage, grade, age, smoking status, and previous cancer. Although all of these models could be used to help identify patients who need neoadjuvant therapy, it has not proved to be better than clinical staging alone.

There have been multiple postcystectomy nomograms and risk stratifications that help identify patients who may benefit from adjuvant therapy.[13–18] Most prediction models include pathologic features from RC, including lymphovascular invasion (LVI), grade, and lymph node involvement, yet there is still only a minimal increase in accuracy of survival or recurrence compared with staging alone.

Karakiewicz and colleagues[13] created probability nomograms including age, T stage, N stage, grade, LVI, CIS, adjuvant radiotherapy, adjuvant chemotherapy, and neoadjuvant chemotherapy, which predict 2-, 5-, and 8-year RFS with 78% accuracy. Shariat and colleagues[14,15] had a similar probability nomogram, using the same categories as Karakiewicz, which predicted 2-, 5-, and 8-year overall survival (OS) and

bladder CSS at 79% and 73% accuracy, respectively. Additional nomograms created by the groups of Bassi and Bochner[16,17] have been found to be able to predict 5-year RFS and 5-year OS at 75% and 76% accuracy, respectively.

One of the best markers for survival is a complete pathologic response (pT0). There is approximately a 15% complete response (CR) from transurethral resection alone, whereas there is about a 35% to 45% CR after neoadjuvant chemotherapy.[18] In the Southwest Oncology Group (SWOG) neoadjuvant trial, 85% of patients with pT0 were alive after 5 years of follow-up.[19]

Biomarkers to help predict response to therapy could possibly increase the CR rate. A 20-gene expression profile has been shown to predict advanced or metastatic UC; however, this requires further prospective validation before incorporating it into routine clinical practice.[20] The COXEN (CO eXpression ExtapolatioN) model has shown promise in the preclinical setting at predicting which cell lines will respond to gemcitabine/cisplatin or MVAC (methotrexate, vinblastine, doxorubicin, cisplatin) therapy. This model is currently being tested in a prospective clinical trial.[21] Recently, Van Allen and colleagues[22] found that mutations in ERCC2, a DNA damage repair protein, correlate with CR in patients receiving cisplatin-based combination neoadjuvant chemotherapy. There is a need to validate these markers and investigate additional novel prediction models in both precystectomy and postcystectomy settings.

NEOADJUVANT STRATEGIES FOR MUSCLE INVASIVE BLADDER CANCER

Advantages

Neoadjuvant chemotherapy has several advantages. The 2 main advantages are the ability to eradicate micrometastases early, and the potential to downstage chemotherapy sensitive tumors.[23] Approximately 38% of patients who are able to receive cisplatin combination chemotherapy have a pathologic CR, compared with the pathologic CR rate of 6% to 15% for patients who did not receive cisplatin-based combination neoadjuvant chemotherapy.[2,19,20] Pathologic CR has been shown to strongly predict outcomes, and is used as an important end point for patient prognosis.[23]

Disadvantages

There are some potential disadvantages to neoadjuvant chemotherapy. Because there are no validated ways to predict response to neoadjuvant chemotherapy, patients with chemoresistant bladder tumors who undergo neoadjuvant chemotherapy are inevitably delayed from receipt of a potentially curative surgical therapeutic option (ie, RC). This delay and its association with survival outcomes remain unclear. In addition, there is some concern that neoadjuvant chemotherapy may subsequently increase the risk of complications during RC, although this has recently been contended in population-based studies.[24,25]

Evidence Summary

Chemotherapy

Randomized clinical trials Multiple clinical trials have demonstrated the benefit of neoadjuvant chemotherapy (**Table 1**). The Nordic I trial included 311 patients with T1-T4NxM0 who were randomized to receive 2 cycles of cisplatin and doxorubicin versus no neoadjuvant treatment before RC. All patients received 20 Gy of irradiation before RC. There was no statistically significant difference in OS or CSS at 5 years. However, in a subgroup analysis of patients with pT3-T4 disease, a 15% survival benefit was seen in patients receiving chemotherapy.[26] In the Nordic II trial, 309 patients were randomized to receive 3 cycles of neoadjuvant cisplatin and methotrexate or to RC alone. Again, no overall significant difference in 5-year survival was seen

Table 1
Randomized trials for neoadjuvant therapy

Series	Study Population	Year	No. of Patients	Chemotherapy	Follow-up[a] (mo) (Range)	Overall Survival[b] (%)	Overall Survival HR (95% CI)	Significant (Yes/No)
Cortesi[74]	T2–T4, N0, M0	(Unpublished)	171	Cisplatin Methotrexate Epirubicin Vinblastine	—	52.4 vs 57.7	—	No
Wallace[75]	T2–T4, Nx, M0	1991	255[c]	Cisplatin	—	71.1 vs 65.8	1.13 (0.80–1.57)	No
Coppin[76]	T2–T4b	1996	102	Cisplatin	78	16 vs 13, $P = .34$	0.75 (90% CI 0.50–1.12)	No
Abol-Enein[77]	T2–T4a, Nx, M0	1997	196	Cisplatin Methotrexate Vinblastine	—	—	—	—
Martinez-Pineiro[78]	T2–T4a, Nx–N2, M0	1995	122	Cisplatin	78.2 (48–101)	35.5 vs 37.3	—	No
Italian Bladder Study (GISTV)[79]	T2–T4a	1996	206	Methotrexate Vinblastine Adriamycin Cisplatin	—	—	—	No
International Collaboration of Trialists[30]	T2–T4a, N0-x, M0	2011	976	Cisplatin Methotrexate Vinblastine	120	36 vs 30, $P = .037$	0.84 (0.72–0.99)	Yes
Malmstrom[80] **Bassi**	T3–T4, N0 Any T, N+	1996	325	Cisplatin Doxorubicin	60	59 vs 51, $P = .1$	—	No
Bassi (GUONE)[81]	T2–T4b, N0-x, M0	2002	153	Cisplatin Methotrexate Vinblastine	—	52 vs 57.6	—	No
Sherif (Nordic II)[27]	T2–T4a, Nx, M0	2002	317	Cisplatin and methotrexate Cisplatin and adriamycin	56.4	56 vs 48	0.80 (0.64–0.99)	Yes
Grossman (SWOG Intergroup)[19]	T2–T4a	2003	317	Methotrexate Vinblastine Adriamycin Cisplatin	104	57 vs 43, $P = .06$	1.33 (1.00–1.76)	Yes

Abbreviations: CI, confidence interval; HR, hazard ratio.

[a] Mean or median follow-up time (in months) as reported by each study during time of publication. Types of range reported include minimum to maximum, interquartile range, and 95% confidence intervals.

[b] Based on number of events out of total number of patients in treatment (neoadjuvant) versus control arm (local treatment: radical cystectomy or radiotherapy).

[c] All 255 patients underwent neoadjuvant chemotherapy, but the control arm received local treatment in the form of radiotherapy in 2 different regimens: (1) 159 patients received 45–50 Gy in 22 fractions and (2) 96 patients received 65 Gy in 22 fractions + 10–15 Gy.

between the treatment groups (53%, neoadjuvant plus RC vs 46%, RC only).[27] One limitation to both Nordic trials is that they both used unconventional regimens that are uncommonly used in current practice (doxorubicin/cisplatin and methotrexate/cisplatin, respectively). However, a combined analysis of the 2 Nordic trials revealed an OS favoring neoadjuvant chemotherapy (5-year survival 56% vs 48%; $P = .049$), highlighting the efficacy of cisplatin-based neoadjuvant chemotherapy.[28]

The largest neoadjuvant prospective trial, published in 1999, included 976 patients (T2-T4N0) who were randomized to receive 3 cycles with the combination chemotherapy regimen of cisplatin, methotrexate, and vinblastine (CMV) or no systemic therapy. Patients were then treated with 1 of the following local therapies: (1) radiation therapy (RT), (2) a combination of low-dose radiotherapy and RC, or (3) RC alone.[29] Although the trial did not initially demonstrate statistical significance in survival, a long-term update, presented in 2002, demonstrated a 10-year OS benefit credited to neoadjuvant chemotherapy (36% vs 30%; hazard ratio [HR] 0.84).[30] The type of localized treatment did not change the survival outcomes. Pathologic CR was attained in 32.5% of patients receiving neoadjuvant therapy, versus 12.3% with RC alone.[30]

The SWOG performed a prospective, randomized controlled trial of 317 patients with T2-T4aN0M0 UC comparing 3 cycles of neoadjuvant MVAC chemotherapy preceding RC with RC alone. Although the trial did not show a statistically significant advantage for neoadjuvant therapy with 5-year OS (57% vs 43%; $P = .06$) or median survival (77 vs 46 mo), the trial is still considered to demonstrate level I superiority of neoadjuvant therapy because the original goal of statistically significant difference, defined as a one-sided $P<.05$, was attained. Patients receiving neoadjuvant therapy had a CR rate of 38%, compared with 15% with RC alone. Most patients (81%–82%) were able to proceed to cystectomy after receiving neoadjuvant chemotherapy. Toxicities of chemotherapy were manageable, with no toxic deaths, grade 4 neutropenia seen in 33%, and grade 3 gastrointestinal toxicities seen in 17%. No increase in postoperative complications was observed.[20]

Finally, single-agent platinum did not yield significantly better outcomes. No single platinum-based combination regimen combined with any local therapy (RC alone, radiotherapy alone, or radiotherapy in combination with RC) has demonstrated superiority over only localized therapy. Cisplatin tends to be the platinum agent used in most patients (>90%), with carboplatin used only in 6% to 7% of patients, owing to carboplatin being shown to be significantly inferior to cisplatin-based treatment.[31]

Nonrandomized prospective and retrospective studies The 2 main neoadjuvant chemotherapy regimens, gemcitabine-cisplatin (GC) and MVAC, have only been compared in retrospective studies. Current data suggest similar rates of pathologic CR and survival outcomes with both regimens (relative risk of CR 0.97, 95% confidence interval [CI] 0.60–1.56; $P = .9$).[32]

Dose-dense MVAC is being used more frequently in the neoadjuvant setting. A phase II study explored the efficacy and safety of this regimen with pegfilgrastim support in patients with muscle-invasive UC. Neoadjuvant chemotherapy resulted in significant pathologic and radiologic downstaging (49% achieved CR defined as $\leq$ pT1N0M0) with a favorable toxicity profile.[33] One advantage of this strategy is the short time to complete the 4 cycles of therapy, thus not delaying surgical treatment in patients who are not sensitive to systemic chemotherapy. Dose-dense therapy is being increasingly investigated by centers of excellence, particularly for bladder UC, and may also be a promising alternative to GC for high-grade upper tract urothelial carcinoma (UTUC).[34]

Meta-analysis The pooling of data from the aforementioned randomized clinical trials using meta-analysis statistical techniques has allowed us to advance our understanding regarding the true utility of neoadjuvant chemotherapy in bladder cancer, in addition to statistically increasing the total number of patients in both arms. The latest published meta-analysis of 11 randomized trials was performed by the Advanced Bladder Cancer Meta-Analysis Collaboration, and included 3005 patients. There was a significant survival benefit (HR 0.86, 95% CI 0.77–0.95; $P = .003$) among those who received neoadjuvant cisplatin-based chemotherapy, compared with those who did not; this translated into a 5% absolute increase in 5-year OS and a 9% absolute increase in 5-year disease-free survival (DFS) in comparison with RC alone.[4] Given this demonstrated survival benefit, in 2012 the National Comprehensive Cancer Network Guidelines recommend the use of neoadjuvant platinum-based combination chemotherapy for cT2 and strongly recommend it for cT3 node-negative disease,[35] similar to guidelines from the European Association of Urology[36] and European Society of Medical Oncology.[37]

At present, there is no effective regimen for patients with poor performance status and/or renal inefficiencies. There has been a meta-analysis comparing carboplatin-based with cisplatin-based chemotherapy regimens, with cisplatin-based therapies showing clear superiority (relative risk 3.54; $P = .005$).[31]

Radiation therapy

UC is relatively radiosensitive, and in the neoadjuvant setting RT may be able to prevent intraoperative seeding of tumor cells in the operative field and to sterilize microscopic extension in the perivesical tissues. There exists only one randomized trial demonstrating the superiority of preoperative radiotherapy over cystectomy alone in 2-year OS in patients with T3 bladder cancer.[38,39] Studies performed in the 1980s investigated the role of preoperative radiotherapy in either T2 or all stages of bladder cancer, and no marked benefits were found. One of the more recent studies was a phase III trial in the United States, which had a total of 140 patients who were randomized to receive 2000 Gy of pelvic irradiation followed by RC within 1 week, or RC alone. The 5-year survival rates were 43% (95% CI 30%–56%) and 53% (95% CI 41%–65%), respectively ($P = .23$).[40] Since then, research into this treatment modality has stagnated. In the contemporary management of bladder cancer, the role of RT in the neoadjuvant setting seems limited. With recent advances in the use of more targeted radiotherapies such as intensity-modulated RT, which has been shown in some studies to significantly reduce the volume of normal tissues affected while treating a variety of abdominopelvic tumors, neoadjuvant radiotherapy may resurface as a potential investigative option for patients with bladder cancer.[41,42]

ADJUVANT STRATEGIES FOR MUSCLE INVASIVE BLADDER CANCER

Advantages

The major advantage of administering adjuvant treatment is the appropriate patient selection according to the risk of recurrence. The adequate pathologic staging reduces the risk of overtreatment and allows for the selection of patients most likely to benefit from systemic therapy.[43] A large retrospective cohort evaluated discrepancies in clinical and pathologic staging in patients who underwent RC for MIBC. Clinical understaging was identified in approximately 50% of the patients, and pathologic downstaging occurred in 18%.[9]

Adjuvant chemotherapy does not delay local treatment for patients with chemoresistant tumors. Moreover, when neoadjuvant was compared with adjuvant chemotherapy, there were no differences in perioperative morbidity.[44] Therefore, adjuvant therapy certainly has its place in contemporary management.

Disadvantages

The major disadvantage to adjuvant treatment is delaying the treatment of micrometastatic disease. In addition, response to treatment measured by pathologic downstaging may provide important prognostic information.[45] With adjuvant chemotherapy, the only way to assess the benefit of this treatment is the absence of disease progression during long-term follow-up.

Another potential disadvantage is the possibility of postsurgical complications that may preclude patients from receiving adjuvant cisplatin-based chemotherapy. Donat and colleagues[46] have found at their high-volume tertiary center that nearly one-third (30%) of patients develop complications after RC of Clavien grade 2 or higher. Although surgical morbidity at their center may reflect the more complicated case mix they encounter, this highlights the importance of considering contributors to postoperative morbidity, as this may delay the administration of adjuvant chemotherapy.

Summary of Evidence

Chemotherapy

Randomized clinical trials Several randomized clinical trials attempted to define the role of adjuvant treatment in MIBC (Table 2). In 1994, Studer and colleagues[47] reported the results of a study designed to evaluate the role of adjuvant cisplatin monotherapy after RC. Seventy-seven patients with nonmetastatic MIBC were stratified based on nodal status (stage pN0 vs pN1–N2) and were randomly assigned to observation or adjuvant chemotherapy. In this study, no differences in OS were observed between the 2 groups in patients with all disease stages. Similarly, patients who had pN1-N2 did not benefit from the adjuvant treatment.[47]

Skinner and colleagues[48] randomized 91 patients with T3/T4 or positive lymph node MIBC to receive adjuvant cisplatin, doxorubicin, and cyclophosphamide or to observation after RC. In this study, median OS was 4.3 years for patients who received chemotherapy versus 2.4 years in the observation group ($P = .0062$). Of note, these results could be explained by several methodological biases.[48]

A German phase III clinical trial showed a benefit in OS and PFS with adjuvant chemotherapy (MVAC or MVEC [methotrexate, vinblastine, epirubicin, cisplatin]). This study was prematurely closed because of suggested striking benefits of adjuvant chemotherapy, so only a small number of patients was included in the final analysis. Of note, patients assigned to the observation arm did not receive any further treatment at the time of recurrence. By contrast, another German study showed that patients treated with adjuvant MVEC versus observation did not show significant differences in OS.[49]

Another clinical trial compared 2 neoadjuvant cycles followed by 3 adjuvant cycles after RC versus 5 adjuvant cycles of MVAC. This study enrolled 140 patients and suggested that neoadjuvant chemotherapy may be more feasible than adjuvant chemotherapy, although no difference in survival outcome was demonstrated.[44]

Recently, trials using cisplatin/gemcitabine-based regimens in the adjuvant setting were performed, based on results of this regimen in the metastatic setting. The prospective Italian trial of 194 patients was underpowered to demonstrate a survival difference in patients receiving 4 cycles of adjuvant GC (HR 1.29).[50] The Spanish Oncology Genitourinary Group trial randomized 340 patients with high-risk disease (T3–T4 or lymph node positive) to receive 4 cycles of paclitaxel, gemcitabine, and cisplatin (PCG) versus observation. Adjuvant PCG resulted in a significant increase in OS compared with no chemotherapy (60% vs 31%, HR 0.44).[51] Of note, both trials were prematurely closed, and the power of these analyses limits the conclusion regarding the efficacy of this strategy.

A biomarker-driven clinical trial, based on altered p53 levels, randomized patients with organ-confined disease (pT1 or pT2, N0M0) to 3 cycles of MVAC versus observation. No statistically significant difference in clinical outcome was identified based on p53 status.[52]

Most recently, the results of the European Organization for Research and Treatment of Cancer (EORTC) intergroup randomized phase III clinical trial was presented. The study's initial plan was to enroll a total of 1344 patients with MIBC to receive 4 cycles of adjuvant chemotherapy according to physician choice (GC, MVAC, or dose-dense MVAC) versus 6 cycles of deferred therapy at the time of recurrence. The trial was prematurely closed after enrollment of 284 patients with pT3-T4 and/or lymph node–positive and M0 disease. Adjuvant chemotherapy resulted in a statistically significant difference in PFS: 46.8% in the adjuvant treatment arm versus 29.5% in patients in the deferred arm. However, the median OS (primary end point) was 53.6% for patients who received immediate treatment versus 47.7% for patients in the deferred chemotherapy group (HR 0.78, 95% CI 0.56–1.10; $P = .13$).[53]

Nonrandomized prospective and retrospective studies Logothesis and colleagues[54] are among the first to report the impact of adjuvant chemotherapy in patients with MIBC. In this study, 71 patients presenting with pT3b, pT4, N1, or vascular/lymphatic invasion were treated with cisplatin, cyclophosphamide, and adriamycin. The 5-year survival rate for patients treated with this strategy was 70%, compared with 37% for those patients who were part of a historical control treated with surgery alone. Similar results in terms of long-term survival were reported from another study in which adjuvant CMV (n = 23) was compared with the same drugs plus doxorubicin (n = 12).[55] These studies supported the rationale for randomized investigation of this therapeutic strategy.

