Sunitinib induces early histomolecular changes in a subset of renal cancer cells that contribute to resistance

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ABSTRACT: Sunitinib is the standard-of-care, first-line treatment for advanced renal cell carcinoma (RCC). Characteristics of treatment-resistant RCC have been described; however, complex tumor adaptation mechanisms obstruct the identification of significant operators in resistance. We hypothesized that resistance is a late manifestation of early, treatment-induced histomolecular alterations; therefore, studying early drug response may identify drivers of resistance. We describe an epithelioid RCC growth pattern in RCC xenografts, which emerges in sunitinib-sensitive tumors and is augmented during resistance. This growth modality is molecularly and morphologically related to the RCC spheroids that advance during in vitro treatment. Based on time-lapse microscopy, mRNA and microRNA screening, and tumor behavior-related characteristics, we propose that the spheroid and adherent RCC growth patterns differentially respond to sunitinib. Gene expression analysis indicated that sunitinib promoted spheroid formation, which provided a selective survival advantage under treatment. Functional studies confirm that E-cadherin is a key contributor to the survival of RCC cells under sunitinib treatment. In summary, we suggest that sunitinib-resistant RCC cells exist in treatment-sensitive tumors and are histologically identifiable.

KEY WORDS: kidney cancer; differential drug response; receptor tyrosine kinase inhibitor

INTRODUCTION

Renal cell carcinoma (RCC) is among the 10 most common cancers in North America, and 85% of RCC cases fall within the clear cell renal cell carcinoma (ccRCC) subtype. The multitargeted receptor tyrosine kinase (RTK) inhibitor sunitinibisthestandard, first-line treatment for the 30% of patients with RCC who develop distant metastases (1). Although 47% of patients with metastatic RCC (mRCC) initially respond to sunitinib (2), long-term response and complete remission are rare because of acquired resistance (3). Since the FDA approved sunitinib for the treatment of mRCC, much work has been done to uncover the molecular mechanisms that contribute to resistance (2, 4–9). The dynamics of the potential sunitinib-adaptation mechanisms, however, and therefore, the root of resistance, remain unexplored. With recent promising results of immunecheckpointtherapies, there is an emerging interest in the combination of different immune therapies as well as the combination of an immunetherapy with RTKs. Teasing outthemostpotentcombinationtreatmentwillbeaidedby preclinicalmodels, and characterization of RTK resistance will be crucial in investigating the interaction between immunetherapyandRTK-resistantcancercells. Previous studies aimed to capture the drivers of sunitinib resistance by comparing sunitinib-sensitive and sunitinib-resistant specimens. That approach, however, neglectsdriversthatmayalreadyoperateatthesensitive stage. We hypothesized that the sensitive and resistant stages were at different time points of the single continuum of molecular events induced by sunitinib. Therefore, studying molecular and related histologic changes that occur early during treatment may identify the relevant alterations that invoke resistance. Inthisstudy, we used in vitro and in vivo models (both allogeneic and syngeneic xenograft models) to examine the effect of sunitinib treatment on kidney cancer cells during both sensitive and resistant stages. Our results show that sunitinib treatment provides selective advantage for RCC cells that are able to form tight epithelioid spheroids in vitro or the corresponding morphology in vivo. Furthermore, we show that the resistant morphology isinducedbythetreatment.

MATERIALS AND METHODS

Cell culturing and in vitro treatment

ACHN, 786-0, and Renca cells were obtained from American Type Culture Collection (Manassas, VA, USA) and were culturedperthedistributor'sdescription.Cells were treated with 1 mM sunitinib-malate (Selleckchem, Houston, TX, USA) dissolved in DMSO or with DMSO only (vehicle). When cells were passaged, floating cells were also collected from the mediumbycentrifugationandweretrypsinizedtogetherwith the adherent cells. RCC spheres were passaged as described below. Resistance index was measured and calculated as previously described (10, 11).

Secondary and tertiary sphere formation

RCC spheres were picked under a microscope and were trypsinized and further disrupted by pipetting through a 200-ml pipette tip or a 30-gauge needle. Sphere disruption was monitoredunderamicroscope. Cells (23104cells/well)were plated on 6-well plates (Sarstedt, N¨umbrecht, Germany) and coated with Matrigel (Thermo Fisher Scientific, Waltham, MA, USA). Cells were then stained with Hoescht-3422, and cell number/well was determined by ImageXpress Micro Acquisition System (Molecular Devices, Sunnyvale, CA, USA), using the IMX software (IMXSoftwareGroup,London, England). The minimum sphere size was 40 mm, and the maximum spheroid size was 250 mm.

Mouse tumor models Syngeneic xenograft model

BALB/c mice (6–8 wk old, female; Charles River Laboratories, Wilmington,MA,USA)wereinjectedwith53105cells,s.c.inthe femur area. Injection contained a 1:1 ratio of Matrigel (Thermo FisherScientific)andsingle-cellsuspension.

Allogenic xenograft model

NOD/SCIDg (NSG) mice (6–8 wk old, female; The Jackson Laboratory, Bar Harbor, ME, USA) were injected with 5 3 105 cells subcutaneously in the femur area. Injection contained a 1:1 ratio of Matrigel (Thermo Fisher Scientific) and single-cell suspension.

