

Intra-tumour molecular heterogeneity of clear cell renal cell carcinoma reveals the diversity of the response to targeted therapies using patient-derived xenograft models

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ABSTRACT

Inter- and intra-tumour molecular heterogeneity is increasingly recognized in clear cell renal cell carcinoma (ccRCC). It may partially explain the diversity of responses to targeted therapies and the various clinical outcomes. In this study, a 56-year-old male ccRCC patient with multiple metastases received radical nephrectomy and resection of the metastatic tumour in chest wall. The surgical specimens were implanted into nude mice to establish patient-derived xenograft (PDX) models with KI2367 model derived from the primary tumour and KI2368 model from the metastatic tumour. The two models were treated with Sorafenib, Sunitinib, Axitinib, combined Sorafenib/Sunitinib, or alternating therapy of Sorafenib and Sunitinib. Significant anti-tumour activity was found in KI2367 treated with Sorafenib/Sunitinib monotherapy, combined Sorafenib/Sunitinib, and alternating therapy of Sorafenib/Sunitinib ($P < 0.05$) but not in that treated with Axitinib monotherapy. In contrast, KI2368 was significantly responsive to Sunitinib monotherapy, combined Sorafenib/Sunitinib therapy and alternating therapy of Sorafenib/Sunitinib but not responsive to Sorafenib and Axitinib monotherapy ($P < 0.05$). RNAseq of the two models demonstrated that the expression levels of 1,725 genes including the drug targeted genes of *PDGFA*, *PDGFB* and *PDGFRA* were >5-fold higher in KI2367 than in KI2368 and the expression levels of 994 genes were > 5-fold higher in KI2368 than in KI2367. These results suggest the presence of intra-tumour molecular heterogeneity in this patient. This heterogeneity may influence the response to targeted therapies. Multiple biopsy, liquid biopsy and genomic analysis of intra-tumour molecular heterogeneity may help guide a more precise and effective plan in selecting targeted therapies for ccRCC patients.

INTRODUCTION

Over the past decade, the high-throughput determination of biological molecules such as whole genome

sequencing, transcriptome sequencing and proteomics has increased our knowledge about the molecular mechanism of tumorigenesis. On the other hand, the overall incidence of cancers is gradually increasing [1]. The modern concept

of cancer treatment emphasizes the reduction of cancer mortality and treatment complications. The second generation sequencing technology has revealed multiple genetic changes in tumours, providing the basis for drug development and personalized cancer treatment. The precision medicine for managing cancers is progressive and attractive. The identification of driver mutations that are critical for tumorigenesis remains a challenge. Several malignant tumours have recently achieved personalized cancer treatment in clinical practice, such as anti-HER2 antibody for *HER2*-positive breast cancer, anti-EGFR therapy for *KRAS* wild-type colon cancer, and BRAF inhibitor for *BRAF* mutant melanoma [2–5].

Renal cell carcinoma (RCC) can be divided into several subtypes according to its histology and molecular differentiation [6–8]. In recent decades, the incidence of RCC has been rapidly increasing at 2.5 percent per year. In 2012, there were about 338,000 new cases of kidney cancer worldwide and an estimated 143,000 cases died of kidney cancer, ranking as the 16th most common cause of cancer death [9–11]. The increased incidence of kidney cancer was partly attributed to the wide use of imaging techniques, such as magnetic resonance imaging (MRI), computed tomography (CT) and ultrasound [12]. Surgery is still the preferred treatment to eradicate the tumour, but approximately 30% of patients undergoing nephrectomy for localized RCC develop metastases [9, 10]. Moreover, RCC is insensitive to chemoradiotherapy. The development of targeted drugs has significantly improved the prognosis of metastatic RCC [13–17]. They target the molecules relating to tumour development to inhibit tumour growth, but few patients got complete or long-term response. Genome-wide studies have confirmed significant genetic diversity among RCCs and found several important driver mutations and multiple of passenger changes in RCCs, which partially explain the heterogeneous clinical outcomes of patients with similar histopathological type [18–21]. In addition to inter-tumour heterogeneity, intra-tumour heterogeneity with diverse genetic subclones within a single tumour has been gradually recognized in RCC [22].

Although a large number of molecular changes in hundreds of renal tumours have been obtained by genome-wide analyses, the effects of these changes on the characteristics of the tumour and clinical presentations have not been clearly elucidated [23, 24]. The therapeutic effect and development of targeted therapies for RCC may be hampered by the inter-tumour and intra-tumour heterogeneity. Single tumour-biopsy may not be able to fully reflect the genetic composition of the tumour due to intra-tumour heterogeneity, causing considerable challenges to the development of individualized precise treatment.

Here we investigated the intra-tumour heterogeneity of gene expression and mutation load in primary and metastatic clear cell renal cell carcinoma (ccRCC) and analyzed the diverse responses to targeted therapies using

the patient-derived xenograft (PDX) models. Surgical samples of primary and metastatic ccRCC were collected from a patient. Two PDX models were established to test their responses to targeted therapies. Transcriptome sequencing (RNA-seq) of PDXs was performed to detect the genetic diversity between the primary and metastatic ccRCC.

RESULTS

Patient prognosis

The patient was discharged 6 days after the operation. His general status was well after the operation for one month. Afterwards, the patient began to take Sorafenib (400 mg oral, twice daily). Partial response was obtained for his pulmonary metastasis, brain metastasis progressed [25]. The patient died after the operation for 6 months.

Results of molecular target drugs in treating the kidney carcinoma model of KI2367

The KI2367 PDX model was derived from the primary ccRCC tumour. We treated this model with three monotherapies (Sorafenib, Sunitinib, and Axitinib), Sorafenib and Sunitinib combined therapy, and Sorafenib/Sunitinib alternating therapy. The results showed that there was obvious anti-tumour activity from Sorafenib/Sunitinib monotherapy and from combined and alternating treatment of Sorafenib and Sunitinib, with the $\Delta T/\Delta C\%$ values of -66.91%, -46.57%, -238.03% and -60.24% respectively. However, Axitinib showed no significant anti-tumour activity to KI2367, with the $\Delta T/\Delta C\%$ value of 82.11%. A statistically significant difference was observed between vehicle/Axitinib and other treatment groups ($P < 0.05$). No significant difference or synergistic tumour growth inhibition was observed between the 4 effective treatment groups (Figure 1; Table 1).