A large retrospective study evaluated 932 patients from 11 centers who received adjuvant chemotherapy after RC, and found that adjuvant chemotherapy was independently associated with longer OS (HR 0.83, 95% CI 0.72–0.97; $P = .017$). As expected, the benefit was higher in patients who presented both pT3 stage and lymph node–positive disease (HR 0.75, 95% CI 0.62–0.90; $P = .002$).[56]

Meta-analysis As the results from the prospective randomized clinical trials were not definitive and have several methodological limitations, meta-analyses have been conducted to help interpret the available data. The Advanced Bladder Cancer Meta-Analysis Collaboration conducted a meta-analysis with individual patient data from 491 patients enrolled in 6 studies. In this analysis, patients who were treated with adjuvant chemotherapy had a relative reduction in the risk of death of 25%.[57]

Recently, a study-level meta-analysis of 9 randomized trials including 945 patients was published.[58] In this updated analysis, patients receiving adjuvant treatment with cisplatin-based regimens had a DFS benefit (HR 0.66, 95% CI 0.45–0.91, $P = .014$) and OS benefit (HR 0.78, 95% CI 0.61–0.99; $P = .044$) compared with those who underwent RC alone. Moreover, lymph node–positive patients seem to have greater benefit with this strategy. Interpretation of these results should be taken cautiously, as individual patient data were not analyzed.[59–61] Therefore, the next study to look out for will be an updated individual patient data meta-analysis including the latest EORTC intergroup study, as the pooled HR is likely to demonstrate OS benefit for adjuvant chemotherapy. Such findings may influence clinical practice substantially.

Radiation therapy

RT has no well-established role in the adjuvant setting. Although the rationale of decreasing local recurrences may lead to subsequently lower rates of distant disease,

Table 2
Randomized trials for adjuvant therapy

Series	Year	Study Population	No. of Patients Total	Treatment Arm (Chemotherapy Regimen)	Control Arm (Locoregional Treatment)	Follow-Up (mo) (Range)[a]	Overall Survival (%)	Overall Survival HR (95% CI)	Significance (Yes/No)
Freiha[82]	1996	T3–T4, Any N	55	Cisplatin and methotrexate Vinblastine	Radical cystectomy	62 (24–96)	63 vs 36	0.74 (0.36–1.53)	No
Otto[83]	2001	T3/N1–N22	108	Methotrexate Vinblastine Epirubicin Cisplatin	Radical cystectomy	44	50.9 vs 54.7	0.82 (0.48–1.38)	No
Skinner[48]	1991	T3–T4, N0	102	Patients 1–17: 16 cisplatin-based, in combinations with doxorubicin, cyclophosphamide, 5-fluorouracil, vinblastine, or bleomycin	Radical cystectomy	—	51.6 vs 28.8	0.75 (0.48–1.19)	Yes
		Any T, N+		Patients 18–91: Cisplatin, doxorubicin, cyclophosphamide					
Lehmann[84]	2006	Any T, N+ T3–T4, Any N	49	MVAC or MVEC (1 patient received carboplatin instead of cisplatin)	Radical cystectomy	120	17.4 vs 26.9	1.75 (0.95–3.23)	No
Studer[47]	1994	Any T, Any N	91	Cisplatin	Radical cystectomy	69 (36–96)	57 vs 54	1.02 (0.57–1.84)	No
Stadler[52]	2011	T1–T2, N0 p53+	114	Methotrexate Vinblastine Doxorubicin Cisplatin	Radical cystectomy	64.8 (61.2–70.8)	20.7 vs 16.1	1.11 (0.45–2.72)	No
Italian trial[50]	2012	T2 (grade 3) T3–T4, N0–N2	194	Gemcitabine Cisplatin	Radical cystectomy	35 (15–57)	46.6 vs 39.9	1.29 (0.84–1.99)	No
Spanish trial[51]	2010	T3–T4, N0 Any T, N+	142	Paclitaxel Gemcitabine Cisplatin	Radical cystectomy	29.8 (1–95)	60 vs 31	0.38 (0.22–0.65)	Yes
EORTC Intergroup Trial[53]	2014	T3–T4, N0 Any T, N+	284	Gemcitabine, cisplatin or MVAC or High-dose MVAC	Radical cystectomy	83.6 (–) vs 86.5 (–)	53.6 vs 47.7	0.78 (0.56–1.08)	No

Abbreviations: EORTC, European Organization for Research and Treatment of Cancer; MVAC, methotrexate, vinblastine, doxorubicin, cisplatin; MVEC, methotrexate, vinblastine, epirubicin, cisplatin.

[a] Mean or median follow-up time (in months) as reported by each study during time of publication. Types of range reported include minimum to maximum, interquartile range, and 95% confidence intervals.

the use of RT after an RC has resulted in suboptimal results and has been associated with higher toxicity levels.

A small randomized trial showed that adjuvant radiotherapy may improve both local control and DFS in comparison with surgery alone.[62] In addition, a retrospective study reported similar results.[63] Results of a phase III randomized clinical trial were reported in 2006 at the American Society of Clinical Oncology Annual Meeting, whereby no statistical differences were observed in DFS rates in high-risk patients with bladder cancer who received adjuvant chemoradiation with cisplatin plus gemcitabine versus radiation alone.[64]

Regarding the limitations of these studies, further evaluation and a better characterization of patients who may benefit from this therapy are warranted. Similarly to the neoadjuvant setting, modern RT techniques may have a role in improving the toxicity profile and adding clinical benefit.

MOLECULAR BIOLOGY AND TARGETED THERAPIES

Our understanding of the molecular pathways underlying bladder cancer has benefited from recent advances in technologies such as high-throughput transcript profiling, microarrays, metabolomics, and proteomics. Intense research efforts in this area have borne fruit through the discovery of numerous molecular markers. These markers may be useful for screening, early diagnosis, and surveillance in addition to staging and prognosis.[65] Leading the effort is The Cancer Genome Atlas project (TCGA), which has identified potential therapeutic targets in 69% of UC tumors, including pathways suitable for further investigation.[66] It has been estimated that at least 60% of genomic alterations could be treated by drugs that are already available or under clinical testing.[67] Some potential new targets for treatment intervention have been described for UC, including the most recurrent reported mutations in the receptor tyrosine kinases (*RTK*)-*RAS*-*RAF*, phosphoinositide 3-kinase (*PI3K*)/*AKT*/mammalian target of rapamycin pathways (mTOR), and regulators of G1-S cell cycle progression such as TP53 and RB1.[67]

Other potential therapeutic targets lie in the mutation and/or gene amplifications present in a large proportion of urothelial tumors, including *FGFR3* mutations,[68] *PTEN* deletions, and *FGFR1*, *CCND1*, and *MDM2* amplifications.[66] More than half of UC have also been found to contain aberrations of the chromatin remodeling genes (*UTX*, *MLL-MLL3*, *CREBBP-EP300*, *NCOR1*, *ARID1A*, and *CHD6*) and, more recently, *STAG2* mutations.[67,69] Nevertheless, it must be cautioned that the functional effect of mutations in these genes encoding epigenomic regulatory proteins remains relatively unknown. It may be possible that identifying these driving genomic alterations, even if occurring in only a small subset of patients with bladder cancer, may lead to the development of patient-specific therapies. For example, recently described mutations in *TSC1* were useful in helping investigators examine the response to mTOR inhibitors such as everolimus, or in the *PIK3CA* gene, mutated in up to 26% of cases, which may predict sensitivity to *PIK3CA*/mTOR inhibitors.[70–73] Cancer immunotherapy also represents an exciting avenue for research, with the Food and Drug Administration recently granting "Breakthrough Therapy Designation" for MPDL3280A (anti-PDL1) in bladder cancer.

ONGOING CLINICAL TRIALS

Several clinical investigations have also been performed to address some open questions in the neoadjuvant and adjuvant treatment scenarios. A phase III trial clinical trial of GC versus high-dose intensity MVAC, with regimen selection decisions driven by

genomic profile, will help to define the optimal chemotherapy regimen in the perioperative setting for patients with locally advanced UC (NCT01812369). In addition, the role of taxanes in the neoadjuvant setting is being evaluated in a phase I study consisting of administering 4 cycles of cabazitaxel and cisplatin before RC. The study's primary end point is response rate (NCT01616875). In the adjuvant setting, a German phase III study was designed to evaluate gemcitabine alone versus nontreatment in the control arm in a subset of patients who are not suitable for cisplatin-based chemotherapy (NCT00146276). This study, like the previous studies in the adjuvant setting, was closed because of poor accrual, but can still be valuable. Another study is evaluating the impact of an immunotherapeutic agent recMAGE-A3 + AS-15 in patients with MIBC who were surgically treated and are positive for the antigen MAGE-A3 (MAGNOLIA) (NCT01435356). Finally, a randomized phase II study is evaluating DN24-02 (a Her2 targeting autologous antigen-presenting cell-based vaccine) as adjuvant therapy in subjects with high-risk HER2+ UC.

SUMMARY AND FUTURE DIRECTIONS

MIBC is an aggressive disease associated with poor survival rates. Although RC alone may result in cure for a subset of patients, the higher rates of relapse suggest that early administration of systemic therapy may improve clinical outcomes. Therefore, contemporary management of patients with MIBC involves the combination of surgery, systemic chemotherapy, and chemoradiation in select patients who are candidates for bladder preservation.

Neoadjuvant treatment with cisplatin-based combination regimens is an established standard of care and has improved long-term survival in MIBC. However, owing to the low rates of adoption of neoadjuvant chemotherapy, clinicians will still face the decision of whether to administer adjuvant chemotherapy to high-risk patients who have not received neoadjuvant chemotherapy. In the absence of definitive evidence justifying the recommendation of adjuvant chemotherapy, administering systemic therapy after an RC in high-risk patients is still an option if clinical trials are not available.

In the genomic era, the biology underlying MIBC has been elucidated. The TCGA has characterized genes and molecular pathways involved in cancer development and tumor progression, providing insights to improve the therapeutic arsenal. In addition, these results may add to the development of biomarkers to select patients for the available or new therapies. Of importance is that immunotherapy strategies have produced encouraging results in patients with advanced disease. However, how this new knowledge will affect the perioperative treatment in MIBC is still undefined, and efforts should be undertaken to integrate molecular aspects in innovative clinical trial designs in this setting.

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APÊNDICE J - Identification of ALK Gene Alterations in Urothelial Carcinoma

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Identification of *ALK* Gene Alterations in Urothelial Carcinoma

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Abstract

Background: Anaplastic lymphoma kinase (*ALK*) genomic alterations have emerged as a potent predictor of benefit from treatment with *ALK* inhibitors in several cancers. Currently, there is no information about *ALK* gene alterations in urothelial carcinoma (UC) and its correlation with clinical or pathologic features and outcome.

Methods: Samples from patients with advanced UC and correlative clinical data were collected. Genomic imbalances were investigated by array comparative genomic hybridization (aCGH). *ALK* gene status was evaluated by fluorescence *in situ* hybridization (FISH). *ALK* expression was assessed by immunohistochemistry (IHC) and high-throughput mutation analysis with Oncomap 3 platform. Next generation sequencing was performed using Illumina Genome Analyzer IIx, and Illumina HiSeq 2000 in the FISH positive case.

Results: 70 of 96 patients had tissue available for all the tests performed. Arm level copy number gains at chromosome 2 were identified in 17 (24%) patients. Minor copy number alterations (CNAs) in the proximity of *ALK* locus were found in 3 patients by aCGH. By FISH analysis, one of these samples had a deletion of the *5'ALK*. Whole genome next generation sequencing was inconclusive to confirm the deletion at the level of the *ALK* gene at the coverage level used. We did not observe an association between *ALK* CNA and overall survival, ECOG PS, or development of visceral disease.

Conclusions: *ALK* genomic alterations are rare and probably without prognostic implications in UC. The potential for testing *ALK* inhibitors in UC merits further investigation but might be restricted to the identification of an enriched population.

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Introduction

Urothelial carcinoma (UC) accounts for 15,210 cancer deaths per year in the United States [1]. Five-year survival for patients with muscle invasive (T2) disease or greater is only 50%.

Advanced UC of the bladder is often associated with mutations and multiple somatic copy number alterations [2]. Comparative genomic hybridization studies of bladder carcinomas and cell lines have revealed a number of recurrent genetic aberrations including amplifications or gains on 8q22-24, 11q13, 17q21, and losses on chromosomes 9, 8p22-23, and 17p6-9 [3,4]. In several clinical cohorts, some of these genomic alterations have also been associated with pathological stage and outcome [5].

In the recent years, potential new targets for treatment intervention have been described in urothelial tumors. The identification of driving genomic alterations as mutations even if occurring in only a small subset of bladder cancer patients, may lead to the development of patient-specific therapies as has been the case of the recently described mutations in *TSC1* predicting response to mTOR inhibitors like everolimus [6-8]. Another example is the *PIK3CA* gene, mutated in up to 26% of cases in the

series by Ross and colleagues that may predict sensitivity to *PIK3CA*/mTOR inhibitors [9].

The ALK (anaplastic lymphoma Kinase) inhibitor crizotinib, has recently shown high efficacy in the treatment of patients with non-small cell lung cancer (NSCLC) with *ALK* translocation which is present in about 4–7% of the tumors [10–12]. In a phase I study of NSCLC patients with an *ALK* translocation, the response rate was 57% independent of performance status or number of previous treatments with a 70% probability of progression free survival at 6 months [13]. In several other tumor types besides lung cancer, *ALK* genomic alterations have been identified as potential oncogenic drivers, meaning that cancers in different organs can be targeted for treatment with *ALK* inhibitors regardless of their cell of origin.

In UC, *ALK* copy number gain, amplification, translocations, mutations, or expression have not been characterized. We therefore investigated *ALK* protein expression and underlying genetic aberrations in a cohort of patients who received chemotherapy in the setting of metastatic disease, focusing on clinical and prognostic implications.

In the present study we show that *ALK* genomic alterations, such as copy number alterations (CNA) and deletions, occur in UC. Additionally, we attempted to identify the impact of these alterations with clinical and outcome features.

Material and Methods

Patients

This project was approved by the local ethics committee (CEIC-IMAS) at Hospital del Mar, and by the Dana-Farber/Harvard Cancer Center (DF/HCC) institutional review board (IRB). Because the majority of patients were died at the time of collecting samples, a waiver of consent was requested and given from IRB of DF/HCC for all participants (requiring complete deidentification of the samples prior the analysis).

A cohort of 96 patients, with metastatic UC treated with platinum-based combination was identified. All patients underwent several treatment regimens, all containing gemcitabine and a platinum compound, with some patients receiving additional paclitaxel as well. Patient clinical data was collected. The final cohort included 70 patients (52 males, 18 females) with available clinical data and sufficient tissue samples to conduct all the genomic studies.

Tumor Samples

The analysis was performed in formalin-fixed paraffin embedded (FFPE) tissue from UC of the urinary tract. Other molecular studies have been performed and reported in these samples in order to characterize the biology of UC [14]. The specimens were retrospectively retrieved from the pathology archive at Hospital del Mar and Mar Biobank in Barcelona, Spain. Slides were reviewed separately by two genitourinary specialist pathologists (MS, DB). All patients had high grade transitional cell carcinoma and no other histological variant was included in this study. Tumor areas were evaluated by a single pathologist (DB) and tumor bearing 0.6 mm cores were punched for DNA extraction and/or tissue microarray (TMA) construction.

ALK analysis

ALK genomic alterations were evaluated by array comparative genomic hibridization (aCGH), fluorescence *in situ* hybridization (FISH), immunohistochemistry (IHC), mass spectrometry mutation analysis and next-generation sequencing. Description of methods can be found in the appendix (**Methods S1**).

Statistical analysis

Statistical analysis of clinical data and molecular features was carried out with SAS version 9.2 (SAS Institute Inc, Cary, NC). Patient and clinical characteristics were summarized as number and percentages for categorical variables and median and interquartile ranges for continuous variables. Overall survival (OS) was defined from the date patients received first line chemotherapy for advanced disease until date of death or censored on the last known alive date. *ALK* copy number alteration was defined as having more than a 4 fold change [15]. Fisher exact test was used to assess the associations of *ALK* copy number alteration with ECOG PS and whether patients developed visceral disease. Cox proportional hazard model was used to assess the associations of *ALK* copy number alteration and overall survival in both univariate and multivariate analyses. Kaplan-Meier estimate was used to summarize median overall survival. All the statistical tests were conducted at the two-sided 0.05 level of significance.

Results

The median OS was 12 months with 45 patients deceased at the time of analysis, with a median follow-up of 23 months. **Table 1** summarizes patient and clinical characteristic for the entire cohort as well as for patients with more than 4 fold copy number gain in the FISH analysis.

Recurrent chromosomal gains and losses by aCGH

Analysis by aCGH of the 70 patients included in the study identified 95 focal and 21 broad (identified as >50% of the chromosome arm) events. The results of the broad alteration analysis were largely consistent with the current literature [16–18]. We observed frequent losses of chromosomes 5q (43%), 8p (69%), 9 (p: 48%; q: 41%), 10q (41%), 11p (49%), 17p (51%), and 22q (40%) and recurrent gains of chromosomes 3q (46%), 5p (48%), 8q (48%), 19q (34%), and 20 (60%). Three specimens out of 70 harbored minor non-significant alterations (log2 ratio 0–0.8) in chromosome 2, where *ALK* gene locus is located. This encouraged us to conduct a more in-depth search of *ALK* genomic alterations and to further characterize the 5′*ALK* deletion seen by FISH in one patient.

FISH analysis of *ALK* gene/copy number gains

To further characterize genomic imbalances on chromosome 2, all samples underwent FISH analysis. One case presented a deletion of the green signal (5′*ALK*), centromeric to the *ALK* gene, and also had gain of the *ALK* gene fusion signals and 3′*ALK* signal (**Figures 1 and 2**). This FISH pattern was interpreted as an *ALK* atypical rearrangement as has been described in *ALK* positive NSCLC because a single orange (3′*ALK*) signal was seen [19]. In these cases it is assumed that the deletion is the result of translocation. Analyses of EML4 as well as other known fusion partners such as TGF and KIF5 were performed without finding any translocation of these genes. Even so, it is possible that the deletion does not cause the *ALK* translocation and other molecular techniques need to be applied to further characterize the FISH findings.

ALK gene copy number gains and amplification were analyzed in all samples. Two cases presented amplification of *ALK*. 90% of samples showed *ALK* copy number gain due to polysomy of chromosome 2. All of them had 3 to 6 copies of CEP2 except one case with high polysomy. Among 70 urothelial tumors, 7 (10%) demonstrated 2F signals (2 intact *ALK* loci), 46 (65.7%) had 3–4F signals present, and 17 (24.3%) had ≥5F signals (range 5F–11F; median 6F) in >10% of nuclei (**Table 2**). The associations of

Table 1. Patients and Clinical Characteristics.