In vivo sunitinib treatment

Miceweretreatedwith40mg/kgsunitinib(Selleckchem)orwith citratebuffer(vehicle-treatedgroup)bygavage,7d/wk.Sunitinib treatment startedonce xenografts reached the estimated volume of 100 mm3 (for Renca xenografts) or 50 mm3 (for ACHN xenografts). Tumor size was monitored daily by manual caliper, and tumorvolumewasestimatedwiththefollowingequation: Volume¼ðwidthÞ2 3ðlengthÞ 2

Spontaneous metastasis model

FiftyACHNor103 Rencacellswithanequalvolumeofmatrigel were subcutaneously injected into the neck area of 6–8-wk-old, female BALB/c or NSG mice. Tumor formation was monitored 3d/wk.Micewereeuthanizedat27d(Renca-recipientmice)or 60d(ACHN-recipientmice)afterinoculation.

Tail-vein injection metastasis assay

Renca(53103)orACHNcellswereinjecteddirectlyintothetail vein of 6–8-wk-old female BALB/c or NSG mice. Metastatic disease was monitoredby observingchanges in bodycondition (hunched back, loss of body weight, and dehydration). Rencarecipient mice were euthanized 40 d after inoculation, and ACHN-recipientmicewereeuthanized80dafterinoculation.

Histologic assessment

Tumorsandorgans(liver,kidneys,lungs,spleen,stomach,guts, andanyareasuspiciousformetastasis)werecollectedandsnapfrozenforRNAanalysisorfixedin10%formalin.Allspecimens were paraffin embedded, and 5–8-mm sections were prepared andstainedwithhematoxylinandeosin.Twopathologists(S.R. andG.M.Y.) independentlyassessedthestainedsections.

Immunohistochemistry

Specimens were fixed in PBS-buffered 10% formalin and embedded in paraffin. The following antibodies were used: Ki-67 [1:100, heat-induced epitope retrieval (HIER), cell conditioner 1(CC1)for32min;BiocareMedical,Concord,CA,USA],b-catenin (1:400, clone 14, HIER, CC1 for 64 min; Cell Marque, Rocklin, CA, USA), E-cadherin (1:50, HIER, CC1 for 64 min; Inter Medico, Markham, ON, Canada), pancytokeratin (pan-CK; prediluted,cloneae1/ae3/pck26,protease1–4,CC1for16min; F.Hoffmann-LaRoche,Basel,Switzerland),low-MWCK(1:20, CAM5.2,protease1–4min;BectonDickinson,FranklinLakes, NJ, USA). Antigen retrieval was performed on automated Ventana Discovery Ultra (F. Hoffmann-La Roche).

Immunocytochemistry

Spheroids were stained as described by Weiswald et al. (12). Anti–E-cadherin and anti–b-catenin antibodies were purchased from Santa Cruz Biotechnology (sc-7963; Dallas, TX, USA) and Cell Signaling

Technology (4627; Danvers, MA, USA) and were used as directed by the manufacturers. DAPI staining was performedtovisualize the nuclei.

RNA isolation and quantitative RT-PCR analysis

Tumors were snap-frozen and stored at 280°C for RNA isolation.TotalRNAwasisolatedwiththemicroRNA(miRNA)kit (miRNeasy Isolation Kit; Qiagen, Hilden, Germany) following the manufacturer's recommendations. Reverse transcription for mRNAanalysiswasperformedwiththeHighCapacityRNA-tocDNA Kit (Thermo Fisher Scientific), as recommended by the manufacturer.RNAqualityandquantityweredeterminedwith aBioanalyzerRNAChip(AgilentTechnologies,SantaClara,CA, USA)andaNanoDrop2000Spectrophotometer(ThermoFisher Scientific).QuantitativePCRreactionswereperformedonViia7 Real-Time PCR System (Thermo Fisher Scientific), using the SYBR Green Master Mix (Thermo Fisher Scientific). Cycle thresholdvalueswerenormalizedagainstthegeometricmeanof glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (PPIA), ribosomal protein lateral stalk subunitP0(RPLP0),andhypoxanthine phosphoribosyltransferase1(HPRT1).TheDDCt methodwasusedtoobtaintherelative quantificationvalues.

Imaging

LiveimagingwasperformedwithanAxioObserverLiveCellwith Fluorescence Lifetime Imaging instrument (Carl Zeiss, Oberkochen,Germany),andimageswereanalyzedwithZenBlack(Carl Zeiss). Confocal microscopy was performed with a Carl Zeiss LSM700. For spheroid counting, cultures were stained with Hoechst33342,andautomatedspheroidcountingwasperformed byImageXpressMicroAcquisitionSystem.Minimalspheroidsize wasadjustedto40 mm,andmaximumsizewas250 mm.

Proliferation and apoptosis assay

AproliferationassaywasperformedbyWST-1(MilliporeSigma, Billerica, MA, USA). Absorbance was measured at 650 and 450 nm with a SpectraMax M5 spectrophotometer (Molecular Devices). Because of the broad fluorescence spectrum of sunitinib,cytotoxicitywasquantifiedwiththeLive/DeadFixableFar RedDeadCellStainKit(ThermoFisherScientific).FlowcytometryanalysiswasperformedonaBDLSRFortessa X-20system.

