

Development of Novel Patient-Derived Preclinical Models from Malignant Effusions in Patients with Tyrosine Kinase Inhibitor-Resistant Clear Cell Renal Cell Carcinoma¹



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Abstract

PURPOSE: Although targeting angiogenesis with tyrosine kinase inhibitors (TKIs) has become standard of care in the treatment of clear cell renal cell carcinoma (RCC), resistance mechanism are not fully understood, and there is a need to develop new therapeutic options overcoming them. **METHODS AND MATERIALS:** To develop a preclinical model that predicts clinical activity of novel agents in 19 RCC patients, we established patient-derived cell (PDC) and xenograft (PDX) models derived from malignant effusions or surgical specimen. **RESULTS:** Successful PDCs, defined as cells that maintained growth following two passages, were established in 5 of 15 malignant effusions and 1 of 4 surgical specimens. One PDC, clinically refractory to TKIs, was implanted and engrafted in mice, resulting in a comparable histology to the primary tumor. The PDC-PDX model also showed similar genomic features when tested using targeted sequencing of cancer-related genes. When we examined the drug effects of the PDX model, the tumor cells showed resistance to TKIs and everolimus *in vitro*. **CONCLUSION:** The results suggest that the PDC-PDX preclinical model we developed using malignant effusions can be a useful preclinical model to interrogate sensitivity to targeted agents based on genomic alterations.

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Introduction

Clear cell renal cell carcinoma (RCC) represents a unique clinical setting for the application of antiangiogenic therapy. Targeting angiogenesis via the vascular endothelial growth factor receptor (VEGFR) or mammalian target of rapamycin (mTOR) pathways has produced robust clinical effects and revolutionized the treatment of metastatic RCC (mRCC) [1]. Multitargeted tyrosine kinase inhibitors (TKIs) against VEGFR such as sunitinib [2], sorafenib [3], and pazopanib [4] have demonstrated improved progression-free survival and/or overall survival compared with interferon and/or supportive care. However, some patients with clear cell mRCC who received these TKIs do not achieve response. Also, in most responders, resistance to therapy will eventually develop. While the mechanisms of resistance to VEGFR TKIs are not yet well understood, there is a need to develop new therapeutic options overcoming TKI resistance. The goal should be met through

preclinical models that reliably predict clinical activity of novel antiangiogenic compounds in patients.

It becomes increasingly clear that novel preclinical models that more closely simulate the heterogeneity of human cancers are needed

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for more efficient oncology drug development. Until recently, drug screening of cancer has emphasized xenograft models derived from the established, conventional cell lines and [5], in some cases, from patient samples [6]. As the limitations of current xenograft models derived from previously established cell lines have been well described [7], patient-derived xenograft (PDX) models may provide more accurate depiction of the human cancers they are derived from than cell line-derived xenografts. As patient-derived models might reflect a clinical response better [8] and the ability to obtain metastatic tumor samples is not always possible, we already have established disease-specific panels of patient-derived cell (PDC) models directly from malignant effusions [9]. Both PDC lines and patient-derived xenografts (PDX) made from malignant effusions are alternative models that may overcome sample challenges.

So far, numerous tumor-specific PDX models have been established, and importantly, they are biologically mostly stable when passaged in mice in terms of gene expression patterns, mutational status, metastatic potential, and drug responsiveness [10]. However, the practical relevance of PDX models for application in clinical oncology is limited owing to the time required for PDX establishment (~4 months) since mRCC patients with refractory disease live less than 1 year [11]. Despite an obvious advantage of PDX over xenografts from cell lines [12], their application has been criticized by the fact that many PDXs are established from the primary tumors or, in some cases, from metastatic sites of previously untreated patients. Thereby, they fail to reproduce the refractory patient population in whom most novel therapeutics will undergo their initial trials [13]. In addition, tumor take rates may be higher for metastases with more aggressive phenotypes than primary tumors.

In an effort to develop a novel PDX model with PDCs, we established a large collection of RCC PDC models directly derived from malignant effusions or ascites collected after TKI failure. This model could be used to develop new therapeutic targets, to better understand the basis of sensitivity of tumors from individual patients, and potentially to help the stratification of patients according to molecular characteristics. TKI-resistant PDCs were selected and tested further using PDX cells.

