

**Original Article** 

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# Treatment strategy for papillary renal cell carcinoma type 2: a case series of seven patients treated based on next generation sequencing data

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**Background:** Papillary renal cell carcinoma type 2 (PRCC2) is refractory to systemic treatment and has a dismal prognosis. Previous studies showed that genetic alterations in PRCC2 were heterogeneous regardless of germline or somatic mutations. In this study, we aimed to perform precision treatment of PRCC2 based on genetic information.

**Methods:** We performed exome and genome sequencing of tumor tissues and matched normal samples. Based on sequencing data, we treated patients with metastatic PRCC2 using precision oncology.

**Results:** Four patients underwent curative surgery of PRCC2 and three patients had metastatic PRCC2. All PRCC2 heterogeneously harbored own driver mutations. Two out of the three patients with metastatic disease had fumarate hydratase (*FH*) germline mutations. One patient with a germline *FH* mutation was diagnosed with hereditary leiomyomatosis RCC. He was treated with bevacizumab and erlotinib combination and showed a durable response. The other metastatic PRCC2 patient harboring a germline *FH* mutation had an additional somatic *FH* mutation and was durably controlled with pazopanib. Other metastatic PRCC2 patient with somatic *PBRM1* and *SETD2* mutations had over 5 years of overall survival with axitinib treatment.

**Conclusions:** We performed precision systemic treatment based on genetic information. Genome sequencing could help identify candidates for targeted therapy in PRCC2, a genetically heterogeneous disease.

**Keywords:** Whole genome sequencing; papillary renal cell carcinoma type 2 (PRCC2); precision oncology; fumarate hydratase (FH); vascular endothelial growth factor-receptor (VEGFR)

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#### Introduction

Renal cell carcinoma (RCC) is a heterogeneous disease comprising a number of different types of cancer (1,2). The genetic background differs among various forms of RCC, and treatment response and prognosis vary according to subtype (3-6).

Papillary renal cell carcinoma (PRCC), which accounts for 15–20% of RCCs, is divided into type 1 and type 2 (7). In terms of clinicopathologic characteristics, type 1 and 2 diseases are different, and type 1 disease has a relatively good prognosis compared to type 2 (1). Genetic alterations also differ between type 1 and type 2 PRCC (7-9). In particular, type 1 PRCC frequently harbors *MET* alteration regardless of sporadic or hereditary disease (7).

PRCC type 2 (PRCC2) comprises a number of different types of non-PRCC type 1 cancers, including hereditary leiomyomatosis and renal cell carcinoma (HLRCC) (10). HLRCC is the most aggressive subtype of PRCC and harbors germline pathologic variants of fumarate hydratase (*FH*), which encodes one of the Krebs cycle enzymes (11). In addition, other genetic alteration, *TFE3* translocation was reported in RCC in children and young adults and was associated with a grave prognosis (12).

There has been a steady effort to find genetic alterations of PRCCs. Exome and genome sequencing data suggest that the driver genetic alterations of PRCCs are varied, and somatic copy number alterations might be pathogenic events during the genetic evolution of PRCCs (13). Furthermore, large-scale genomic characterization of PRCC demonstrated that PRCC2 is divided into three subgroups according to genetic alterations. *CDKN2A* silencing, *SETD2* mutation, *TFE3* fusion, and NRF2-antioxidant response element (ARE) pathway activation were found in PRCC2 including *FH* mutation (7). Moreover, these genetic alterations are significantly related to cancer prognosis.

However, current treatment guideline could not reflect the advance of comprehensive genomic analysis in PRCC2 (14). Current treatment guideline suggested sunitinib or clinical trial as the first line treatment for metastatic non-clear RCC, including PRCC2 (8,15). Treatment guideline

recommended only bevacizumab plus erlotinib treatment for HLRCC among PRCC2 (14). Moreover, there have been no clinical trials of treatment based on genomic information of PRCC2 because of disease rarity.

In this study, we performed exome and genome sequencing of PRCC2 patient samples to characterize their genomic landscapes. Potentially functional rare germline mutations in a wide range of known cancer genes as well as somatic mutations of a few PRCC-related genes such as *ALK*, *CSF1R*, *NF2*, *SETD2*, and *FH* were identified from our sequencing analysis. We also found previously reported recurrent copy gains of chr7, chr16, and chr17 in PRCC from our data as well. Our analysis confirmed the heterogeneous nature of PRCC2 genomic characteristics and identified genetic biomarkers for the prediction of prognosis and drug response in PRCC2. We present the following article in accordance with the MDAR checklist (available at http://dx.doi.org/10.21037/atm-20-3466).

