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Derivative Chromosome 3 Loss from t(3;6)(q12;q14) Followed by Differential *VHL* Mutations Underlie Multifocal ccRCC

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Abstract. *Background/Aim: The Von Hippel-Lindau (VHL) gene encodes a protein (pVHL) that plays an important role in proteasome degradation of hypoxia inducible factor α (HIFα) through E3 activation. Accumulation of HIFα by loss of functional pVHL promotes tumorigenesis, thus, VHL has tumor suppressor gene capability in clear cell renal cell carcinoma (ccRCC). VHL is the most frequently mutated gene in ccRCC. The complete loss of VHL is mainly achieved by loss of chromosome 3p, which has a VHL coding region in combination with mutation or hypermethylation of the remaining copy of VHL. Given the risk of constitutional chromosome 3 translocation for RCC, it is important to detect the translocation and understand the mechanism underlying the development of multifocal ccRCC. Case Report: A 67-year-old female patient diagnosed with multifocal RCC underwent robot-assisted partial nephrectomy (RAPN) for three kidney tumors. A cancer gene panel test using next generation sequencing (NGS) detected differential VHL mutations (c.533T>G; p.L178R, c.465_466insTA; p.T157Ifs*3, c.343C>A; p.H115N), while VHL mutation was not detected in peripheral blood DNA. A tendency toward copy number loss of genes on der(3) was also detected in all tumors, but not in the germline one. A karyotype analysis revealed a germline translocation between 3 and 6, t(3;6)(q12;q14). Conclusion: Chromosome 3 translocation and loss of derivative chromosome containing 3p and subsequent somatic differential VHL mutations in this case strongly support the previously proposed three-step model to explain the development of familial conventional ccRCC.*

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Key Words: Cancer gene panel test, chromosomal translocation t(3;6)(q12;q14), multifocal ccRCC, hereditary renal cell carcinoma, VHL.

Renal cell carcinoma (RCC) is one of the most lethal malignant tumors (1). The most common pathological type of RCC is clear cell renal cell carcinoma (ccRCC) (1). Recent advances in RCC treatment have provided new options for advanced RCC, but mortality remains high (2). Meanwhile, genomic studies employing cancer gene panel (CGP) tests using next-generation sequencing (NGS) have rapidly risen to improve prognosis of patients with advanced cancer, including ccRCC (3, 4). Genomic studies revealed that the von Hippel-Lindau (VHL) gene alteration is the most frequent gene alteration in ccRCC (5, 6). *VHL* is located on chromosome 3p and is responsible for encoding the VHL protein (pVHL), which is involved in degradation of hypoxia-induced factors (HIFs) (7). Dimerized HIF2α translocates to the nucleus to upregulate transcription of hypoxia-responsive genes (*e.g.*, vascular endothelial growth factor, platelet-derived growth factor, and others) through binding with hypoxia-response elements. Increased HIF2α-induced factors promote tumorigenesis and progression; thus, *VHL* has tumor suppressor gene capability in ccRCC (7, 8). In most cases of sporadic ccRCC with *VHL* gene alteration, tumor development

is explained by a 2-hit model (9, 10). *VHL* loss of heterozygosity (LOH) following a second hit on another *VHL* allele causes biallelic loss of *VHL*. Then, pVHL loses its function and the tumor develops through induction of HIFs. The first hit, LOH in *VHL*, is thought to occur due to deletion of chromosome 3p; this typically occurs decades before the diagnosis of RCC. In contrast, a three-step model for the development of familial conventional RCC has been proposed. In this model, germ line-balanced chromosome 3 translocation and loss of the derivative chromosome 3, der(3), occurs as a first step prior to the 2-hit phenomenon (11-13).

One study showed that the spectrum of *VHL* alterations in bilateral, multifocal ccRCC from a single patient detected by genetic test using NGS will improve the accuracy of differentiation between primary tumors and metastatic disease (14). In this report, we show a case of multifocal ccRCCs with germline translocation, t(3;6)(q12;q14) and differential *VHL* mutation patterns with a consistent trend of *VHL* loss that were not detected in the germ line. These results strongly support a three-step mechanism of multifocal ccRCC development.

