Determinants of renal cell carcinoma invasion and metastatic competence

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Metastasis is the principal cause of cancer related deaths. Tumor invasion is essential for metastatic spread. However, determinants of invasion are poorly understood. We addressed this knowledge gap by leveraging a unique attribute of kidney cancer. Renal tumors invade into large vessels forming tumor thrombi (TT) that migrate extending sometimes into the heart. Over a decade, we prospectively enrolled 83 ethnically-diverse patients undergoing surgical resection for grossly invasive tumors at UT Southwestern Kidney Cancer Program. In this study, we perform comprehensive histological analyses, integrate multi-region genomic studies, generate in vivo models, and execute functional studies to define tumor invasion and metastatic competence. We find that invasion is not always associated with the most aggressive clone. Driven by immediate early genes, invasion appears to be an opportunistic trait attained by subclones with diverse oncogenic status in geospatial proximity to vasculature. We show that not all invasive tumors metastasize and identify determinants of metastatic competency. TT associated with metastases are characterized by higher grade, mTOR activation and a particular immune contexture. Moreover, TT grade is a better predictor of metastasis than overall tumor grade, which may have implications for clinical practice.

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Metastasis is the major cause of cancer-related deaths. It is a complex process involving tumor cell invasion, dissemination through the vasculature or lymphatic vessels, extravasation, and colonization of distant organs. While invasion is thought to be a rate limiting step, its relative contribution to the metastatic process is poorly understood. An important limitation in delineating determinants of metastatic competence associated with invasion is a lack of suitable physiologically relevant experimental systems and the inability to track metastatic progression in real time. In vitro systems hardly recapitulate the physiological complexity of the metastatic process, and in vivo studies are limited by the often microscopic nature of intravascular capillary tumor extensions. Constraints imposed by necessarily reductionist experimental approaches and laboratory models have impeded progress in delineating determinants of invasion and metastatic competence.

The epithelial to mesenchymal transition (EMT) developmental program is known to be hijacked by cancer cells to invade and initiate metastases. Cancer cell invasion is not restricted to single cells and collective cell migration may be observed in histopathological samples; however, its contribution to metastatic progression is poorly understood. It is believed that invasive cells exist in several distinct cellular states described as hybrid or partial EMT depending on the activation status of EMT transcription factors.

Renal cancer offers a unique experimental paradigm to dissect the link between vascular invasion and metastases. Renal cell carcinoma (RCC), the most common kidney tumor, grossly invades into large vessels. Tumor extensions (so-called tumor thrombi [TT]) into the renal vein are observed in approximately 15% of RCC patients. These TT are able to traverse extensive distances arriving sometimes to the heart. This directional process offers a unique, yet untapped opportunity to understand the relationship between invasion, migration and metastases.

Importantly, the extent of TT invasion drives tumor staging and prognosis. Level I thrombi invade into the renal vein and its branches (stage pT1a); level II TT extend into the inferior vena cava (IVC) up to the hepatic veins (pT3b); level III TT approximate the diaphragm (also pT3b); and level IV TT extend into the heart (pT3c). The level of TT has been shown to have prognostic significance with 5-year survival rates of 43%, 37% and 22% for pT1a, pT3b and pT3c, respectively.

RCC with TT are typically managed surgically with removal of the kidney tumor and TT. This sometimes requires extracorporeal circulation with cardiac bypass to remove the TT from the heart. Remarkably, despite gross invasion into the largest vein in the body (the IVC), and extensive migration sometimes into the heart, not all RCC TT lead to metastasis.

Here, we report the results of extensive analyses of a prospectively enrolled cohort of 83 ethnically diverse patients with a renal mass and associated TT scheduled to undergo surgical resection at UT Southwestern Medical Center Kidney Cancer Program over a 10-year period. We performed extensive histological and integrated genomic analyses, implanted TT in mice and executed functional studies. The study is divided into two parts. Initially we focus on evaluating determinants of invasion by comparing TT and the corresponding primary tumors (PT). Subsequently, we explore determinants of metastatic competence by comparing TT from patients that developed metastases to the ones that did not.

Results

Spatially resolved multi-region sampling and co-registered integrated genomic analyses of RCC with intravascular tumor extension. We prospectively enrolled 83 patients with a renal mass and TT scheduled to undergo surgical resection. Supplementary Table 1 summarizes the clinicopathological features of this cohort. All but one tumor (a leiomyosarcoma) were RCCs (73 Clear Cell, 1 Papillary, 1 Chromophobe, and 7 Unclassified) (Fig. 1a, b). We performed extensive histological analyses of RCC PT and TT. RCCs are notoriously heterogeneous and exhibited multiple architectural patterns (Fig. 1c and Supplementary Fig. 1a, b).