Results of molecular target drugs in treating the kidney carcinoma model of KI2368

The KI2368 PDX model was derived from the metastatic ccRCC in left chest wall of the patient. We observed obvious anti-tumour activity from Sunitinib monotherapy and combined and alternating treatments of Sorafenib and Sunitinib, with the $\Delta T/\Delta C\%$ values of 40.09%, 12.25%, and 23.12% respectively. However, there were no significant anti-tumour activities from Sorafenib and Axitinib monotherapy, with the $\Delta T/\Delta C\%$ values of 79.02% and 85.45% respectively. A statistically significant difference was observed between vehicle/Sorafenib/Axitinib and the other treatment groups ($P < 0.05$). Synergistic tumour growth inhibition was observed in the Sorafenib and Sunitinib combined therapy

Table 1: The TV and T/C data of applying molecular target drugs in treating kidney carcinoma model KI2367.

| Treatment | TV ^a day 0 (mm ³) ^b | TV post final treatment (mm ³) | TGI(%) ^c | ΔT/ΔC (%) ^d | P Value ^e |
|--|---|--|---------------------|------------------------|----------------------|
| Group 01, Negative control | 142.3±5.8 | 265.3±33.3 | — | 100 | — |
| Group 02, Sorafenib, 50 mg/kg, Qd*4w, p.o. | 140±13.6 | 67.2±11.5 | 166.91 | -66.91 | 0.002 |
| Group 03, Sunitinib, 60 mg/kg, Qd*4w, p.o. | 144±18.3 | 28.2±17.1 | 146.57 | -46.57 | 0.002 |
| Group 04, Axitinib, 15mg/kg, Qd*4w, p.o. | 143.3±29.3 | 244±39.2 | 17.89 | 82.11 | 0.917 |
| Group 05, Sorafenib, 50 mg/kg, Qd*4w, p.o., Sunitinib, 60 mg/kg, Qd*4w, p.o. | 145.3±28.2 | 79.5±16.2 | 338.03 | -238.03 | 0.001 |
| Group 06, Sunitinib(1,3w), 60 mg/kg, Qd*2w, p.o., Sorafenib(2,4w), 50 mg/kg, Qd*2w, p.o. | 148.7±29.5 | 49±24.9 | 160.24 | -60.24 | 0.002 |

a. Total Volume; b. Mean ± SEM; c. Tumor Growth Inhibition (TGI) = (1-%ΔT/ΔC)*100; d. ΔT/ΔC(%) = (mean(T)-mean(T0))/(mean(C)-mean(C0)) * 100%; e. Compare to Vehicle Control.

groups compared with the monotherapy groups (P<0.05) (Figure 2; Table 2).

RNaseq analysis of KI2367 and KI2368 PDXs

The expression of 53598 genes was examined in KI2367 and KI2368 by RNaseq. We found 1,725 genes with > 5-fold higher expression levels in KI2367 than in KI2368; these genes included drug target-related genes such as *PDGFA*, *PDGFB* and *PDGFRA* (Supplementary Table 1). A total of 994 genes had > 5-fold higher expression levels in KI2368 than in KI2367 (Supplementary Table 2). In addition, 5,539 and 5,827 mRNA changes predicted

to result in protein variants were found in KI2367 and KI2368 respectively, and 4,023 mRNA changes predicted to result in protein variants were found both in KI2367 and KI2368. The variant detection by RNaseq largely depends on the gene expression level, and it is likely that many of the variants are false positives. We also found 20 and 4 in-frame gene fusions in KI2367 and KI2368, respectively, but no common in-frame fusion was detected (Table 3; see Supplementary Figure 1-5 for validation).

PDGFRA was highly expressed in KI2367, but not expressed in KI2368. The expression level of *PDGFRB* was higher in KI2367 than in KI2368 (Table 4). The frameshift insertion in the *HIF1A* gene was observed in

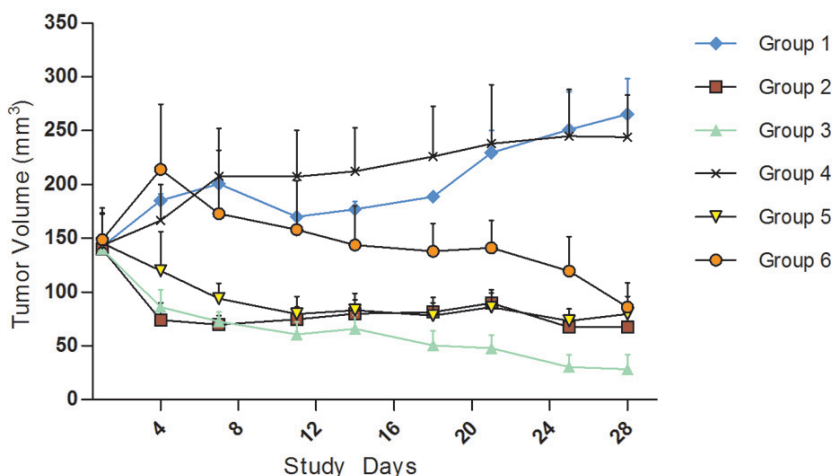


Figure 1: Tumour volume trends for molecular target drugs in treating kidney carcinoma model, KI2367. The result showed obvious anti-tumour activity for Sorafenib/Sunitinib monotherapy, combination and alternating treatment. The tumour volume decreased in all of the above treatment groups following treatment. However, no significant anti-tumour activity with Axitinib monotherapy was seen for the KI2367 model. A significant difference was observed between vehicle/Axitinib and other treatment groups (P<0.05). No significant differences or synergistic tumour growth inhibition was observed between the Sorafenib, Sunitinib, combined therapy and alternating treatment groups. Group 1, Negative Control Group 2, Sorafenib, 50 mg/kg p.o. QD*4 weeks Group 3, Sunitinib, 60 mg/kg p.o. QD*4 weeks, Group 4, Axitinib, 15 mg/kg, p.o. QD*4 weeks, Group 5, Sorafenib 50 mg/kg+sunitinib 60 mg/kg p.o. (QD*4 weeks) Group 6, Sorafenib 50 mg/kg and Sunitinib 60 mg/kg alternated (Weeks 1 and 3, dose with Sunitinib QD, p.o.; Weeks 2 and 4, dose with Sorafenib QD, p.o.).

Table 2: The TV and T/C data of applying molecular target drugs in treating kidney carcinoma model KI2368.

| Treatment | TV ^a day0 (mm ³) ^b | TV post final treatment (mm ³) | TGI(%) ^c | ΔT/ΔC (%) ^d | P Value ^e |
|--|--|--|---------------------|------------------------|----------------------|
| Group 01, Negative control | 140.99±16.78 | 793.89±87.38 | — | 100 | — |
| Group 02, Sorafenib, 50 mg/kg, Qd*4w, p.o. | 140.23±22.0 | 656.33±99.5 | 20.98 | 79.02 | 0.654 |
| Group 03, Sunitinib, 60 mg/kg, Qd*4w, p.o. | 140.37±17.68 | 407.00±51.22 | 59.91 | 40.09 | 0.035 |
| Group 04, Axitinib, 15mg/kg, Qd*4w, p.o. | 140.9±10.69 | 698.67±15.68 | 14.55 | 85.45 | 0.912 |
| Group 05, Sorafenib, 50 mg/kg, Qd*4w, p.o., Sunitinib, 60 mg/kg, Qd*4w, p.o. | 140.89±11.79 | 220.67±18.84 | 87.75 | 12.25 | 0.001 |
| Group 06, Sunitinib(1,3w), 60 mg/kg, Qd*2w, p.o., Sorafenib(2,4w), 50 mg/kg, Qd*2w, p.o. | 139.43±17.65 | 290.33±20.17 | 76.88 | 23.12 | 0.001 |

a. Total Volume; b. Mean ± SEM; c. Tumor Growth Inhibition (TGI) = (1-%ΔT/ΔC)*100; d. ΔT/ΔC(%) = (mean(T)-mean(T0))/(mean(C)-mean(C0)) * 100%; e. Compare to Vehicle Control.

KI2368, which may impact the expression of *PDGFRB* and other genes (Table 5; Supplementary Figure 6). Moreover, a frameshift mutation in *RICTOR* that may affect HIF1A function and subsequently *PDGFR* expression was found in KI2368, and a frameshift mutation in *VEGFB* was detected in KI2368 but not in KI2367 (Table 5; Supplementary Figure 7).