Case Report

Patient. A 67-year-old female patient was diagnosed with multifocal RCC [1 tumor in the left kidney (tumor #1) and 2 tumors in the right kidney (tumor #2 and tumor #3)] (Figure 1). She had no remarkable personal medical history; however, her father had suffered from metachronous bilateral RCC, and her brother had died of RCC. She first underwent robot-assisted partial nephrectomy (RAPN) for the left kidney tumor (#1) and subsequently underwent RAPN for the right kidney tumors (#2 and #3). The pathological diagnosis of all renal tumors was ccRCC with Fuhrman nuclear grade 1. Paraffin embedded tissues retrieved from each ccRCC were tested with the GeneRead Human Comprehensive Cancer Panel (Qiagen, Hilden, Germany) using NGS, and variants in *VHL* were detected (tumor #1; c.533T>G: p.L178R, tumor #2; c.465_466insTA: p.T157Ifs*3, tumor #3; c.343C>A: p.H115N). These loss of function or missense variants were differential mutations; thus, this multifocal ccRCC did not rely on a germline variant of *VHL*. Indeed, no *VHL* variant was detected by germline testing of DNA isolated from peripheral blood leukocytes using CGP (SureSelect PrePool custom Tier2, Agilent, Santa Clara, CA, USA). A copy number alteration analysis showed a trend of gene loss, suggesting segmental loss of chromosome 3p in comparison to the germ line (Figure 2A and B). In addition to 3p, we observed possible segmental loss of 6q (Figure 2A). Several studies on the translocation of chromosome 3 in familial RCC were previously reported (13, 15), and therefore we used karyotype analysis because of her familial history of RCC in addition to possible loss of chromosome 3p. The patient was shown to have a germline balanced translocation between chromosomes 3 and 6, t(3;6)(q12;q14) (Figure 3). The loss of der(3) accounts for the tendency to lose genes in both 3p and der(3), which were meant to be on chromosome 6 (in the absence of translocation) (Figure 2B).

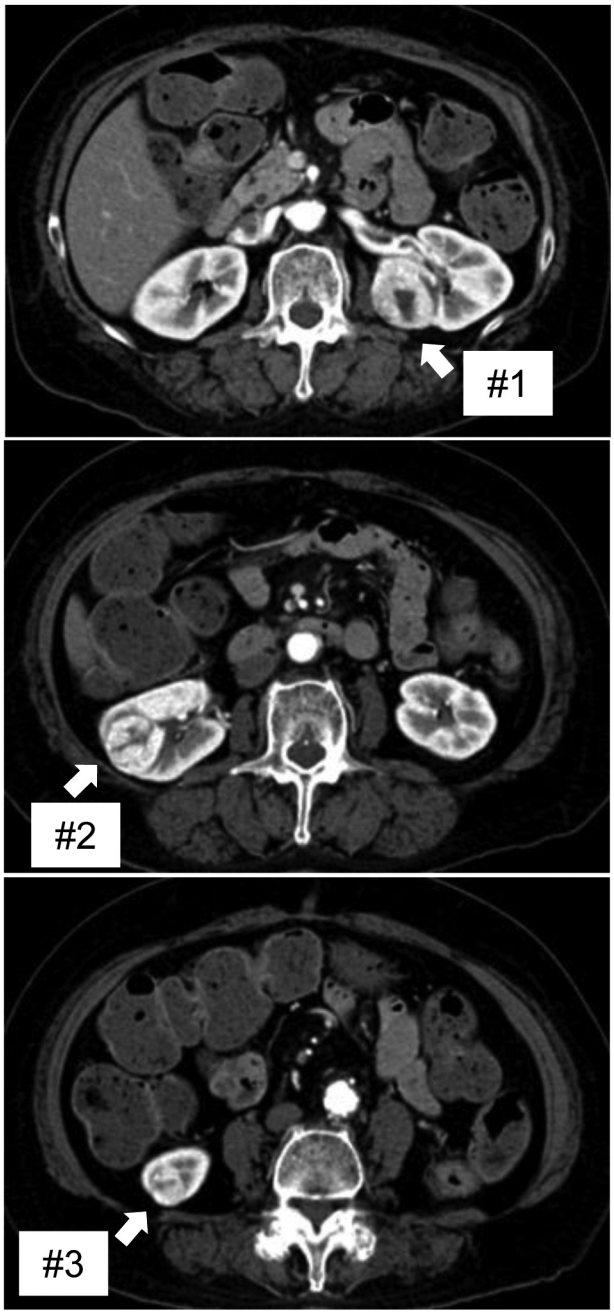

Figure 1. *Computed tomography (CT) images showing bilateral multifocal clear renal cell carcinoma (mCRCC). One tumor was located in the right kidney (tumor #1) and two tumors were located in the left kidney (tumor #2 and tumor #3).*