We then focused on tumor grade, which is a critical determinant of prognosis. Tumor grade is largely based on nucleolar prominence and scored from low (grade 1 and 2) to high (grade 3 and 4). As for architectural patterns, tumor grade varies from area to area in tumors. In routine clinical practice, overall tumor grade is based on the highest-grade area observed. Comparative analyses of tumor grade between PT and TT revealed discrepancies in tumor grade (Supplementary Fig. 1d). Unexpectedly, TT were not always colonized by the highest grade clone in the PT. In fact, among PT of grade 3 and 4, the TT was of lower grade in 45% and 66% of cases, respectively (Fig. 1d). These data show that TT are not always seeded by the most aggressive clone.

We collected 277 spatially resolved paired PT and TT samples. Samples were selected from macroscopically diverse areas (Fig. 1a, b). We leveraged the directionality afforded by TT. A sample was often collected adjacent to the primary tumor (TT4), at the distal leading tip (TTD) and somewhere in the middle (TTM) (Fig. 1e).

Several samples from the PT were also gathered. When accessible, matched metastatic lesions (M; Met) were similarly collected (n = 6 patients).

Tumor samples were used for integrated genomic analyses and transplanted into mice to generate live tumor models (patient-derived xenograft [PDX] or tumorgraft [TG] models; Fig. 1e). To accurately integrate genomic and transcriptomic findings and enable histological co-registration, simultaneous isolation of DNA and RNA was performed from individual samples characterized histologically through immediately flanking sections. Whole-exome sequencing (WES) analyses were performed on 260 matched PT/TT from 78 patients and RNA-seq from 275 matched PT/TT from 82 patients (Fig. 1e).

Oncogenically diverse RCCs in proximity to the renal vein are invasion competent. Whole-exome profiles of spatially resolved TT samples did not reveal significant differences compared to PT in overall mutation burden, number or enrichment of driver mutations, or copy number variations (CNVs) (Fig. 1f and Supplementary Fig. 1e). 3p and 8p loss were prominent followed by 5q gain, 14q loss and 9p loss (Fig. 1f). 3p and 8p loss were prominent followed by 5q gain, 14q loss and 9p loss (Fig. 1f).
Fig. 1 Characterization of invasive intravascular RCC tumors. a MRI scan of a patient with a clear cell renal cell carcinoma (ccRCC) and inferior vena cava (IVC) thrombus. Coronal T1-weighted fat saturated contrast-enhanced spoiled gradient echo image acquired during the nephrographic phase shows a large left renal mass (white dotted line) extending into the proximal renal vein (black arrowhead). Tumor thrombus extends into the IVC (yellow dotted line) with its distal tip (yellow arrowhead) above the diaphragm and entering the right atrium (Level IV). A large heterogeneous left adrenal metastasis (green dotted line) is present.

b Macroscopic image of a primary tumor (PT) and tumor thrombus (TT) from the same patient.

c Summarized architectural patterns (indolent (microcystic) to aggressive (solid sheet)) within PT (inner circle) and TT (outer circle) in 71 ccRCC patients with intravascular TT.

d Representation of microscopic analysis of PT-TT paired samples based on grade. e Illustration showing multi-region sampling of invasive tumor samples for sequencing, PDX generation, and histological analyses. PT primary tumor, TT-A adjacent thrombus, TT-M middle thrombus, TT-D distal thrombus.

f Overview of mutations, CNVs, and clinical parameters for ccRCC patients.
(28.5%±15.6%); BAP1 (9.9%±10.4%); and SETD2 (6.6%±11.9%) (Fig. 1f). As we reported earlier, BAP1 and PBRM1 mutations were mutually exclusive (Fig. 1f).

Known ccRCC driver mutations were observed in approximately 65% of invasive TT. Based on driver mutations, TT could be classified into PBRM1 mutant (22%); BAP1 mutant (20.6%); SETD2 mutant (13.2%); PBRM1-SETD2 mutant (7.4%); and BAP1-SETD2 mutant (1.5%) (Fig. 2b).

Non-ccRCC PT and TT (1 Chromophobe RCC, 1 Papillary RCC, 7 unclassified RCC, and 1 leiomyosarcoma) were also similar in somatic alterations and driver mutations (Supplementary Fig. 2d). 7p gain was observed across most non-ccRCC (Supplementary Fig. 1e). TP53 mutations were observed in 40% (4/10) and SETD2 mutations in 20% (2/10) of non-ccRCC TT (Supplementary Fig. 2e). ARID1A mutations were private to TT in 2/3 non-ccRCC patients, and so was a TSC2 mutation (Supplementary Fig. 2f).