#### **Methods**

#### Patients and sample preparation

We performed genetic analysis of PRCC2 using prospectively collected, surgically removed, fresh frozen samples of RCC and paired normal tissues in the Seoul National University Hospital tissue bank. Two other formalin-fixed paraffin-embedded samples from patients with metastatic PRCC2 were also included. These seven samples were reviewed by qualified pathologists and histologically classified as PRCC2. We obtained informed consent from all subjects, and this study was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 1204-026-403) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Genomic DNA was extracted from FFPE samples using a QIAgen FFPE Tissue DNA kit. DNA and RNA were extracted from the fresh frozen samples using GeneAll exgene cell SV kit and Ambio PureLink RNA mini kit,

Table 1 Clinical and pathological baseline characteristics of PRCC2

No.	Age (years)	Sex	Prognostic risk group	Stage	Grade	FU (months)	Status	Tumor	Normal	Sample type
1	30	М	NA	3	4	95.20	NED	Kidney*	Kidney	Fresh frozen
2	74	М	NA	1	3	46.83	NED	Kidney*	Kidney	Fresh frozen
3	82	М	Intermediate	4	3	69.40	Dead	Kidney*	Kidney	Fresh frozen
4	63	М	NA	1	3	81.17	NED	Kidney*	Kidney	Fresh frozen
5	51	М	NA	1	3	108.60	NED	Kidney*	PB	Fresh frozen
6	27	М	High	4	NA	14.50	Dead	Bone#	PB	FFPE
7	26	F	High	4	3	35.83	Dead	Kidney#	РВ	FFPE

<sup>\*,</sup> surgical specimen; \*, percutaneous needle biopsy specimen. PRCC2, PRCC type 2; NED, no evidence of disease; PB, peripheral blood; FFPE, formalin fixed paraffin embedded.

respectively. Concentrations of DNA and RNA were measured using Qubit.

## Exome capture, library construction and sequencing

Up to 3 µg of genomic DNA was sheared with a Covaris SS Ultrasonicator and adaptors were then ligated to both ends of the fragments. Adaptor-ligated templates were purified using Agencourt AMPure SPRI beads, and fragments with an insert size of ~200 bp were isolated. Exons were captured from adaptor-ligated DNAs using SureSelect Human All Exon v4 + UTRs kit (71 Mb) according to the manufacturer's instructions (Agilent Technologies). PCR amplification of the libraries was carried out for four cycles in the pre-capture step and for ten cycles after capture. Paired-end sequencing, resulting in sequences of 101 base pairs from each end of the fragments, was performed on the HiSeq 2000 platform (Illumina) following the manufacturer's instructions. Image analysis and base calling were performed using the Illumina pipeline with default settings.

# Bioinformatics analysis

Whole-genome sequencing analysis was performed for the five pairs of PRCC2 and matched control samples (four adjacent normal tissues and one blood sample). Whole-exome sequencing analysis was conducted for the two pairs of PRCC2. Reads were mapped to human reference genome (hg19) using Burrows-Wheeler Alignment (BWA) tool 0.7.12 (16). The mapped reads were processed using GATK best practice pipeline (http://fp.amegroups.cn/cms/59e8a8bf8e7cf142d5298efd3f7e76e6/atm-20-3466-1.docx) (17). We used the Mutect2 (version 3.6) (18) and

GATK HaplotypeCaller (version 3.6) algorithms in order to identify somatic and germline single nucleotide variants (SNVs)/short insertions and deletions (indels). Germline variants were categorized by the American College of Medical Genomics and Genetics (ACMG) guidelines using InterVar (19,20). Germline variants classified as benign or uncertain significance by InterVar were discarded if their allele frequencies (AFs) are higher than 0.01 based on both 1,000 genomes project (21) and gnomAD (22). Somatic variants were further filtered by visual inspection of panel of normal data using Integrative Genomics Viewer (23). Both germline and somatic variants were annotated using Variant Effect Predictor (version 92) (24), and only the protein-altering variants were used for subsequent analyses (http://fp.amegroups.cn/cms/cf916fe8d d33be9ffb96d81d425f881e/atm-20-3466-2.docx).