	All patients (N = 70)		Patients with copy number alteration (N = 17)	
	N	**% or median (q1, q3)**	**N**	**% or median (q1, q3)**
Age	61	63 (54, 68)	15	66 (58, 68)
Sex				
Male	52	74%	15	88%
Female	18	16%	2	12%
ECOG PS				
0	22	31%	4	24%
1, 2	48	69%	13	76%
Visceral diseases				
No	41	59%	7	41%
Yes	29	41%	10	59%
Pathological stage				
Stage 0 (Ta)	5	7%	2	12%
Stage I (T1)	5	7%	0	0%
Stage II (T2)	36	51%	8	47%
Stage III (T3, T4)	22	31%	7	41%
Stage IV (L, M)	1	1%	0	0%
Missing	1	1%	0	0%

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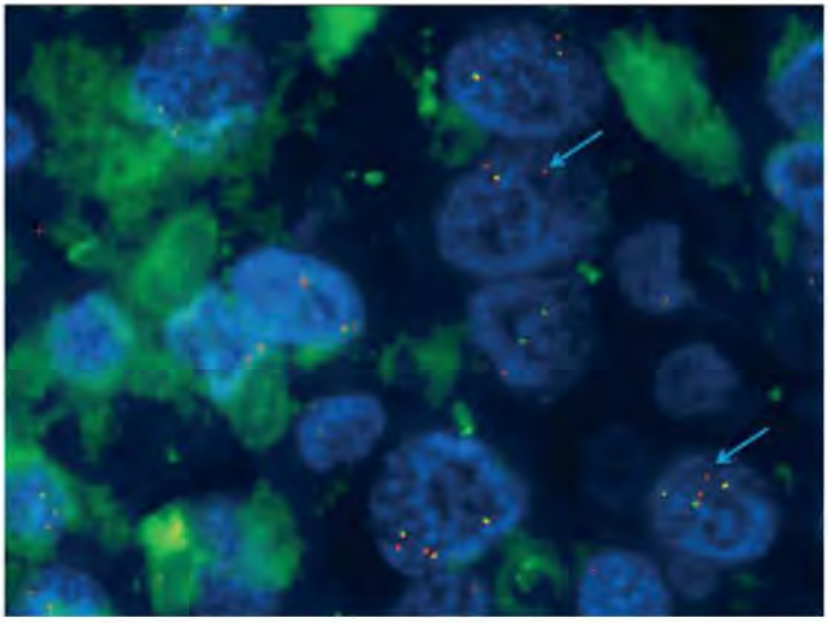

Figure 1. 1298case –FISH + for ALK variant (green probe missing).
doi:10.1371/journal.pone.0103325.g001

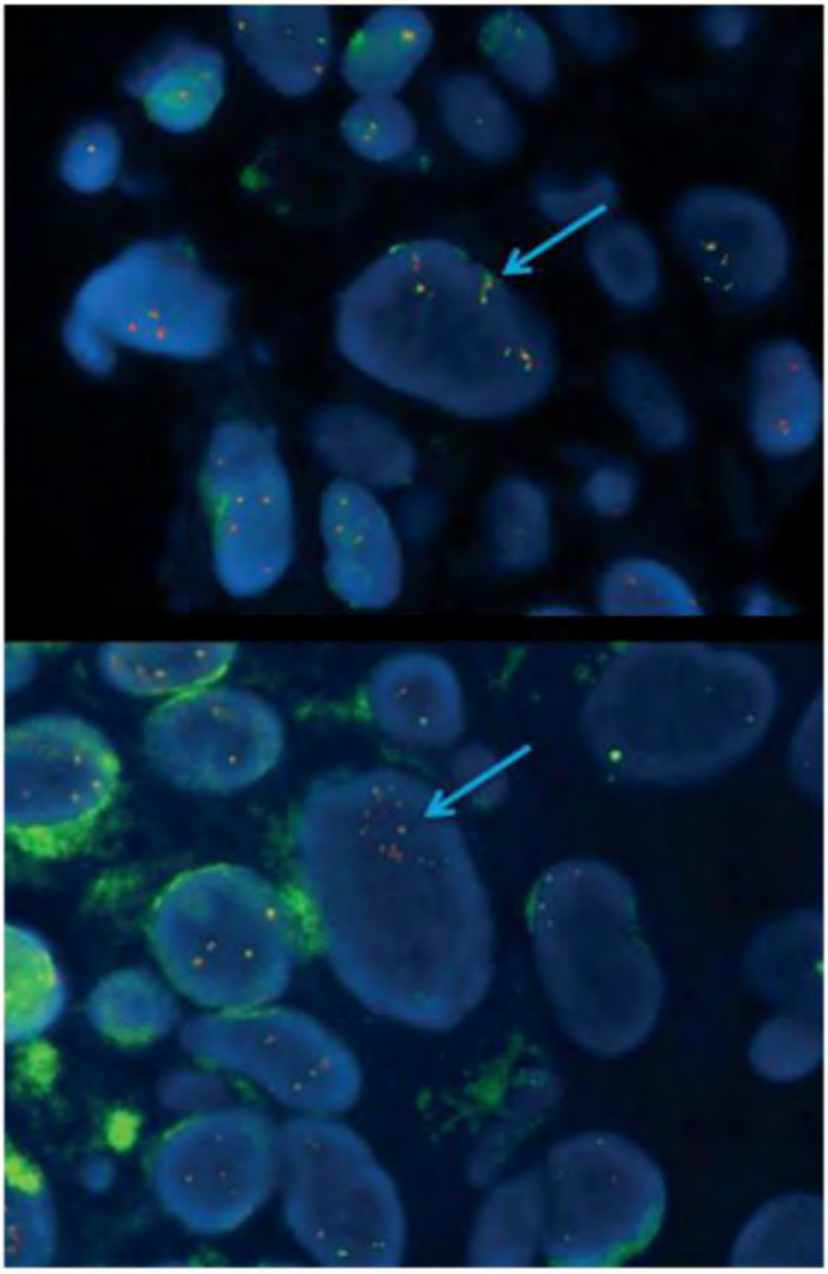

Figure 2. 1298case –FISH copy gain (a) & amplified (b).
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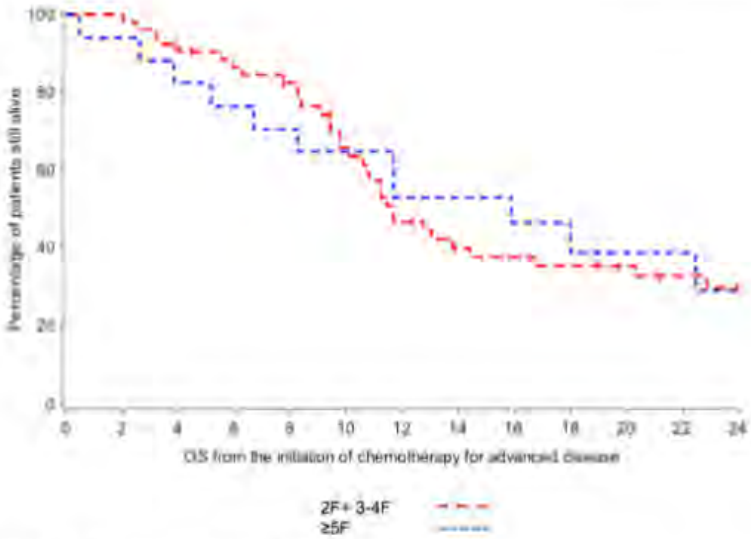

Figure 3. Comparison of OS between ≥5F patients and 2F+3−4F.
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ALK copy number alteration with ECOG PS, visceral disease, and OS are summarized in **Tables 3 and 4**. No significant association between *ALK* copy number alteration and clinical features or overall survival was observed (**Figure 3**).

Comparison of *ALK* gene copy number gains to clinical and pathological features for the 70 patients are summarized in **Table 1**. There were no differences between *ALK* gene copy number gains and clinical features in all the subgroups (2F, 3–4F and ≥5F). OS rates for patients with 2F+3−4F and >5F were 12 and 16 months respectively. There was no statistically significant difference between these groups (**Figure 3**).

ALK protein expression by immunohistochemistry

To further characterize whether *ALK* protein expression was affected, immunohistochemistry analysis of all FFPE samples was performed using the Cell Signaling antibody. Immunohistochemistry staining was negative in the tumor with *ALK* FISH positive test. Similarly, among tumors with *ALK* gene copy gain or amplification, *ALK* protein expression was not detected. None of the tumors classified as *ALK* negative by FISH showed *ALK* protein expression by immunohistochemistry.

Table 2. Copy Number Alteration.

	N	%
2F	7	10
3-4F	46	66
≥5F	17	24

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High-throughput mutational analysis using Oncomap

To have more accurate information on genetic alteration in these UC samples, mass spectrometry mutation analysis was also performed for all samples. Ninety-six samples were submitted for OncoMap: 87/96 (91%) passed all quality control steps. 79% (69/87) passing samples harbored candidate mutations. In total, 150 candidate mutation calls were made across 47 genes. Overall, 39% (58/150) of candidate mutations in passing samples were conservative and 61% (92/150) were aggressive. No mutations in *ALK* were found using this platform. *ALK* P496L candidate mutation was found in one of the sample but was not confirmed with IHC.

Next-generation sequencing of *ALK* gene

Since FISH technique gives no information of the specific sequence and the exact size of the deleted fragment in *ALK*, directed analysis of *ALK* gene was performed by next generation sequencing (Illumina). Analysis of the region containing P496 only showed base changes at rates below 1%, reflecting the expected sequencing error rate. Thus, only the wild-type sequence for the position P496 was detected and no mutations on *ALK* were detected by this technique.

We then extended the search space to the centromere with the intention to explore potential deletions according to FISH results. In the new analysis performed on the FISH positive patient, one read of a pair should match within the ALK locus, 29.37 Mb - 32 Mb, and one read should match at some place towards the centromere (>10 kb up to position 93.3 Mb). However, at the coverage level used no deletions could be confirmed with this approach.

Table 3. Association of *ALK* copy number alteration with ECOG PS and visceral disease.

	ALK >4 copies		P-value
	No	Yes	
ECOG PS			0.55
0	18	4	
1, 2	35	13	
Visceral disease			0.16
No	34	7	
Yes	19	10	

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Discussion

In the present study we interrogate whether the *ALK* genomic alterations are of potential clinical relevance in patients with UC. Our study shows that *ALK* amplification and copy number gain but not fusions and translocations occurs in UC but is not associated with poor outcome in our patients with already bad prognosis.

ALK gene is located in 2p23 and encodes a transmembrane tyrosine kinase receptor involved in the development of nervous system during embryogenesis [20,21]. *ALK* gene was first shown to have a role in cancer as part of the fusion gene nucleophosmin (NPM)-*ALK* in anaplastic large cell lymphomas(9, 10). Preclinical studies show that tumors with aberrant activation of *ALK* tyrosine kinase are oncogene addicted to *ALK* intracellular signaling, and inhibition of the kinase by specific *ALK* targeting drugs results in tumor growth arrest and cell death (25).

The best well studied genomic alteration is the translocation seen in NSCLC patients. The majority of *ALK* rearrangements come from an interstitial deletion and inversion in chromosome 2p resulting in EML*A*–*ALK* fusion gene product [22–27].Although translocation is the most commonly identified mechanism for *ALK* activation, amplification and mutation have also been shown to act as oncogenic events [28–30]. The role of amplification and of copy number gain, as well as the role of deletion found in tumors like RMS remains to be determined [27,31–34].

The finding that several tumor types have been identified that have *ALK* as an oncogenic driver regardless of their cell of origin has prompted the creation of the term "*ALK*omas" implying a "beyond organ" concept classification assuming consequently responses to *ALK* inhibitors such as crizotinib [10,35]. Based on that, exploration of this concept is worthwhile in UC even if the frequency happens to be low.

In our cohort, aCGH-A found only some minor focal events in 3/70 specimens harboring non-significant alterations in *ALK* gene locus region. Since copy number gain has been recently associated with poor prognosis in several tumors like RMS, RCC and colorectal cancer (CRC), FISH analysis to assess the impact of copy number variations of *ALK* in our cohort was performed. In our patients, polysomy was frequently found in 90% of the cases [15,34]. The biological relevance of such finding is uncertain but could reflect genomic instability. The OS for patients with (2F+3−4F) vs. >5F was found to be 12 and 16 months respectively, however did not reach statistical significance (**Figure 1**). Likewise, there were no differences between *ALK* gene copy number gains and clinical features in all the different subgroups (2F, 3–4F and ≥ 5F). A plausible explanation for this lack of a significant difference between these groups is that it could be related to the natural history and the aggressive phenotype of our analysis cohort (metastatic disease requiring chemotherapy) with other genetic abnormalities beyond *ALK* gene copy number having a greater functional role in oncogenesis. Similarly, arm level ALK gene copy number gain as observed in this analysis may be unrelated to the driver oncogenic events.

Generally, patients with *ALK* copy gain have not shown to have detectable *ALK* protein expression as assessed by IHC except for a recent publication by van Gaal and colleagues [27,29,34,36]. In our series, no patient with gene copy gain or amplification tested positive by IHC. This is similar to that observed in CRC where increased *ALK* gene copy number did not translate to increased *ALK* protein expression [37]. However, this is not the case for patients being categorized as FISH positive, where this positivity strongly correlates with IHC. Of note, in lung cancer, a positive *ALK* FISH test and *ALK* IHC have been proposed as screening tools to detect *ALK* alterations being considered sufficiently sensitive to indicate treatment with crizotinib [37]. Moreover, in NSCLC, abnormal FISH signal patterns have varied from a single split signal to more complex signal patterns, such as deletions of the green 5′ end of the *ALK* probe, gain of the split or 5′*ALK* signal or both. These variant *ALK* FISH signals usually, but not always, represent an *ALK* translocation and therefore the finding of a loss of the 5′*ALK* signal has been considered to be a presumptive evidence of an *ALK* gene rearrangement [37].

In our series, the patient with a FISH positive result had a variant signal pattern that did not correlate with *ALK* protein expression as assessed by IHC. The case was interpreted as having a deletion in the *ALK* region due to loss of the green 5′ end of the *ALK* signal, after excluding the possibility it could be related to alternative translocation partners [Kinesin family 5B (*KIF5B*) and TRK-fused gene (*TFG*)]. In our patient we did not test for the rearrangement of other fusion partners to *ALK* such as C2orf44, *KIF*5B, *NPM*1, *VCL*, *TFG*, *RET*, *ROS*, and *VCL* [38–43]. These genes have all been shown to be partners of *ALK* in lung cancer [44].

Finally, *ALK* Mutations have been described in 10.4% of neuroblastoma samples but not in other pediatric tumors like RMS, Ewing sarcoma, or DSRCT and only occasionally in other solid tumors like CRC [45,46]. In lung cancers, *ALK* mutations appear to develop during clinical treatment with crizotinib and their generation probably renders EML*A*-*ALK* resistant not only to crizotinib but also to other *ALK* inhibitors [47]. In our series, no *ALK* P496L mutation was observed. In our study the limitations of the platform used limits our conclusions of the mutation analysis. The absence or very low percentage of activating mutation of *ALK* described in the majority of adult solid tumors tested support our analysis that these alterations are not relevant events in UC.

Table 4. Comparison of OS between ≥5F patients and 2F+3−4F.

	N	Death	Median OS	Hazard ratio	P-value	Adjusted hazard ratio	P-value
AKL >4 copies					0.80		0.38
2F+3−4F	53	34	12	1.1 (0.55, 2.16)		1.36 (0.60, 2.72)	
≥5F	17	11	16	1 (reference)		1 (reference)	

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Unfortunately, the suspected deletion in the *ALK* region was not confirmed with the sequencing approach used. Discordantly, mapping read pairs suggesting deletions resolved into correctly mapping read pairs that were in agreement with the insert size of the library when a single mismatch between read and reference genome was tolerated. Thus, these pairs do not support deletions at the *ALK* locus. The average read coverage across the *ALK* region was 5× and if only a small proportion of cells contained a deletion, we would not have been able to detect it. Because we suspect the deletion was close to the centromere, we might have missed it and might not have been able to confirm it by next generation sequencing.

To summarize, the increasing evidence that *ALK* alterations are seen in tumors from different origins highlights the concept of stratifying tumors according to oncogenic genotypes as opposed to tissue type when considering treatment strategies. The finding of the absence of *ALK* rearrangement together with no activating mutation in *ALK* suggests that these alterations might not be pathogenic events in UC. The utility of testing ALK inhibitors in UC is not supported by this data, although in the absence of effective alternative agents testing ALK inhibitors may still be warranted.

In conclusion, *ALK* genomic alterations are rare and probably without prognostic implications in UC. The potential for testing *ALK* inhibitors in patients with deletions and copy number changes UC merits further investigation in a larger expanded cohort of UCs, but might be restricted to the infrequent finding of a FISH positive patient.

Supporting Information

Methods S1 Supplementary Methods.
(DOCX)

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Author Contributions

Conceived and designed the experiments: JB TC JR. Performed the experiments: SS SR MS SM BB AM SS HH DB. Analyzed the data: JB SS SR MS SM BB LW AF SS CM HH DB PK TC JR. Contributed reagents/materials/analysis tools: JB IC SM RO JB PK TC JR. Contributed to the writing of the manuscript: JB AF SS CM PK TC JR.

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APÊNDICE K - A Systematic Review and Meta-analysis of Adjuvant and Neoadjuvant Chemotherapy for Upper Tract Urothelial Carcinoma

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Review – Urothelial Cancer

A Systematic Review and Meta-analysis of Adjuvant and Neoadjuvant Chemotherapy for Upper Tract Urothelial Carcinoma

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Abstract

Context: The role of adjuvant chemotherapy (AC) or neoadjuvant chemotherapy (NC) remains poorly defined for the management of upper tract urothelial carcinoma (UTUC), although some studies suggest a benefit.
Objective: To update the current evidence on the role of NC and AC for UTUC patients.
Evidence acquisition: We searched for all studies investigating NC or AC for UTUC in Medline, Embase, the Cochrane Central Register of Controlled Trials, and abstracts from the American Society of Clinical Oncology meetings prior to February 2014. A systematic review and meta-analysis were performed.
Evidence synthesis: No randomized trials investigated the role of AC for UTUC. There was one prospective study ($n = 36$) investigating adjuvant carboplatin–paclitaxel and nine retrospective studies, with a total of 482 patients receiving cisplatin-based or non-cisplatin-based AC after nephroureterectomy (NU) and 1300 patients receiving NU alone. Across three cisplatin-based studies, the pooled hazard ratio (HR) for overall survival (OS) was 0.43 (95% confidence interval [CI], 0.21–0.89; $p = 0.023$) compared with those who received surgery alone. For disease-free survival (DFS), the pooled HR across two studies was 0.49 (95% CI, 0.24–0.99; $p = 0.048$). Benefit was not seen for non-cisplatin-based regimens. For NC, two phase 2 trials demonstrated favorable pathologic downstaging rates, with 3-yr OS and disease-specific survival (DSS) ≤93%. Across two retrospective studies investigating NC, there was a DSS benefit, with a pooled HR of 0.41 (95% CI, 0.22–0.76; $p = 0.005$).
Conclusions: There appears to be an OS and DFS benefit for cisplatin-based AC in UTUC. This evidence is limited by the retrospective nature of studies and their relatively small sample size. NC appears to be promising, but more trials are needed to confirm its utility.
Patient summary: After a comprehensive search of studies examining the role of chemotherapy for upper tract urothelial cancer, the pooled evidence shows that cisplatin-based adjuvant chemotherapy was beneficial for prolonging survival.

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1. Introduction

Upper tract urothelial carcinoma (UTUC) accounts for <5% of all urothelial cancers [1]. Presently, no definitive recommendations exist regarding the use of perioperative chemotherapy in the management of UTUC, and current practice is mainly derived from evidence related to muscle-invasive bladder cancer (BCa) [1–3]. UTUC portends a poor prognosis overall, and the majority of patients are typically ineligible to receive nephrotoxic cisplatin-based chemotherapy after definitive surgical removal of the kidney, ureter, and bladder cuff resulting from the decline of kidney function. Although UTUC is similar morphologically to lower tract bladder tumors, there are occasional phenotypic and genotypic (genetic and epigenetic) differences between transitional cell carcinoma of the upper and lower urinary tracts.