Methods

This prospective, pilot study is a part of the Samsung Medical Center Oncology Biomarker study (ClinicalTrials.gov identifier: NCT01831609). The Institutional Review Board at Samsung Medical Center (Seoul, Korea) approved the study. After obtaining a written informed consent, we collected effusions or ascites from 15 adult (>18 years), clear cell mRCC patients who failed treatment with sunitinib or pazopanib as a first-line TKI therapy. Effusions were obtained for a therapeutic purpose and in accordance with the Declaration of Helsinki. In addition, four RCC samples from surgical resection of metastases were collected for primary culture. Details of patient characteristics are listed in Table 1. Patients had either intrinsic (i.e., primary; $n = 14$) or developed secondary ($n = 5$) resistance to TKIs. We defined patients with intrinsic resistance as those who had progressive disease (PD) as their best response during the first 8 weeks after starting TKI treatment. In accordance with the Response Evaluation Criteria in Solid tumors, PD was defined as a >20% increase in the sum of the diameters of the target lesions, and the sum must also demonstrate an absolute increase of at least 5 mm. Patients who showed complete response (CR), partial response (PR), or stable disease (SD) as their best response to TKIs but eventually progressed were classified as those with secondary resistance.

Table 1. Patient Characteristics

No.	Age/Sex	Therapy	Resistance	Survival	Origin of PDC	Cell Number	Passage
123	48/M	Sunitinib	Intrinsic	6 mo	Surgery		0
161	55/F	Sunitinib	Intrinsic	8 mo	Ascites		P1
162	66/M	Pazopanib	Intrinsic	6 mo	Surgery		0
166	71/M	Sunitinib	Secondary	14 mo	Surgery		P2
189	59/F	Sunitinib	Secondary	21 mo	Surgery		0
192	52/F	Sunitinib	Secondary	16 mo	Ascites		P1
391	53/M	Sunitinib	Secondary	13 mo	Ascites	1.26E+ 07/ml	P1
395	39/M	Sunitinib	Intrinsic	4 mo	Pleural effusion	1.20E+ 07/ml	P2
413	67/M	Sunitinib	Secondary	27 mo	Pericardial effusion	1.73E+ 07/ml	0
426	55/M	Sunitinib	Intrinsic	9 mo	Pleural effusion	2.00E+ 06/ml	0
483	53/M	Sunitinib	Intrinsic	5 mo	Pleural effusion	2.30E+ 06/ml	0
491	62/M	Pazopanib	Intrinsic	11 mo	Pleural effusion	1.22E+ 07/ml	P1
518	65/M	Pazopanib	Intrinsic	19 mo	Pleural effusion	1.20E+ 06/ml	0
539	63/F	Sunitinib	Secondary	26 mo	Ascites	5.00E+ 06/ml	P3
573	36/M	Pazopanib	Intrinsic	8 mo	Pleural effusion	3.40E+ 07/ml	P3
575	44/M	Pazopanib	Intrinsic	13 mo	Pleural effusion	5.00E+ 05/ml	0
590	42/F	Sunitinib	Intrinsic	6 mo	Ascites	6.08E+ 06/ml	P2
603	61/M	Sunitinib	Secondary	37 mo	Pleural effusion	8.83E+ 06/ml	P2
624	54/F	Sunitinib	Intrinsic	9 mo	Ascites	6.39E+ 06/ml	0

Primary cultures of human effusions and metastatic tumors were conducted according to a method described previously [9]. In brief, collected effusions (1 to 5 l) were divided into 50-ml tubes, centrifuged at 1500 rpm for 10 minutes, and washed twice with PBS. Cell pellets were resuspended in culture medium and divided into 75-cm² culture flasks. Cells were grown in RPMI 1640 supplemented with 10% FBS and 1% antibiotic-antimycotic solution (Gibco BRL, Paisley, UK). The media were changed every 3 days, and cells were maintained at 37°C in a humidified 5% CO₂ incubator. PDCs were passaged using TrypLE Express (Gibco BRL) to detach cells when the culture was at 80% to 90% confluence. A similar process of tumor primary culture was conducted on snap-frozen tumor samples collected immediately after surgery.