Copy-number alterations (CNAs) were identified using Control-FREEC (25). For WES data, exonic regions were used as initial windows, and for WGS data, we used 5 kb for both initial window and step size for CNA detection (http://fp.amegroups.cn/cms/02ef9eaf1b6b4d30901c76d9e c6d4557/atm-20-3466-3.docx). Structural variations (SVs) were detected from WGS data using Meerkat (version 0.189) program (26). We set the standard deviation cutoff to call and cluster discordant repairs as 5 and the number of supporting split reads to call a SV event as 3 (http://fp.amegroups.cn/cms/b7f5aa78e9c0e90bf1e94315b355 bc81/atm-20-3466-4.docx).

#### **Results**

# Clinical characteristics of patients with PRCC type 2

We obtained a total of seven PRCC2 tissues and paired

Table 2 Medical treatment of stage IV PRCC2 patients

No.	Metastasis sites	Treatment	No. of cycle	Time to progression (weeks)	Best response	Treatment off	Overall survival
3	Lung	Temsirolimus	Week 12	23	SD	AE	69.4 months
	Adrenal gland	Axitinib	22	87	PR	Patient wish	
6	Bone, liver	Temsirolimus	Week 4	4	PD	PD	14.5 months
	Lymph node	Axitinib	1	4	PD	PD	
		Gemcitabine/ cisplatin	1	4	PD	PD	
		Pembrolizumab	2	2	PD	PD	
		Bevacizumab + erlotinib <sup>‡</sup>	13	40	SD	PD	
		Nivolumab + ipilimumab	1	2	PD	PD	
7	Bone	RTx1*	-	-	-	PD	35.8 months
	Liver	Temsirolimus	Week 26	26	SD	PD	
	Adrenal gland	High dose interleukin-2	6	19	SD	PD	
	Peritoneum	RTx2 <sup>†</sup>	-	-	-	PD	
		Pazopanib <sup>‡</sup>	14	52	PR	PD	

<sup>\*,</sup> tomotherapy at bone metastasis; †, tomotherapy at bone, adrenal gland, abdomen wall, pelvis metastasis; †, precision treatment using NGS data. PRCC2, PRCC type 2; SD, stable disease; PR, partial response; PD, progressive disease; AE, adverse event.

normal samples. The clinical characteristics of the patients are described in *Tables 1,2*. Of seven patients, only one was female. Median age at cancer diagnosis was 51 (range, 25–82). Four patients underwent curative resection of PRCC2 and these patients were alive without metastasis, however, three patients with metastatic disease received systemic chemotherapy and ultimately died due to disease progression. Two patients had poor prognostic risk groups and one of intermediate risk group according to Memorial Sloan Kettering Cancer Center Prognostic Model (27).

The systemic treatments administered to patients with metastatic disease are listed in *Table 2*. All three patients received temsirolimus as first-line systemic treatment. After disease progression, two patients received axitinib and one patient received high-dose interleukin-2 as second-line treatment. In terms of patients with metastatic disease, we performed genome sequencing at disease progression after first line systemic treatment. In other patients who had stage I–III disease, genome sequencing was performed after curative surgery.

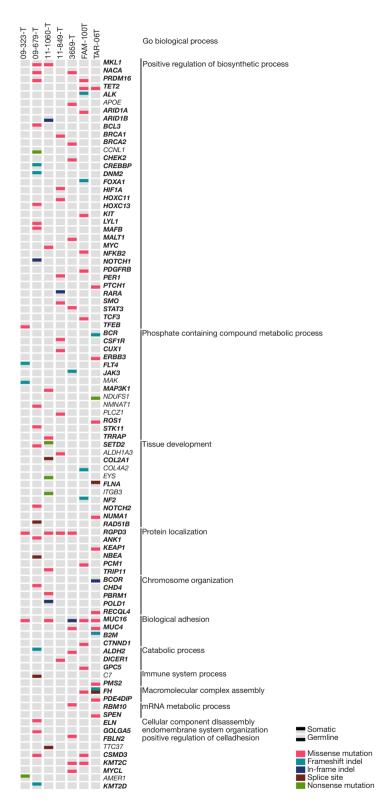
#### Germline and somatic SNVs/indels

From genome and exome sequencing analysis, we determined germline and somatic SNVs/indels (Figure 1,

