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

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 mechanisms 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 as a xenograft 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.

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...
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 | Asciites | 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 | Asciites | P1 |
| 391 | 53/M   | Sunitinib | Secondary | 13 mo | Asciites | 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 |
| 481 | 51/M   | Pazopanib | 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 | Asciites | 5.00E + 06/ml | P3 |
| 573 | 36/M   | Pazopanib | Intrinsic | 8 mo | Pleural effusion | 3.40E + 07/ml | P3 |
| 574 | 44/M   | Pazopanib | Intrinsic | 13 mo | Pleural effusion | 5.00E + 05/ml | 0 |
| 590 | 42/F   | Sunitinib | Intrinsic | 6 mo | Asciites | 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 | Asciites | 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 in 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, Zenoaq, Japan), transferred into cryovials (Nalgé 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 x 10⁶ 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).
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).

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

In the primary tumor, clear cells are arranged in an alveolar pattern, while granular cells are in a solid pattern. PDX tumor showed similar
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, metastatic lesions showed progression, and large amount of bilateral pleural effusions.

To confirm the similarity of genomic features between the primary tumor and PDX, we compared variant allele frequency results of the therapy that only supportive care was given until death. A subsequent scan after 6 weeks of sunitinib therapy showing disease progression and the development of large amount of bilateral pleural effusions.

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  | 3.754165    | 15        |
| Unknown      | SRC             | Amplification | 0.931351  | 3.834123    | 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 (μM) |
|---------------|----------------------------|-----------|
| 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-0239804) 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       |

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.
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

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**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 μM and 0.79 μM, respectively. (C) The western blot for mTOR and FGFR phosphorylation and targeted downstream pathways. Cells were treated with 1 μM BEZ235 and 1 μM BGJ-398 for 5 days, respectively. Control cell was treated with DMSO.
(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

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

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