Once the cells reached 80% to 90% confluence, they were washed and detached using TrypLE Express, and incubated for 3 minutes at 37°C with 5% CO₂. Following detachment, 4 ml of complete culture media was added to stop the activity of trypsin, and cells were transferred to a 15-ml sterile centrifuge tube. After centrifugation, cells were resuspended in 1 ml of freezing medium (Cellbanker, Zenoq, Japan), transferred into cryovials (Nalge Nunc, Naperville, IL), and slowly frozen in a -80°C freezer overnight.

PDCs were received frozen and were transferred to Oncotest GmbH (Germany) for the establishment of PDX models [9]. On site, cells were thawed, the freezing media removed, and the cells resuspended and transferred into T75 flasks. Cells were grown for 3 to 7 days in RPMI/10% FBS until the culture reached around 80% confluence. Cells were collected and counted, and 5×10^6 cells were injected into the hind flanks of NOD scid gamma (NSG) mice (Jackson Laboratories). Tumors developed within 25 to 85 days postinjection. These tumors were explanted, and viable portions of the tumors were cut into pieces and implanted subcutaneously into female NMRI nu/nu mice (Harlan Laboratories). This process was repeated to serially passage the respective models. From each passage, formalin-fixed, paraffin-embedded blocks were prepared, and tumor slices were stained with hematoxylin and eosin. Slides were scanned with a Hamamatsu slide scanner, and images were extracted using the Nanozoomer program from Hamamatsu. All animal handling and experiments with animals were in accordance with the guidelines set by the Samsung Biological Research Institute (Seoul, Korea).