To assess TT evolution, we generated phylogenies using 332 defined driver mutations. Comparative analysis of matched PT and TT revealed four distinct patterns of clonal evolution (Fig. 2c). In more than 50% of cases (n = 43) different driver mutations were observed in the PT and matched TT suggesting divergent or independent evolution (Fig. 2c). Driver mutations private to either the PT or invasive TT were also observed in 10% and 27%, respectively. In 8% of the cases, PT and TT had the same mutational profile indicating co-evolution or clonal expansion. Detailed analyses of tumors with 2 or more TT samples (n = 51 patients) identified additional driver mutations in 92% of the TT cases (n = 47). Branched heterogenous evolution was observed in 45% (n = 23) of the cases. Linear homogenous evolution was observed in 35% (n = 18) while linear heterogenous evolution was observed in 20% (n = 10) (Fig. 2d).

These studies were further extended through analyses of metastases, which were available from 6 patients (29 multi-region samples from 3 ccRCC and 3 non-ccRCC patients). No significant differences in mutational frequencies or number of driver mutations were observed between the TT and metastatic samples.
Invasion driven by immediate early genes represents a reversible adaptive response. To identify gene programs associated with invasion, we analyzed transcriptomic profiles of invasive TT (Fig. 3a). We discovered a set of transcription factors that were differentially expressed between TT and PT (p-value < 0.05, FDRq < 0.2). The majority were immediate early genes (IEGs), including genes encoding components of the AP-1 complex (FOSB, FOS, and JUNB) as well as EGR1, EGR3, IER2, ATF3, and NRB4 (Fig. 3b). These genes are expressed as an adaptive response to external signals and are expressed in gliomas and prostate tumors (Supplementary Fig. 3a). Additionally, motif enrichment analysis revealed significant enrichment for genes with binding sites for AP-1 and EGR transcription factors in TT (Fig. 3c).

The TGF-beta responsive EMT determinant gene PRRX1 was overexpressed in invasive TT (Fig. 3b). PRRX1 together with TWIST1, an EMT promoting transcription factor, drives cell invasion and dissemination.22–25 Moreover, high expression of PRRX1 was associated with lower survival in renal cancer. In contrast, FOSB and EGR1 overexpression was favorable (Supplementary Fig. 3b–d). Pathway enrichment analysis of differentially expressed genes in TT showed enrichment for AP1 and TGF-beta driven responsive genes like RUNX (AP-1 interacting TF) and SMAD2/3 (Fig. 3d; Supplementary Fig. 3e; and Supplementary Data File. 1). These differentially expressed genes were also enriched in TGs, which were available from 8 patients, 8 PT-grafts, and 9 TT-grafts (Supplementary Fig. 4a).

Cellular plasticity driven by AP1 and EGR transcription factors is dependent on cyclic AMP response element binding protein (CREB)26. To test if increased CREB activity was associated with augmented expression of IEGs and invasion, we assayed pCREB levels in ccRCC cell lines. Caki-1 had the least pCREB activity, while 769-P and 786-O cells had moderate pCREB levels compared to A498 and UMRC-2 cells (Fig. 3e). Expression of IEGs strongly correlated with pCREB status in these cells (Fig. 3e; Source Data file. 1). Pharmacological inhibition of c-Fos/AP-1 using T-5224 reduced migration of ccRCC cell lines but did not affect cell proliferation (Fig. 3f and Supplementary Fig. 4b–d).27,28 Similar results were observed with siRNA knockdown of the AP1 transcription factor JUNB (Supplementary Fig. 4e–i).

A closer look at differentially expressed genes in TT identified several key factors. Kruppel like factor 2 (KLF2) and β-globin genes (HBB and HBA2), which are induced in response to fluid shear stress and maintain cellular redox, were overexpressed in TT.29,30 Likewise, expression of CXCL2, a cytokine implicated in migration, and THBD, which interferes with blood clotting, was increased in TT (Supplementary Fig. 4j).

As intravascular tumor cells extravasate and colonize distal organs, the IEG driven adaptive response is likely to be diminished. We tested this notion by assessing the expression of IEGs in metastatic lesions. Relative to the intravascular outgrowth, there was a significant reversal in expression of IEGs in metastatic lesions (M). The expression of β-globin and clotting blocking factors enriched in TT was also attenuated in metastases (Fig. 3g). Next, we examined transcriptomic profiles of matched TT and M samples. EMT and TGF-β signaling was enriched in metastatic samples as expected while metabolic gene sets were enriched in TT compared to matched M clones (Supplementary Fig. 5a, b).

Taken together, these data implicate CREB/AP-1 driven IEG expression in TT cell migration as a reversible adaptive response to fluid shear stress in the vasculature.