DISCUSSION

In recent years, the incidence of RCC has rapidly increased. On the other hand, adjuvant therapies for RCC have also been greatly improved and several randomized trials for RCC have been carried out. The early studies included trials of chemotherapy, hormonotherapy, immunotherapy such as interferon alpha and interleukin-2

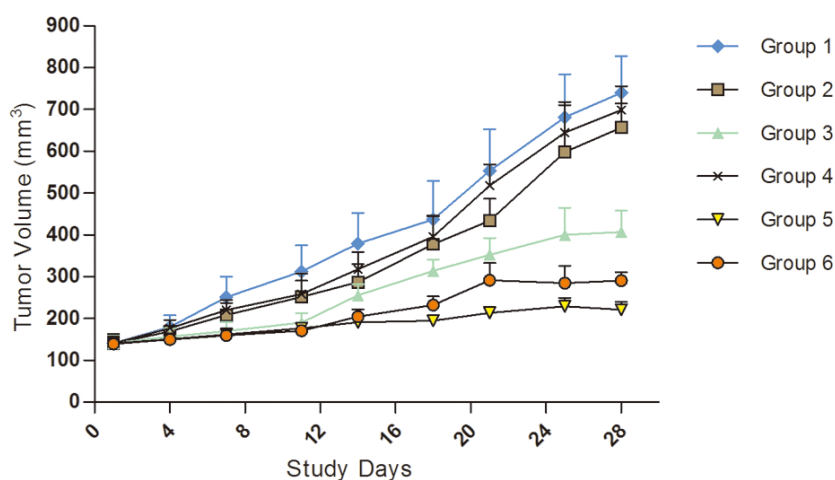


Figure 2: Tumour volume trends for molecular target drugs in treating kidney carcinoma model, KI2368. The result showed obvious anti-tumour activity for Sunitinib monotherapy, Sorafenib and Sunitinib combination or alternating treatment. However, no significant anti-tumour activity for Sorafenib or Axitinib monotherapy was seen in the model. A significant difference was observed between vehicle/Axitinib/Sorafenib and other treatment groups ($P < 0.05$). Synergistic tumour growth inhibition was observed in the Sorafenib and Sunitinib combination therapy groups compared with the drug monotherapy groups ($P < 0.05$). Group 1, Negative Control Group 2, Sorafenib, 50 mg/kg p.o. QD*4 weeks Group 3, Sunitinib, 60 mg/kg p.o. QD*4 weeks, Group 4, Axitinib, 15 mg/kg, p.o. QD*4 weeks, Group 5, Sorafenib 50 mg/kg+sunitinib 60 mg/kg p.o. (QD*4 weeks) Group 6, Sorafenib 50 mg/kg and Sunitinib 60 mg/kg alternated (Weeks 1 and 3, dose with Sunitinib QD, p.o.; Weeks 2 and 4, dose with Sorafenib QD, p.o.).

Table 3: In-frame gene fusions in KI2367 and KI2368.

| Sample | Up-gene | Up- chr | Up- Genome- pos | Dw-gene | Dw- chr | Dw- strand | Dw- Genome- pos |
|--------|----------|---------|-----------------------|---------|---------|------------|-----------------------|
| KI2367 | ACSS1 | chr20 | 24988402 | APMAP | chr20 | - | 24964655 |
| KI2367 | ACSS1 | chr20 | 24988404 | APMAP | chr20 | - | 24964654 |
| KI2367 | ALDH1A1 | chr9 | 75524545 | GAPDH | chr12 | + | 6647073 |
| KI2367 | ASL | chr7 | 65557650 | CRCP | chr7 | + | 65592691 |
| KI2367 | CFI | chr4 | 110687748 | ALDOA | chr16 | + | 30081492 |
| KI2367 | CLCF1 | chr11 | 67140995 | POLD4 | chr11 | - | 67120548 |
| KI2367 | CP | chr3 | 148904356 | ALDOA | chr16 | + | 30080934 |
| KI2367 | EEF1A1 | chr6 | 74228900 | RPL35A | chr3 | + | 197678056 |
| KI2367 | FABP1 | chr2 | 88425695 | COL1A2 | chr7 | + | 94045741 |
| KI2367 | FGG | chr4 | 155527982 | GAPDH | chr12 | + | 6646833 |
| KI2367 | FTL | chr19 | 49468781 | APOL1 | chr22 | + | 36661638 |
| KI2367 | IMMP2L | chr7 | 111161369 | DOCK4 | chr7 | - | 111387499 |
| KI2367 | P4HB | chr17 | 79803483 | CALR | chr19 | + | 13050364 |
| KI2367 | P4HB | chr17 | 79804351 | CALR | chr19 | + | 13054623 |
| KI2367 | PKM | chr15 | 72501143 | FTH1 | chr11 | - | 61732905 |
| KI2367 | PKM | chr15 | 72511365 | GAPDH | chr12 | + | 6647094 |
| KI2367 | SDHA | chr5 | 254621 | CLPTM1L | chr5 | - | 1323026 |
| KI2367 | SERPINA1 | chr14 | 94844887 | CP | chr3 | - | 148930369 |
| KI2367 | SERPINA1 | chr14 | 94844899 | EEF1A1 | chr6 | - | 74228848 |
| KI2367 | VIM | chr10 | 17271889 | ENO1 | chr1 | - | 8930567 |
| KI2368 | C3 | chr19 | 6712322 | SPP1 | chr4 | + | 88901222 |
| KI2368 | HDAC8 | chrX | 71571583 | CITED1 | chrX | - | 71522784 |
| KI2368 | SDHA | chr5 | 254621 | CLPTM1L | chr5 | - | 1323026 |
| KI2368 | SLC28A1 | chr15 | 85467341 | PDE8A | chr15 | + | 85607601 |

[26–32], and autologous vaccination strategies [33–36]. These were the alternatives for treatment at the time but were less effective for metastatic RCC, and the overall outcomes were unsatisfactory [37, 38]. With the development of cancer therapy, we are now in the era of second-generation adjuvant studies [39]. Targeted therapies inhibit the tumour progression by interfering with tumour-associated signaling molecules involved in cancer growth, invasion and metastasis [13, 40-45].

The targeted drugs of tyrosine kinase inhibitors (TKIs) and anti-VEGF antibodies, such as Sorafenib, Sunitinib, Pazopanib and Axitinib are now recommended as the first- or second-line treatment for RCC. Sunitinib has been approved by the United States Food and Drug Administration (FDA) and European Medicines Agency as a first-line treatment for RCC [46]. The mammalian target

of kanamycin (mTOR) inhibitor Temsirolimus has been approved for the first-line treatment of RCC patients with poor-prognosis, and Everolimus has been recommended for patients with advanced RCC or unresponsive to anti-VEGF therapies [42]. The targeted therapies have obtained better progression-free survival compared with either placebo, IFN or IL-2 treatment [13, 40-45].