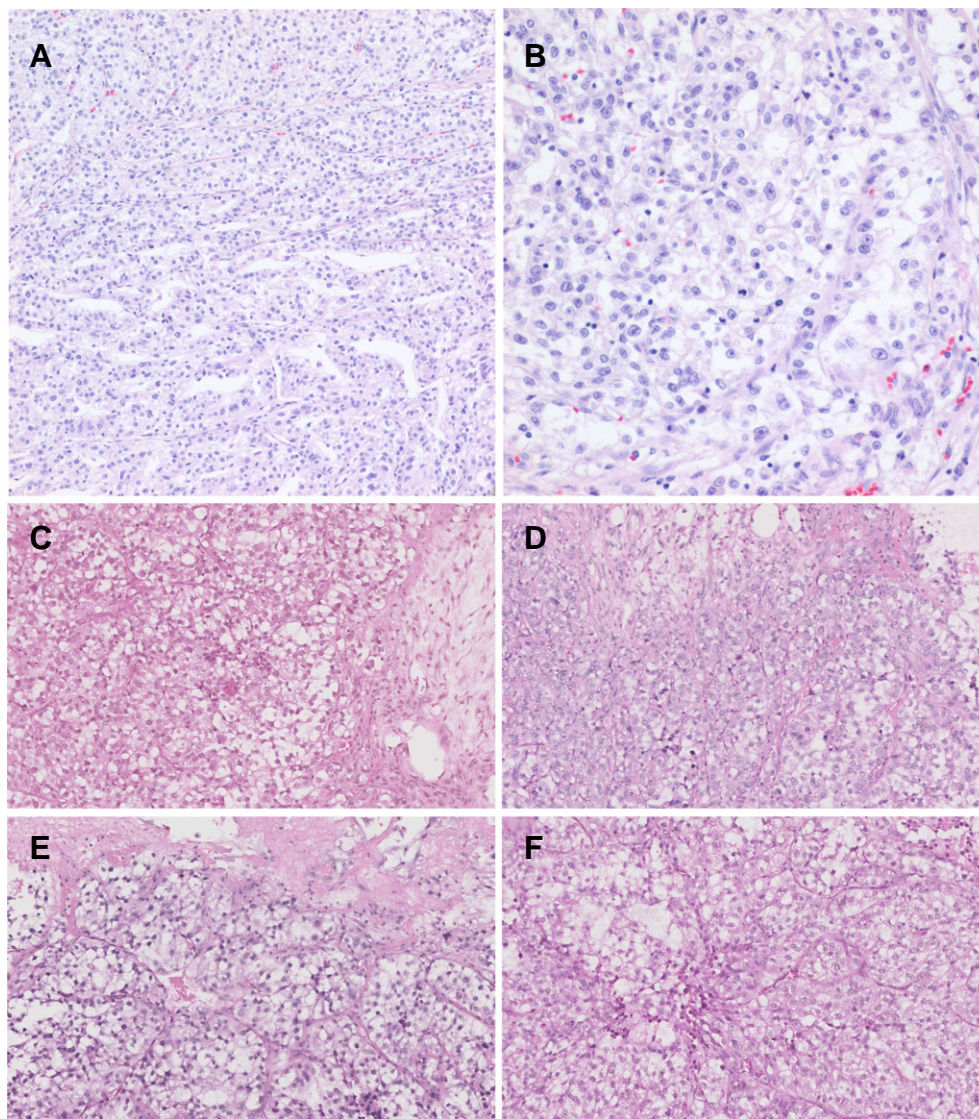


Figure 1. Morphology of primary tumor (A and B) and xenograft (P1, C; P2, D; P3, E; P4, F).

To genomically compare the final PDX model to the primary tumor, we performed targeted deep sequencing using Illumina HiSeq 2500. Genomic DNA was extracted from a single cell suspension of PDX cells and sequenced for capturing cancer-related genes (Supplement file 1) [9]. Concurrently, copy number variations were analyzed using the nCounter Copy Number Variation Assay Kit (NanoString Technologies, Seattle, WA). Average count numbers of greater than 3 were called and confirmed by immunohistochemistry, fluorescent *in situ* hybridization, or real-time polymerase chain reaction.

For exploratory purposes, we examined the drug effects on cell viability with single cell suspension using the PDX tumor at passage 5. The overall process of cell proliferation inhibition assay and the high-throughput screening (Samsung Electro-Mechanics CO [SEMCO], Suwon, Korea) is summarized in Supplement file 2 [14], and cell viability was determined with the CellTiter 96 Aqueous One Solution assay (Promega, Madison, WI) according to the manufacturer's protocol. Agents including sunitinib, pazopanib, lapatinib, and everolimus were purchased from Selleck Chemicals (Houston, TX).

Results

PDCs were derived from malignant effusions ($n = 15$) and surgical specimens ($n = 4$) of metastatic disease. All patients had clear cell carcinoma in either alveolar or tubular configurations. These tumors were aggressive in clinical behavior; 12 patients experienced intrinsic resistance to first-line sunitinib or pazopanib, whereas 7 patients had secondary resistant tumors (Table 1). Cells from all patients were stained with either hematoxylin and eosin or Papanicolaou, and micrographs were prospectively stored in our internal database. Successful PDCs, defined as cells that maintained growth following 2 passages, were established in 5 of 15 effusions and 1 of 4 surgical specimens.

After successful PDC establishment from malignant effusions, we implanted one PDC (no. 395) in NSG mice to evaluate whether PDCs could successfully be converted to a PDX model. The PDX was serially passaged for five times before it was used for experiments. The PDC-PDX model exhibited histological features and immunohistochemistry findings similar to those of the primary tumor (Figure 1). In the primary tumor, clear cells are arranged in an alveolar pattern, while granular cells are in a solid pattern. PDX tumor showed similar

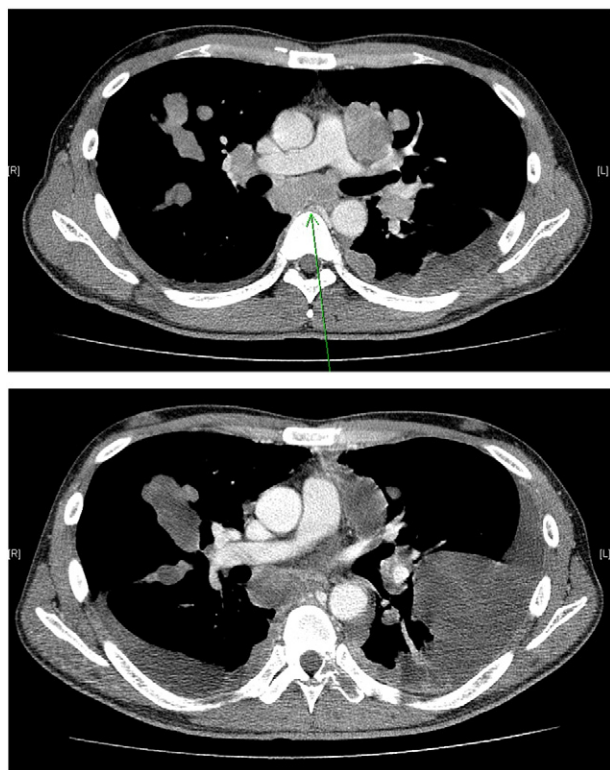


Figure 2. Chest computed tomography scans of the patient no. 395 with refractory RCC. (A) Baseline scan showing multiple lung and lymph node metastases. (B) Subsequent scan after 6 weeks of sunitinib therapy showing disease progression and the development of large amount of bilateral pleural effusions.