Identification of determinants of metastatic competence. To delineate the relationship between invasion and metastatic competence, next we performed comparative analyses between patients with TT that metastasized and those that did not. At a median follow-up of 3 years, ~50% of patients in our cohort (n = 41) developed metastases (Fig. 4a; Supplementary Fig. 6a; and Supplementary Table. 1). Among the patients with metastasis (TTM), 23 presented with synchronous metastases and 18 developed metachronous metastases. Metastases mostly involved the lungs followed by bone and liver (Fig. 4a), a pattern which is characteristic of RCC. Forty-two patients with TT did not metastasize (TTNM). These two cohorts are remarkably different as illustrated also by Kaplan–Meier analyses, which showed substantial differences in overall survival (HR 4.29; 95% CI:1.24–14.82; p = 0.01) (Supplementary Fig. 6b). We systematically examined clinicopathological features, mutations, DNA copy number alterations and gene expression across the TTM and TTNM cohorts. However, no significant differences in somatic alterations were identified between TTM and TTNM (Supplementary Fig. 6c).

From a clinicopathological standpoint, we evaluated the impact of tumor size, foci, surgical margins, stage, tumor grade, sarcomatoid differentiation, tumor necrosis, invasion into the kidney parenchyma (non-intravascular) and lymph node involvement. These analyses revealed an association between TTM with stage at presentation, tumor grade, sarcomatoid differentiation, tumor necrosis, kidney parenchyma invasion and lymph node involvement (Supplementary Table. 3). In addition, aggressive tissue architectural patterns were also enriched in TTM relative to TTNM (Fig. 4b) and in metastasis from paired ccRCC patients (Supplementary Fig. 6d).

To identify the most critical variables, we performed a Multivariate Cox proportional hazards analysis using time to metastasis. Unsurprisingly, these analyses showed that stage, sarcomatoid differentiation, and lymph node involvement were independent predictors of time to metastasis. Provocatively, however, grade in the TT but not overall tumor grade was also predictive. Furthermore, as determined by the hazard ratio, TT grade was the strongest predictor (HR, 6.42; p = 0.003). We observed higher tumor grade in TTM relative to TTNM (Fig. 4c). We focused on patients with grade 3 PT, which accounted for 52.1% in our cohort. Among these patients, 20 had TT that were grade 3 and 17 had TT that were grade 2. Interestingly, despite similarly high grade in the PT, patients with a TT of lower grade had significantly better metastasis-free survival than those with grade 3 TT (Fig. 4d).

Extending our previous observations that the most aggressive clone with the highest grade does not always seed the TT, these data show that the grade of the clone seeding the TT is a more important determinant of metastatic competency than overall tumor grade (Fig. 4e).

Inflammatory immune contexture associated with metastatic competence. Next, we compared transcriptomic profiles of TTM

mutational profiles similar to the TT suggesting that tumor cell dispersion was through the intravascular extension (Fig. 2e). Linear evolution was observed in one ccRCC patient with metastasis to a lymph node. Driver phylogenies of non-ccRCC patients with synchronous metastasis showed overall greater similarity between metastases and the PT, suggesting early dissemination and divergent evolution with respect to TT (Fig. 2e).

These observations show that PT with diverse oncogenic status irrespective of their association with disease aggressiveness can invade and extend as TT. Although TT evolve and acquire additional mutations, we found no association between overall somatic mutation status in PT with invasion and outgrowth in the vasculature.
and TT\textsuperscript{NM}. We identified 70 genes overexpressed and 61 under expressed in TT\textsuperscript{M} relative to TT\textsuperscript{NM} (Fig. 5a). These studies were complemented with studies of publicly available datasets where we hypothesized that genes upregulated in TT\textsuperscript{M} would be associated with poor prognosis. This was the case among different kidney cancer datasets, including for nccRCC histologies, as well as in other tumor types (Fig. 5a and Supplementary Fig. 7a, b). Gene ontology analysis of TT\textsuperscript{M} genes identified leukocyte homeostasis and attenuated angiogenesis (Supplementary Fig. 7c).
inflammation and mTOR signaling in TT<sup>M</sup> (Supplementary Fig. 7d). Overall, attenuated expression of genes associated with angiogenesis and increased chemokine/cytokine expression was observed in TT<sup>M</sup> (Supplementary Fig. 7e).

We validated these observations by immunohistochemistry (IHC) studies on flanking sections of TT<sup>M</sup> and TT<sup>NNM</sup> samples used for gene expression analyses. As determined by ERG staining, endothelial cells were decreased in TT<sup>M</sup> relative to TT<sup>NNM</sup> (Fig. 5b). A significant increase in pro-tumorigenic M2 macrophages (CD163<sup>+</sup>) was observed in the flanking sections of TT<sup>M</sup> (Fig. 5c). In contrast, no significant differences in PD-L1 staining were observed (Supplementary Fig. 7f).