Unfortunately, it is difficult to achieve complete and long-term tumour control with targeted therapy. Tumours may adapt to chronic drug use and escape from drug-mediated growth control [47]. Only a portion of patients responded well to the targeted therapies. With the recognition of more key molecules in the tumourigenic signalling pathway of RCC including VEGF, PDGF and EGF, new agents against these targets have been developed [48]. New targeted drugs such as cabozantinib

Table 4: Gene expression of drug targets and their ligands.

| | KI2367 | KI2368 |
|--------|----------|----------|
| VEGFA | 8.661 | 10.360 |
| VEGFC | 3.053 | 1.294 |
| VEGFB | 6.072 | 5.821 |
| PDGFB | 1.818 | -0.118 |
| PDGFRB | -0.675 | -4.659 |
| PDGFRA | 6.117 | -3.478 |
| PDGFC | 2.693 | 3.859 |
| PDGFD | -1.443 | -10.483 |
| PDGFA | 4.780 | -1.381 |
| KDR | -5.978 | -6.862 |
| FLT4 | -4.530 | -5.238 |
| FLT1 | -3.897 | -5.317 |
| FLT3 | -5.274 | -8.522 |
| KIT | -0.95918 | 0.841965 |

Expression in \log_2 (FPKM).

Table 5: Gene variants found in MTOR pathway.

| Gene | KI2367-P9 | KI2368-P7 |
|--------|-----------|-----------|
| RPTOR | H126Y | not found |
| PIK3R3 | not found | N329K |
| RICTOR | not found | N1473fs |
| HIF1A | not found | S190fs |
| VEGFB | not found | P126fs |

*Variants found by RNA-seq.

and lenvatinib have recently been approved for treating advanced renal cell carcinoma [49, 50]. Sequential therapy is considered to be an innovative option that provides maximal efficacy with a minimum risk of therapeutic failure [51, 52]. The agent that is still useful after resistance to a TKI-based regimen remains unclear.

Another hypothesis for the poor long-term effect of targeted therapies is the heterogeneity of RCC. The high-throughput sequencing have identified significant genetic diversity and screened out several important driver mutations and multiple of passenger changes in RCC [53, 54]. The diversity of drug response, resistance to targeted therapies and varied clinical outcomes may be partly attributed to the molecular heterogeneity of RCC.

Inter- and intra-tumour molecular heterogeneity are intriguing and important characteristics of tumours. Intra-tumour heterogeneity with diverse genetic subclones in

a single tumour has been gradually recognized in RCC [22]. Nowell reported that subclones have the same origin and share common genetic changes; but each subclone also harbours unique somatic mutations [55]. Gerlinger *et al.* applied next-generation sequencing to characterize intra-tumour heterogeneity in primary and metastatic ccRCC [23, 24]. Genomics analyses from multiple regions of a primary tumour identified a common clonal origin. Somatic mutation and allele imbalance in different subclones showed considerable variability. Of note, the alteration of phosphoinositide 3-kinase (PI3K)–mTOR appeared in different subclones of RCC, possibly relating to resistance to mTOR inhibitors. Gerlinger *et al.* also compared the genetic alterations between primary and metastatic RCCs, and demonstrated that metastatic RCC had genetic features different from those of primary tumour [23, 24]. Therefore, metastatic tumours may originate from rare primary tumour clones, consistent with the findings in other malignancies [56–59].

Our results also indicate that molecular heterogeneity especially the intra-tumour heterogeneity is an important factor for the diversity of response to targeted therapies. This patient had multiple metastases in brain, lung, and skin and underwent right radical nephrectomy. After the operation, the patient received Sorafenib therapy. During the follow-up period, metastases in lung became stable but metastases in brain progressed. Finally, the patient died of brain metastases. Subsequently, Sorafenib had a diverse effect on metastases at different locations in the same patient. The PDX models of KI2367 and KI2368 showed different responses to Sorafenib monotherapy (in group 2), also indicating that metastases in different locations may have different molecular changes that may relate to the diverse responses to targeted therapies. Consequently, metastatic tumours could have diverse responses and some may become resistant to the targeted therapies[60].

We observed that KI2367 and KI2368 had different response to the TKIs, indicating that primary and metastatic tumours have different genomic profiles. RNAseq analysis reveals that PDGFRA is highly expressed in KI2367 but not expressed in KI2368. The expression of PDGFRB is higher in KI2367 than in KI2368. Conversely, KIT is expressed in KI2368 but not expressed in KI2367. HIF1 is known to induce the transcription of many angiogenesis-related genes. The frameshift insertion in HIF1A may impact on the expression of PDGFRB and other genes in KI2368. The frameshift mutation of RICTOR in KI2368 may also affect the function of HIF1A and subsequently PDGFR expression. It is possible that the difference in PDGFR expression affects the drug responses in KI2367 and KI2368. We also found a VEGFB frameshift mutation in KI2368 but not in KI2367. VEGFB is dispensable for blood vessel growth but is critical for their survival of blood vessels. The VEGFB mutation may reduce the effectiveness of drugs such as Axitinib on

VEGF receptors. Sorafenib, Sunitinib and Axitinib target the PDGFR and VEGFR. Axitinib is reported to inhibit KIT and the BCR-ABL fusion protein. Sorafenib also inhibits RAF family kinases, and Sunitinib inhibits KIT,

RET, CD114 and CD135. It is likely that the activation of kinases other than PDGFR and VEGFR influences the different efficacy of the three drugs. In KI2368, we observed that the anti-tumour activity of combined or