findings where the cytoplasm was eosinophilic and the nuclei had prominent nucleoli and coarse chromatin. The patient (no. 395, 39-year-old male) presented with a diagnosis of clear cell carcinoma of the right kidney. Fifteen months after his surgical resection, the patient developed multiple lung and lymph node metastases (Figure 2A). He had been treated with sunitinib at 50 mg per day for 4 weeks followed by 2 weeks of rest. Six weeks after starting the sunitinib therapy, metastatic lesions showed progression, and large amount of bilateral pleural effusions developed (Figure 2B). After thoracentesis for a therapeutic purpose, at which a PDC was established, everolimus had been administered but failed to achieve any clinical benefit. The clinical condition of the patient deteriorated rapidly after 1 month of everolimus therapy that only supportive care was given until death.

To confirm the similarity of genomic features between the primary tumor and PDX, we compared variant allele frequency results of the primary tumor and single cell suspensions from the PDX tumor at passage 5 (Table 2). With targeted sequencing for 381 cancer-related genes, these 2 samples showed surprisingly similar genomic alterations. Sequencing of primary tumor and PDX revealed mutual deletions in TP53, as well as cyclin-dependent kinase inhibitor 2A (CDKN2A) and von Hippel–Lindau (VHL), both of which are thought to be related with RCC carcinogenesis.

We then examined the drug effects on cell viability as described in the Methods section. Antitumor activity of 18 drugs (pazopanib, sunitinib, everolimus, lapatinib, BEZ235, BGJ-398, and others) was tested against no. 395 PDCs (Table 3). In accordance to his clinical resistance to VEGFR TKIs and everolimus, we confirmed that cells

Table 2. Copy Number Variations Seen in Patient (No. 395)-Derived Xenograft

Type	Gene Name	Alteration	Sig. Ratio	Copy Number	Sig. Exon
Known	CDKN2A	Deletion	-9.8149	0.00222	5
Known	TP53	Deletion	-1.0027	0.998127	13
Known	VHL	Deletion	-1.07139	0.951723	3
Unknown	DDR2	Amplification	0.900786	3.734165	15
Unknown	SRC	Amplification	0.931351	3.814123	11
Unknown	TOP1	Amplification	0.987788	3.966284	21
Unknown	AURKB	Deletion	-0.83248	1.12313	8
Unknown	FLT3	Deletion	-0.88982	1.079362	25
Unknown	MTOR	Deletion	-0.80689	1.143227	45
Unknown	PTCH2	Deletion	-0.83586	1.120501	19

Table 3. Drug Sensitivity Profiles Seen in Patient (No. 395)-Derived Xenograft

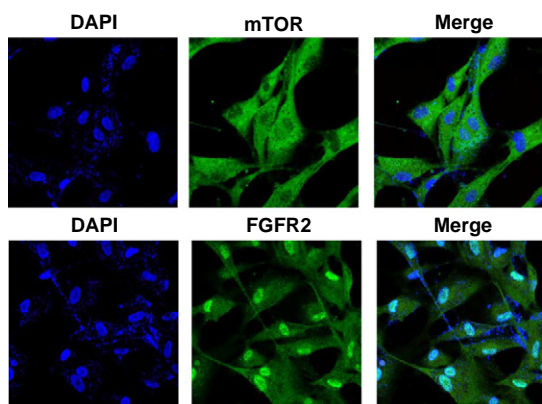
Drug	Mechanism	IC50 (μ M)
AZD1775	Wee1 kinase inhibitor	5.1
Everolimus	MTOR inhibitor	>10
Crizotinib	ALK and ROS1 inhibitor	>10
Pazopanib	VEGFR kinase inhibitor	>10
Sorafenib	VEGFR kinase inhibitor	>10
Sunitinib	VEGFR kinase inhibitor	>10
Vemurafenib	Anti-BRAF antibody	>10
Ceruximab	Anti-EGFR antibody	>10
Trastuzumab	Anti-HER2 antibody	>10
Gefitinib	EGFR inhibitor	>10
Dacomitinib (PF-0299804)	EGFR inhibitor	>10
Lapatinib	Pan-HER inhibitor	>10
BEZ235	MTOR/PI3K inhibitor	3.3
AZD2014	MTOR inhibitor	>10
LEE011	CDK4/6 inhibitor	>10
5-Fluorouracil	Cytotoxic	>10
Neratinib	Pan-HER inhibitor	1.0
BGJ-398	FGFR inhibitor	1.2