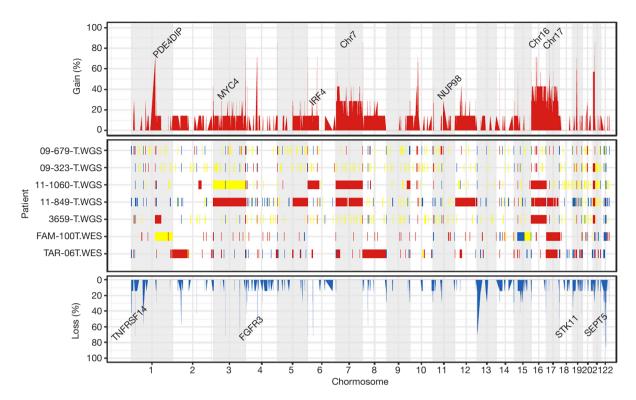
http://fp.amegroups.cn/cms/cf916fe8dd33be9ffb96d81d425f 881e/atm-20-3466-2.docx). In terms of germline mutations, a wide range of known cancer genes involved in various kinds of biological processes such as positive regulation of biosynthetic process, phosphate containing compound metabolic process, tissue development, and others were seemed to be affected by potentially functional rare germline variants reflecting the heterogeneous nature of PRCC2.

Among a few recurrently mutated genes by germline variants, *FH* germline mutations were detected in two patients (*Figure 1*). For the patients with metastatic disease, one with familial leiomyoma had a germline missense mutation of *FH* (p.Lys230Glu) (Figure S1A), and the other patient without family history of HLRCC harbored a germline splicing site mutation (c.1108+1G>A) in *FH* (Figure S1B).

Other germline mutations were also found in PRCC2 (Figure 1, http://fp.amegroups.cn/cms/cf916fe8dd33be 9ffb96d81d425f881e/atm-20-3466-2.docx). Germline MKL1, NACA, PRDM16, TET2, RGPD3, CSMD3 and KMT2C alterations were found in more than one PRCC2 sample. Genetic alterations were present in different loci of coding region. In addition, BRCA1, BRCA2 and PMS2 germline mutations were also detected. In terms of functional alteration according to base change, BRCA1



**Figure 1** Germline and somatic mutations in PRCC2. Known cancer-related gene based on the Cancer Gene Consensus of COSMIC database are shown in bold letters. PRCC2, PRCC type 2.



**Figure 2** Copy number alterations detected from the seven PRCC2 sequencing data. WGS, whole-exome sequencing; WES, whole-genome sequencing. PRCC2, PRCC type 2.

alteration (p.Lys181Gln), *BRCA2* (p.Gly2508Ser) and *PMS2* (p.Val196Phe) resulted in amino acid changes be deleterious by SIFT prediction (28). In addition, 16 genes were affected by pathogenic or likely pathogenic germline mutations based on the ACMG criteria in at least one patient. Among them *ALDH2*, *BCR*, *FH* and *NBEA* genes were known cancer driver genes according to COSMIC Cancer Gene Census database (29) identified (http://fp.amegroups.cn/cms/cf916fe 8dd33be9ffb96d81d425f881e/atm-20-3466-2.docx).

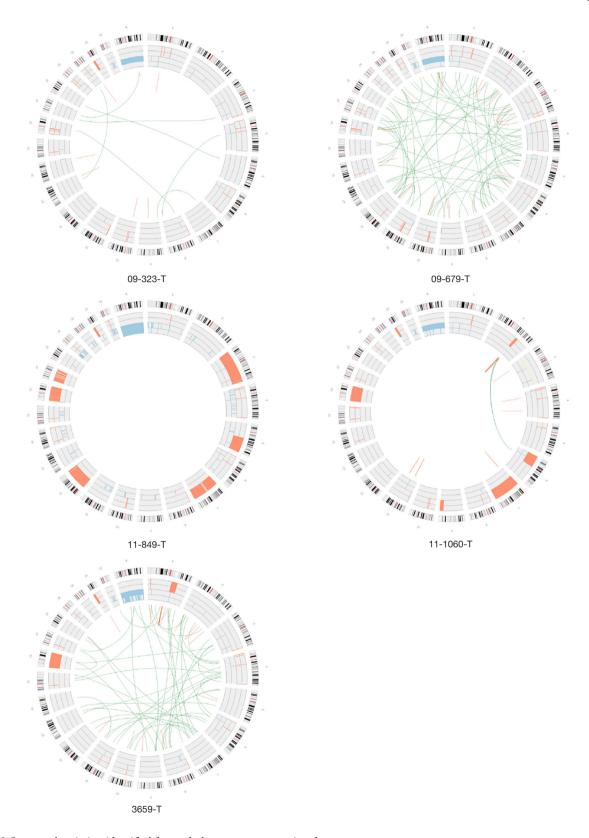
A significant number of somatic mutations were detected from tissue development-related genes such as *SETD2*, *COL2A1*, *FLNA*, *NF2*, *NOTCH2*, and *RAD51B*. Especially, *SETD2* was altered also by germline mutation in other samples (*Figure 1*). Interestingly, a somatic *FH* frameshift deletion (p.Leu132Ter) was detected from the patient with metastatic disease who already harbored the germline splicing site mutation (c.1108+1G>A) (Figure S1B).