Besides the different management, anatomic location, and patterns of dissemination, there are interesting differences at the embryologic and molecular levels [4]. First, bladder and ureter urothelium arise from different embryologic tissues [5]. Second, studies have demonstrated that bladder and ureter urothelial tissues differ in uroplakin content, keratin expression pattern, and propensity to keratinize [6]. In addition, extracellular matrix–associated proteins with counteradhesive properties react differently in bladder and ureter urothelial cells [7]. Third, microsatellite instability [8,9] and hypermethylation [10,11] are more common in upper tract cancers than lower tract cancers. Perhaps consequently, the natural history of UTUC is different from that of BCa, with >60% of UTUCs and only 15–25% of BCa presenting with invasion at diagnosis [1,12].

In terms of chemotherapy response, differences have been suggested in post hoc analyses of large randomized clinical trials when comparing responses between upper and lower urinary tract tumors using two different regimens [13]. These findings suggest that data generated from BCa studies cannot always be extrapolated to patients with upper urinary tract tumors [14]. In addition, unique patterns of genomic alterations in UTUC have been recently identified [15].

In light of these inherent differences between upper and lower urothelial cancers and the paucity of data on chemotherapy for UTUC, the aim of this paper was to perform a systematic review and meta-analysis of currently available evidence to evaluate the contemporary role of chemotherapy for patients with UTUC and provide practical recommendations.

2. Evidence acquisition

We performed this study according to the Preferred Reported Items for Systematic Reviews and Meta-analysis guidelines [16]. As the majority of available studies were retrospective observational cohorts, we followed guidelines for meta-analysis of observational studies [17].

2.1. Literature search

We performed a systematic literature search of PubMed, Embase, the Cochrane Central Register of Controlled Trials, ClinicalTrials.gov, and the American Society of Clinical Oncology meeting abstracts to identify observational cohort studies and controlled trials performed prior to February 2014. All studies examined the role of chemotherapy for UTUC. Patients received adjuvant chemotherapy (AC) after definitive surgical treatment with nephroureterectomy (NU), neoadjuvant chemotherapy (NC) before NU, or NU alone. Search terms used included *ureteral neoplasms, urothelium, ureter, upper tract urothelial, chemotherapy, adjuvant, neoadjuvant*, and relevant variants. Search results were independently reviewed by two authors (J.J.L., J.B.). Full articles were retrieved for further qualitative review (Fig. 1).

2.2. Statistical analysis

Effect measures for the outcomes of overall survival (OS), disease-free survival (DFS), and disease-specific survival (DSS) were hazard ratios (HRs) and 95% confidence intervals (CIs), which were extracted from published studies. DFS was defined as the time from the date of surgery to the date of documented relapse or recurrence, while DSS was commonly defined as the time from surgery to death from disease or urothelial cancer. For studies with Kaplan-Meier log-rank or Wilcoxon p values available but no published HRs or 95% CIs, we employed a widely used method to estimate HRs and 95% CIs [18]. Choice of primary statistical model for pooled HRs was based on level of statistical heterogeneity among included studies, which was assessed using the Cochran Q statistic (p value for heterogeneity) and the I_2 statistic (total percentage of variation resulting from heterogeneity) [19]. Significant heterogeneity was denoted by a Cochran Q p value <0.05 and an I^2 >50% [19], in which case random-effects models were used to report HRs via the DerSimonian and Laird method [19–22]. When substantial heterogeneity was not observed, fixed-effects models were used to estimate the pooled HR via the inverse variance method.

2.3. Sensitivity analyses and publication bias

We first examined studies that used only cisplatin-based chemotherapy regimens (ie, 100% of the study's patients received cisplatin-based chemotherapy). Subsequently, we performed a subgroup analysis that included not only studies using cisplatin-based regimens but also those allowing other non-cisplatin–based regimens (herein referred to as *non-cisplatin based* or *other*). We also performed influence analysis, where we removed each study one at a time to determine the impact on the overall pooled result. In addition, we performed sensitivity analyses on studies depending on the proportion of patients receiving cisplatin-based regimens. Publication bias was evaluated using both the Egger's linear regression approach and funnel plots [23]. All statistical analyses were performed using Stata/SE v.12.0 software (StataCorp, College Station, TX, USA).

3. Evidence synthesis

3.1. Search results

We identified a total of 1555 studies using the search criteria. We extracted 994 based on the following keywords

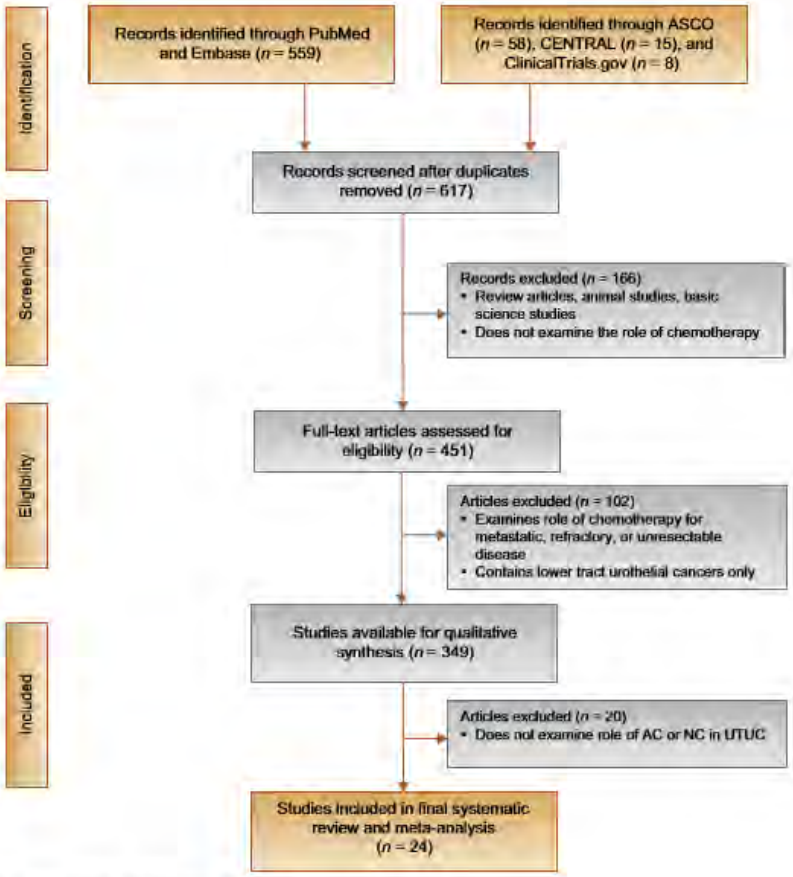

Fig. 1 – Preferred Reporting Items for Systematic Reviews and meta-analysis flowchart.
AC = adjuvant chemotherapy; ASCO = American Society of Clinical Oncology; CENTRAL = Cochrane Central Register of Controlled Trials; NC = neoadjuvant chemotherapy; UTUC = upper tract urothelial carcinoma.

present in the title or abstract: *upper*, *urothelial*, and *chemotherapy*. Thereafter, we examined each article qualitatively.

3.2. *Studies evaluating the role of adjuvant chemotherapy*

No randomized trials investigated the role of AC for UTUC. We found one prospective study that treated all 36 patients with adjuvant carboplatin–paclitaxel [24] and nine retrospective studies with a total of 482 patients receiving cisplatin-based or other non-cisplatin–based AC after NU and 1300 patients receiving NU alone [25–33]. Among these nine studies, there were five single-institution series from Korea [26,27,32] and Japan [25,28], in which 100% of patients received cisplatin-based AC after NU. Cisplatin-based regimens were most commonly methotrexate, vinblastine, adriamycin, and cisplatin (M-VAC) or gemcitabine–cisplatin (GC). The 5-yr OS, DFS, and DSS rates appear favorable to AC compared with those who did not receive AC (Table 1).

Four other studies consisted of patients who received other non-cisplatin–based AC regimens—namely, Canadian [33], French [30], and Japanese [31] multicenter studies and the multinational UTUC collaboration [29] (Table 1). Patients received a variety of AC regimens, including cisplatin-based, carboplatin-based, and non-platinum–based regimens. Study-level HRs for outcomes used to calculate pooled results are shown in Table 2.

3.2.1. *Meta-analyses and sensitivity analyses for adjuvant chemotherapy*

3.2.1.1. Overall survival. For OS, five studies had sufficient data for meta-analysis, three of which evaluated cisplatin-based

Table 1 – Studies investigating cisplatin-based* and other, non–cisplatin-based adjuvant chemotherapy for upper tract urothelial carcinoma**

Study	Year	Study type	No. of patients			Tumor stage or grade		Age		5-yr OS rates (%)		5-yr DFS rates (%)		5-yr DSS rates (%)	
			AC	No AC	AC regimen*	AC	No AC	AC	No AC	AC	No AC	AC	No AC	AC	No AC
Studies investigating cisplatin-based AC*															
Soga [28]	2008	Retrospective single center	24	22	M-VAC (100%)	pT2 N0: 7 (29%) pT3 N0: 17 (71%)	6 (27%) 16 (73%)	<70 yr: 15 of 24 (63%) ≥70 yr: 9 of 24 (37%)	10 (45%) 12 (55%)	96	87			–	–
Kwak [26]	2006	Retrospective single center	32	11	M-VAC (72%) GC (22%) CISCA (6%)	pT2 N0: 4 (13%) pT3 N0: 28 (87%)	0 (0%) 11 (100%)	<60 yr: 17 of 32 (53%) ≥60 yr: 15 of 32 (47%)	5 (82%) 6 (18%)	78	36	63	36	–	–
Suzuki [25]	2004	Retrospective single center	16	15	M-VAC or MEC (100%)	≤pT2: 2 (13%) ≥pT3: 14 (87%) pN1: 6 (38%) pN2: 10 (63%)	3 (20%) 12 (80%) 6 (40%) 9 (60%)	 Median (range): 56 (41–78)	67 (47–84)	63***	27	56	10	–	–
Kim [32]	2013	Retrospective single center	36	29	GC (100%)	≤pT2 N1–3: 2 (6%) ≥pT3–4 N0–3: 34 (94%)	0 (0%) 29 (100%)	<60 yr: 22 of 36 (61%) ≥60 yr: 14 of 36 (39%)	12 (41%) 17 (59%)			–	–	68	54
Lee [27]	2006	Retrospective single center	16	11	M-VAC (100%)	G1: 1 (6%) G2: 7 (44%) G3: 8 (50%)	0 (0%) 6 (55%) 5 (45%)	<60 yr: 8 of 16 (50%) ≥60 yr: 8 of 16 (50%)	5 (45%) 6 (55%)			–	–	75	71
*Studies investigating other, non–cisplatin-based** AC*															
UTUC Collaboration [29]	2009	Retrospective multicenter	121	421	M-VAC (59%) GC (20%) Other (21%)	pT3N0: 62 (16%) pT4N0: 7 (22%) pN+: 52 (41%)	321 (76%) 25 (6%) 75 (18%)	<60 yr: 33 (34%) ≥60 yr: 88 (66%)	64 (15%) 357 (85%)	41	45	–	–	45	53
Canadian [33]	2013	Retrospective multicenter	59	249	Unknown	≤pT2: 1 (2%) ≥pT3: 57 (98%) pN0: 9 (15%) pN+: 27 (46%) pNx: 23 (39%)	10 (4%) 237 (96%) 44 (18%) 51 (20%) 154 (62%)	 Median (range): 65 (57–71)	 72 (63–78)	37	43	–	–	38	52
Japanese [31]	2012	Retrospective multicenter	38	55	M-VAC (71%) GC (11%) Other (18%)	pT3; G1: 2 (5%) pT3; G2: 12 (32%) pT3; G3: 24 (63%)	4 (7%) 19 (35%) 32 (58%)	 Median (range): 66 (49–80)	 71 (34–87)	–	–	–	–	81	64
French [30]	2011	Retrospective multicenter	140	487	GC (25%) M-VAC (14%) CMV (12%) Carbo-pac (22%) Carbo-gem (16%)	≤pT2: 69 (49%) ≥pT3: 71 (51%) N+ M0: 38 (27%) M+: 31 (22%)	162 (33%) 325 (67%) 86 (17%) 76 (16%)	 Median (range): 67 (61–73)	 71 (66–85)	43	–	–	–	60	54†
Bamias [24]	2004	Prospective single center	36	–	Carbo-pac (100%)	pT2 N0: 2 (6%) pT3N0: 26 (72%) pT4N0: 8 (22%)	 – 	 Median (range): 69 (36–78)	 –	52		–	–		

AC = adjuvant chemotherapy; Carbo-pac = carboplatin, paclitaxel; Carbo-gem = carboplatin, gemcitabine; CISCA = cisplatin, cyclophosphamide, doxorubicin; CMV = cisplatin, methotrexate, vinblastine; CSS = cancer-specific survival; DFS = disease-free survival; DSS = disease-specific survival; GC = gemcitabine, cisplatin; HR = hazard ratio; M-VAC = methotrexate, vinblastine, Adriamycin, cisplatin; MEC = methotrexate, epirubicin, cisplatin; OS = overall survival; UTUC = upper tract urothelial carcinoma.

* Studies evaluating only cisplatin-based AC regimens (100% of patients treated with a cisplatin-based regimen).

** Non–cisplatin-based regimens allowed carboplatin- and/or nedaplatin-based regimens (not exclusively and 100% cisplatin based).

*** All rates for this study were 3-yr rates.

† Estimated based on CSS for AC and HR for AC versus no AC.

Table 2 – Overall, disease-free, and disease-specific survival hazard ratios of studies investigating cisplatin-based* and other, non–cisplatin-based adjuvant chemotherapy for upper tract urothelial carcinoma**

Study	Year	OS		DFS		DSS	
		HR (95% CI)***	p value	HR (95% CI)	p value	HR (95% CI)	p value
Studies investigating cisplatin-based AC*							
Soga [28]	2008	0.76 (0.09–6.8)[†]	0.81	–	–	–	–
Kwak [26]	2006	0.11 (0.02–0.53)	0.01	0.66 (0.19–2.27)	0.51	–	–
Suzuki [25]	2004	0.60 (0.25–1.45)	0.26	0.42 (0.18–1.00)	0.05	–	–
Kim [32]	2013	–	–	–	–	0.52 (0.17–1.82)	0.21
Lee [27]	2006	–	–	–	–	0.81 (0.18–3.65)[‡]	0.78
*Studies investigating other, non–cisplatin-based** AC*							
UTUC Collaboration [29]	2009	1.06 (0.80–1.40)	0.69	–	–	1.26 (0.93–1.71)	0.13
Canadian [33]	2013	0.70 (0.29–1.66)[§]	0.41	–	–	0.78 (0.40–1.50)	0.45
Japanese [31]	2012	–	–	–	–	0.21 (0.06–0.66)	0.01
French [30]	2011	–	–	–	–	1.14 (0.42–3.13)[§]	0.80

AC = adjuvant chemotherapy; CI = confidence interval; DFS = disease-free survival; DSS = disease-specific survival; HR = hazard ratio; OS = overall survival; UTUC = upper tract urothelial carcinoma.

* Studies evaluating only cisplatin-based AC regimens (100% of patients treated with a cisplatin-based regimen).

** Non-cisplatin-based regimens allowed carboplatin- and/or nedaplatin-based regimens (not exclusively and 100% cisplatin based).

*** All HRs and 95% CIs are reported as AC compared with no AC. These are obtained by inversing the HR and 95% CI if the original study reported as no AC compared with AC.

[†] Estimated from Parmar equations.

[‡] For high-risk patients (≥pT3 or pTxN+ or both): HR for OS: 1.085 (0.499–2.359; $p = 0.8363$), HR for DSS: 1.021 (0.438–2.379; $p = 0.9625$).

[§] Study reported risk of "cancer-specific mortality" separately for platinum-based chemotherapy and for gemcitabine-based chemotherapy. HR used represents that for platinum-based chemotherapy only and was reversed (yes vs no).

[25,26,28] and two non-cisplatin–based regimens (mainly being carboplatin based) [29,33]. Across the three cisplatin-based studies, the pooled HR was 0.43 (95% CI, 0.21–0.89; $p = 0.023$), representing a 57% benefit in OS among those treated with AC compared with those who received surgery alone (Fig. 2a). Between-study heterogeneity was not significant based on the Cochran Q statistic ($p = 0.15$) and $I^2 = 46\%$, so a fixed-effects model was used in the primary analysis.

Sensitivity analyses are shown in Table 3. Including all studies yielded a nonsignificant pooled HR of 0.65 ($p = 0.17$) in a random-effects model. Assuming that the Canadian study was 100% cisplatin based, the inclusion of this study resulted in a pooled HR of 0.52 (0.30–0.92; $p = 0.02$). When all studies with >75% of patients receiving cisplatin-based regimens were included (study added: UTUC Collaboration), this yielded a nonsignificant HR trending toward benefit for AC (HR: 0.58; 95% CI, 0.25–1.37; $p = 0.21$). Influence analyses revealed that if the UTUC Collaboration study [29] were omitted, it had the greatest impact on the overall HR, while the Kwak et al. study [26] was most impactful on the cisplatin-based HR.

3.2.1.2. Disease-free survival. Only two studies reported DFS outcomes, and both used cisplatin-based AC [25,26]. The pooled HR was 0.49 (95% CI, 0.24–0.99; $p = 0.048$), representing a 51% benefit in OS among those treated with AC compared with those who received surgery alone (Fig. 2b). Between-study heterogeneity was not significant based on the Cochran Q statistic ($p = 0.57$) and $I^2 = 0\%$, so a fixed-effects model was used. Influence analysis revealed that the Suzuki et al. study [25] had the greater impact on the pooled HR.

3.2.1.3. Disease-specific survival. For DSS, six studies had sufficient data for meta-analysis, three of which evaluated cisplatin-based [27,32] and four non-cisplatin–based regimens [29–31,33]. Regardless of stratification into cisplatin-based or non-cisplatin–based studies, pooled HRs remained nonsignificant although suggestive of a possible benefit toward AC, with an overall HR of 0.77 (Fig. 2c). Further sensitivity analyses did not substantially change results (Table 3). Influence analyses revealed that if the Canadian study [33] were omitted, it had the greatest impact on the overall HR, while the Kim et al. study [32] was most impactful on the cisplatin-based HR.

3.3. Studies evaluating the role of neoadjuvant chemotherapy

A total of four retrospective studies examined the role of NC for UTUC [34–37] (Table 4). A total of 123 patients received NC, while 1724 did not. Two studies reported 5-yr OS rates of 13–14% [34,37]. The UTUC Collaboration reported a 5-yr DFS rate of 49% (vs 64% and 30% among N0 and N+ patients who received NU only, respectively) and a 5-yr DSS of 44% (vs 64% and 35% among N0 and N+ patients who received NU only, respectively) [34].