DNA isolation. Prior to DNA extraction, pathologists investigated the tissue tumor cell content in hematoxylin and eosin-stained slides. The area that predominantly included tumor cells was then macro-dissected from 10-μm-thick formalin-fixed paraffin-embedded (FFPE) tissue sections and DNA was extracted using a Maxwell RSC DNA FFPE Kit-PKK, Custom (Promega, Fitchburg, WI, USA). DNA from peripheral blood was extracted using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions.

Next-generation sequencing-based multiplex gene assay. The multiplex gene assay, which is based on NGS, was reported previously (6). Briefly, purified genomic DNA was quantified using an Agilent 4200 TapeStation. DNA libraries were prepared with DNA with a DNA integrity number of >3.1 for subsequent genomic sequencing following gene amplification using GeneRead Human Comprehensive Cancer Panel (160 genes, NGHS-501X; Qiagen) for tumor DNA and SureSelect PrePool custom Tier2 (Agilent) for peripheral blood DNA. Targeted amplicon exome sequencing for cancer-related genes was performed using the Illumina Miseq sequencing platform (Illumina, San Diego, CA, USA). Genome annotation and curation was performed using GenomeJack (Mitsubishi Electric Software Corporation, Tokyo, Japan). Single nucleotide variations, insertions/deletions and copy number variations (CNVs) were identified as cancer-related gene alterations.

Karyotype analysis. Chromosomal preparation from peripheral lymphocytes and the G-banding methodology have been described elsewhere (16, 17). Metaphases were analyzed and the karyotype was written according to the International System for Human Cytogenomic Nomenclature 2009 guidelines.

Ethics statement. This study was approved by the research Ethics Committee of Central Japan International Medical Center (No. 2022-013). Written informed consent for this study was obtained from the patient.

Discussion

Several cases of hereditary RCC with translocation of chromosome 3 have been reported (13, 15). We described the first case, to our knowledge, of multifocal ccRCC with chromosome translocation t(3;6)(q12;q14). To date, CGP tests are widely used to obtain better outcomes in cancer patients; however, the identification of patients with chromosome translocation underlying RCC by CGP testing is not established nor has it been reported, despite the potential application of the CGP test for this purpose. CGP can detect the following genomic alterations: base substitutions, insertions and deletions, copy number alterations and rearrangement or fusions. In this patient, all tumors showed differential *VHL* mutations, and the trend of gene loss in a segment of chromosome 3p harboring *VHL* was observed. However, no 3p loss was detected in the germ line, either by CGP or by karyotype analysis, and furthermore, the translocation of the breakpoint did not seem to interrupt VHL. These results led us to speculate that a mechanism destroying pVHL, which did not involve a breakpoint, may underlie multifocal ccRCC.

The *VHL* gene is located on 3p25. It was discovered in a patient with von Hippel-Lindau disease with RCC and is now widely known as a tumor suppressor gene (7, 8). Gene alteration in *VHL*, which plays a critical role in tumor development, is most frequently observed in ccRCC (5, 6); thus, this may constitute a therapeutic target.

Chromosome 6 is one of the most frequent partners for chromosome 3 translocation in familial RCC; however, there are only few reports on t(3;6)-associated RCC (18-21). The breakpoints were t(3;6)(p13;q25.1), t(3;6)(q23; q16.2), t(3;6)(q12;q15) and t(3;6)(q22;q16.1), and our case is the first case of RCC with t(3;6)(q12;q14) to our knowledge. Since different 3p breakpoints accompanying a tumor-associated loss of either der(3) or derivative chromosome carrying the segment containing *VHL* have been reported in familial ccRCC (13), a three-step model for tumorigenesis of familial RCC has been proposed (11-13, 22): first, balanced translocation of germline chromosome 3; second, nondisjunctional loss of the derivative chromosome containing the 3p segment; and third, a somatic mutation in the remaining 3p allele of *VHL* causing functional loss of pVHL, thus promoting the development of RCC. In the present patient, translocation t(3;6)(q12;q14) was revealed by a karyotype analysis, and CGP suggested the loss of der(3) in all tumors. Among the three tumors, the estimated tumor content (40-50%) may not have had sufficient power to show the significance of the loss of genes on der(3). Although the change in the tumors was not significant, it was not observed in germline DNA at all. Relatively neutral copy numbers of other genes and SNP frequency imbalance in der(3) may represent the loss of der(3) (data not shown). In combination with this corroboration, the trend of gene loss on der(3) strongly suggests non-disjunctional loss of the der(3) containing *VHL*: the second step in the three-step model.