#### Copy number alterations and structure variations

CNAs were analyzed using both whole-exome and wholegenome sequencing data. Recurrent copy number gains of chromosome 7, 16, and 17 were observed in our data (Figure 2), which is well concordant with that reported by The Cancer Genome Atlas (7). Other than chromosomal copy number alterations, recurrent focal amplifications of potential oncogenes such as MYC4, IRF4 and NUP98 were identified. Frequent copy number losses of TNFRSF14, FGFR3, STK11, and SEPT5 that are known to have tumor suppressor properties were also found. For SV analysis, we used whole-genome sequencing data and identified average 34.3 SV events (sd: 29.7) per samples. We identified a number of gene fusions in PRCC2 samples but none affecting known cancer driver genes (Figure 3).

# Precision patient treatment strategy according to genetic alterations

We evaluated the effect of genetic alterations on patient prognosis (*Tables 1,2*). Regardless of genetic alterations, primary carcinoma that received curative surgery did not recur. Three patients with metastatic carcinomas were treated with systemic chemotherapy and eventually died due to disease progression. All patients had received



 $\textbf{Figure 3} \ \textbf{Structural variation identified from whole-genome sequencing data}.$ 

temsirolimus (mTOR inhibitor) as their first line treatment according to treatment guideline (14). After disease progression, sequencing results were reported and two of the three patients with metastatic disease had genetic alteration of *FH*.

One patient with familiar history of leiomyoma had a germline missense mutation of FH. Among his family, his mother and her sisters have been diagnosed with myoma, thyroid cancer, and early gastric cancer (Figure S2). Germline FH mutation testing of his family members revealed germline missense mutation of FH at the same locus in his maternal family members but absent in his father (Figure S1A). In clinical course, this patient did not respond to mTOR inhibitor, VEGFR TKI, cytotoxic chemotherapy, or the anti-PD1 antibody pembrolizumab (Table 2). After we found the FH germline mutation, the patient was treated with a combination of bevacizumab [an anti-VEGF antibody) and erlotinib (an epithelial growth factor receptor (EGFR) TKI) and showed a durable response of 40 weeks (Figure 4A). Disease progression after bevacizumab and erlotinib treatment, there was no effective treatment strategy. In this patient, overall survival (OS) duration between diagnosis to death was 14.5 months.

A young patient with germline and somatic FH mutations was treated with pazopanib, a selective multi-targeted receptor TKI, after her genetic alteration was revealed (Table 2). Pazopanib resulted in a decrease in the tumor over one year, but grade 3 lethargy developed and resulted in treatment discontinuation. Time to progression of pazopanib treatment was 52weeks. Her disease also harbored PMS2 alteration (Figure 4B). However, her performance status did not permit further treatment, such as immune check point inhibitor. She had 35.8 months of overall survival.

The patient without genetic alteration of FH showed a durable response to temsirolimus and axitinib [a tyrosine kinase inhibitor (TKI), which targets vascular endothelial growth factor-receptor 1–3 (VEGFR 1–3)]. We observed PBRM1 and SETD2 mutations in his disease and he had relatively long survival duration. He responded axitinib treatment for a long duration (87 weeks) until adverse events forbade him from receiving treatment (Table 2, Figure 4C).