Two prospective single-center phase 2 trials accrued patients with locally advanced urothelial cancers, including UTUC. One trial using sequential triplet ifosfamide, doxorubicin, gemcitabine–cisplatin, gemcitabine, ifosfamide had five patients who received NC, with a 60% pathologic downstaging (to ≤pT1N0) rate [38], while the other using dose-dense (DD)-MVAC with bevacizumab had 16 patients, with a 75% pathologic downstaging (to ≤pT1N0) rate [39]. Across two retrospective studies investigating NC [34,40], there was a DSS benefit, with a pooled HR of 0.41 (95% CI,

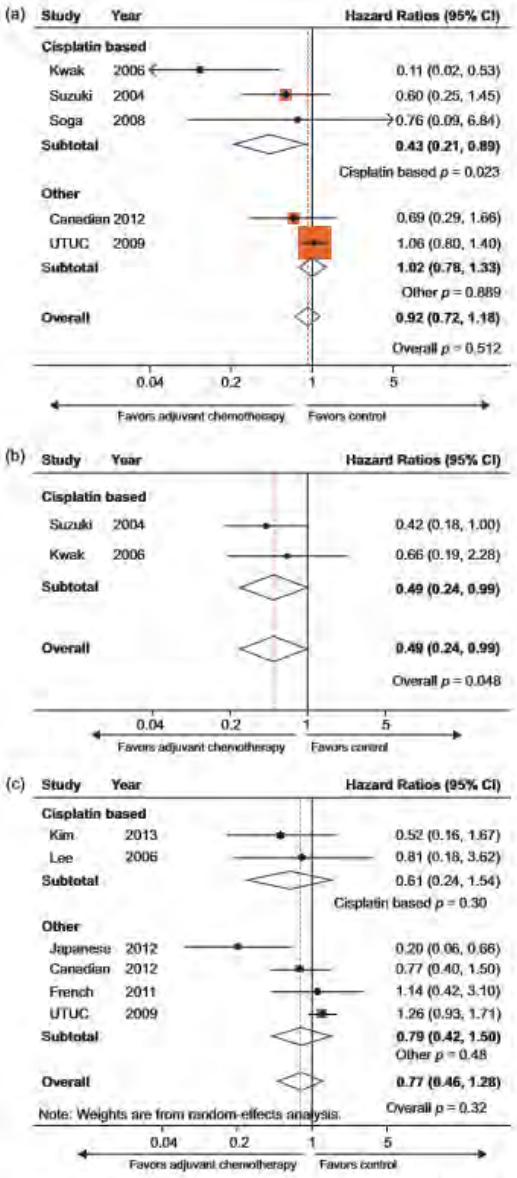

Fig. 2 – Forest plots: (a) overall survival, (b) disease-free survival, and (c) disease-specific survival hazard ratios of studies investigating cisplatin-based* and other, non-cisplatin–based adjuvant chemotherapy for upper tract urothelial carcinoma. Fixed-effects models were used for the primary analysis for cisplatin-based studies.**
AC = adjuvant chemotherapy; CI = confidence interval; UTUC = upper tract urothelial carcinoma.
*** Studies evaluating only cisplatin-based AC regimens (100% of patients treated with cisplatin-based regimen).**
**** Other non-cisplatin–based regimens allowed carboplatin- and/or nedaplatin-based regimens (not exclusively and 100% cisplatin based).**

Table 3 – Sensitivity analyses for meta-analyses of overall, disease-free, and disease-specific survival hazard ratios of studies investigating cisplatin-based* and other, non–cisplatin-based adjuvant chemotherapy for upper tract urothelial carcinoma**

Sensitivity analyses	Pooled HR (95% CI)	*p* value
OS		
Primary analysis (100% cisplatin-based regimens)	0.43 (0.21–0.89)	0.023
Including Canadian study (assuming 100% of study's patients received cisplatin-based regimens) [25,26,28,33]	0.52 (0.30–0.92)***	0.023
Including studies with >75% of patients receiving cisplatin-based regimens (UTUC Collaboration study added) [25,26,28,29]	0.58 (0.25–1.37)†	0.212
Including all studies [25,26,28,29,33]	0.65 (0.35–1.20)††	0.166
Influence analysis: identifies which study had the largest impact on the pooled HR if omitted	Cisplatin based: Kwak [26] Overall: UTUC Collaboration [29]	—
DFS		
Primary analysis (100% cisplatin-based regimens)	0.49 (0.24–0.99)	0.048
Including studies where >75% of patients received cisplatin-based regimens [25,26]	0.49 (0.24–0.99) Same (both studies were cisplatin-based regimens)	0.048
Including all studies [25,26]		
Influence analysis: identifies which study had the largest impact on the pooled HR if omitted	Suzuki [25]	
DSS		
Primary analysis (100% cisplatin-based regimens)	0.61 (0.24–1.54)	0.299
Including Canadian study (assuming 100% of study's patients received cisplatin-based regimens) [27,32,33]	0.72 (0.42–1.22)†	0.222
Including studies with >75% of patients receiving cisplatin-based regimens (UTUC Collaboration and Japanese studies added) [27,29,31,32]	0.62 (0.25–1.54)††	0.305
Including all studies [27,29–33]	0.77 (0.46–1.28)§	0.318
Influence analysis: identifies which study had the largest impact on the pooled HR if omitted	Cisplatin based: Kim [32] Overall: Canadian [33]	

CI = confidence interval; DFS = disease-free survival; DSS = disease-specific survival; HR = hazard ratio; OS = overall survival; UTUC = upper tract urothelial carcinoma.
AC = adjuvant chemotherapy.
* Studies evaluating only cisplatin-based AC regimens (100% of patients treated with a cisplatin-based regimen).
** Non–cisplatin-based regimens allowed carboplatin- and/or nedaplatin-based regimens (not exclusively and 100% cisplatin based).
*** Fixed-effects model was used (heterogeneity: $p = 0.22$; $I^2 = 32\%$).
† Random-effects model was used (heterogeneity: $p = 0.03$; $I^2 = 66\%$).
†† Random-effects model was used (heterogeneity: $p = 0.05$; $I^2 = 57\%$).
‡ Fixed-effects model was used (heterogeneity: $p = 0.82$; $I^2 = 0\%$).
‡‡ Random-effects model was used (heterogeneity: $p = 0.02$; $I^2 = 71\%$).
§ Random-effects model was used (heterogeneity: $p = 0.04$; $I^2 = 56\%$).

0.22–0.76; $p = 0.005$; Fig. 3). Between-study heterogeneity was not significant based on the Cochran *Q* statistic ($p = 0.28$) and $I^2 = 16\%$, so a fixed-effects model was used.

3.4. Publication bias

There was no significant evidence of publication bias for any of the three outcomes based on Egger's graph and funnel plots (data not shown).

3.5. Discussion

3.5.1. The role of adjuvant chemotherapy

This meta-analysis has several important findings regarding the role of AC. First, we found an OS and DFS benefit among patients treated with cisplatin-based AC for UTUC compared with those who had surgery alone (Fig. 1a and 1b). Second, these findings remained relatively consistent for OS, with an HR approaching 1 (ie, lesser benefit) as we included more studies in sensitivity analyses (Table 3) but nevertheless a nonsignificant trend toward OS benefit. These findings are congruent with the strengths of AC, which include accurate postoperative pathologic staging and the possibility of eradicating any subclinical metastases to maximize the patient's survival. A recent meta-analysis of nine randomized trials investigating postcystectomy adjuvant cisplatin-based chemotherapy for BCa found benefit in both OS and DFS [3], but AC's utility in UTUC is limited because of the decline in renal function following NU given cisplatin's renal excretion and inherent nephrotoxicity. Independent NU series reveal that only about 20% of patients have a postoperative glomerular filtration rate (GFR) >60 ml/min [41,42], consistent with findings from the Canadian multicenter study [33]. Another recent study found that in patients who had normal preoperative GFR (>60), renal function decrease by a third after NU [43].

Consequently, possibly because of the difficulty in accruing patients for randomized clinical trials, all but one study examining AC were retrospective. These studies have generally reported that the use of AC had limited survival outcomes benefit compared with patients observed following definitive surgery, but it is important to interpret these conclusions in light of these studies' numerous limitations. Nonrandomized retrospective studies suffer

Table 4 – Studies investigating neoadjuvant chemotherapy for upper tract urothelial carcinoma

Study	Year	Type	Tumor stage	NC regimen	No. of patients		5-yr OS rates (%)		5-yr DFS rates (%)		5-yr DSS rates (%)	
					NC	No NC	NC	No NC	NC	No NC	NC	No NC
Porten [49]	2013	Retrospective single center	pT0–1 (*n* = 40; 36%) pT2 (*n* = 24; 21%) pT3 (*n* = 39; 35%) pT4 (*n* = 9; 8%) N0/x (*n* = 95; 84%) N1 (*n* = 10; 8%) N2 (*n* = 9; 8%)	Ifosfamide (77%) mCisplatin (13%) NS combi (16%)	31	81	80	62			90	62
UTUC Collaboration–Youssef [34]	2011	Retrospective multicenter	pT0–1 (*n* = 12; 4%) pT2 (*n* = 83; 26%) pT3 (*n* = 181; 58%) pT4 (*n* = 37; 12%) N0 (*n* = 184; 59%) N1 (*n* = 98; 31%) N2 (*n* = 31; 10%)	GC (77.8%) M-VAC (22.2%)	18	295	–	–	49	30 (N+) 64 (N0)	44	35 (N+) 69 (N0)
UTUC Collaboration–Margulis [36]	2009	Retrospective multicenter	–	M-VAC (34%) CMV (29%) GC (20%) Other (17%)	47	1322	–	–	–	–	–	–
Matin [35]	2010	Retrospective single center	pT0–2 (*n* = 92; 61%) pT3 (*n* = 49; 33%) pT4 (*n* = 9; 6%) pN+ (*n* = 23; 16%)	M-VAC (44%) GC/GCI (33%) Other (23%)	43	107	14	–	–	–	–	–
Igawa [37]	1995	Retrospective single center	pT2 (*n* = 6) pT3 (*n* = 4) pT4 (*n* = 5)	M-VAC (33%) MVEC (40%) MEC (27%)	15	–	13	–	–	–	–	–

Study	Year	Type	Tumor stage	NC regimen	NC	No NC	Pathologic downstaging rates (%)	
							≤pT0N0	≤pT1N0
Hoffman-Censits [54]	2013	Prospective single-center phase 2 trial	High grade	Accelerated M-VAC	10		1	4
Siefker-Radtke [38]	2013	Prospective single-center phase 2 trial	High grade (100%)*	IAG followed by CGI	5	–	–	60
Siefker-Radtke [39]	2012	Prospective single-center phase 2 trial	High-grade or radiographically measurable sessile mass	DD-MVAC with bevacizumab	16		38	75

CMV = cisplatin, methotrexate, vinblastine; DD-MVAC = dose-dense M-VAC; DFS = disease-free survival; DSS = disease-specific survival; GC = gemcitabine, cisplatin; GCI = GC, ifosfamide; IAG = ifosfamide, doxorubicin, gemcitabine; M-VAC = methotrexate, vinblastine, Adriamycin, cisplatin; mCisplatin = modified dose of cisplatin; MEC = methotrexate, epirubicin, cisplatin; MVEC = methotrexate, vinblastine, epirubicin and cisplatinum; NC = neoadjuvant chemotherapy; NS combi = nephron-sparing combination; OS = overall survival; UTUC = upper tract urothelial carcinoma.

* Trial investigated NC in patients who had locally advanced urothelial cancer; this included clinical T2 (*n* = 37), T3b (*n* = 18), and T4a (*n* = 5) patients, as well. Only five patients had renal pelvis or ureter disease, and we considered these UTUC. Numbers reported in Table 4 pertain only to UTUC.

from substantial selection biases that are difficult to adjust for, where patients with the worst prognostic factors are selected to receive AC compared with counterparts undergoing observation. This is evident especially when one examines the proportion of patients receiving AC; this proportion is three to four times smaller than those who received surgery alone (Table 1). In addition, the proportion of patients who had N+ disease receiving AC was higher than those not receiving AC in some studies [29,33]. Therefore, these limitations may restrict our ability to detect larger differences in survival outcomes and hence may underestimate the true efficacy of AC in each of these studies. Conversely, an alternative interpretation could be that counterbalancing biases in favor of AC exist when analyzing observational data, because patients receiving AC may have better renal function and performance status or may have received AC without baseline muscle-invasive disease. Given the importance of N+ disease for prognosis, we believe that the negative selection bias described above would likely prevail, tending to cause a net underestimate of AC efficacy. These factors are important to consider when interpreting the results of our meta-analysis, which nevertheless still showed a statistically significant benefit for OS and DFS among studies using cisplatin-based AC.

3.5.1.1. Cisplatin-based adjuvant chemotherapy studies. Examining each study in detail may shed further light. Smaller studies have generally shown positive results, perhaps attesting to the strengths of better-run single-center studies [44],

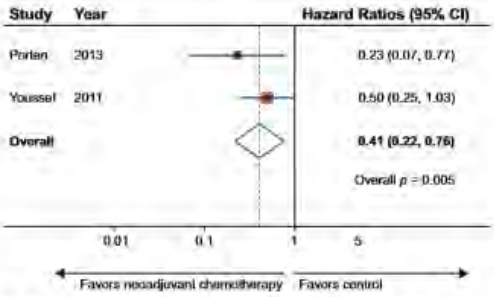

Fig. 3 – Forest plot: disease-specific survival hazard ratios of studies investigating neoadjuvant chemotherapy for upper tract urothelial carcinoma.
CI = confidence interval.

including the fact that the vast majority of their patients received cisplatin-based AC.

Soga and colleagues retrospectively examined 132 UTUC patients who underwent NU between 1986 and 2005 [28]. A total of 46 patients with locally advanced disease (pT2/3 N0M0) were selected, of whom 24 received adjuvant M-VAC therapy and 22 did not. There were no significant differences in 5- and 10-yr OS rates.

Kwak et al. examined 43 NU-treated patients, of whom 32 received more than four cycles of cisplatin-based therapy [26]. At a median follow-up of 30 mo, only 28.1% of patients in the AC group had died compared with 81.8% of those who did not receive AC. Multivariable Cox proportional hazard analyses revealed that the use of AC was strongly associated with OS (HR: 0.11; $p = 0.01$).

3.5.1.2. Other non-cisplatin–based adjuvant chemotherapy studies.
Other larger studies were not included in our primary analysis because they included patients who received non-cisplatin-based chemotherapy regimens (most commonly, carboplatin-based regimens), likely diluting the effect of AC (Table 1).

The first large study used the French Collaborative National Database on UTUC, which identified 627 high-risk patients (pT3–4 N0 and/or lymph node positive) [30]. A minority ($n = 140$; 22.6%) received AC, of whom only 52.8% were treated with cisplatin-based regimens. Overall, there were no significant differences in 5-yr OS and cancer-specific survival (CSS) rates between the groups. Of note, 87% of those who received AC ($n = 122$) had grade 3 disease compared with only 38% in the control arm ($n = 487$; $p = 0.001$). There were also significantly more patients in the AC group who had node-positive (27% vs 17%) and metastatic (22% vs 16%) disease ($p = 0.001$), clearly underlining selection bias in assignment of patients to AC. The presence of patients who had metastatic disease also contradicts the guiding principle of AC, which is strictly given to patients without clinical evidence of nodal or metastatic disease. In addition, nearly 40% of patients received carboplatin-based regimens (either paclitaxel or gemcitabine), contributing an additional possible explanation for why no survival benefit was seen.

The second large series by the UTUC Collaboration [29] impressively amassed 1390 patients from different centers treated with NU, of whom 542 (39%) were classified as high risk (pT3–4 N0 and/or lymph node positive). However, only 22% of these high-risk patients received AC (11% of which was non-cisplatin based). Patients receiving adjuvant therapy had higher tumor grade and stage. There were no significant differences in OS or DSS between the two groups. Although this study has the merit of a large sample size, nearly 80% of patients did not receive AC, and those who did were higher-risk patients. Therefore, the counter-intuitive conclusions likely arise from nonoptimal patient selection criteria and selection biases in the AC arm, restricting the value of its conclusions.

The third large Canadian multicenter study consisted of 1029 patients treated with NU [33]. The 59 patients (5.7%) who received AC were more likely to have concomitant carcinoma in situ, presence of lymphovascular invasion, and node-positive disease compared with those who did not receive AC. Multivariable-adjusted survival analyses did not find that AC translated into a significant improvement in OS or DSS, even for patients who had high-risk disease (≥pT3 or pTxN+ or both). The type of AC regimen was unreported, but given that the median preoperative estimated GFR was 59 ml/min per 1.73 m^2, for this analysis, we assumed that because of the post-NU decline, not all patients received cisplatin-based AC.

The only prospective study investigating AC for UTUC was a phase 2 trial performed by the Hellenic Cooperative Oncology Group, which treated 36 patients who had ≥pT3b and/or N+ disease with four cycles of adjuvant paclitaxel and carboplatin [24]. Carboplatin is sometimes used in preference over cisplatin because dose adjustments based on GFR can be made, targeting a predefined area under the curve (usually of 5) for patients who have some degree of renal impairment (creatinine clearance rate < 60 ml/min). However, carboplatin-based regimens are considered inferior to cisplatin-based approaches for urothelial cancers [45]. With a median follow-up of 40.6 mo, the Hellenic investigators found a 5-yr OS and DFS of 52% and 40.2%, respectively [24]. It is pertinent to note that none of the 20% of patients who had grade 2 disease relapsed, while the remaining 80% who had grade 3 disease had a much higher relapse rate of 60%. Only 17% of all patients developed distant metastases. This study concluded that the use of adjuvant paclitaxel and carboplatin was well tolerated in ≥pT3 UTUC patients, and some benefit was seen for preventing distant metastases, but the effect of AC on OS could not be seen. Therefore, carboplatin-based regimens, although less nephrotoxic, may be considered less efficacious. Their use is not uncommon in practice; nearly 40% of patients in the French multicenter study received carboplatin with paclitaxel or gemcitabine [30]. Extrapolation from bladder studies indicates that inferior outcomes are associated with carboplatin-based regimens compared with cisplatin regimens in the adjuvant setting [3].

3.5.2. *Adjuvant vs neoadjuvant chemotherapy*

Compared with AC, NC makes even more sense to pursue in UTUC, given challenges in administering cisplatin-based chemotherapy following NU because of the renal function decline. Most of the UTUC experience with NC has been guided by overwhelming level 1 evidence in BCa [2]. Potential benefits include the treatment of early micrometastases, better patient tolerability, and the ability to administer full-dose cisplatin with curative intent when patients still have functioning kidneys. Furthermore, pathologic downstaging in response to chemotherapy may be useful in predicting patients who may have a decreased risk of relapse following neoadjuvant chemotherapy and definitive surgery, as in the bladder experience [2]. In contrast, some downsides exist, including a delay to definitive surgical management leading to inadvertent disease progression in chemoresistant patients [46]. Moreover, there may be theoretical increased perioperative morbidity, although this has not been shown in BCa [47,48]. Last, overtreatment can occur in patients without pathologically proven muscle-invasive disease [2].

3.5.2.1. The role of neoadjuvant chemotherapy. Published studies addressing NC for UTUC are summarized in Table 4. The M.D. Anderson Cancer Center (MDACC) reported outcomes for 43 UTUC patients treated with NC [35]. When compared with a historical cohort of 107 patients who underwent initial surgery, pathologic downstaging was significantly higher in patients receiving NC. In addition, 14% of patients had complete pathologic response, congruent with that seen in the study of 15 patients by Igawa et al. [37]. The study's main limitation was lack of long-term follow-up, but a recent update with a median OS of 78 mo showed an impressive 3- and 5-yr DSS of 77% and 67%, respectively [49]. On multivariable analysis, receipt of NC was significantly associated with improved DSS (HR: 0.23; 95% CI, 0.07–0.79; $p = 0.02$) (Fig. 3).