To further characterize differences in stromal composition between TT<sup>M</sup> and TT<sup>NNM</sup>, we deployed an empirically derived gene signature of the RCC tumor microenvironment (eTME)<sup>31</sup>. Supporting our findings, eTME analyses revealed an enrichment of macrophages and a reduction in endothelial cell markers in TT<sup>M</sup> (Supplementary Fig. 7g). Monocytes and regulatory T cells were enriched, and NK cell markers were reduced in TT<sup>M</sup>. Similar observations were made with QUANTISEQ, EPIC, MCPCOUNTER, and Tumor Immune Estimation Resource (TIMER)<sup>32,33</sup> (Fig. 5d and Supplementary Fig. 7h–j). Macrophages and dendritic cell associated genes from QUANTISEQ are listed (Supplementary Fig. 7k–m). We are referring to our findings in Fig. 4d where higher TT grade is associated with metastatic outcome. Likewise, inflammation and immune signatures are high in TT grade 3/4 compared to TT grade 2. Analogous to our observations with TT<sup>M</sup>, TT grade 3–4 tumors were enriched for inflammation and immune signatures and deprived of angiogenesis related gene sets (Supplementary Fig. 8a, b).

**Augmented mTORC1 signaling determines metastatic potential.** To further evaluate whether mTOR (referring to mTOR complex 1, or mTORC1) was differentially activated between TT<sup>M</sup> and TT<sup>NNM</sup>, we performed immunohistochemical analyses. p-S6, a broadly used marker of mTORC1 activity, was significantly higher in TT<sup>M</sup> (compared to TT<sup>NNM</sup>). p-S6 was also increased in high grade TT (compared to low-grade TT) (Fig. 6a and Supplementary Fig. 9a). The mTOR gene signature was also enriched in TT compared to PT and M (Supplementary Fig. 9b, c).

To evaluate the functional consequences of mTOR activation, we tested the impact of mTORC1 inhibition in PDX models. PDX models were generated from 5 TT<sup>M</sup> and 9 metastases (XP373 and XP453 from this cohort and 12 additional ones). Mice bearing size-matched tumors (<i>n</i> = 3–5) were administered sirolimus, an allosteric mTORC1 inhibitor, and tumor progression was monitored compared to vehicle controls (<i>n</i> = 3–5) (Fig. 6b). 13/14 PDXs were sensitive to sirolimus resulting in a median tumor growth inhibition of 80.5% (Fig. 6c and Supplementary Fig. 9d, e). Next, we assessed the impact of mTOR inhibition on metastasis (Fig. 6d). For these experiments, mice were injected intracardially with 786-O ccRCC cells transduced with
GFP-luciferase to monitor metastatic colonization. Post injection, mice were randomized into two groups and treated with either vehicle or everolimus (a sirolimus analogue). Metastatic incidence was tracked using bioluminescent imaging. We observed a significant reduction in total body photon flux in mice treated with the mTOR inhibitor (Fig. 6e). Five weeks post-injection, mice were euthanised. Ex vivo imaging and luciferase staining on sections showed a markedly reduced incidence of liver, lung, and bone metastases (Fig. 6f and Supplementary Fig. 9f–i). Overall, these data show that targeted inhibition of mTOR signaling in cancer cells impedes tumor progression and metastatic outbreaks.

Discussion
Intratumor heterogeneity and sub-clonal evolution is characteristic of RCC. Yet, metastases are more homogenous, and harbor fewer driver mutations and somatic alterations than PT, indicating that an evolutionary bottleneck process governs metastatic colonization. The acquisition of invasion determinants is considered to be such a bottleneck. However not all patients with invasive tumors develop metastases.

Using a patient cohort assembled over a decade, we present studies to delineate invasion and metastatic competence employing paradigmatic RCC tumors with TT. We show that TT can be seeded by PT clones with diverse oncogenic status (see model in Fig. 7). While BAP1, SETD2, TSC1/2, TP53 mutations are associated with high tumor grade, disease aggressiveness, and poor survival, we did not observe an enrichment of such mutations in TT. Both high- and low-grade clones were present in TT. These data support a contrasting model where intravascular invasion represents an opportunistic adaptive trait acquired by tumor clones in proximity to the renal vein segmental branches. Accordingly, invasion and cancer cell dispersion could be an early or late event depending on PT proximity to vasculature.

Analyzing clearly defined invasive intravascular RCC tumor extensions revealed enrichment for a cellular plasticity program driven by immediate early genes (IEGs). Our study suggest that this IEG program represents an adaptive and transient response to fluid shear stress during intravascular invasion.

Analyzing clearly defined invasive intravascular RCC tumor extensions revealed enrichment for a cellular plasticity program driven by immediate early genes (IEGs). Our study suggest that this IEG program represents an adaptive and transient response to fluid shear stress during intravascular invasion. Analyses of paired PT, TT and metastatic samples, show that the majority of genes enriched in TT were attenuated in metastatic lesions. This is in agreement with the transient nature of the invasion program and a lack of fixed irreversible somatic alterations dictating invasion. Only a subset of the differentially expressed genes in TT were retained in metastatic lesions. This subset included PRRX1, CXCL2 and IER2, and is consistent with their role in other tumor types, where they have been implicated in metastasis. Understanding the downstream effectors of IEGs may provide mechanistic insights and strategies to limit RCC invasion and metastases.