## Discussion

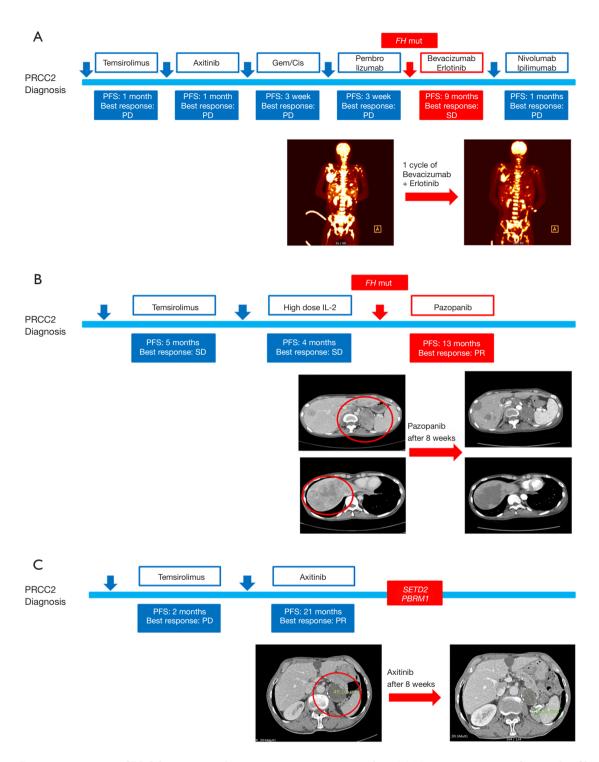
In this study, we identified genetic alterations in PRCC2. Mutation in the gene encoding *FH*, either germline or somatic, was found in patients younger than 30 years and indicated poor prognosis. In terms of treatment, patients

harboring FH mutation responded to anti-angiogenesis agents including bevacizumab and pazopanib.

PRCC is defined as RCC with a papillary pattern based on pathologic finding (1). Type 2 PRCC is lined by large cells with abundant eosinophilic cytoplasm compared to the small cells with clear to basophilic cytoplasm present in type 1 PRCC. The best-known genetic alteration of type 1 PRCC is MET mutation, whereas FH nutation is common in type 2, especially in familial cases (30). However, with the exception of familial cases of PRCC, genetic alterations that drive tumorigenesis of PRCC2 have not been revealed. Recent genetic analysis showed that PRCC2 could be classified into three subgroups according to molecular differences (7). One subgroup represented by FH mutation and CDKN2A silencing due to hypermethylation of the CDKN2A promoter had a relatively small number of PRCC2 (13.3%). This subgroup was characterized by relatively young patients and extremely short survival duration compared to other subtypes. In our study, two young patients with FH mutated PRCC2 had similar clinical characteristics to this subtype.

FH, a tricarboxylic acid cycle (TCA) enzyme, catalyzes the hydration of fumarate into malate. Deficiency of FH causes accumulation of fumarate and activation of hypoxia inducible factor (HIF) under normal oxygen levels (31). VEGF and glucose transporter-1 (GLUT-1) are upregulated by an excess of intracellular fumarate through the HIF-dependent pathway. Therefore, inhibition of the VEGF pathway and glucose transport has been suggested as a therapeutic approach in FH mutant cancer (31,32). In our study, we identified two patients with PRCC2 harboring FH mutation characterized by rapid progression. One patient with germline FH mutation did not respond to axitinib, a selective VEGFR inhibitor, but did respond to bevacizumab and erlotinib combination therapy (33). Another patient was treated using pazopanib, a multi-targeted receptor TKI whose targets include VEGFR, and she only responded to this treatment. The mechanism of action of pazopanib does not include glucose metabolism, but hypoglycemia frequently occurs during pazopanib treatment (34). With regard to this adverse event, pazopanib might modify glucose metabolism in FH mutant PRCC2. Therefore, based on our clinical experience, a therapeutic strategy for FH mutant PRCC2 should consider targeting glucose metabolism in addition to inhibition of the VEGF pathway.