Statistics

Data were analyzed with the Prism 7 package (GraphPad Software, La Jolla, CA, USA). We used 1-way ANOVA to visualize the tumor-growth curves and to analyze mRNA expression values.

Microarray and nanostring expression analysis

Microarray gene expression was performed at Centre for AppliedGenomicsofTheHospitalforSickChildren(Toronto,ON, Canada). A Nanostring Encounter Human miRNA Expression AssayKitwasusedtoprofiletheexpressionof800miRNAs.Data were processed with the R Bioconductor 3.2.3 software (R Foundation for Statistical Computing,Vienna,Austria). Affymetrix Mouse Gene 2.0 ST transcriptome array (Thermo Fisher Scientific) data were processed using R functions contained withinaffypackage1.48.0,andthebackgroundofthearrayswas correctedbyrobustmultiarrayaveraging.TheHT-12Expression BeadChip Kit (Illumina, San Diego, CA, USA) array data were processed using R functions contained within the Beadarray 2.20.1 package, whereas the NanoString data were processed withfunctionswithinNanoStringNormpackage(RFoundation), and probes with ,30 read count for all the samples were excluded. In all 3 studies—human arrays, mouse arrays, and Nanostring—between-sample/arrayquantilenormalization was performed, followed by the gene expression changes between groups using a t test (for 2 groups) and subjected to correction for multiple testing with the Benjamini-Hochberg false-discovery rate, in the limma package 3.26.9. The data sets supporting the conclusions in this article are available in the Gene Expression Omnibus database (National Center for Biotechnology Information, Bethesda, MD, USA). Lentiviral transduction

Third-generation, self-inactivating lentiviral vectors (LVs) were produced by cotransfection of human embryonic kidney 293T cells with polyethylenimine and packaging plasmids pMDL-g/pRRE, pMD2-VSVg, and pRSV-Rev (Addgene, Cambridge,MA,USA)aswellastheLVtransfervectorscarryingthe short hairpin RNA (shRNA) sequences against E-cadherin (TRCN0000039665, TRCN0000039664, and TRCN0000039667; Dharmacon,Lafayette,CO,USA).Oligonucleotidesequenceand the position of the shRNAs were as follows TRCN0000039664 forward, 59-CCGGCCAGTGAACAACGATGGCATTCTCGAGAATGCCATCGTTGTTCACTGGTTTTTG-39,reverseoligo sequence, 59-AATTCAAAAACCAGTGAACAACGATGGCATTCTCGAGAATGCCATCGTTGTTCACTGG-39, match position, 1562; TRCN0000039665 forward, 59-CCGGCCAAGCAGAATTGCTCACATTCTCGAGAATGTGAGCAATTCTGCTTGGTTTTTG-39, reverse, 59-AATTCAAAAACCAAGCAGAATTGCTCACATTCTCGAGAATGTGAGCAATTCTGCTTGG-39, match position, 682; and TRCN0000039667 forward, 59-CCGGCCAACCCAAGAATCTATCATTCTCGAGAATGATAGATTCTTGGGTTGGTTTTTG-39, reverse, 5 9-AATTCAAAAACCAACCCAAGAATCTATCATTCTCGAGAATGATAGATTCTTGGGTTGG-39, match position, 2210. Subsequently, LVs were concentrated by ultracentrifugation of the human embryonic kidney 293T cell medium at 20,000 rpm for 2 h at 4°C and stored at 280°C. Cells were transduced with lentivector with a multiplicity of infection of 10.

RESULTS

Sunitinib-treated tumor xenografts exhibit aggressive tumor behavior that starts early during response phase and is marked by unique histomorphologic changes

Weusedxenograftmodelstoassesstheeffectofsunitinibon cancer cells in vivo. BALB/c mice were xenografted with Renca RCC cells and were used as the immunocompetent model (n = 20). The Renca cell line was established from a tumor that arose spontaneously as a renal cortical adenocarcinoma in BALB/cCr mice. This cell line gives a high number of spontaneous metastases to the lung and liver, accurately mimicking human adult renal cell carcinoma, thus making Renca the most used immunocompetent murine model for RCC. To assess the behavior of human RCC, 786-0 and ACHN cell lines were used in this study, with the 786-0 cell line derived from a primary RCC site, and showing canonical clear cell histology, depicting the mostcommonRCCsubtype.The786-0cellslackfunctional von Hippel-Lindau tumor suppressor protein expression, whichisawidelyaccepteddriverofccRCC.TheACHNcell line was isolated from a malignant pleural RCC effusion. NSG mice were used as a host for human-derived tumor xenografts.Inadditiontothe scid mutation,whichrenders the strain deficient in T and B cells, NSG mice are also deficient in functional NK cells, minimizing rejection. NSG mice were xenografted with 786-0 RCC cells and were used as an immunocompromised model (n = 12). Mice were randomized to vehicle-treated and sunitinib-treated groups.Treatmentresponsewasassessedbasedonthetumor growth curve. Most xenografts exhibited initial drugsensitivity(flattumorgrowthcurve),followedbyresistance (steep increase in growth tumor curve). The tumors that were harvested at the time of rapid growth after an initial responsewereconsideredtreatmentdefiantandwereused as the sunitinib-resistant cohort (Fig. 1). Some xenografts, however, showed an extended drug-sensitive period and wereusedasthesunitinibsensitivecohort(Fig.1A). Sunitinib-sensitive xenografts of both Renca and 7860 cell lines showed early signs of aggressive behavior, which manifested as irregular invasive borders, local invasion,andextensivemetastaticdeposits.Multinucleated giant cells, which are described as indicators of genomic instability (13), were also frequent in the sensitive xenografts.Thesefeaturesbecameevenmoreprominentinthe sunitinib-resistant xenografts (Supplemental Fig. S1 and SupplementalTableS1). Strikingly, sunitinib-treated tumors showed viable, compact tumor islands (tongues of tumors within necrotic spaces), which contrasted with the confluent necrosis observed in vehicle-treated tumors. These tumor tongues were epithelioid and expressed mesenchymal and epithelial markers (vimentin, CKs). PAX8 stainingconfirmedtheirrenalorigin(Fig.1B).Budding of those epithelioid nests was observed at the tumor front. Such a phenomenon is associated with invasive potential and poor prognosis in other cancers and indicates the aggressive potential of the surviving RCC tumor tongues.