Interestingly, despite gross invasion into the vasculature, we show that not all RCC TT tumors metastasize. By comparing TTNM and TTNM, we identified metastatic competence determinants. We identified TT grade as an independent predictor of metastatic outcome. Accordingly, patients with lower grade TT had significantly reduced rates of metastases and better prognoses, than patients with higher grade TT irrespective of the grade of the PT. Whereas current prognosis/metastatic risk
assessment tools consider the highest-grade area in the tumor, our findings suggest that grade of TT (where applicable) is a more important determinant of metastatic potential and survival. These data are consistent with the notion that the invasive clone, rather than the most aggressive clone, is the primary driver of survival. Thus, determinants of invasion could be effective therapeutic targets to reduce metastases.

TTM were characterized by augmented mTORC1 signaling. Increased mTORC1 activity may be driving the higher grade observed in TTM compared to TTNM. Several findings support this notion. Grade is largely determined by the prominence of the nucleolus, which is implicated in ribosome assembly, and protein translation, processes regulated by mTORC1. Furthermore, mTORC1 has been shown to regulate nucleolar size. In addition, a correlation between higher grade and mTORC1 activation has been previously reported in RCC. Perhaps most telling, disruption of Tsc1 with consequent mTORC1 activation in Vhl/Pbrm1-genetically engineered mouse models is sufficient to increase tumor grade. The importance of mTORC1 is highlighted through studies in animal models where mTORC1 inhibition reduced tumor growth and metastases. Accordingly, it is not surprising that mTORC1 inhibitors have also been proven to be effective for RCC patients, and two sirolimus analogues are approved by the FDA, temsirolimus and everolimus.

Beyond mTORC1 activation, TTM were also characterized by pro-tumorigenic macrophages and reduced angiogenesis. Comprehensive dissection of the tumor and immune contexture within the TT using single cell sequencing and proteomic based analyses may provide mechanistic insight into how invading tumor cells achieve metastatic competence and evade immune surveillance.

Fig. 6 mTOR signaling is induced in metastatic tumor thrombus. a Quantification (left) and representative immunohistochemistry images (right) of phospho-S6 in TTNM and TTM (n = 65 patients, 81 samples). Data are presented as scatter dot plots and lines are at mean values ±SEM. Two-sided unpaired Student t test. b Schematic highlighting experimental protocol to inhibit mTOR in RCC patient-derived xenografts (PDXs). c Tumor growth inhibition rates of TT- and metastasis-derived PDX lines by mTOR inhibitor sirolimus (3-5 mice for each PDX). d Schematic of experimental protocol to inhibit mTOR in experimental metastasis assay. e, f Luciferase transduced 786-O ccRCC cells were intracardially injected and metastatic incidence was tracked by whole-body bioluminescent imaging (BLI: Total photon flux (p/s)) in mice treated with either vehicle or everolimus daily by oral gavage. BLI quantification and representative images of whole-body are shown (e). Quantification of metastatic burden in organs by ex vivo imaging (f). n = 4 mice each group. Data are presented as mean ± SD. Two-tailed Mann-Whitney U-test.

Fig. 7 Determinants of invasion and metastatic competence in invasive intravascular RCC. Model highlighting key findings from the study. IEG overexpression in TT was based on gene expression analysis of biologically independent PT (n = 114) and TT (n = 161) samples. Tumor grade was analyzed using matched PT and TT from 71 ccRCC patients. Representative IHC images shown for p-S6 (n = 65 patients, 81 samples), Endothelial Cells (EC, anti-ERG, n = 20 patients, 36 samples), and Macrophages (Mϕ, anti-CD163 n = 20 patients, 36 samples).
In summary, we report that invasion is an opportunistic trait attained by subclones with diverse oncogenic states in geopatral patioly to the vasculature. We show that invasion is not necessarily driven by the most aggressive subclones in the tumor, and that the invasive subclones (rather than the most aggressive) appear to be the main determinants of metastatic competence. These results suggest that progression for RCC with TT is most accurately determined based on TT features, which has implications for clinical practice.

Methods

Patient selection. All patients that enrolled in the study provided informed consent allowing the use of discarded surgical samples for research purposes and genetic de-identified by UT Southwestern Institutional Review Board. The University of Texas Southwestern Medical Center Tissue Resource [STU 10201-051], Kidney Cancer New Pathway Discovery Project [STU 012011-190], and Comprehensive Database for Patients with Kidney Tumor [STU 022015-015)]. Surgical schedules were screened weekly for RCC patients undergoing tumor excisions or biopsies. Inclusion criteria were largely based on imaging.