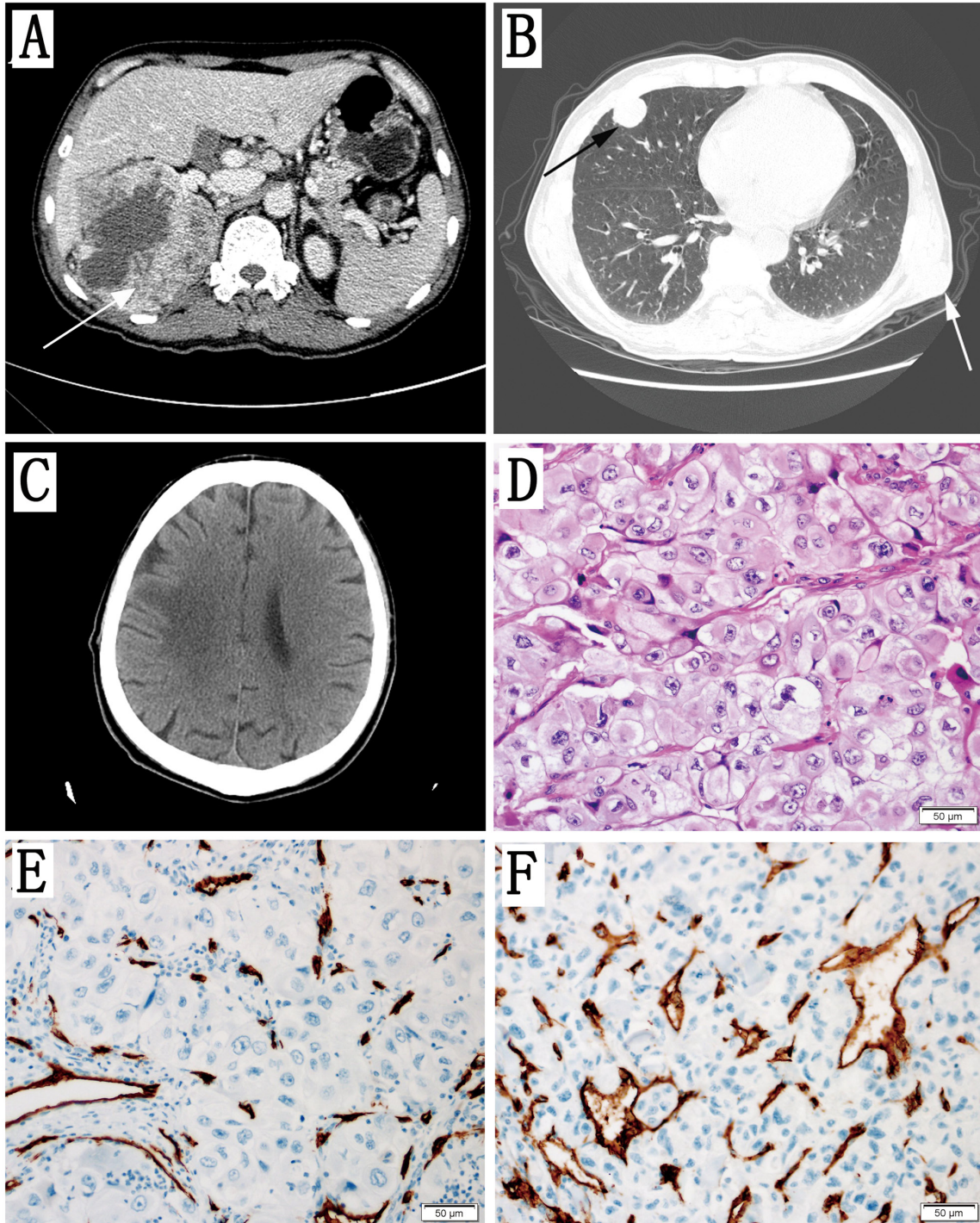


Figure 3: Clinical data of the Patient. (A) Axial contrast-enhanced CT scan showing a large mass in the right kidney (arrow). (B) Axial noncontrast CT scan showing multiple metastases in the lung (black arrow) and one metastasis in the chest wall (white arrow). (C) Axial noncontrast CT scan showing brain metastases. (D) The pathology results reported that both primary the tumour of the kidney and metastatic tumour of the chest wall were ccRCC. The Fuhrman grade was 4. (E) Vascular density stained with CD34 in the primary renal tumour. (F) Vascular density stained with CD34 in the subcutaneous metastatic tumour of chest wall.

alternating therapies was stronger than monotherapy. The combined or alternating therapies can inhibit more targets and block the tumour growth.

Taken together, these findings confirm again that there is intra-tumour molecular heterogeneity in ccRCC. The intra-tumour heterogeneity could influence the clinical outcome of targeted therapies and explain the diverse drug response among metastatic tumours at different sites from a ccRCC patient. Therefore, intra-tumour heterogeneity may lead to tumour evolution and becomes a huge challenge to the development of personalized therapy for RCC. A single tumour-biopsy may not be able to accurately reflect the features of a tumour. Multiple biopsy, liquid biopsy and genomic analysis could further identify intra-tumour molecular heterogeneity. Multiple biopsies in patients with multiple metastases are not clinically feasible. However, with the development of minimally invasive technology and imaging technology, it will be possible to collect multiple biopsies. Furthermore, liquid biopsy is also a very popular method and may be used in the future. Thereafter, precise and comprehensive results could help us more effectively plan targeted therapies. Further studies are needed to understand the cause of intra-tumour heterogeneity in ccRCC to guide the clinical treatment of patients.

MATERIALS AND METHODS

Mice

Female BALB/c nude mice (4-6 weeks, 16-20g, SPF degree) were supplied by Beijing FuKang Bioscience (Beijing, China, Animal Certificate No.: 11401300025891). Procedures related to animal handling, care, and treatment in this study were performed according to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of Crown Bioscience following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Collection of tumour specimens

The patient was diagnosed with ccRCC at the Department of Urological Oncology of Peking University Cancer Hospital. All protocols in this study were reviewed and approved by the Institutional Medical Ethics Committee, and written informed consent was obtained from the patient. After operation the specimens were examined by pathologists, and some of the samples were used for laboratory study. The specimens for PDX models were preserved in HTK solution (CUSTODIOT, XISAIER Technology, Beijing, China) at 4°C and treated within 6 hours..

Preparation of PDX of ccRCC

Methods and parameters regarding PDX and tumour inhibition assay using the PDX have been described

previously [61, 62]. The specimens were sliced into approximately 3×3×3 mm³ fragments (n=20), and 5 mice were implanted with 4 blocks each. The dorsal surface of the mouse was prepped with betadine solution, and the specimens were subcutaneously inoculated into the flanks of mice with a 16-G needle. The tumour growth was monitored twice weekly using a calliper. The established tumour models (passage 0 or P0) were serially re-engrafted to maintain the tumours in vivo. These subsequent passages were called P1, P2, P3, etc. When the tumours reached 500–700 mm³ (1/2 length x width²), they were harvested for the next round of engraftment for serial passage or conducting studies of efficacy and molecular analyses.

Clinical data of the patient

This 56 years old male patient had a right renal tumour (Figure 3A) with a subcutaneous soft tumour in his left chest wall (Figure 3B) and multiple pulmonary and brain metastases (Figure 3, 3B and 3C). An open radical nephrectomy was performed using the subcostal flank approach, and the subcutaneous metastatic tumour in left chest wall was resected as well. ccRCC metastases were found in the 2 lymph nodes of the 6 resected kidney pedicle lymph nodes. The peritoneum was also invaded by the tumour. The adrenal was normal. The pathological type was ccRCC (Figure 3D). The Fuhrman grade was 4 and the pathological stage was pT4N1M1. Immunohistochemical staining of vascular density (CD34) is shown in Figure 3 (3E and 3F).

Treatment of PDX lines with targeted therapies

Treatment for mice was initiated when the tumours from 2 ccRCC PDX models reached 100 mm³ to 150 mm³. The established PDX models were KI2367 from the primary tumour and KI2368 from the subcutaneous metastatic tumour of the chest wall. Each PDX model was divided into 6 experimental groups with 4 mice in every group. The control group (Group 1) was treated orally with vehicle daily (QD)*4 weeks, and the treatment groups were treated with one of following dosing regimens: Sorafenib (Group 2, 50 mg/kg p.o. QD*4 weeks, Dalian Meilun Biotech Co., Ltd), Sunitinib (Group 3, 60 mg/kg p.o. QD*4 weeks, Dalian Meilun Biotech Co., Ltd), Axitinib (Group 4, 15 mg/kg, p.o. QD*4 weeks, Dalian Meilun Biotech Co., Ltd), Sorafenib 50 mg/kg+sunitinib 60 mg/kg p.o. (Group 5, QD*4 weeks) and Sorafenib 50 mg/kg and Sunitinib 60 mg/kg in alternation (Group 6, Weeks 1 and 3, dose with Sunitinib QD, p.o.; Weeks 2 and 4, dose with Sorafenib QD, p.o.). The %ΔT/ΔC value was calculated for assessing the tumour response to the treatment. (%ΔT/ΔC= (mean(T)-mean(T0))/(mean(C)-mean(C0)) *100%, T- Treatment group value, T0 - Treatment group initial value, C - control group

value, and C0 - control group initial value). The tumour growth inhibition (TGI) = $(1 - \frac{\Delta T}{\Delta C}) * 100$.