In vitro sunitinib treatment induced formation of RCC spheroids with increased colonization potential and expression of stem cell markers

Tobetterunderstandsunitinib'searlyeffectoncancercells, ACHN and Renca cells were treated with sunitinib in vitro.Sunitinibsensitivityorresistancewasassessedbythe resistanceindex(10,11).Treatedculturesshowedincreased expression of stem cell–related markers, such as OCT4, NANOG, Kruppel-like factor 4 (KLF4), and stem cell markersofkidneyandothercancers,suchasneuronalcell adhesion molecule (NRCAM), CD105, leucine-rich repeat containing G protein-coupled receptor 5 (LGR5), ABCC9, andGLIS2(SupplementalFig.S2)(14).Thesechangeswere detected early, within 1 wk of treatment and before the onsetofresistance.QuantitativePCRanalysisdidnotsuggestepithelial–mesenchymaltransition(EMT)changes. We previously described the formation of RCC spheroid structures with low efficiency under standard culturing conditions (15). Compared with the DMSO control, sunitinib increased the spheroid formation rate by 8-fold, starting as early as d 2 after treatment (Fig. 2A–C). To investigate whether sunitinib also affected the ability of spheroids to differentiate into adherent cells, we dissociated and propagated RCC spheroids in the presence of DMSO or sunitinib and quantified the number of spheroids that were formed and the ratio of cells that engaged in spheroid formation. Sunitinib did not significantly increase the number of secondary and tertiary spheroids. The rate of cells that participated in spheroid formation, however, significantly increased under sunitinib treatment,comparedwiththeDMSO-treatedcontrol.This indicates that sunitinib created an imbalance between 2-dimensional (2D) and 3-dimensional (3D) growth patterns (Fig. 2D, E), either by inhibition of adherent growth orbyselectingforthespheroidarchitecture. Sunitinib has previously been reported to enhance RCCmetastasisinaneoadjuvantsetting(16,17).Inour xenograftmodel,ACHNandRencacellsretainedtheir tumorigenic and metastatic ability, regardless of sunitinib treatment and growth modality. We thought to separately examine extravasation potential of RCC cells because it is a crucial step in metastasis. During tail-vein assay, cancer cells are directly injected into the host's circulatory system. This assay tests the potential of cancer cells to exit the vasculature and generate metastatic deposits. To explore the relation among the extravasation potential of RCC cells, sunitinib treatment, and spheroid formation, the following 4 subpopulations of ACHN and Renca cells were injected into mice tail-vein: 1) vehicle-treated, adherent; 2) vehicle-treated, RCC spheroids; 3) sunitinib-treated, adherent; and 4) sunitinib-treated, RCC spheroids. Mice injected with the ACHN spheroid-derived suspension formed 10 times moremetastaticdepositsinthelungsandlivercompared withadherentcells.Additionally,spheroidsmetastasized to the kidney, which was rarein the case of the adherent RCC cells. In line with the more aggressive nature of the Renca cells, all mice developed metastases. However, spheroid-injectedmicehadsignificantlymoredepositsin

thelungsandhadshortersurvival(SupplementalFig.S3).
Takentogether,sunitinibledtotheaccumulationofRCC spheroids with increased stem cell–related marker expression, and enhanced extravasation potential. Of note, the overall metastatic ability of RCC spheroids was similar to that of the adherent patterns, rather than the extravasation, and/or subsequent survival of metastases wasincreased.Increasedextravasationpotentialofother cancerspheroidshasbeenreported (18).