were resistant to most VEGFR TKIs tested, with an IC50 of more than 10 μ M for everolimus, pazopanib, sorafenib, and sunitinib. The drugs with IC50 values below 4.0 μ M included neratinib, BEZ235, and BGJ-398. To define the role of these drugs in TKI-resistant clear cell RCC, cells were treated again to assess cell viability using CellTiter-Glo reagents (Supplement file 2). As expected, BEZ235 and BGJ-398 inhibited cell proliferation with IC50 values of 0.015 μ M and 0.79 μ M, respectively (Figure 3B). A subsequent immunofluorescence assay showed mTOR protein expression in the cytoplasm and FGFR2 in the nuclei (Figure 3A). Western blot confirmed the expression of mTOR/PI3K and FGFR2 proteins in the cells (Figure 3C). We observed that the inhibition of mTOR/PI3K pathway with BEZ235 resulted in the downregulation of phosphorylation of the pathway. BGJ-398 also inhibited FGFR and downstream phosphorylation of AKT, MEK, and ERK1/2. However, the results with BEZ235 and BGJ-398 were not reproduced when we tested with an *in vivo* PDX model (Figure 4).

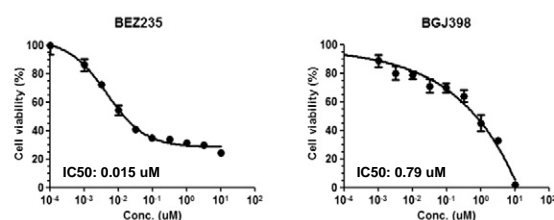
Discussion

Treatment strategies targeting angiogenesis via the VEGFR TKIs or mTOR inhibitors have dramatically improved the treatment outcomes for patients with clear cell mRCC [1]. While most patients would develop adaptive or secondary resistance to these targeted agents after months or even after years of clinical benefit, a substantial number of patients exhibit intrinsic resistance to VEGFR TKIs. Once this has happened, limited treatment options remain. Furthermore, this subset of patients with intrinsic resistance rarely responds to subsequent therapy such as mTOR inhibitors or other VEGFR TKIs

A) immunofluorescence



B) Cell viability assay



C) Western blot

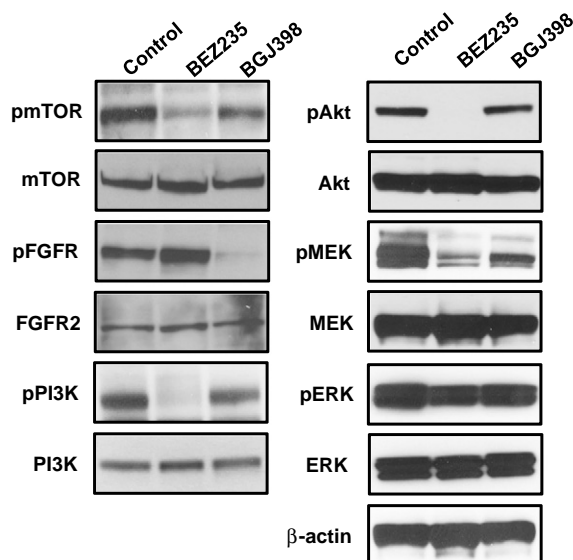


Figure 3. Antitumor activity of BEZ235 and BGJ-398. (A) mTOR and FGFR2 protein expression by immunofluorescence. (B) The viability of RCC PDCs was measured by CellTiter-Glo assay after treatment with various concentrations of BEZ235 and BGJ-398 for 5 days. The cell viability (%) represents the percent growth as compared to the control (no treatment), and IC₅₀ values are 0.015 μ M and 0.79 μ M, respectively. (C) The western blot for mTOR and FGFR phosphorylation and targeted downstream pathways. Cells were treated with 1 μ M BEZ235 and 1 μ M BGJ-398 for 5 days, respectively. Control cell was treated with DMSO.