The mice were kept in individually ventilated cage (IVC) systems at constant temperature and humidity, with 4 animals in each cage. The data for tumour growth and normal behaviour including mobility, visual estimation of food and water consumption, body weight gain/loss measured twice weekly, eye/hair matting and any other abnormal effects, were collected every 3 to 4 days. Tumour sizes were measured every 3 to 4 days using the formula: $V = 0.5 \text{ length} \times \text{width}^2$. Samples of mice treated with vehicle were stored for future analysis. Any remaining tumour was re-implanted for maintenance of a PDX model.

Genomic analysis of PDXs

Snap frozen PDX tumour samples were used to extract RNA for transcriptome sequencing (RNA-seq). The purity and integrity were checked with an Agilent Bioanalyzer prior to RNA sequencing. Only RNA samples with RIN >7, 28S/18S >1 and mouse content <10% were used for library construction and sequencing. The sequencing was performed at PE125 on an Illumina HiSeq2500 platform (KI2367) and PE100 on an Illumina HiSeq2500 platform (KI2368) by certified Illumina service providers. The RNAseq raw data were first cleaned by removing reads that were preferentially mapped to a mouse genome (UCSC MM9). Transcript expression was estimated by MMSEQ [63] and represented by $\log_2(\text{FPKM})$. SNP and INDELs were detected by STAR [64] mapping software and GATK [64] variant discovery toolkit, and gene fusions were detected by SOAP fuse [65] and deFuse [66].

Statistical analysis and software

All statistical analyses were performed using SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). Two sample t-tests were used to check the equality of means between two groups. One-way ANOVA was used to check the equality of means among 3 and more groups. Differences were considered statistically significant at $P < 0.05$.

Author's contributions

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CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

REFERENCES

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2015. *CA Cancer J Clin.* 2015; 65: 5-29.
2. Joensuu H, Bono P, Kataja V, Alanko T, Kokko R, Asola R, Utraiainen T, Turpeenniemi-Hujanen T, Jyrkkio S, Moykkynen K, Helle L, Ingalsuo S, Pajunen M, et al. Fluorouracil, epirubicin, and cyclophosphamide with either docetaxel or vinorelbine, with or without trastuzumab, as adjuvant treatments of breast cancer: final results of the FinHer Trial. *J Clin Oncol.* 2009; 27: 5685-5692.
3. Spielmann M, Roche H, Delozier T, Canon JL, Romieu G, Bourgeois H, Extra JM, Serin D, Kerbrat P, Machiels JP, Lortholary A, Orfeuvre H, Campone M, et al. Trastuzumab for patients with axillary-node-positive breast cancer: results of the FNCLCC-PACS 04 trial. *J Clin Oncol.* 2009; 27: 6129-6134.
4. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD, Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. *J Clin Oncol.* 2008; 26: 1626-1634.
5. Chapman PB, Hauschild A, Robert C, Haanen JB, Ascierto P, Larkin J, Dummer R, Garbe C, Testori A, Maio M, Hogg D, Lorigan P, Lebbe C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. *N Engl J Med.* 2011; 364: 2507-2516.
6. Dalgliesh GL, Furge K, Greenman C, Chen L, Bignell G, Butler A, Davies H, Edkins S, Hardy C, Latimer C, Teague J, Andrews J, Barthorpe S, et al. Systematic sequencing of renal carcinoma reveals inactivation of histone modifying genes. *Nature.* 2010; 463: 360-363.
7. Guo G, Gui Y, Gao S, Tang A, Hu X, Huang Y, Jia W, Li Z, He M, Sun L, Song P, Sun X, Zhao X, et al. Frequent mutations of genes encoding ubiquitin-mediated proteolysis pathway components in clear cell renal cell carcinoma. *Nat Genet.* 2011; 44: 17-19.
8. Sato Y, Yoshizato T, Shiraishi Y, Maekawa S, Okuno Y, Kamura T, Shimamura T, Sato-Otsubo A, Nagae G, Suzuki

- H, Nagata Y, Yoshida K, Kon A, et al. Integrated molecular analysis of clear-cell renal cell carcinoma. *Nat Genet.* 2013; 45: 860-867.
9. Frank I, Blute ML, Leibovich BC, Cheville JC, Lohse CM, Zincke H. Independent validation of the 2002 American Joint Committee on cancer primary tumour classification for renal cell carcinoma using a large, single institution cohort. *J Urol.* 2005; 173: 1889-1892.
 10. Leibovich BC, Blute ML, Cheville JC, Lohse CM, Frank I, Kwon ED, Weaver AL, Parker AS, Zincke H. Prediction of progression after radical nephrectomy for patients with clear cell renal cell carcinoma: a stratification tool for prospective clinical trials. *Cancer.* 2003; 97: 1663-1671.
 11. Znaor A, Lortet-Tieulent J, Laversanne M, Jemal A, Bray F. International variations and trends in renal cell carcinoma incidence and mortality. *Eur Urol.* 2015; 67: 519-530.
 12. Kay FU, Pedrosa I. Imaging of Solid Renal Masses. *Radiol Clin North Am.* 2017; 55: 243-258.
 13. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, Negrier S, Chevreau C, Solska E, Desai AA, Rolland F, Demkow T, Hutson TE, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. *N Engl J Med.* 2007; 356: 125-134.
 14. Pal SK, Haas NB. Adjuvant therapy for renal cell carcinoma: past, present, and future. *Oncologist.* 2014; 19: 851-859.
 15. Ravaud A, Motzer RJ, Pandha HS, George DJ, Pantuck AJ, Patel A, Chang YH, Escudier B, Donskov F, Magheli A, Carteni G, Laguerre B, Tomczak P, et al. Adjuvant Sunitinib in High-Risk Renal-Cell Carcinoma after Nephrectomy. *N Engl J Med.* 2016; 375: 2246-2254.
 16. Ravaud A, Motzer RJ, Pandha HS, Staehler M, George D, Pantuck AJ, Patel A, Chang Y, Escudier B, Donskov F, Magheli A, Carteni G, Laguerre B, et al. Phase III trial of sunitinib (SU) vs placebo (PBO) as adjuvant treatment for high-risk renal cell carcinoma (RCC) after nephrectomy (S-TRAC). *Annals of Oncology.* 2016; 27: 1-36.
 17. Haas NB, Manola J, Uzzo RG, Atkins MB, Wilding G, Pins M, Jewett MAS, Kane CJ, Cella D, Wagner LI, Coomes B, Wood CG, Dutcher JP, et al. Initial results from ASSURE (E2805): Adjuvant sorafenib or sunitinib for unfavorable renal carcinoma, an ECOG-ACRIN-led, NCTN phase III trial. *J Clin Oncol.* 2015; 33: 403.
 18. Davis CF, Ricketts CJ, Wang M, Yang L, Cherniack AD, Shen H, Buhay C, Kang H, Kim SC, Fahey CC, Hacker KE, Bhanot G, Gordenin DA, et al. The somatic genomic landscape of chromophobe renal cell carcinoma. *Cancer Cell.* 2014; 26: 319-330.
 19. Durinck S, Stawiski EW, Pavia-Jimenez A, Modrusan Z, Kapur P, Jaiswal BS, Zhang N, Toffessi-Tcheuyap V, Nguyen TT, Pahuja KB, Chen YJ, Saleem S, Chaudhuri S, et al. Spectrum of diverse genomic alterations define non-clear cell renal carcinoma subtypes. *Nat Genet.* 2015; 47: 13-21.
 20. Scelo G, Riazalhosseini Y, Greger L, Letourneau L, Gonzalez-Porta M, Wozniak MB, Bourgey M, Harnden P, Egevad L, Jackson SM, Karimzadeh M, Arseneault M, Lepage P, et al. Variation in genomic landscape of clear cell renal cell carcinoma across Europe. *Nat Commun.* 2014; 5: 5135.
 21. Varela I, Tarpey P, Raine K, Huang D, Ong CK, Stephens P, Davies H, Jones D, Lin ML, Teague J, Bignell G, Butler A, Cho J, et al. Exome sequencing identifies frequent mutation of the SWI/SNF complex gene PBRM1 in renal carcinoma. *Nature.* 2011; 469: 539-542.
 22. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. *Nature.* 2013; 501: 338-345.
 23. Gerlinger M, Horswell S, Larkin J, Rowan AJ, Salm MP, Varela I, Fisher R, McGranahan N, Matthews N, Santos CR, Martinez P, Phillimore B, Begum S, et al. Genomic architecture and evolution of clear cell renal cell carcinomas defined by multiregion sequencing. *Nat Genet.* 2014; 46: 225-233.
 24. Gerlinger M, Rowan AJ, Horswell S, Larkin J, Endesfelder D, Gronroos E, Martinez P, Matthews N, Stewart A, Tarpey P, Varela I, Phillimore B, Begum S, et al. Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *N Engl J Med.* 2012; 366: 883-892.
 25. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, Dancey J, Arbuck S, Gwyther S, Mooney M, Rubinstein L, Shankar L, Dodd L, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). *Eur J Cancer.* 2009; 45: 228-247.
 26. Pizzocaro G, Piva L, Di Fronzo G, Giongo A, Cozzoli A, Dormia E, Minervini S, Zanollo A, Fontanella U, Longo G, Maggioni A. Adjuvant medroxyprogesterone acetate to radical nephrectomy in renal cancer: 5-year results of a prospective randomized study. *J Urol.* 1987; 138: 1379-1381.
 27. Yagoda A, Abi-Rached B, Petrylak D. Chemotherapy for advanced renal-cell carcinoma: 1983-1993. *Semin Oncol.* 1995; 22: 42-60.
 28. Fyfe G, Fisher RI, Rosenberg SA, Sznol M, Parkinson DR, Louie AC. Results of treatment of 255 patients with metastatic renal cell carcinoma who received high-dose recombinant interleukin-2 therapy. *J Clin Oncol.* 1995; 13: 688-696.
 29. Gore ME, Griffin CL, Hancock B, Patel PM, Pyle L, Aitchison M, James N, Oliver RT, Mardiak J, Hussain T, Sylvester R, Parmar MK, Royston P, et al. Interferon alfa-2a versus combination therapy with interferon alfa-2a, interleukin-2, and fluorouracil in patients with untreated metastatic renal cell carcinoma (MRC RE04/EORTC GU 30012): an open-label randomised trial. *Lancet.* 2010; 375: 641-648.
 30. McDermott DF, Regan MM, Clark JI, Flaherty LE, Weiss GR, Logan TF, Kirkwood JM, Gordon MS, Sosman JA, Ernstoff MS, Tretter CP, Urba WJ, Smith JW, et al.