Sunitinib preferentially affects adherent RCC cells compared with spheroid-forming cells

We have shown that sunitinib treatment favored RCC spheroidgrowthinvitro.Toverifythedifferentialresponse of RCC spheroids and adherent cells to sunitinib, we monitored RCC growth dynamics under treatment by real-time microscopy for 55 h. We tested 4 conditions: 1) cells untreated for 1 wk that remained untreated during the time-lapse imaging; 2) cells pretreated with sunitinib for 1 wk with continued treatment during imaging; 3)cellsuntreatedfor1wkandthenswitchedtosunitinibtreatment during imaging; and 4) cells pretreated with sunitinib for 1 week and switched to no treatment duringimaging(Fig.3A).Untreatedcellspredominantly grew as an adherent, 2D monolayer, whereas sunitinibtreated cells showed significantly more spheroid formations (Fig. 3B, C). Untreated cells reached confluence at 25–32 h of imaging, indicating high proliferation andmigrationability(condition1).Conversely,sunitinib treatment enhanced spheroid formation, whereas adherent colonies were unable to advance (condition 2). Likewise, when cells were switched to sunitinib-treatment (condition 3), RCC spheroids grew in diameter, whereas adherent cell growth was inhibited. In condition 4, sunitinib withdrawal promoted 2D adherent growth, whereas the spheroids lost their compact morphology andwerereplacedbyamonolayergrowthpattern(Fig.3). QuantificationbyImageXpressconfirmedthatsunitinibhad a contrasting effect on the different RCC growth patterns, favoring3Dspheroidsandlimiting2Dmonolayergrowth.

Overlapping molecular signatures mark spheroid formation and sunitinib treatment

To gain information about the molecular pathways associated with spheroid formation and with sunitinib treatment and to assess whether those were related, we compared global mRNA and miRNA expression between the aforementioned 4 subpopulations in ACHN and Renca cell lines. Comparison of DMSOtreated ACHN spheroids and DMSO-treated adherent cells with stringent criteria (fold change, .3; falsediscovery rate, ,4%) indicated the up-regulation of cell adhesion and its auxiliary gene ontology (GO) categories, such as membrane trafficking, vesicular transport, and cell polarity in the spheroids. These results suggest that cell aggregation through the relocalization of adhesion factors is a primary contributor to spheroid formation (Fig. 4A). Adherent ACHN cells were treated with vehicle or sunitinibfor2dtoinvestigateearlyresponse.Wenoteda significant overlap between pathways that were induced in the adherent ACHN cells upon treatment and pathways that were induced during spheroid formation. Cell-adhesion–relatedGOcategories,suchascytoskeletal dynamics, vesicular transport, and disruption of cell polarity were overrepresented in the treated adherent cells. Additionally, sunitinib induced the small GTPasemediated signaling along with several developmental processes,suchastubedevelopment,negativeregulation of development, and anatomic structure morphogenesis. Down-regulated categories included kinetochore organization, regulation of microtubule cytoskeleton organization,andregulationofmitoticnucleardivision.Increased presenceofthemultinucleatedgiantcellsinthesunitinibtreated xenografts morphologically supported these results.Theoverlapbetweenmoleculareventsthatoccurin spheroids and during early sunitinib treatment of adherent cells indicate that sunitinib may generate a cell status that endorses spheroid formation and opposes the 2D adherentstate.Thissuggestionissupportedbytheinvitro observationthatsunitinibincreasedthespheroidnumber. Among the signaling pathways, PI3K signaling/AKT activation, cell–cell interaction, and G protein-coupled receptors(GPCRs)activationwerethemostsignificantlyupregulated under treatment. AKT is activated by RTKs, GPCRs,andcell–celladhesionmolecules,suchascadherins (19–21). Because sunitinib reportedly inhibits most RTKs(22),itisplausiblethatPI3K/AKT-basedsurvivalrelies on alternative inputs under treatment. For example, GPCR and cadherin-mediated activation could compensate for the lostinput,thuspropellingsurvival(Fig.4B).Additionally, sunitinib-treated spheroids overexpressed cytoskeletal/ microtubularproteinsandsmallGTPasesandalsoshowed overrepresentation of developmental categories, such as anatomic structure and morphogenesis. Transcriptome analysis of the 4 Renca subpopulations showed similar results. Overall, transcriptome analyses revealed the operation of overlapping molecular mechanisms during early response to sunitinib and spheroid formation. Our results suggest that sunitinib treatment supports spheroid formation by promoting cell–cell contact andvesicle-mediatedtransport.

miRNA expression analysis reveals different treatment responses in adherent vs. spheroid-forming RCC cells

Vehicle-treated adherent, vehicle-treated spheroid, sunitinib-treatedadherent,andsunitinib-treatedspheroid AHCNcellswereevaluatedformiRNAexpression.Ofthe 825 screened miRNAs, 246 miRNAs were significantly anddifferentiallyexpressedin$1comparison.Sunitinibtreatment had a greater effect on adherent cells than on spheroid-forming cells. miR-1268b, miR-302b, miR-579, andmiR-1185-2-3pweresignificantlygreaterinadherent cells, whereas miR-7-5p was up-regulated in the DMSOtreated adherent cells only. We have not identified miRNAsthatwerespecificforthespheroidsbutwerenot relatedtosunitinibtreatment,supportingourhypothesis that sunitinib treatment promoted spheroid formation andcounterbalanced adherentgrowth. OurdataindicatedthatmiRNAshadasignificanteffect on ubiquitin-mediated proteolysis under sunitinib treatment,andmiR-579appearedtobeakeyregulatorofthat process.TheBMPreceptor-andactivinreceptor-mediated branchesofTGF-b signalingappearedtobedifferentially regulated in adherent RCC cells vs. spheroids. DifferentiationdrivenbytheNODAL(nodalgrowthdifferentiation factor)/ACVR2 (activin A receptor type 2A)/SMAD2/ SMAD4 axis appeared to be under miRNA inhibition in theRCCspheresbutnotinadherent ACHNcells. Overall, mRNA and miRNA expression converged on the regulation of intracellular vesicular trafficking (SupplementalFig.S4). Tumor islands that appear during early sunitinib treatment in vivo share characteristics with RCC spheroids that emerge under sunitinib treatment in vitro