The international UTUC Collaboration (including MDACC patients) had 313 NU patients, of whom 18 received "concept-modified" NC because of biopsy-proven locoregional nodal metastases [34]. These 18 patients had favorable 5-yr DFS and CSS rates of 49% and 44%, respectively, but it should be noted again that this strictly deviates from the true concept of neoadjuvant intent, where patients should have clinical N0 disease. Another, earlier study from the UTUC Collaboration reported that only 3% of 1363 NU patients ($n = 41$) received NC [36].

Limited prospective data exist for NC in UTUC. A phase 2 trial of neoadjuvant cisplatin-based sequential triplets for UC enrolled 65 patients, of whom only 5 had primary tumors in the renal pelvis or ureter. Pathologic downstaging to ≤pT1N0 disease was seen in three of these patients [38]. The same investigators from MDACC recently presented the results from a trial investigating neoadjuvant DD-MVAC with bevacizumab in patients who had UC of the bladder and upper tract [39]. Of the 60 patients enrolled, 16 had tumors of the renal pelvis and/or ureter. Keeping in mind the limitations and difficulties associated with accurate staging, this study found pathologic downstaging to ≤pT0N0 and ≤pT1N0 in 38% and 75% of UTUC patients, respectively, with a 3-yr OS and DSS of 93% for all patients. Based on these findings, they concluded that neoadjuvant DD-MVAC appears to be an acceptable alternative to M-VAC, but limitations on accurate prechemotherapy staging preclude any definitive conclusion.

3.5.3. *Future perspectives*

Even in lower tract urothelial cancer, despite the availability of level 1 evidence, use of NC and AC for lower tract BCa remains low, at ≤20% of patients [2,3,50]. Expectedly, in the absence of corresponding strong evidence, chemotherapy use is even lower for UTUC. Therefore, results from ongoing phase 2/3 trials are eagerly expected (Table 5). For example, the phase 3 POUT trial (ISRCTN98387754) randomizing patients undergoing NU for UTUC to either adjuvant platinum-based chemotherapy or surveillance, with an estimated enrollment of 345 patients, will guide whether AC is actually required [51], but this trial may be limited by inadequate stratification of local or locoregional disease.

Development of less toxic and more tolerable regimens may obviate the need for nephrotoxic cisplatin-based

Table 5 – Ongoing trials examining adjuvant and neoadjuvant chemotherapy for upper trace urothelial carcinoma

ClinicalTrials.gov identifier	Phase	Estimated enrollment	Estimated completion	Tumor stage	Timing of chemotherapy	Chemotherapy arm	Primary outcomes	Secondary outcomes
ISRCTN98387754	3	345	March 2017	pT2–pT4 pN0–3 M0 or pTany N1–3 M0	Adjuvant	GC (for GFR 30–49 ml/min postoperative)	–	–
NCT00028860	2	30	October 2004	T3b–4, N0, M0 or Any T, N1–3, M0	Adjuvant	Paclitaxel, ifosfamide, carboplatin, and gemcitabine	–	–
NCT01663285	2	55	August 2016	High grade	Neoadjuvant	GC	2-yr RFS	(1) Rate of BCa stage (2) Adverse events (3) Tumor sequencing
NCT01261728	2	54	December 2013	T2–4a N0/X M0	Neoadjuvant	GC	Pathologic response rate (<pT2)	Time to disease progression; OS; safety and tolerability

BCa = bladder cancer; GC = gemcitabine, carboplatin; GFR = glomerular filtration rate; OS = overall survival; RFS = recurrence-free survival.

regimens. Two trials are investigating the platinum-based regimen of GC (NCT01663285, NCT01261728). DD-MVAC with bevacizumab appears to have favorable results [39] and so should be considered in future trials. Trials investigating non-cisplatin combinations (eg, carboplatin) will likely not occur, as current data from both UTUC and BCa do not support their use.

As mentioned earlier, upper tract cancers differ from lower tract cancers at the embryologic and molecular level [8,10]. Investigators may be able to take advantage of these molecular alterations to predict chemosensitivity. For example, investigators have found that microsatellite instability predicts improved response to adjuvant therapy with irinotecan, fluorouracil, and leucovorin in stage III colon cancer [52]. The potential to personalize chemotherapy regimens based on predictive biomarkers of resected urothelial tissue (precision medicine) represents a promising area of further research [14].

3.5.4. Treatment recommendations

We concur with the recommendations from the 2013 update of the European Guidelines on UTUC [1]. In addition, based on our meta-analysis results and bearing in mind the limitations associated with these studies, we believe that there might be a role for cisplatin-based AC in UTUC patients who have adequate postoperative renal function and high-risk disease (eg, pT3, pN+, positive surgical margins), but insufficient evidence currently exists to recommend routine use of AC for all UTUC patients. There appears to be more robust evidence for the use of AC over NC. Despite the limited data, NC appears to be a promising approach, especially for those who have good renal function and histologic evidence of high-grade muscle-invasive disease, as we have learned from the experience in bladder primaries [53]. Based on the positive experiences from BCa NC trials [2], we advocate for continued multicenter collaborative prospective trials to investigate DD-MVAC, M-VAC, or GC in the neoadjuvant setting for UTUC patients.

4. Conclusions

Our meta-analysis of retrospective studies demonstrates significant OS and DFS benefits, with a nonsignificant trend toward benefit in DSS for cisplatin-based AC in UTUC. Current studies are limited by small numbers and selection biases secondary to their retrospective design; therefore, although there might be a benefit, there is insufficient evidence to recommend routine use of cisplatin-based AC for UTUC. NC appears promising, with favorable pathologic response rates, but additional trials are needed for confirmation.

Author contributions: Joaquim Bellmunt had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: Leow, Bellmunt.
Acquisition of data: Leow, Martin-Doyle, Bellmunt.
Analysis and interpretation of data: Leow, Martin-Doyle, Fay, Choueiri, Chang, Bellmunt.
Drafting of the manuscript: Leow, Martin-Doyle, Bellmunt.
Critical revision of the manuscript for important intellectual content: Leow, Martin-Doyle, Fay, Choueiri, Chang, Bellmunt.
Statistical analysis: Leow, Martin-Doyle.
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APÊNDICE L - 10% Tumor Diameter Shrinkage on the First Follow-Up Computed Tomography Predicts Clinical Outcome in Patients With Advanced Renal Cell Carcinoma Treated With Angiogenesis Inhibitors: A Follow-Up Validation Study

The Oncologist

Genitourinary Cancer: Renal, Bladder, and Testicular

10% Tumor Diameter Shrinkage on the First Follow-Up Computed Tomography Predicts Clinical Outcome in Patients With Advanced Renal Cell Carcinoma Treated With Angiogenesis Inhibitors: A Follow-Up Validation Study

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Renal cell carcinoma • VEGF-targeted therapy • RECIST 1.0 • Choi • Tumor shrinkage

ABSTRACT

Background. Vascular endothelial growth factor (VEGF)-targeted agents are standard therapies for metastatic renal cell carcinoma (mRCC), associated with variable tumor shrinkage. Response Evaluation Criteria in Solid Tumors (RECIST) is of limited utility in this setting, and other imaging changes are sought to reliably predict outcome early. We aim to validate 10% tumor shrinkage as the best early indicator of outcome.

Methods. In this institutional review board-approved, Health Insurance Portability and Accountability Act-compliant study, 66 mRCC patients with 165 lesions on clinical trials of VEGF-targeted agents underwent thoracic and abdominal computed tomography at baseline and at first follow-up after therapy. Measurements were performed according to RECIST and tumor shrinkage of ≥10% decrease in sum of the longest diameter (−10%SLD). Correlation with time-to-treatment failure (TTF) and overall survival (OS) were compared and stratified by response to the radiologic criteria. Receiver-operating characteristics (ROC) analysis yielded the optimal threshold change in SLD, defining patients with prolonged survival.

Results. More than −10%SLD significantly differentiated responders from nonresponders (median TTF 8.4 vs. 4.1 months, $p = .001$), whereas partial response by RECIST did not (median TTF 6.9 vs. 5.5 months in responders vs. nonresponders, $p = .34$). −10%SLD was also significantly predictive of OS (median OS 35.1 vs. 15.0 months in responders vs. nonresponders, $p = .003$). ROC curve analysis yielded −9.3% in SLD as the optimal threshold for response/no response.

Conclusion. Ten percent tumor shrinkage is validated as a reliable early predictor of outcome in mRCC patients receiving VEGF-targeted therapies and may provide a practical measure to guide therapeutic decisions. ***The Oncologist*** 2014;19:507–514

Implications for Practice: Tumor shrinkage of 10% on first follow-up computed tomography after initiation of therapy predicts time-to-treatment failure and overall survival in this validation cohort of metastatic renal cell carcinoma patients treated with antiangiogenic therapies. Given the utility of this response indicator in multiple reports, 10% tumor shrinkage may provide a simple and practical aid for therapeutic decision making if used in clinical trials and practice with a prospective evaluation.

INTRODUCTION

Molecular targeted therapies have revolutionized the treatment of metastatic renal cell carcinoma (mRCC), resulting in significant survival benefits in treated patients despite variable amounts of tumor shrinkage [1]. Antiangiogenic therapies are known to decrease tumor vascularization rather than result in direct cytotoxicity and have been associated with lesser degrees of tumor shrinkage than traditional antitumor agents, with typical objective response rates ranging from 10% to 40% [2–6]. Many patients do not achieve 30% tumor shrinkage to indicate partial response in these reports, and it is commonly accepted that response evaluation criteria in solid tumors (RECIST) may be of limited utility in discriminating patients responding to such therapies.

Computed tomography (CT) is a robust, widely available imaging modality routinely used in the assessment of patients' tumor burden throughout treatment and in monitoring

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patients for therapy-related toxicities. Several investigators have sought to optimize tumor response assessment via imaging, such that imaging indicators better correlate with clinical benefit and survival outcomes early in the treatment course. Although the shortcomings of the conventional RECIST criteria in this setting are known, there is no consensus as to the preferred response assessment method. Several alternative tumor shrinkage thresholds have been proposed, namely 10% and 20% long axis diameter reductions, either alone [7, 8] or in combination with other CT imaging response criteria, such as attenuation [9–13]. Ten percent tumor shrinkage as an indicator of response has been supported in a study of patients treated with sunitinib in a multicenter phase III trial [7] as well as in another study of multiple imaging indicators of response in patients treated with bevacizumab, sorafenib, and sunitinib [8]. Ten percent reduction in the long axis diameter of target lesions is also a component of response according to Choi criteria [14], recently applied to patients with mRCC [9, 12, 13]. Twenty percent long axis reductions are components of favorable response in the recently developed size and attenuation CT (SACT) and morphology, attenuation, size, and structure (MASS) criteria [10, 11].

In a previous study of 70 mRCC patients treated with first-line sunitinib, sorafenib, or bevacizumab, four criteria of early postimaging changes were compared, including RECIST 1.0, 10% tumor diameter shrinkage, CT attenuation of tumor, and Choi criteria in assessing time-to-treatment failure (TTF) and overall survival (OS) [8]. Ten percent tumor shrinkage at first follow-up was demonstrated statistically significant in predicting TTF and OS, whereas other criteria were not. However, given the retrospective nature of the study, it is necessary to validate the results in an independent cohort and reproduce the association between 10% tumor shrinkage and survival, to propose use in future prospective clinical trials and clinical practice.

The purpose of the present study is to determine whether 10% tumor shrinkage can differentiate responders from nonresponders in an independent validation cohort of mRCC patients enrolled in prospective clinical trials of vascular endothelial growth factor (VEGF) inhibitors. We hypothesize that 10% tumor shrinkage at first follow-up, as demonstrated in our previous report and others [7, 8], represents a more clinically meaningful response assessment than RECIST 1.0. If found reproducible, a 10% tumor shrinkage criterion could be widely applicable to mRCC patients, to identify patients likely to benefit from treatment early after initiation of therapy.

Patients and Methods

Patients and Treatment

The study sample consists of patients with metastatic renal cell carcinoma enrolled in six recent and ongoing multicenter, open-label studies of VEGF-targeted agents (vatalanib, sunitinib, sorafenib, pazopanib, foretinib, and tivozanib) (3, 15–19). Patients were part of approved Institutional Review Board (IRB) protocols for advanced renal cell carcinoma (RCC) at the institution in which baseline and follow-up clinical data were prospectively collected, and this study was approved by the IRB and is Health Insurance Portability and Accountability Act compliant. Patients enrolled in these trials who had been included in a previous study cohort were excluded from this validation cohort [8]. All patients had histologically confirmed stage IV mRCC. Patients were treated at standard doses of study drug according to the assigned protocol until they experienced disease progression, severe or intolerable toxicity, or withdrew consent. Compliance was checked after each cycle with a treatment diary.

Imaging and Image Analysis

Patients eligible for analysis included those with target lesions by RECIST who underwent noncontrast-enhanced or contrast-enhanced CT of the chest, abdomen, and pelvis before and after VEGF-targeted therapy initiation, with pre- and post-therapy scans at the same institution. The routine oncology protocol was used on multidetector CT scanners (64 detector row) using oral contrast in all patients and intravenous contrast in patients with adequate estimated glomerular filtration rate (eGFR) and no known allergy to the contrast. Contrast-enhanced scans were performed after 75–100 cc nonionic contrast administration (Ultravist; Bayer HealthCare, Leverkusen, Germany, http://www.bayer.com; based on eGFR), empirically timed with chest images obtained in the arterial phase (30-second delay) and abdominal images obtained in the portal venous phase (70-second delay). Images were reviewed and measured on a Picture Archiving and Communication System (Centricity; General Electric, Milwaukee, WI, http://www.gehealthcare.com).

Up to three target lesions in each patient were selected on the baseline CT, according to RECIST 1.0 [20], by a single board-certified radiologist with 8 years of experience in cancer imaging, blinded to patient outcomes. At baseline and first follow-up, the longest axial axis of each target was recorded to the nearest millimeter, as described previously [8]. Sum of the longest diameter (SLD) of the targets was recorded at baseline and at first follow-up of all patients.

RECIST response, including partial response (PR) and progressive disease (PD), was recorded at CT follow-up, based on ≥30% decrease in SLD for PR and ≥20% increase in SLD for PD [20]. Patients not meeting either were tabulated as stable disease (SD). Whereas RECIST response according to RECIST 1.0 was studied in this report, the RECIST categorizations are thought to be comparable to those according to the updated RECIST 1.1 criteria, because the definitions of PR, PD, and SD are unchanged [21, 22]. Ten percent decrease in SLD was also examined as an independent predictor of outcome.

Clinical outcome data were gathered by review of medical records, including date of treatment failure (progression as determined by RECIST) and date of death. TTF was defined as time from treatment initiation until failure, including progression and death. Patients who did not progress were censored on the date of last follow-up. OS was defined as the time from treatment initiation until death. Alive patients were censored on the date of last follow-up.

Statistical Analysis

Patients were classified into responders (PR) versus SD or PD, according to RECIST 1.0. The survival distribution for patients in the two groups was estimated according to the Kaplan-Meier method [23]. The difference in the survival distribution between the groups was evaluated using the log-rank test. An analysis of RECIST 1.0 clinical benefit, PR or SD versus PD, was not performed as only one patient had PD at the time of first follow-up, rendering this invalid. To explore any effect modifier among baseline characteristics in the relationships

Table 1. Patient demographic and disease characteristics of the 66 patients

Characteristics	Frequency	Percent
Age (median, range)	60.9	(32–84)
Gender		
Male	46	70
Female	20	30
Histology		
Clear cell	39	59
Papillary	22	33
Chromophobe	2	3.0
Sarcomatoid with clear cell	1	1.6
Not specified	2	3.0
Medication		
Vatalanib	21	31.8
Foretinib	17	25.8
Pazopanib	10	15.2
Sorafenib	6	9.1
Sunitinib	10	15.2
Tivozanib	2	3.0
Reason for off study		
PD	38	57.6
On trial	2	3.0
Toxicity	24	36.4
Withdrew consent	2	3.0
CT size change from baseline to first follow-up CT (%) (median, range)	−10.8	(−100, 20)
Interval from baseline CT to treatment initiation (days) (median, range)	13	[3, 27]
Interval from baseline CT to post-treatment CT (days) (median, range)	62	(20, 172)
Follow-up from registration to the last documentation or death (months) (median, range)	22.9	(1.3, 94.4)

Abbreviations: CT, computed tomography; PD, progressive disease.

between decrease in SLD and survival outcomes, bivariate analyses were performed using Cox proportional hazard models in an exploratory fashion.

Receiver-operating characteristics (ROC) analysis was used to determine the optimal threshold change in SLD at first CT follow-up to define patients with survival end point (death or censored at the end of the study), similar to Thiam et al. [7]. After confirming validity of the area under the ROC curve (AUC) in an accuracy test using a traditional point-system classification, Youden's index in the ROC analysis was calculated for the series of Ki-67 values observed in the data. We identified the threshold that minimized the false-positive and false-negative rates for best detecting prolonged survival, selecting patients with treatment benefit.

Results

Patients

Sixty-six patients with 165 target lesions were eligible for the study and evaluated (Table 1). Thirty-nine patients of the cohort had clear cell RCC histology (59%), 22 patients had papillary (33%), 2 patients each had chromophobe and unavailable histologic subtype, and 1 patient had predominant sarcomatoid features in addition to having clear cell histology.

The median age at targeted therapy initiation was 60.9 years. Twenty-one patients (32%) were treated with vatalanib, 17 patients (26%) with foretinib, 10 patients (15%) each with pazopanib and sunitinib, 6 patients (9%) with sorafenib, and 2 patients (3%) with tivozanib. Median time from baseline CT to drug initiation was 13 days, and median time from baseline to first post-treatment CT was 62 days (range 20–172 days). The observed outlier of 172 days in time from baseline to first post-treatment CT lay in 7 times the interquartile range (52 to 67 days) above the third quartile among all samples. All 66 patients were evaluated according to RECIST 1.0 and 10% tumor shrinkage thresholds.

Using the selected target lesions for this study, most patients (59 patients, 89%) were SD according to RECIST 1.0 at first post-treatment CT, whereas 6 were PR (9%) and 1 was PD (2%). Using 10% tumor shrinkage to indicate response, 33 patients achieved 10% tumor shrinkage and were considered responders (50%; Fig. 1), whereas 33 patients achieved less than 10% tumor shrinkage, deemed nonresponders. Proportional changes of SLD in reference to baseline in all but one of the study patients at the time of first follow-up CT ranged from 52% decrease in SLD to 20% increase in the SLD of target lesions (Fig. 2). One patient with a single measurable target lesion in the peritoneum at baseline experienced significant tumor shrinkage such that the target lesion was no longer measurable at first follow-up CT, although nontarget ascites fluid remained present, rendering the patient a PR according to RECIST.

No patient who achieved 10% tumor shrinkage at first follow-up on retrospective assessment developed a new lesion or was deemed as having progressive disease at the time of the original response assessment.

Association Between Early Post-Therapy Imaging Changes and Clinical Outcome

The TTF for patients with PR (median 6.9 months) was not significantly different from patients with SD or PD (median 5.5 months, $p = .34$; Fig. 3A). Similarly, the OS for patients with PR (median not reached, NR) was not significantly different from patients with SD or PD (median 25.5 months, $p = .72$; Fig. 3B).