- Randomized phase III trial of high-dose interleukin-2 versus subcutaneous interleukin-2 and interferon in patients with metastatic renal cell carcinoma. *J Clin Oncol.* 2005; 23: 133-141.
31. Negrier S, Perol D, Ravaud A, Chevreau C, Bay JO, Delva R, Sevin E, Caty A, Escudier B. Medroxyprogesterone, interferon alfa-2a, interleukin 2, or combination of both cytokines in patients with metastatic renal carcinoma of intermediate prognosis: results of a randomized controlled trial. *Cancer.* 2007; 110: 2468-2477.
 32. Pyrhonen S, Salminen E, Ruutu M, Lehtonen T, Nurmi M, Tammela T, Juusela H, Rintala E, Hietanen P, Kellokumpu-Lehtinen PL. Prospective randomized trial of interferon alfa-2a plus vinblastine versus vinblastine alone in patients with advanced renal cell cancer. *J Clin Oncol.* 1999; 17: 2859-2867.
 33. Galligioni E, Quaia M, Merlo A, Carbone A, Spada A, Favaro D, Santarosa M, Sacco C, Talamini R. Adjuvant immunotherapy treatment of renal carcinoma patients with autologous tumor cells and bacillus Calmette-Guerin: five-year results of a prospective randomized study. *Cancer.* 1996; 77: 2560-2566.
 34. Jocham D, Richter A, Hoffmann L, Iwig K, Fahlenkamp D, Zakrzewski G, Schmitt E, Dannenberg T, Lehmacher W, von Wietersheim J, Doehn C. Adjuvant autologous renal tumour cell vaccine and risk of tumour progression in patients with renal-cell carcinoma after radical nephrectomy: phase III, randomised controlled trial. *Lancet.* 2004; 363: 594-599.
 35. Repmann R, Goldschmidt AJ, Richter A. Adjuvant therapy of renal cell carcinoma patients with an autologous tumor cell lysate vaccine: a 5-year follow-up analysis. *Anticancer Res.* 2003; 23: 969-974.
 36. Repmann R, Wagner S, Richter A. Adjuvant therapy of renal cell carcinoma with active-specific-immunotherapy (ASI) using autologous tumor vaccine. *Anticancer Res.* 1997; 17: 2879-2882.
 37. Stillebroer AB, Mulders PF, Boerman OC, Oyen WJ, Oosterwijk E. Carbonic anhydrase IX in renal cell carcinoma: implications for prognosis, diagnosis, and therapy. *Eur Urol.* 2010; 58: 75-83.
 38. Surfus JE, Hank JA, Oosterwijk E, Welt S, Lindstrom MJ, Albertini MR, Schiller JH, Sondel PM. Anti-renal-cell carcinoma chimeric antibody G250 facilitates antibody-dependent cellular cytotoxicity with in vitro and in vivo interleukin-2-activated effectors. *J Immunother Emphasis Tumor Immunol.* 1996; 19: 184-191.
 39. Powles T, Chowdhury S, Jones R, Mantle M, Nathan P, Bex A, Lim L, Hutson T. Sunitinib and other targeted therapies for renal cell carcinoma. *Br J Cancer.* 2011; 104: 741-745.
 40. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Staehler M, Negrier S, Chevreau C, Desai AA, Rolland F, Demkow T, Hutson TE, Gore M, et al. Sorafenib for treatment of renal cell carcinoma: Final efficacy and safety results of the phase III treatment approaches in renal cancer global evaluation trial. *J Clin Oncol.* 2009; 27: 3312-3318.
 41. Escudier B, Pluzanska A, Koralewski P, Ravaud A, Bracarda S, Szczylik C, Chevreau C, Filipek M, Melichar B, Bajetta E, Gorbunova V, Bay JO, Bodrogi I, et al. Bevacizumab plus interferon alfa-2a for treatment of metastatic renal cell carcinoma: a randomised, double-blind phase III trial. *Lancet.* 2007; 370: 2103-2111.
 42. Motzer RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grunwald V, Thompson JA, Figlin RA, Hollaender N, Kay A, Ravaud A. Phase 3 trial of everolimus for metastatic renal cell carcinoma : final results and analysis of prognostic factors. *Cancer.* 2010; 116: 4256-4265.
 43. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Oudard S, Negrier S, Szczylik C, Pili R, Bjarnason GA, Garcia-del-Muro X, Sosman JA, Solska E, et al. Overall survival and updated results for sunitinib compared with interferon alfa in patients with metastatic renal cell carcinoma. *J Clin Oncol.* 2009; 27: 3584-3590.
 44. Rini BI, Escudier B, Tomczak P, Kaprin A, Szczylik C, Hutson TE, Michaelson MD, Gorbunova VA, Gore ME, Rusakov IG, Negrier S, Ou YC, Castellano D, et al. Comparative effectiveness of axitinib versus sorafenib in advanced renal cell carcinoma (AXIS): a randomised phase 3 trial. *Lancet.* 2011; 378: 1931-1939.
 45. Rini BI, Halabi S, Rosenberg JE, Stadler WM, Vaena DA, Ou SS, Archer L, Atkins JN, Picus J, Czaykowski P, Dutcher J, Small EJ. Bevacizumab plus interferon alfa compared with interferon alfa monotherapy in patients with metastatic renal cell carcinoma: CALGB 90206. *J Clin Oncol.* 2008; 26: 5422-5428.
 46. Sidana A, Srinivasan R. Therapeutic Strategies for Hereditary Kidney Cancer. *Curr Oncol Rep.* 2016; 18: 50.
 47. Juengel E, Kim D, Makarevic J, Reiter M, Tsaour I, Bartsch G, Haferkamp A, Blaheta RA. Molecular analysis of sunitinib resistant renal cell carcinoma cells after sequential treatment with RAD001 (everolimus) or sorafenib. *J Cell Mol Med.* 2015; 19: 430-441.
 48. Park K, Lee JL, Park I, Park S, Ahn Y, Ahn JH, Ahn S, Song C, Hong JH, Kim CS, Ahn H. Comparative efficacy of vascular endothelial growth factor (VEGF) tyrosine kinase inhibitor (TKI) and mammalian target of rapamycin (mTOR) inhibitor as second-line therapy in patients with metastatic renal cell carcinoma after the failure of first-line VEGF TKI. *Med Oncol.* 2012; 29: 3291-3297.
 49. Singh H, Brave M, Beaver JA, Cheng J, Tang S, Zahalka E, Palmby TR, Venugopal R, Song P, Liu Q, Liu C, Yu J, Chen XH, et al. U.S. Food and Drug Administration Approval: Cabozantinib for the Treatment of Advanced Renal Cell Carcinoma. *Clin Cancer Res.* 2017; 23: 330-335.
 50. Glen H. Lenvatinib therapy for the treatment of patients with advanced renal cell carcinoma. *Future Oncol.* 2016; 12: 2195-2204.
 51. Mulders P. Vascular endothelial growth factor and mTOR pathways in renal cell carcinoma: differences and synergies of two targeted mechanisms. *BJU Int.* 2009; 104: 1585-1589.