To establish the link between the in vitro spheroids and the surviving tumor islands in vivo, we performed immunohistochemistry (IHC) for E-cadherin and bcatenin expression and localization, based on our in vitromRNAexpressiondatashowingcell–celladhesionas the most significant difference between adherent and spheroid-forming cells. Both spheroids (in vitro) and epithelioid tongues (in vivo) showed membranous positivity forE-cadherinandstainedpositiveforb-catenin,whereas DMSO-treated adherent cells (in vitro) and other xenograft tumor areas (in vivo) exhibited low levels of cytoplasmic (but not membranous) E-cadherin staining and werenegativefor b-catenin(Fig. 5A,B). Further, we injected either adherent cells or spheroids into mice to evaluate the histology of their xenografts. ACHN spheroid xenografts exhibited a spectrum of morphologic patterns, including epithelioid nests, cytoplasmic clearing (typical ccRCC morphology), and spindled pattern (23). Additionally, compact, epithelioid tumor tongues were frequently observed. Tumors were invasive to fat and had multiple small, distant metastases and vascular co-option in the lungs (Fig. 5). Overall, the growth pattern was similar to that of the sunitinibsensitive xenografts. In contrast, adherent RCC-derived xenografts were mostly spindle shaped with no morphologic diversity and with minimal presence of tumor tongues (Fig. 5C–E and Supplemental Table S2). Weconcluded that the in vitro and in vivo growth patterns were related,andE-cadherinthatexpressedlivetumortongues in sunitinib-treated xenografts reflected the in vitro RCC spheroidsthat emergedundersunitinibtreatment.

E-cadherin-based cell–cell contact protects RCC spheres from sunitinib-mediated cell death with vehicle, and apoptosis and proliferation were quantifiedafter72h.Sunitinibhadasignificantcytotoxic effect on adherent cells, whereas it did not increase apoptosis in the RCC spheroids (Fig. 6A–C). Consistent with our real-time microscopy data, spheroid cultures showedincreased proliferation under sunitinib treatment, whereas the proliferation of adherent cells did not change significantly (Fig. 6D). Because

Sunitinib exerted a contrasting effect on the different growth modalities of RCC. To evaluate whether disparate response was reflected by alterations in commonly tested tumor characteristics, adherent and spheroid ACHN cultures were treated with sunitinib or cadherin-mediated cell–cell adhesion was a top upregulated GO category in sunitinib-treated cells and spheroids, we thought to assess whether cadherinmediated cell–cell contact could promote survival of RCC spheres under treatment. Cells were switched to Ca2+-free condition to disrupt cadherin-mediated adhesions (24) and were treated with sunitinib for 72 h. Ca2+-free condition sensitized RCC spheroids to sunitinibtreatment,whichmanifestedasincreasedratio of apoptotic cells in addition to decreased proliferative capacity (Fig. 6B, D). To determine whether E-cadherin was specifically involvedinprotectingspheroidsundersunitinibtreatment, cultures were transduced by lentiviral particles carrying appropriate shRNAs. Adherent and spheroid cultures weresubsequentlytreatedwithsunitinibfor72h.Viability andproliferationofRCCspheroidsdecreasedsignificantly in response to E-cadherin inhibition. In contrast, sunitinib's cytotoxicity did not change when spheroids were transduced with control enhanced green fluorescent protein lentiviral particles, whereas a nonsignificant increase in proliferation was observed (Fig. 6C, E). In vivo, the proliferative marker Ki-67 showed markedly strong positivity within the epithelioid tumor tongues of sunitinib-sensitive xenografts, compared with the spindledmorphology of thesametumor (Fig.6F, G). Overall,ourdataindicatedthatspheroidformationvia cadherin-mediatedcell–cellcontactenhancedtheviability and proliferation of RCC spheres under sunitinib treatment.Thisobservationisinagreementwiththeemergence of E-cadherin–positive tumor areas on sunitinib-treated xenografts.