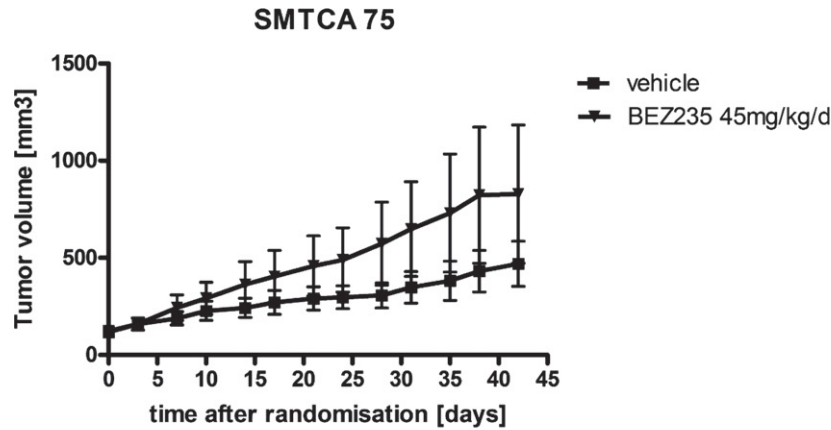
[15–17]. This raises a need for the development of novel therapeutics to overcome VEGFR TKI resistance. Historically, in cancer research, the development of new agents is predominantly based on preclinical studies of drug activity, typically tested with *in vitro* cell lines and *in vivo* murine model systems. While conventional cell lines are convenient and easily accessible, their predictive power has been poor [18,19]. The ability to establish preclinical models that closely resemble actual tumor *in vivo* in terms of molecular profiles and clinical behaviors holds enormous promise in oncology drug development.

In this study, we developed PDC and PDX models using cancer cells derived from malignant effusions that exhibited morphologic features and genomic profiles similar to those of tumor tissue samples in patients with clear cell mRCC. PDC from malignant effusions has more than a few advantages over conventional cell lines [9]. First, PDC models faithfully recapitulated primary patient tumors, with the molecular and gross phenotypic characteristics of the primary tumor retained. Second, the median time from specimen collection to PDC passage 1 takes only 3 weeks, which is more feasible for clinical application than xenograft models. Third, we found that the success rate is as high as >70% of attempted cases. Fourth, although only

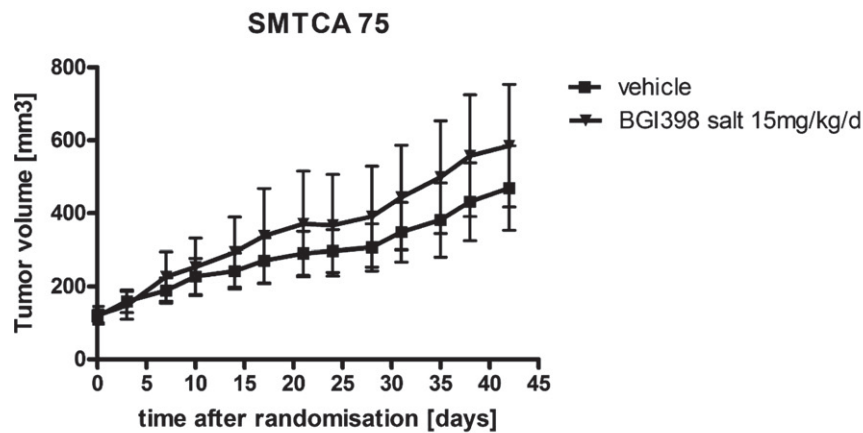
tested in a limited setting so far, PDC could be successfully engrafted into immunocompromised mice; thus, PDCs can be used both *in vitro* and as a cell source for further *in vivo* analyses. Although tested for exploratory purposes, we assessed the sensitivity of tumor to a number of targeted drugs including sunitinib and pazopanib. As a result, the tumor cells were resistant to VEGFR TKIs as well as to everolimus, concordant with the actual clinical responses to sunitinib and everolimus.