52. Iacovelli R, Palazzo A, Trenta P, Mezi S, Pellegrino D, Naso G, Cortesi E. Management of metastatic renal cell carcinoma progressed after sunitinib or another antiangiogenic treatment. *Am J Clin Oncol*. 2014; 37: 611-615.
53. Brannon AR, Reddy A, Seiler M, Arreola A, Moore DT, Pruthi RS, Wallen EM, Nielsen ME, Liu H, Nathanson KL, Ljungberg B, Zhao H, Brooks JD, et al. Molecular Stratification of Clear Cell Renal Cell Carcinoma by Consensus Clustering Reveals Distinct Subtypes and Survival Patterns. *GenesCancer*. 2010; 1: 152-163. doi: 10.1177/1947601909359929.
54. Creighton CJ, Morgan M, Gunaratne PH, Wheeler DA, Gibbs RA, Robertson A, Chu A, Beroukhi R, Cibulskis K, Signoretti S, Vandin F, Wu HT, Raphael BJ, et al. Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature*. 2013; 499: 43-49.
55. Nowell PC. The clonal evolution of tumor cell populations. *Science*. 1976; 194: 23-28.
56. Shah SP, Morin RD, Khattra J, Prentice L, Pugh T, Burleigh A, Delaney A, Gelmon K, Guliany R, Senz J, Steidl C, Holt RA, Jones S, et al. Mutational evolution in a lobular breast tumour profiled at single nucleotide resolution. *Nature*. 2009; 461: 809-813.
57. Jiang JK, Chen YJ, Lin CH, Yu IT, Lin JK. Genetic changes and clonality relationship between primary colorectal cancers and their pulmonary metastases--an analysis by comparative genomic hybridization. *Genes Chromosomes Cancer*. 2005; 43: 25-36.
58. Hovey RM, Chu L, Balazs M, DeVries S, Moore D, Sauter G, Carroll PR, Waldman FM. Genetic alterations in primary bladder cancers and their metastases. *Cancer Res*. 1998; 58: 3555-3560.
59. Campbell PJ, Yachida S, Mudie LJ, Stephens PJ, Pleasance ED, Stebbings LA, Morsberger LA, Latimer C, McLaren S, Lin ML, McBride DJ, Varela I, Nik-Zainal SA, et al. The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature*. 2010; 467: 1109-1113.
60. Cruz SM, Tang YZ, Sarker SJ, Prevoo W, Kiyani I, Beltran L, Peters J, Sahdev A, Bex A, Powles T, Gerlinger M. Heterogeneous response and progression patterns reveal phenotypic heterogeneity of tyrosine kinase inhibitor response in metastatic renal cell carcinoma. *BMC Med*. 2016;14:185.
61. Yang M, Shan B, Li Q, Song X, Cai J, Deng J, Zhang L, Du Z, Lu J, Chen T, Wery JP, Chen Y, Li Q. Overcoming erlotinib resistance with tailored treatment regimen in patient-derived xenografts from naive Asian NSCLC patients. *Int J Cancer*. 2013; 132: E74-E84.
62. Zhang L, Yang J, Cai J, Song X, Deng J, Huang X, Chen D, Yang M, Wery JP, Li S, Wu A, Li Z, Li Z, et al. A subset of gastric cancers with EGFR amplification and overexpression respond to cetuximab therapy. *Sci Rep*. 2013; 3: 2992.
63. Turro E, Su SY, Goncalves A, Coin LJ, Richardson S, Lewin A. Haplotype and isoform specific expression estimation using multi-mapping RNA-seq reads. *Genome Biol*. 2011; 12: R13.
64. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013; 29: 15-21.
65. Jia W, Qiu K, He M, Song P, Zhou Q, Zhou F, Yu Y, Zhu D, Nickerson ML, Wan S, Liao X, Zhu X, Peng S, et al. SOAPfuse: an algorithm for identifying fusion transcripts from paired-end RNA-Seq data. *Genome Biol*. 2013; 14: R12.
66. McPherson A, Hormozdiari F, Zayed A, Giuliany R, Ha G, Sun MG, Griffith M, Heravi MA, Senz J, Melnyk N, Pacheco M, Marra MA, Hirst M, et al. deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data. *PLoS Comput Biol*. 2011; 7: e1001138.