DISCUSSION

Theantiangiogenicsunitinibremainsthestandardofcare for mRCC. We followed initial molecular and morphologic changes under sunitinib treatment to identify alterationsthat couldberelevant todevelopingresistance. Sunitinibresistancehasbeenstudiedextensivelyusing xenograftmodels.However,inmostexperimentaldesigns the sunitinib-sensitive phase was represented by tumors thathavebeenterminatedveryearly,toavoidbuildingup resistance.Inthatsetup,drug-sensitivetumorsweregiven farlesstimetogrowinthehost.Moreover,thetimewhen each sunitinib-sensitive xenograft would enter the resistant phase cannot be foreseen, creating an essentially diverse pool of the drug-sensitive tumor cohort. We, therefore, believe that the comparison between sunitinibresistanttumorsandxenograftsthatgrowataslowerpace under treatment (sunitinib sensitive in our experimental setup) is a more relevant representation of sunitinib sensitivity because it allows the study of the dynamics of treatment response. Additional limitations of the xenograft model are the use of immunodeficient mice and the preferentialselectionforaggressivetumorswhencelllines areestablished. Sunitinib response was characterized based on the tumor growth curve, and treatment resistance was defined as a rapid growth phase after an initial response. Using 3 RCC model cell lines, we did not find truly sunitinib-sensitive xenografts that completely halted theirgrowthundertreatment.Alltumorsbecamelarger over the duration of the experiment, mimicking the clinical scenario, in which the median of progressionfreesurvivalinpatientsis8–9mo,anddurableresponse is rare. The most prominent histologic change between theinvivosunitinib-andvehicle-treatedxenograftswas thepresenceofepithelioidtumortonguesvs.thediffuse necrotic spaces observed in the control tumors. The extentofsurvivingepithelioidislandscorrespondedto the sensitive or resistant states. Morphologic and IHCbasedsimilaritiesbetweenthein vitrospheroidsandin vivo tumor islands that survive sunitinib treatment provided initial evidence that these patterns were related. Additionally, among the different model cell lines, a close correlation was apparent among the spheroid-forming ability, the xenograft's histologic appearance, and the duration of in vivo drug response. DMSO-treated Renca cells formed many spheroids in vitro, which were reflected by the high frequency of spheroid-like islands in the vehicle-treated xenografts. Accordingly, Renca tumors quickly developed spectacular epithelioid tumor tongues upon sunitinib treatment, which was pairedwitha veryshortsunitinibsensitive phase. ACHN and 786-O cell lines had a moderateorlowcapacityforspontaneousspheroidformation invitro,whichparalleledtherareepithelioidorspheroidlikemorphologyintheuntreatedxenograftsandwithan extended sunitinib-sensitive period. Our results are in agreement with previous reports that suggested a nongenetic base for sunitinibresistance(25). Time-lapse microscopy, mRNA analysis, and a metastatic colonization assay show that sunitinib hasapreferential effect on the adherent RCC pattern, compared withthespheroids.Allourresultspointedinthesame direction: the spheroid architecture is beneficial for cancercellsurvivalundertreatment.Thepresenceofa spheroid-associated signature in adherent sunitinibtreated cells suggests that sunitinib primes RCC cells for spheroidformation.Ourdataimplythatcellsthatsurvive sunitinib treatment switch the RTK-based activation of PI3K/AKT to alternative inputs, such as GPCRs and cell-adhesion complexes, to ensure continued activity and survival. Indeed, GPCRs, such as the calcitonin receptor-like receptor and GPCR 162, are significantly up-regulated in RCC, their expression correlates with poor prognosis (26–28), and they affectthe carbohydrate metabolism–AKT-mTOR axis, the quintessential RCC driver(29).Morespecifically,our resultsindicatetheupregulation of the GPCR CDC42, a primary regulator of cell polarity and cytoskeletal organization in renal epithelialtubularcellsduringembryogenesis(30).Similarly, sunitinib-treated gastrointestinal tumors showed elevatedphospho-AKT(31). RCC spheroids showed epithelioid characteristics and highly expressed Ca^{2+}-dependent cell–cell adhesion proteins,suchasE-cadherin.Thisresultseeminglycontradicts reportsthatstressthenecessityofEMTandmesenchymal features to gain aggressive tumor features and drug resistance(6,32–35).Recently,however,severallaboratories reported a more-complex relation between EMT and cancer (36). Several studies suggest that EMT is not a prerequisite for metastasis. Genetic tracing of cells that underwent EMT and, in an independent study, the deletion of Snail and Twist revealed that EMT is not compulsoryfortheinitiationofprimaryandmetastatic luminal breast adenocarcinoma and pancreatic ductal adenocarcinoma (36, 37).