Tumor shrinkage of ≥10% decrease in SLD significantly differentiated responders ($n = 33$, median TTF 8.4 months) from nonresponders ($n = 33$, median TTF 4.1 months, $p = .001$; Fig. 4A). Ten percent decrease in SLD was also significantly predictive of overall survival (with responders OS of 35.1 months vs. nonresponders OS of 15.0 months, $p = .003$; Fig. 4B). Bivariate Cox proportional hazards models showed that 10% decrease in SLD was a significantly robust predictor of TTF ($p = .001$) and OS ($p = .01$), respectively, after controlling for either baseline age, gender, drug, histology, or Memorial Sloan-Kettering Cancer Center risk (low/intermediate vs. high), whereas none of the controlled factors was significant.

Threshold Evaluation by ROC Analysis

For tumor shrinkage threshold evaluation, ROC curve analysis yielded −9.3% in SLD (maximum Youden index score 0.28) as the optimal threshold for response/no response with respect to OS.

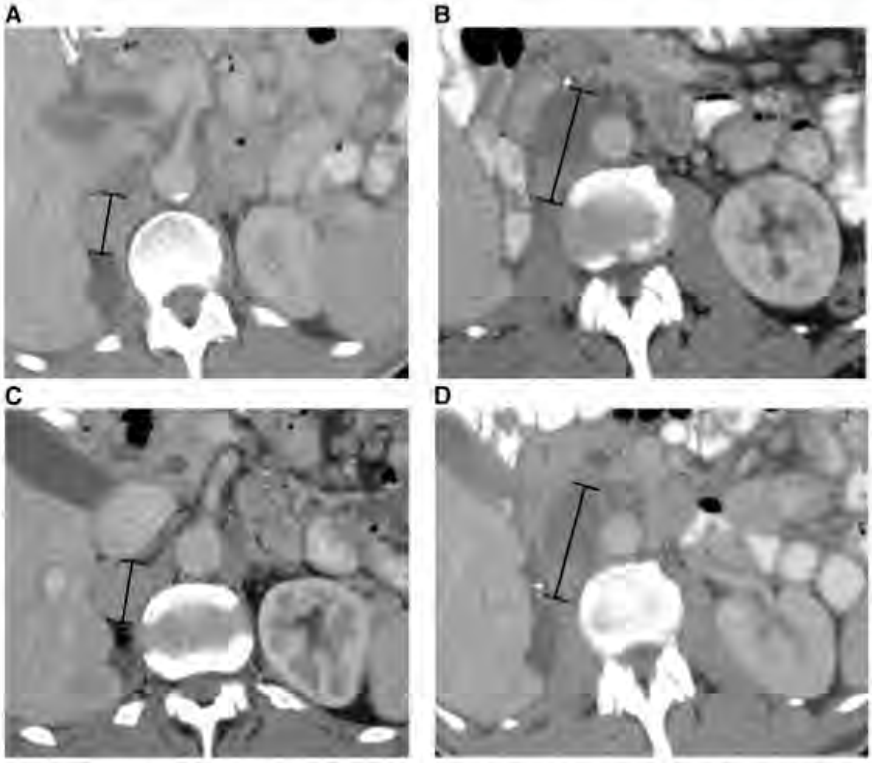

Figure 1. A 73-year-old man with metastatic renal cell carcinoma treated with foretinib. Axial contrast-enhanced computed tomography (CT) images at baseline (**A** and **B**) demonstrate target right adrenal and retroperitoneal metastases (black measurement lines), measuring 31 mm and 58 mm in long axis, respectively. Axial contrast-enhanced CT at first follow-up after treatment initiation (**C** and **D**) demonstrates approximately 10% decrease in the sum of the longest diameter of the targets (black measurement lines), measuring 28 mm and 52 mm, respectively.

Discussion

We evaluated RECIST response and tumor shrinkage as practical and reproducible imaging predictors of benefit from various VEGF-targeted therapies in mRCC patients treated in recent and ongoing clinical trials. In our study of 66 patients, a sizable cohort with respect to similar literature, a 10% decrease in target SLD was a reliable predictor of TTF and OS outcomes on first follow-up CT. This finding lends further support to the 10% tumor shrinkage threshold as an indicator of response in this setting, as previously advocated in the literature [7, 8]. Such early changes in post-therapy imaging enable us to distinguish responder patients and aid in patient management in three ways: (a) permitting patients who achieve this degree of tumor shrinkage to continue treatment with greater confidence, (b) limiting unnecessary toxicities to patients without evidence of treatment benefit, and (c) enabling these patients to consider other therapies.

The 10% tumor shrinkage threshold as an indicator of response to VEGF-targeted therapies in mRCC patients has been supported by analysis of a prior phase III study of sunitinib in which 10% tumor shrinkage was the best predictor of survival in 334 treated patients, including ROC analysis of various other shrinkage thresholds [7]. The advocated −10% threshold in this study and the −30% threshold for PR according to RECIST were reached after the first cycle of therapy in 73% and 19% of cases, respectively [7]. Ten percent tumor shrinkage was also a significant predictor of TTF and OS, whereas RECIST and Choi criteria were not predictive in another study of patients treated with bevacizumab, sunitinib, and sorafenib [8]. The current study lends further support to the applicability and utility of 10% tumor shrinkage as an early indicator of response in mRCC patients treated with a variety of VEGF-targeted therapies, as class-specific tumor response criteria rather than drug-specific.

Choi criteria have been applied to mRCC patients treated with VEGF-targeted therapies, with differing conclusions on the applicability of these criteria in different reports. Choi criteria similarly incorporate 10% tumor shrinkage into "response," or 15% decrease in mean tumor CT attenuation. In one such report by van der Veldt et al. [9], Choi criteria better delineated responders and nonresponders to therapy compared with RECIST, but did not indicate early progression. Thereafter, Choi criteria were found to early discriminate patients benefiting

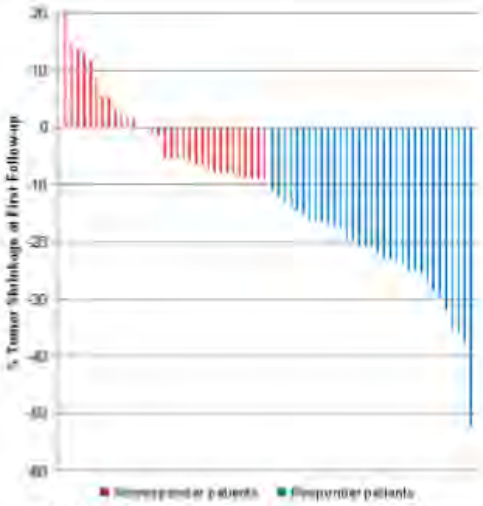

Figure 2. Range of change in the sum long axis diameter of target lesions at first follow-up computed tomography (CT). Responder patients with 10% tumor shrinkage at the time of the first follow-up CT (blue bars) had median time-to-treatment failure (TTF) and overall survival (OS) of 8.4 and 35.1 months, respectively, whereas nonresponder patients who did not achieve at least 10% tumor shrinkage (red bars) had median TTF and OS of 4.1 and 15.0 months ($p = .0014$ and .0032, respectively).

from treatment in at least one other report [12], although they did not significantly correlate with survival outcomes in others [8, 13, 24]. In each of these studies, Choi criteria were more likely to characterize patients as responders than other criteria in general, regardless of outcome. Technical CT parameters, contrast, CT attenuation measurement variability, and patient variables most likely contribute to the differences in results observed. In addition, no singular attenuation change threshold has been found to correlate with survival [8], raising questions regarding the applicability of the 15% decreased attenuation component of this response schema in the mRCC population.

Similarly, the SACT and subsequent MASS criteria were developed to delineate response in mRCC patients treated with sunitinib and sorafenib. These criteria include 20% tumor shrinkage, marked central necrosis, or marked decreased attenuation as components of favorable response, whereas 20% increase in size, new lesions, marked central fill-in, or new enhancement are features of unfavorable response [10, 11]. Pretherapy schema have been recently combined with the MASS criteria to improve accuracy [24], offering a unique clinical and imaging response model. However, drawbacks of these criteria include arbitrary correlation with progression-free survival >250 days, exclusion of lung lesions, and volumetric measurements necessitating another workstation in SACT. In contrast to these methods, the 10% tumor shrinkage assessment method described in this work may be advantageous because of the simplicity of single long axis diameter measurements of only three target lesions, which can be performed on a routine clinical basis without added time or a separate additional workstation, as is needed in volumetric measurements.

The unmet clinical need and utility of more generalized tumor response criteria are emphasized by various criteria evaluated in recent literature. A tumor response criterion that is generalizable as well as indicative of treatment benefit, such as one based on a tumor shrinkage threshold, is preferred and sought in this population. CT scanning is widely available and serves multiple purposes in the care of patients with widespread metastatic renal cell carcinoma. It is commonly used to assess the following: (a) patients' overall tumor burden in terms of growth patterns and distribution; (b) individual sites of metastatic disease to anticipate and limit troublesome disease-related complications via alternative, focused treatments (such as surgery or radiation to spinal metastases that may result in cord compression); and (c) evidence of treatment-related toxicities, potentially before the patient develops symptoms (such as pneumatosis intestinalis and/or hemorrhage in VEGF-targeted treatments) (25, 26). A simple, reliable measurement schema that has a better correlation with survival than RECIST can easily be incorporated into routine clinical practice at any center, regardless of scan protocol. Furthermore, single kidney patients with elevated eGFR or those with allergy may be assessed accordingly using the 10% tumor shrinkage, without the necessity of intravenous contrast.

There are several limitations to this study. For instance, the imaging review was performed retrospectively, by a single oncoradiologist, and patients were treated at a single institution. Given the small number of patients and the retrospective nature of the study, comparison of the 10% tumor shrinkage threshold with the RECIST criteria is limited and should not be overinterpreted. In fact, in this study, we applied the RECIST definitions of partial response, stable disease, and progressive disease to the time of first follow-up CT rather than assess RECIST response at all study time points. Of note, most RECIST responses do happen within 2 months (60 days median). According to Thiam et al. [7], 64% of partial responses were achieved within two cycles of sunitinib treatment. Therefore, it is likely that we captured most patients who achieved partial response for the best response during their treatment course, as a result of the timing of the first follow-up CTs.

Our purpose was to evaluate early changes in tumor burden on first CT follow-up and correlate these imaging changes with survival. Although nadir tumor burden or "best overall response" is important, this information is not available until a patient has finished therapy. Early tumor shrinkage is useful because it can be assessed prospectively in all patients. Our results show that early tumor shrinkage is informative, although the results of our study should be interpreted in the context of the study design. Another persistent limitation in this validation cohort is that it may be difficult to place patients close to the threshold (9% decrease) in the appropriate response category, but this would be true for any criteria using a threshold.

Measurement reproducibility in this cohort was separately evaluated [27]. In this study, we found 10% tumor shrinkage was a reproducible radiologic response indicator when baseline and follow-up studies were measured by a single radiologist. However, 10% tumor shrinkage was within the range of interobserver measurement variability. The validation study findings must be considered in this context. Some of the benefits of the patient cohort in this study include fairly homogeneous timing of the baseline and follow-up scans. Patients in this study

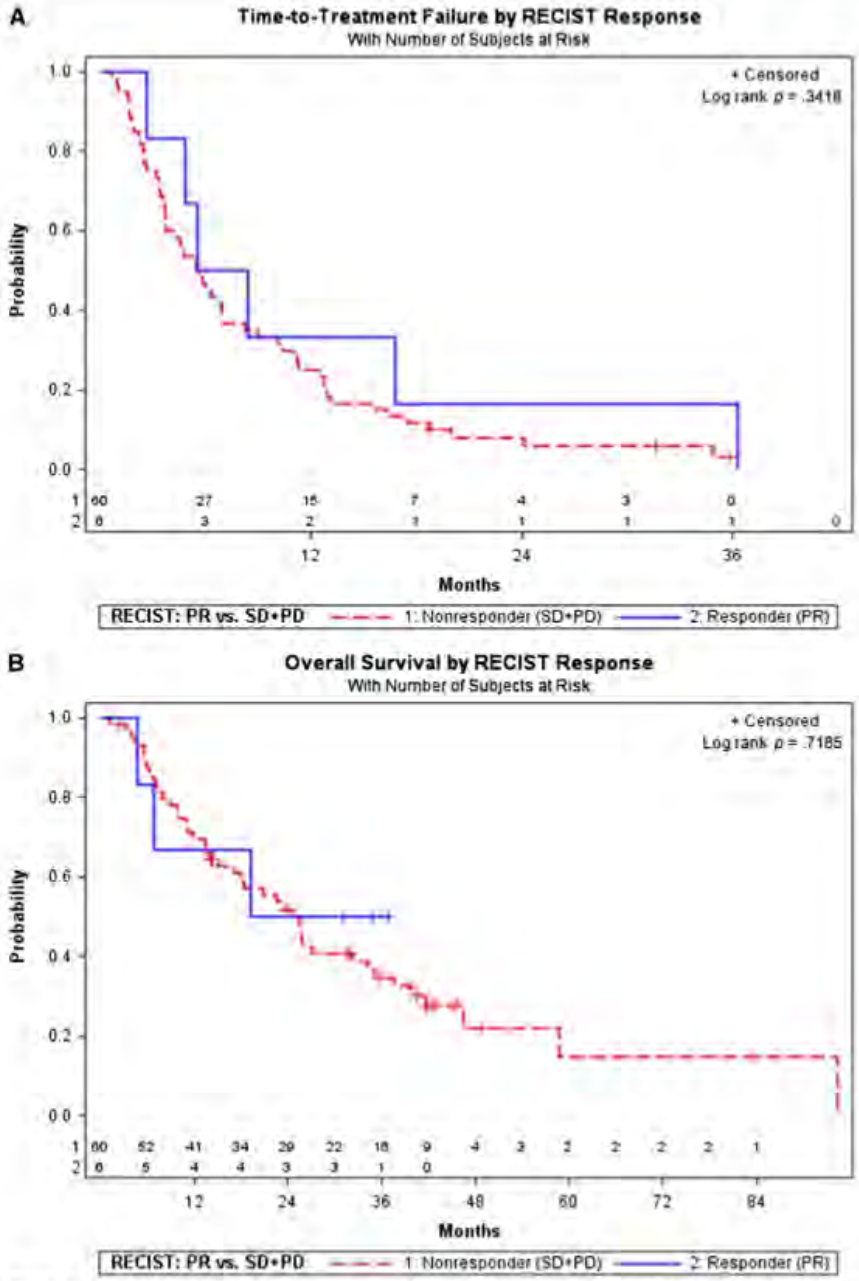

Figure 3. Time to treatment failure (TTF) and overall survival (OS) stratified by RECIST response at first follow-up computed tomography. **(A):** TTF stratified by RECIST response (PR) versus no response (SD + PD). TTF was not significantly different between RECIST responders and nonresponders (6.9 vs. 5.5 months, $p = .3418$). Partial response occurred in 6 patients at first follow-up, 6 events; 60 patients were nonresponders (SD + PD; 57 events and 3 censored). **(B):** OS stratified by RECIST response (PR) versus no response (SD + PD). OS was not significantly different between RECIST responders and nonresponders (not reached vs. 25.5 months, $p = .7185$). Partial response occurred in 6 patients at first follow-up, with 3 patients deceased and 3 censored; 60 patients were nonresponders (SD + PD; 43 deceased and 17 censored).
Abbreviations: PD, progressive disease; PR, partial response; RECIST, Response Evaluation Criteria In Solid Tumors; SD, stable disease.

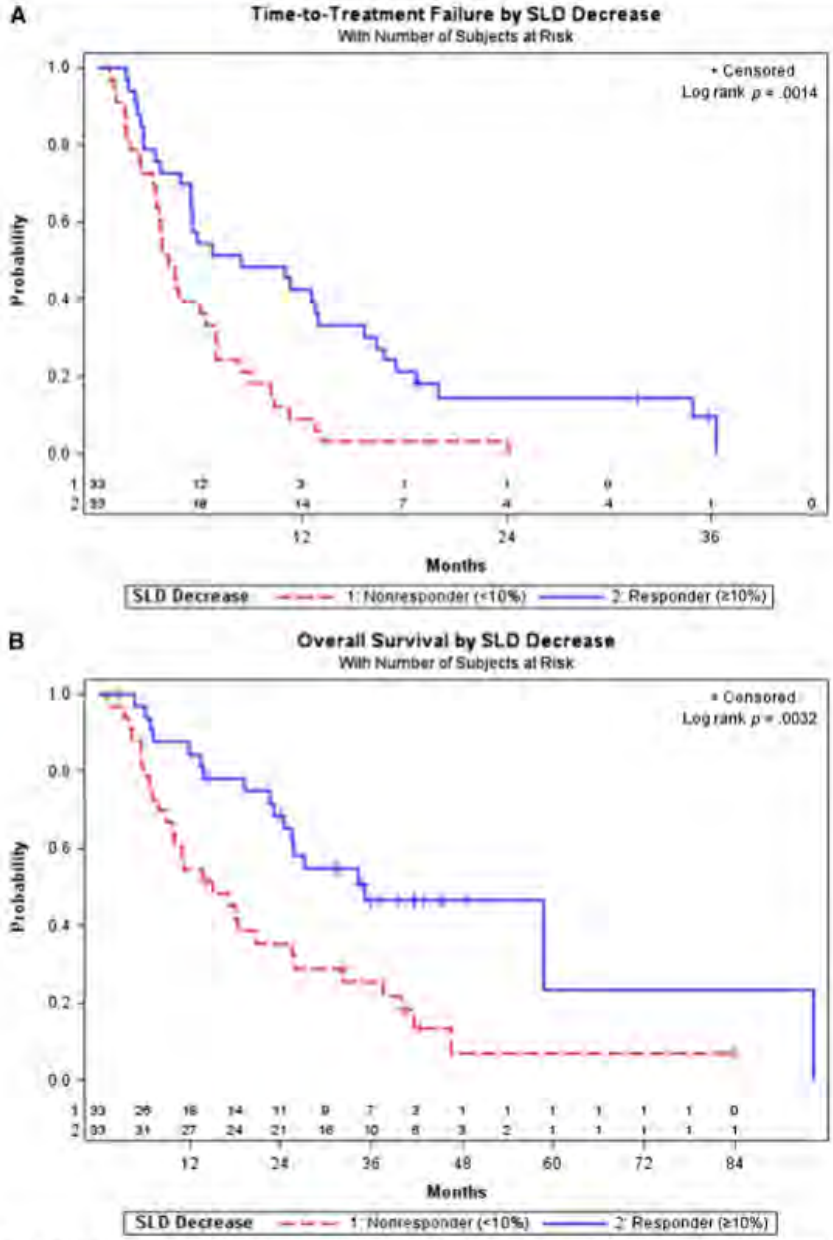

Figure 4. Time-to-treatment failure (TTF) and overall survival (OS) stratified by first follow-up computed tomography response according to >10% decrease in size versus no response. **(A):** TTF stratified by response according to ≥10% decrease in size versus no response (<10% decrease in size). TTF was significantly different between responders who achieved 10% decrease in size and nonresponders who did not (8.4 vs. 4.1 months, $p = .0014$). The ≥10% tumor shrinkage occurred in 33 patients at first follow-up, with 30 events and 3 censored; 33 patients were nonresponders (33 events). **(B):** OS stratified by response according to ≥10% decrease in size versus no response (<10% decrease in size). OS was significantly different between responders who achieved 10% decrease in size and nonresponders who did not (35.1 vs. 15.0 months, $p = .0032$). The ≥10% tumor shrinkage occurred in 33 patients at first follow-up, with 18 patients deceased and 15 censored; 33 patients were nonresponders, with 28 patients deceased and 5 censored.