As stated above, the limitations of current preclinical models have been well described [12]. In contrast, PDX may represent more meaningful models for the development of novel therapeutic agents. As they normally display similar heterogeneity to the tumors they are originally derived from, they can predict the subsequent clinical outcomes more accurately. This allows for mechanistic studies of clinical agents that cannot be done in the patient themselves. Establishing PDX models from surgical specimens faces certain limitations. There is a need for a sufficient amount of fresh tumor tissue, and unless the patient is scheduled for surgery, available tumor material might not be sufficient. Moreover, depending on the originating histology, the failure rate in PDX establishment can be substantial. In addition, the use of “personalized” PDX models

A) BEZ235



B) BGJ-398



C) Temeirolimus

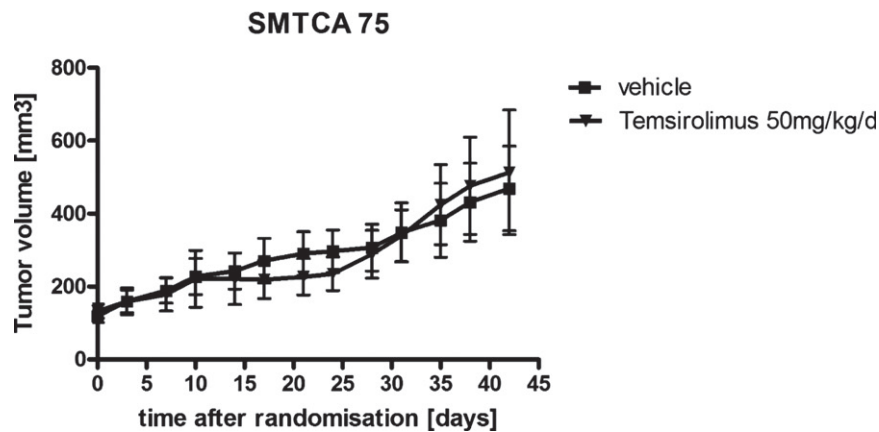


Figure 4. Tumor responses to BEZ235 (A), BGJ-398 (B), and temsirolimus in a PDX model.

(Avatar trials) is most likely limited due to the need for sufficient time to generate *in vivo* study results. Very often, oncologists need to decide much quicker which second-line or third-line therapy the patient has to be switched to. The time required for generating a PDX model, as well as the success rate, directly correlates with tumor aggressiveness, invasiveness, and the quality and quantity of malignant tissue received [20]. Malignant effusions in mRCC are known to be seen only in patients with multiple metastatic disease

[21]. Therefore, PDCs from malignant effusions can be preferred to surgical specimens in PDX establishment.

One may argue that passages and the establishment of a PDX may allow additional DNA alterations to occur within the tumor. In addition, malignant body fluids such as effusions or ascites could only be collected from patients with far-advanced disease, and most of them already acquired clinical resistant to targeted agents. While the mechanisms of resistance to TKIs are not fully understood, it is

generally acknowledged that secondary genetic alterations after repeated exposure to targeted agents may be responsible for the development of resistance [22]. In the present study, the results from the PDC drug test with BEZ235 and BGJ-398 were not reproduced in subsequent PDX model. Further investigations are needed if it may be due to a different tumor microenvironment or secondary mutations. However, we observed the similarity of genomic features between the primary tumor and single cell suspensions from the PDX tumor at passage 5. Still, it also is advantageous having not only PDX models of primary tumors at hand but also models generated from tumors after therapy. It is of increasing interest to pharmaceutical companies getting their hands on models that have arisen from tumor material that has received initial chemotherapy. This way, second-line and third-line therapeutic agents can be developed. Taken together, our study demonstrated that the preclinical model we developed can be a useful preclinical model to interrogate sensitivity to targeted agents based on genomic alterations [9]. Although the results are based on an observation of a single case, the PDC model from malignant effusions of patients was successfully converted to a PDX model. While larger studies are required to further evaluate and confirm the usefulness of the PDC-PDX model, this pilot study suggests that our novel patient-derived preclinical model from malignant effusions represents an important and feasible platform for future cancer research.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.tranon.2017.01.016>.

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