RCC cells likely reside in anintermediate gray zone of the EMT spectrum and are known to coexpress mesenchymal (vimentin) and epithelial (low-MW CK, panCK, and EMA) markers. Recent data show that in some cancers, including RCC, expression of the mesenchymal signature and lack of epithelial signature correlated with better disease-free and overall survival (38). Additionally,RCCcaseswithamore-mesenchymalgenesignature responded better to compounds targeting microtubule dynamics, indicating that the epithelioid compartment has a differential response for various drugs (38). Lastly,itispossiblethatabalanceexistsbetweenthelessproliferative, but more therapy-resistant epithelioid pattern,and the more-proliferativemesenchymalpattern. Despite membranous E-cadherin expression, RCC spheroid cells did not appear to be differentiated, polarized epithelia. RCC spheroid cells were tightly packed, which may indicate loss of polarity and deficiency of microtubular structure and cytoskeletal network. Alternatively, these epithelioid islands and the budding phenotype on the tumor front may reflect a discrete form of invasion. For example, during renal embryonicdevelopment,epithelioidcellclusterscollectively migrateduringnephricductelongation,highlightingthe migratorypotential of epithelioidcells(39–41). Ourresultsshowthatsunitinib-resistanceisinducedin thetreatment-sensitivestage,andnonrespondingcellscan be morphologically identified at this early time point. These findings mayhavefuture implications where sunitinib is used as part of a combination therapy. Several ongoing trials assess sunitinib or other antiangiogenic RTKsincombinationwithnivolumaborpembrolizumab (42). Reportedly, sunitinib itself has immune modulatory effect by suppressing regulatory T (Treg) cells and could, therefore, be used in sequential therapeutic strategies to prime antitumor immune response (43). Antitumor immuneresponsecanalsobegeneratedbyoncolyticviruses, and that response is augmented by sunitinib (44). However, our data and that of others show that, in sunitinib resistance, the tumor acquires molecular signatures that promote tumor survival and may affect the tumor's immunologic properties and thus the effectiveness of the subsequent immunotherapy. During sunitinib resistance, the tumor acquires molecular signatures that promote tumor survival under treatment and may affect the tumor's immunologic properties and thus the effectiveness ofthesubsequentimmunotherapy.Understanding which histologic and molecular characteristics dominate during early and late phases of sunitinib response may be exploited in the future to optimize interference between TKIandimmunetherapy(42).

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Figure Legends

Figure 1. Common histologic changes are present in sunitinib-sensitive and resistant xenografts compared with vehicle-treated tumors. A) Tumor growth curves of vehicle-treated, sunitinib (SU)-sensitive, and SU-resistant Renca and 786-0 xenografts. Lines depict the fitted slope of tumor growth curves. B) Compact cancer tongues interrupted necrotic spaces in SU-sensitive and resistant xenografts. Arrows indicate live tumor islands. Surviving tumor areas within the necrotic spaces expressed epithelioid markers, such as PanCK and low-MW CK (LMWCK). Tumors also stained positively for the mesenchymal marker vimentin (VIM) and the renal marker PAX8.

Figure 2. Sunitinib increases tumor spheroid formation in vitro. Renca and ACHN cells were treated with sunitinib (SU) or with vehicle. A, B) Treated cells showed significant increase in tumor spheroid formation. Number of spheroids, adherent cells, and spheroid-forming cells were quantified by ImageXpress, on Hoescht-3422–stained cultures. A representative picture of vehicleand SU-treated cultures. Cell nuclei were stained by Hoechst 3342 to enable automated spheroid and cell counting by ImageXpress (B). Spheroids are highlighted as light-blue areas. C) Number of tertiary spheres formed under vehicle- or SU treatment. D) Percentage of cells that participate in spheroid formation under vehicle- or SU treatment.

Figure 3. Sunitinib has a distinct effect on adherent and spheroid growth patterns of RCC in vitro. The effect of sunitinib on adherent and spheroid growth patterns of ACHN cellswas followed by time-lapse microscopy. A) Four conditions were evaluated. Condition 1 was vehicle-treated cells; condition 2, sunitinib treatment; condition 3, vehicle-treated cells were switched to sunitinib treatment; and condition 4, sunitinib-treated cells were switched to vehicle-treatment. Time-lapse microscopy start point was at 0 h, and cultures were followed for 55 h. B) Representative photographs of the time-lapse microscopy. Yellow dotted line outlines the border of the adherent RCC areas. Spheroids are shown with orange arrows, whereas adherent growth is denoted by white arrows. C) Quantitative representation of changes in the percentage of area covered by adherent and spheroid patterns under the 4 conditions.

Figure 4. Overlapping cellular processes are predicted to operate under sunitinib treatment and spheroid formation. Transcriptome analysis indicates that cell–cell adhesion, cytoskeletal organization, vesicular transport, and cellular polarity are similarly altered during spheroid formation (A) and sunitinib treatment (B). Additionally, sunitinib treatment is predicted to induce the PI3K/AKT survival pathway through GPCRs.

Figure 5. In vitro RCC spheroids and in vivo tumor islands surviving sunitinib (SU) treatment are related. A) 786-0 spheroids (786) or adherent cells were assessed for E-cadherin (E-CAD) and b-catenin expression by immunocytochemistry. Nuclei were visualized with DAPI staining. B) Vehicle-treated, SU-sensitive, and SU-resistant xenografts were assessed for E-CAD and b-catenin expression by IHC. Arrowheads indicate membranous positive staining. C–M) Adherent (adh) RCC cells or spheroids were xenografted. Tumor morphology was compared on hematoxylin and eosin–stained sections. Adh ACHN xenografts appear to be high-grade tumors with diffuse necrotic cores and necrotic cores with mainly spindled morphology (C–E). Spheroid (sph)-initiated tumors show large, compact tumor tongues, similar to the SUtreated xenografts (F–H). The border of sph-based xenografts shows epithelial nests invading adjacent tissue (H). Black arrows indicate epithelial tumor nests (G, H). Sph-derived xenografts exhibit

different histologic patterns, such as cytoplasmic clearing and areas with giant multinucleated cells (I, J). Black arrows indicate giant cells. Sphderived and SU-treated Renca xenografts show multifocal tumors with compact sph-like patterns (K–M). In contrast, adherent Renca cell xenografts have uninterrupted necrotic space.