Sample collection. Following surgical specimen acquisition, tumors were processed within 2 h. They were frozen for genomic studies or placed in 1% (vol/vol) formalin. Debris was cleared with QIAshredder (Qiagen). Lysates were loaded on a 4%–12% polyacrylamide gel, transferred to a PVDF membrane, and probed with antibodies specific for various proteins. The membranes were developed using ECL (Amersham) and imaged on a phosphor screen. Image analysis was performed using ImageJ software (version 1.48). Densitometry was performed on all Western blots and quantified using the Image software (version 4.4). DMSO or Everolimus (5 mg/kg) in 1% methocel solution was given by oral gavage daily prior to intraductal injection.

Nucleic acid isolation from tissues. DNA and RNA were collected simultaneously as described before 10 using AllPrep DNA/RNA mini kit (Qiagen). Tumor tissues were recovered in sterile 1.5 mL tubes with RLT buffer and stored with pristine. Debris was cleared with QIAshredder (Qiagen). Lysates were loaded onto a DNA spin column and washed, DNA was collected in 10 mM Tris-Cl, pH 8.5 buffer. RNAs were isolated using the Qiagen RNeasy mini kit. RNA integrity was confirmed using the Agilent 2100 Bioanalyzer (Agilent). RNAs were isolated with the RNase-free Trizol Reagent (Invitrogen) and RNA pellets were resuspended in 100 μL 1X PBS and intracardially injected into the right ventricle. Metastatic tumors were detected by non-invasive bioluminescence imaging using an IVIS spectrum. Bioluminescence images were quantified using ROI tool in living Image software (version 4.4). DMSO or Everolimus (5 mg/kg) in 1% methocel solution was given by oral gavage daily prior to intraductal injection.

Animal experimental procedures. Animal experiments were performed in accordance with the Guide for Care and Use of Laboratory Animals approved by the UT Southwestern Animal Care and Use Committee (IACUC). Tumor fragments from stably engrafted tumorgrafts were implanted subcutaneously in 4–6 weeks-old NOD-SCID mice. Tumors were routinely reviewed by a dedicated genitourinary pathologist (P.K.) to select areas with viable tumor cells for study. When tumor volumes reached ~100–300 mm³, mice were allocated into different treatment groups. The basis of following tumoral volumes and mouse weight
did not allow the use of expired experimental metastasis assay. Luciferase labeled 2.0 × 10⁵ cells were resuspended in 100 μL 1X PBS and intracardially injected into the right ventricle. Metastatic tumors were detected by non-invasive bioluminescence imaging using an IVIS spectrum. Bioluminescence images were quantified using ROI tool in living Image software (version 4.4). DMSO or Everolimus (5 mg/kg) in 1% methocel solution was given by oral gavage daily prior to intraductal injection.

Morphologic assessment and immunohistochemistry. Tissue samples for analysis were obtained from the primary renal tumor, tumor thrombus, metastatic sites, and matched normal tissue. Both fresh frozen and formalin-fixed, paraffin-embedded (FFPE) tissue were used. Tissues were dehydrated with 100% ethanol, embeddded in paraffin, and cut into 8 to 10 μm sections, which were subsequently stained with hematoxylin and eosin (H&E) and a panel of IHC markers. The WHO/ISUP4 grade were determined on routine hematoxylin and eosin–stained tumor sections by two pathologists blinded to clinical data. Confirmation of ccRCC diagnosis and grade was performed for each case (as assigned by a group of specialists in the GU pathology division) by two GU pathologists. In each case, both reviewers (an experienced uropathologist (P.K.) and a GU pathology fellow (S.C.) determined WHO/ISUP grading system for the thrombus independently. In cases with interobserver variations, slides were reevaluated, and grade was assigned. Before performing this review, both pathologists reviewed WHO/ISUP grading systems in 10 representative ccRCC cases to evaluate interobserver agreement.

IHC staining for CD31 (1:100, Agilent technology, Cat# M082301-2), ERG (1:100, Biocare Medical, Cat# CM 421 A, C), CD163 (1:100, Biocare Medical, Cat# CM 353 AK, CK), and phosphorylated S6 ribosomal protein (Ser235/236) (1:100, Cell Signaling, Cat# 2211) was performed on FFPE tissue using Autostainer Link 48 (Dako, Glostrup, Denmark). The slides were counterstained with methyl green as the product of staining intensity (0–3) and extent of immunostaining (percentage of cells with staining). Appropriate positive and negative controls were used for each run and checked for validation.