Abbreviation: SLD, sum of the longest diameter.

were on clinical trials of various VEGF-targeted agents, whereas patients in a previous study were not treated on trials [8]. Second, data and conclusions from this study may be applicable to mRCC patients treated with VEGF-targeted therapies in general.

Conclusion

The 10% tumor shrinkage threshold is predictive of survival in this validation cohort of mRCC patients treated with VEGF-targeted therapies. ROC analysis identified 9.3% decrease in SLD at first follow-up as the best size predictor of time-to-treatment failure. Thus, a reduction of 10% in the sum long axis diameter of targets is validated as a reliable, early predictor of outcome in this setting. Given the utility of this response indicator in multiple reports, the 10% tumor shrinkage may provide a simple and practical aid for therapeutic decision making if used in clinical trials and practice with a prospective evaluation.

Author Contributions

Conception/Design: Katherine M. Krajewski, Nikhil Ramaiya, Toni K. Choueiri
Provision of study material or patients: Toni K. Choueiri
Collection and/or assembly of data: Katherine M. Krajewski, Mizuki Nishino, André P. Fay, Toni K. Choueiri
Data analysis and interpretation: Katherine M. Krajewski, Yoko Franchetti, Mizuki Nishino, André P. Fay, Nikhil Ramaiya, Annick D. Van den Abbeele, Toni K. Choueiri
Manuscript writing: Katherine M. Krajewski, Yoko Franchetti, Mizuki Nishino, Nikhil Ramaiya, Annick D. Van den Abbeele, Toni K. Choueiri
Final approval of manuscript: Katherine M. Krajewski, Yoko Franchetti, Mizuki Nishino, André P. Fay, Nikhil Ramaiya, Annick D. Van den Abbeele

Disclosures

Katherine M. Krajewski: General Electric (RF); **Toni K. Choueiri:** Pfizer, Aveo, Exilixis, Novartis (C/A). The other authors indicated no financial relationships.

(C/A) Consulting/advisory relationship; (RF) Research funding; (E) Employment; (ET) Expert testimony; (H) Honoraria received; (OI) Ownership interests; (IP) Intellectual property rights/inventor/patent holder; (SAB) Scientific advisory board

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Editor's Note: See the related commentary by Helen X. Chen et al. on page 439.

APÊNDICE M - Activating genomic mutations in the mTOR pathway to predict responses to everolimus and temsirolimus in patients with metastatic renal cell carcinoma (mRCC): Results from a large multi-institutional cohort. 2015 MERIT AWARD RECIPIENT – American Society of Clinical Oncology

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Activating genomic mutations in the mTOR pathway to predict responses to everolimus and temsirolimus in patients with metastatic renal cell carcinoma (mRCC): Results from a large multi-institutional cohort.

Meeting:
2015 ASCO Annual Meeting

Category:
Genitourinary (Nonprostate) Cancer

Subcategory:
Kidney Cancer

Session Type and Session Title:
Poster Discussion Session, Genitourinary (Nonprostate) Cancer

Abstract Number:
4519

Citation:
J Clin Oncol 33: 2015 (suppl; abstr 4519)

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Background: Mammalian target of rapamycin (mTOR) inhibitors are approved in mRCC, but only a subset of patients derives clinical benefit. Recently, case reports have suggested that mutations in mTOR pathway genes might be associated with response to everolimus and temsirolimus in several malignancies, including mRCC. **Methods:** We amassed a large international cohort of mRCC patients with available tumor specimens who received mTOR inhibitors and had distinct clinical outcomes: responders were defined as complete response (CR), partial response (PR) or stable disease with any tumor shrinkage or no tumor growth for at least 6 months (R); non-responders were defined as disease progression within the first 3 months of therapy (NR). Tumor DNA from 94 patients was analyzed using a targeted next-generation sequencing panel covering 504 cancer genes. We performed a blinded analysis to investigate

the correlation between mutations in mTOR pathway genes and response status. **Results:** Samples from 79 of 94 patients were successfully sequenced and were included in the analysis. Mutations are summarized in **Table 1**. Mutations in *MTOR*, *TSC1* or *TSC2* were more common in R (12/43) than NR (4/36) (OR: 3.05; *p = 0.06; primary hypothesis*). Similarly, mutations in *TSC1* or *TSC2* were more common in R (9/43) than NR (2/36) (OR: 4.42; *p = 0.05; secondary hypothesis*). In an exploratory analysis, 5/12 with PR/CR had mutations in *MTOR*, *TSC1* or *TSC2* vs 4/35 NR (OR: 5.28; *p = 0.04*). **Conclusions:** In this large cohort of mRCC patients, mutations in *MTOR*, *TSC1* or *TSC2* were more common in patients with clinical benefit from everolimus or temsirolimus than in NR. Mutations in those 3 genes were associated with PR/CR to mTOR inhibitors. In contrast, neither PTEN nor PIK3CA mutations showed any association with response. These findings suggest that a personalized medicine approach has value for selection of mTOR inhibitors in mRCC.

Gene Mutation	NR(n = 36) n(%)	R(n = 43) n(%)	Total(N = 79) n(%)
MTOR(activating)	2(6)	3(7)	5(6)
PTEN(inactivating)	5(14)	5(12)	10(13)
TSC1(inactivating)	2(6)	8(19)	10(13)
TSC2(inactivating)	0	1(2)	1(1)
PIK3CA(activating)	0	1(2)	1(1)

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APÊNDICE N - PD-L1 expression in non-clear cell renal cell carcinoma. 2014 MERIT AWARD RECIPIENT – American Society of Clinical Oncology

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PD-L1 expression in non-clear cell renal cell carcinoma.

Meeting:
2014 Genitourinary Cancers Symposium

Category:
Genitourinary Cancer

Subcategory:
Renal Cell Cancer

Session Type and Session Title:
General Poster Session C: Renal Cancer

Abstract Number:
424

Citation:
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Background: Programmed death-1 (PD-1) receptor negatively regulates T cell-mediated responses.PD-1 ligand (PD-L1) is aberrantly expressed in clear cell renal cell carcinoma (ccRCC) and is associated with worse prognosis. Levels of PD-L1 expressions in non-ccRCC and its association with clinicopathological features and survival are unknown. **Methods:** Formalin-fixed paraffin-embedded (FFPE) specimens were obtained from 97 patients with chromophobe (CHR), papillary (PAP), translocation Xp11.2 (TrL) RCC and oncoytoma (ONC) and were included in the analysis. PD-L1 expression was evaluated by immunohistochemistry using a mouse monoclonal anti-PD-L1 antibody (405.9A11). The assay was validated using FFPE cell line controls known to be positive or negative for PD-L1 expression by flow cytometry. PD-L1 tumor positivity (PD-L1+) was defined as ≥5% tumor cell membrane staining. For PD-L1 expression in immune cells, a combined score based on the intensity of infiltrate and percentage of positive cells was used. Baseline characteristics including stage/grade, and survival data were collected. Comparisons between PD-L1 expression and clinicopathological features were evaluated using chisq or wilcoxon rank sum tests. Cox model tests for association of PD-L1 expression with OS in univariate and multivariable analysis. **Results:** Among 97 patients, 12 (12.4%) were considered PD-L1+ in tumor cells: 2/36 (5%) of CHR RCC, 5/50 (10%) of PAP RCC, 3/7 (43%) of TrL RCC, and 2/4 (50%) of ONC. PD-L1 positivity in tumor cells was significantly associated with higher stage (p=0.026) and grade (p=0.046), as

well as lower OS on univariate (p=0.02) but not multivariate analysis (p=0.29). On the other hand, PD-L1 positivity by immune cells was observed in 50 (51.5%) patients: 13/36 (36%) of CHR RCC, 30/50 (60%) of PAP RCC, 6/7 (86%) of TrL RCC, and 1/4 (25%) of ONC. PD-L1 positivity in immune cells did not correlate with stage (p=0.7), grade (p=0.1) or OS (p=0.8). **Conclusions:** PD-L1 expression is variable in non-ccRCC and depends on histology and tumor vs. immune cells scoring. Only PD-L1 positivity in tumors cells was associated with aggressive features. Patients with non-ccRCC should not be automatically excluded from trials of agents that target the PD-1/PD-L1 pathway.

APÊNDICE O - Whole-exome sequencing (WES) predicting two extreme phenotypes of response to VEGF-targeted therapies (VEGF-TT) in patients with metastatic clear cell renal cell carcinoma (mRCC). 2015 MERIT AWARD RECIPIENT – American Society of Clinical Oncology

ASCO University

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Whole-exome sequencing (WES) predicting two extreme phenotypes of response to VEGF-targeted therapies (VEGF-TT) in patients with metastatic clear cell renal cell carcinoma (mRCC).

Meeting:
2015 Genitourinary Cancers Symposium

Category:
Genitourinary Cancer

Subcategory:
Renal Cell Cancer

Session Type and Session Title:
General Poster Session C: Renal Cancer

Abstract Number:
422

Citation:
J Clin Oncol 33, 2015 (suppl 7; abstr 422)

Author(s):
Andre Poisl Fay, Eliezer Mendel Van Allen, Bradley Murray, Laurence Albiges, Sabina Signoretti, Thai Huu Ho, A. Ari Hakimi, Suzanne S Mickey, Melissa L. Stanton, Joaquim Bellmunt, David F. McDermott, Michael B. Atkins, Levi A. Garraway, David J. Kwiatkowski, Toni K. Choueiri; Dana-Farber Cancer Institute, Boston, MA; Broad Institute, Boston, MA; Brigham and Women's Hospital, Harvard Medical School, Boston, MA; Mayo Clinic, Scottsdale, AZ, Scottsdale, AZ; Urology Service, Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY; Mayo Clinic, Phoenix, AZ, Phoenix, AZ; Dana-Farber Cancer Institute, Brigham and Women's Hospital, Boston, MA; Beth Israel Deaconess Medical Center, Boston, MA; Lombardi Comprehensive Cancer Center, Washington, DC

Background: There is significant variability in the response to VEGF-TT in mRCC with no validated predictive biomarkers. We hypothesized that whole exome analysis might identify markers of response and resistance to VEGF-TT in mRCC. **Methods:** mRCC patients who received first-line sunitinib or pazopanib and were in two extreme phenotypes of response were identified. Extreme responders (ER) were defined as PR or CR for ≥3 years (n=10) and primary refractory patients (PRP) were defined as PD within the first 3 months of therapy (n=10). WES was performed in pre-treatment specimens and established Broad Institute analytical pipelines were utilized to identify point mutations and copy number alterations across the exome. ER (n=4) and PRP (n=4) who were part of TCGA project for clear-cell RCC were included in this analysis. Nonsense, missense and indel mutations in established or novel RCC genes were investigated. **Results:** IMDC prognostic scores were not significantly different between the two groups. *VHL* mutations were observed at similar frequency in ER and PRP, overall 57%. Mutations in *PBRM1* were identified in 7 ER (50%) vs. 1 PRP (7%) (p=0.03). In addition, mutations in *TP53* were only found in PRP (p=0.09). No other gene had mutations that were associated with either response or primary refractory disease (Table). **Conclusions:** In this pilot study, *PBRM1* mutations were associated with

extreme response to VEGF-TT. However, this was exploratory, and multivariable analysis was not performed. Analysis of additional patient samples is ongoing to confirm or refute this association. If true it suggests that epigenetic effects of *PBRM1* mutation may contribute to response to VEGF-TT in mRCC.

Gene mutation	ER n=14 n (%)	PRP n=14 n (%)	p value*
VHL	9 (64.3)	7 (50)	p=0.70
PBRM1	7 (50)	1 (7.1)	p=0.03
SETD2	6 (42.8)	2 (14.2)	p=0.20
BAP1	2 (14.2)	2 (14.2)	
KDM5C	1 (7.1)	2 (14.2)	
HIF1A	0 (0)	1 (7.1)	
MTOR	2 (14.2)	1 (7.1)	
PTEN	1 (7.1)	2 (14.2)	
TSC2	1 (7.1)	0 (0)	
AKT2	0 (0)	0 (0)	
NF2	1 (7.1)	0 (0)	
ATM	2 (14.2)	0 (0)	p=0.48
TP53	0 (0)	4 (28.5)	p=0.09

* Fisher's Exact Test.

ANEXO A – Carta de Aprovação

SIPESQ

Sistema de Pesquisas da PUCRS

PROPESQ

Código SIPESQ: 6832 Porto Alegre, 8 de setembro de 2015.

Prezado(a) Pesquisador(a),

A Comissão Científica da FACULDADE DE MEDICINA da PUCRS apreciou e aprovou o Projeto de Pesquisa "Correlação dos Níveis de Expressão de PD-L1 em Células Tumorais e Infiltrados Linfocitários com Características Clínico-patológicas em Pacientes com Carcinoma Renal Não de Células Claras" coordenado por CARLOS E POLI DE FIGUEIREDO. Caso este projeto necessite apreciação do Comitê de Ética em Pesquisa (CEP) e/ou da Comissão de Ética no Uso de Animais (CEUA), toda a documentação anexa deve ser idêntica à documentação enviada ao CEP/CEUA, juntamente com o Documento Unificado gerado pelo SIPESQ.

Atenciosamente,

Comissão Científica da FACULDADE DE MEDICINA

ANEXO B – Parecer Consubstanciado

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Correlação dos níveis de expreessão do PDL-1 em células tumorais e infiltrados linfocitários com características clínico patológicas em pacientes com carcinoma renal não de células claras.

Pesquisador: Carlos Eduardo Poli de Figueiredo

Área Temática:

Versão: 1

CAAE: 49155015.6.0000.5336

Instituição Proponente: UNIÃO BRASILEIRA DE EDUCACAO E ASSISTENCIA

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 1.227.201

Apresentação do Projeto:

Na última década, esforços tem sido feitos para elucidar a biologia do CCR de células claras. O estabelecimento da inativação do gene de VHL como peça chave na carcinogênese, permitiu o desenvolvimento de terapias anti-angiogências que resultam em respostas clínicas em até 80% dos pacientes com doença metastática31 Entretanto, a progressão da doença irá ocorrer em determinado período durante o tratamento. Recentemente, imunoterapias tem mostrado respostas objetivas duradouras no tratamento de diversos tumores sólidos, incluindo CCR de células claras46. Apesar de quando diagnosticados em estágio precoce apresentarem melhor prognóstico, o tratamento do CCR de não células claras metastático é desafiador. Pacientes portadores desta doença não apresentam respostas como as citadas anteriormente e a doença avançada possui prognóstico reservado.

As neoplasias renais são classicamente imunogênicas e apresentam infiltrados leucocitários de forma abundante40. A expressão de PD-L1 tem sido associada a fatores indicadores de alto risco e desfechos clínicos adversos no CCR de células claras. Contudo, dados em CCR de não células claras ainda não foram investigados.

Os grandes estudos clínicos que norteiam o tratamento do CCR não incluem um número significativo de pacientes com esta histologia. Desta forma, dados consistentes para o tratamento

deste subtipo histológico são escassos. O estudo da expressão de PD-L1 neste subgrupo de tumores visa elucidar aspectos relacionados a fisiopatologia da doença, resultando em potenciais estratégias terapêuticas baseadas na resposta imune associada a tal tumor.

Objetivo da Pesquisa:

Objetivo Primário.

O presente estudo visa caracterizar os níveis de expressão de PD-L1 em células tumorais e infiltrados leucocitários presentes em CCR não de
células claras.

Objetivo Secundário:

1. Correlacionar os níveis de expressão de PD-L1 com características clínico-patológicas dos pacientes portadores de CCR não de células claras no
momento do diagnóstico.

2. Correlacionar os níveis de expressão de PD-L1 com risco de recorrência da doença.

3. Correlacionar os níveis de expressão de PD-L1 com a sobrevida global dos pacientes portadores de CCR não de células claras.

Avaliação dos Riscos e Benefícios:

Riscos:

Os riscos deste estudos estão relacionados ao uso de dados clínicos e material biológico previamente coletado sob consentimento livre e esclarecido. Não serão coletados novos materiais biológicos.

Benefícios:

A caracterização da expressão de PD-L1 neste subtipo histológico especial pode desencadear o desenvolvimento de futuros estudos clínicos com agentes bloqueadores desta via de sinalização. Tais agentes tem demonstrado um importante benefício clínico em outras histologias.

Comentários e Considerações sobre a Pesquisa:

O presente projeto foi executado no Centro de Oncologia Genito-urinaria do Dana-Farber (Harvard Medical School, USA) sob orientação do Dr. Toni Choueiri,investigando aspectos clínicos e biomarcadores em câncer de rim, com 104 pacientes. O CEP da instituição avaliou e aprovou o

PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

Continuação do Parecer: 1.227.201

estudo para execussão.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos de apresentação obrigatória e outros necessários para esclarecimentos foram apresentados.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências

Considerações Finais a critério do CEP:

O projeto foi executado na íntegra em outra instituição fora do país e o mesmo foi submetido, avaliado e aprovado pelo CEP institucional. Assim, o CEP-PUCRS considerou o mesmo aprovado sem restrições.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Declaração de Instituição e Infraestrutura	Documentos Doutorado Fay.pdf	13/04/2015 16:56:03		Aceito
Projeto Detalhado / Brochura Investigador	Projeto Doutorado.doc	13/04/2015 16:56:27		Aceito
Declaração de Instituição e Infraestrutura	Fay letter.pdf	13/04/2015 16:59:37		Aceito
Outros	Choueiri_Toni CV .doc	13/04/2015 17:00:16		Aceito
Outros	PB_XML_INTERFACE_REBEC.xml	27/07/2015 07:56:42	André Poisl Fay	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	27/07/2015 08:18:40		Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE Inglês.pdf	27/07/2015 08:19:14		Aceito
Folha de Rosto	Folha de Rosto Plataforma Brasil.pdf	27/07/2015 08:06:53		Aceito
Declaração de Pesquisadores	Links_CV_Lattes.docx	20/08/2015 08:14:51	André Poisl Fay	Aceito
Declaração de	PROPESQ.pdf	20/08/2015	André Poisl Fay	Aceito

Endereço: Av Ipiranga, 6681, prédio 40, sala 505
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UF: RS **Município:** PORTO ALEGRE
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PONTIFÍCIA UNIVERSIDADE CATÓLICA DO RIO GRANDE DO SUL - PUC/RS

Continuação do Parecer: 1.227.201

Instituição e Infraestrutura	PROPESQ.pdf	08:15:12	André Poisl Fay	Aceito
Declaração de Instituição e Infraestrutura	Comissao_Cientifica.pdf	20/08/2015 08:15:33	André Poisl Fay	Aceito
Declaração de Pesquisadores	carta_orientador_portugues.pdf	20/08/2015 08:15:52	André Poisl Fay	Aceito
Declaração de Instituição e Infraestrutura	AndreFayAprovacaoComissaoCientifica.pdf	10/09/2015 07:20:36	André Poisl Fay	Aceito
Declaração de Instituição e Infraestrutura	SIPESQ.pdf	10/09/2015 07:29:51	André Poisl Fay	Aceito
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_491194.pdf	10/09/2015 07:31:24		Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não.

PORTO ALEGRE, 14 de Setembro de 2015

Assinado por:
Denise Cantarelli Machado
(Coordenador)

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