The Warburg effect (35), a metabolic shift to aerobic glycolysis in normoxia status, is one of the characteristics of *FH* mutant RCC (33). This ineffective metabolism



**Figure 4** Precision treatment of PRCC2 patient with next generation sequencing data. (A) Treatment response after 1 cycle of bevacizumab with erlotinib treatment in patient with HLRCC (PRCC type 2); (B) treatment response after pazopanib in PRCC2 with somatic *FH* mutation; (C) treatment response after axitinib in PRCC2 with *PBRM1* and *SETD2* mutation. PRCC2, PRCC type 2.

in tumor cells resulting from the rapid proliferation of cancer cells might induce cachexia of patients (36). In our study, the patient treated with pazopanib suffered cancer-related cachexia even though her disease was well controlled. Consequentially, her general condition gradually deteriorated, and she could not undergo further treatment.

Pathogenic germline alteration of C7, FH, MAK, NBEA, NDUFS1 and TTC37 were also detected in this study. Mutations in NDUFS1, the largest subunit of mitochondrial complex I, are known to reduce the activity of complex I (37). We also identified ten likely pathogenic germline mutations in ALDH1A3, ALDH2, APOE, BCR, CCNL1, COL4A2, EYS, ITGB3, NMNAT1 and PLCZ1. Gao et al. have found that von Hippel-Lindau tumor suppressor (VHL) was directly binding to the promoter of ALDH2 to regulate the transcriptional activity of hepatocyte nuclear factor 4 alpha (HNF4A) in clear-cell RCCs (38). Loss of HNF4A binding activity was often observed in renal cell carcinogenesis (39).

Germline alteration of BRCA1, BRCA2 and PMS2 were also detected in this study. Germline BRCA1 and/or BRCA2 deleterious mutation caused hereditary breast and ovarian cancer as well as other types of familiar cancer syndrome (40). Moreover, cancer caused by BRCA1 mutation was sensitive to PARP1 inhibitors, such as olaparib and talazoparib. Recent clinical trials showed that PARP1 inhibitor prolonged duration of survival in patients with metastatic breast cancer with pathogenic BRCA1 and/or BRCA2 mutations (41,42). We found a deleterious mutation of PMS2, one of mismatch repair (MMR) genes associated with Lynch syndrome (43). With regard to immune checkpoint blockade had more clinical benefit in MMR deficient cancers compared to MMR proficient cancers, anti-PD-1 antibody wound be considered as a therapeutic option in PRCC2 with PMS2 mutation.

Recent genetic study using targeted deep sequencing determined that approximately 20% of advanced RCCs had a germline mutation even though almost all cases were sporadic rather than hereditary cancer. That study found *BRCA1*, *BRCA2*, and *CHEK2* germline mutations and suggested that half of germline mutations could be potential targets for direct systemic treatment (44). Therefore, these germline mutations are potential therapeutic targets in advanced PRCC2.

Somatic mutations in PRCC2 also indicated disease prognosis. One patient with metastatic disease harboring *SETD2* and *PBRM1* somatic mutations had overall survival of about 5 years despite having stage IV PRCC2. His disease showed a durable response to axitinib and had a relatively

good prognosis. Both *SETD2* and *PBRM1* were SWI/SNF complex genes associated with chromatin remodeling. In clear cell RCC, *SETD2* was associated to poor prognosis while *PBRM1* had no impact on patient's survival or also indicated poor prognosis (45,46). Moreover, in terms of prognostic risk group according to clinical characteristics, this patient was categorized into intermediate risk group, not poor risk group in which other two patients with metastatic PRCC2 harboring *FH* mutation (27). Therefore, we treated PRCC2 as clear cell RCC with axitinib and he might be having favorable treatment outcome in this case.

The relationship between genetic alteration and clinical characteristics including risk groups have not been revealed neither previous studies nor our study. Further large scaled genetic studies of metastatic PRCC2 would give the information of the relationship between genetic and clinical characteristics to guide treatment of metastatic PRCC2.

#### **Conclusions**

Our results indicate the role of genetic alterations in precision systemic treatment of PRCC2. *FH* alterations were found in not only HLRCC, but also in sporadic PRCC2. Sporadic PRCC2 with somatic *FH* frameshift deletion had similar clinical outcome to HLRCC. In addition, genetic alterations of *PBRM1* and *SETD2* had a predictive role of PRCC2 prognosis.

In this genetic study, we successfully applied genetic information to patient treatment and prolonged survival. Therefore, genome sequencing should be performed to identify candidates for targeted therapy in PRCC2, a genetically heterogeneous disease.

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#### **Footnote**

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was reviewed and approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 1204-026-403) and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. We obtained informed consent from all subjects.

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