In vitro studies. Cell lines were obtained from ATCC and confirmed by mycoplasma free using Universal Mycoplasma Detection Kit (ATCC). A498, Caki-1 and UMRC-2 cells were provided by Qing Zhang, UT Southwestern. Cells were maintained in RPMI-1640 (769-P and 786-O) or DMEM (A498, Caki-1 and UMRC-2) supplemented with penicillin/streptomycin, -glutamine, and 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO. JUNB siRNAs was obtained from Dharmacon. ccRCC cell lines were transfected with 25 μM siRNAs and DharmaFECT reagent (Dharmacon) for 24 h and knockdown efficiency was checked after 48 h of transfection by qRT-PCR.

qRT-PCR. RNAs were collected from cultured ccRCC cell lines using RNAeasy mini plus kit (Qiagen). cDNAs were generated from 1μg of total RNA using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative RT-PCR was performed using SYBR Green Super mix (Bio-Rad) with specific primers and cDNAs. Specific primer sequences used are listed in Supplementary Table. 5.

Western blot. Cultured ccRCC cell lines were washed with ice-cold phosphate-buffered saline (PBS), RIPA buffer with complete™, Mini Protease Inhibitor Cocktail (Roche) and PhosSTOP (Roche) was added to each 10 cm cell culture plate. Cells were scraped and incubated on ice for 15 min. Lysates were centrifuged

RNA sequencing. TruSeq RNA Sample Preparation kit (Illumina) was used to generate RNA-seq libraries. The libraries were multiplexed and sequenced using HiSeq2500 platform to obtain ~100 million paired-end (2 × 75 bp) reads per sample on average. FastQC was applied to control quality with the parameters –extract --threads 48 –q. RNA-seq reads were aligned to the human reference genome GRCh38 (hg38) utilizing STAR with the parameters --runThreadN 48 --outSAMType BAM Unsorted --outRealignUnmapped Fasta. featureCounts19 with parameters --primary-O -t exon -g transcript_id_s >=0 -T 48 –largestOverlap --minOverlap 3 –ignoreDups -p -P -B -C was then used to get expression level of genes. The human genome annotation file was downloaded from the University of California Santa Cruz (UCSC) table browser in the RefSeq Gene track. The file was employed by featureCounts (1.6.0). Read per kilobase million (RPKM) values were determined from read counts of genes. RPKM values were then transformed by log2. Next generation sequencing data was analyzed using Morpheus (Broad Institute) and R software (version 3.6.0).
for 10 min at 16,000 g and supernatants were collected into new tubes and protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo). 10 μg of protein was loaded on a SDS-PAGE gel and lysates were transferred to a nitrocellulose membrane. Membranes were blocked for 1 h with 5% non-fat milk in TBST and incubated with primary antibodies overnight. Primary antibodies for Phospho-CREB (Ser133) (1:1000, Cell Signaling, Cat# 9198) and Beta Actin (1:25000, Abcam, Cat# ab9900) were used. After secondary antibody incubation with goat anti-rabbit IgG (H - L) HRP conjugated antibody (1:5000, Milipore, Cat# AP307P), the membranes were developed using chemiluminescence. Uncropped scans of the blots are supplied in Source Data file 1.

**Scratch wound healing assay.** After cCRCC cells reached confluency in 96-well tissue culture plates, IncuCyte Woundmaker (Essen BioScience) was used to create a wound in the cell monolayers. The AP1 inhibitor T-5224 was added as indicated. Cell migration was followed by Incucyte every 4 h for 48 h. Cell migration was quantified using Incucyte software.

**Gene Set Enrichment Analysis.** Ranked Gene Set Enrichment Analysis (GSEA) was performed with software version 4.0.3. Default settings were used except for “No Collapse” and “Phenotype” permutation type to “Gene_set”. Defined lists from the Broad Institute were used: h.all.v.7.0.symbol.gmt [Hallmarks], c2.all.v.7.0.symbol.gmt [Gene Ontology]. Statistical criteria are described in results and figure legends.

**Clonal evolution pattern analysis.** For each tumor, a matrix was generated based on WES results including frameshift substitutions, non-frameshift substitutions, stop gain, stop loss, splicing mutations, non-synonymous SNVs, and known mutations. 332 driver genes with mutations (Supplementary Table 6) were used to generate phylogenetic trees for each patient based on following rules: clones having the same mutation profiles were filled with same color whereas clones with additional mutations were filled with different colors. Branching points were decided based on the presence of distinct mutations. The length of lines does not represent mutational similarity or duration to acquire mutations among clones.

**Quantification and statistical analysis.** Data was analyzed and statistics were performed in Prism software v8.0 (Graphpad). Statistical significance was determined using a one-tailed or two-tailed Mann–Whitney U test or a Student t test. Kaplan–Meier (log rank) test was used for survival analysis. Fisher’s exact test was used to test associations between categorical characteristics and the metastasis status. Multivariate Cox proportional hazards model was used for multivariate analysis.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** For patients explicitly consenting to depositing of genomic information in a public database, the raw WES/RNA-seq data is deposited in European Genome-phenome Archive (EGA) under the accession numbers EGAS00001005511 and EGAS00001005512. Data deposited in EGA database can be accessed based on data access agreement. All 83 patients consent for publication of their data. Source data are provided as Source Data file. Source data are provided with this paper.

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