A mutation in tumor #2 was p.T157Ifs*3, a truncating mutation, and the other mutations in tumor #1 (p.L178R) and tumor #3 (p.H115N) were missense mutations. The variant allele frequency (VAF) of the *VHL* mutations was 29.0% in tumor #1, 30.2% in tumor #2 and 25.7% in tumor #3. This VAF suggested that the mutations occurred in one allele resulting in a functional loss of pVHL due to the loss of another alle of *VHL* on der(3) prior to the mutations: the third step in the three-step model. Eventually, functional loss of pVHL failed to inhibit HIFs, thus resulting in development of RCC. In tumor #2, a mutation in *BAP1* (p.A92V) with a VAF of 17.1% was also observed. *BAP1* is located on chromosome 3p and is an important tumor


A
Copy Number
0.50
0.75
1.00
1.20
1.40
1.60
1.80
2.00
2.20
2.40
2.60
2.80
3.00
3.20
3.40
3.60
3.80
4.00
5.00
6.00
7.00
8.00
FANCD2
VHL
XPC
MLH1
MYD88
CTNNB1
SETD2
BAP1
PBRM1
CBLB
GATA2
PIK3CA
BCL6
DAXX
FANCE
PRDM1
ROS1
TNFAIP3
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MAP3K4
Germline

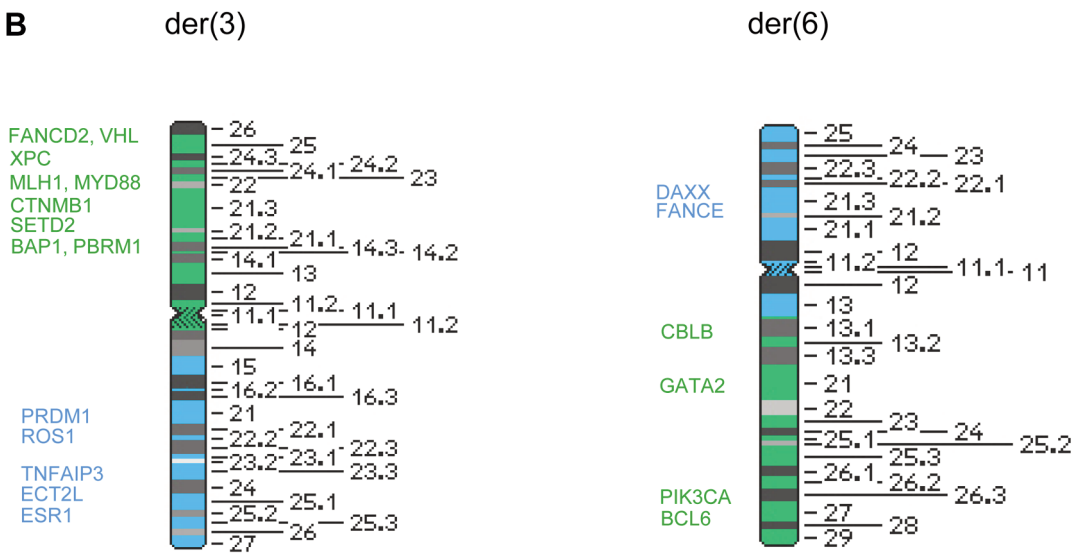

Figure 2. *Copy number variation analysis and diagrams of derivative chromosomes 3 and 6. (A) Copy number variation analysis of the clear cell renal cell carcinomas and the germ line using cancer gene panel (CGP) testing. In the absence of translocation, the gene names in green and blue are considered to be located on chromosomes 3 and 6, respectively. (B) Diagrams of derivative chromosomes 3 and 6 with the relative locations of genes detected by a CGP test. In the absence of translocation, the gene names and diagrams in green and blue are considered to be located on chromosome 3 and 6, respectively.*

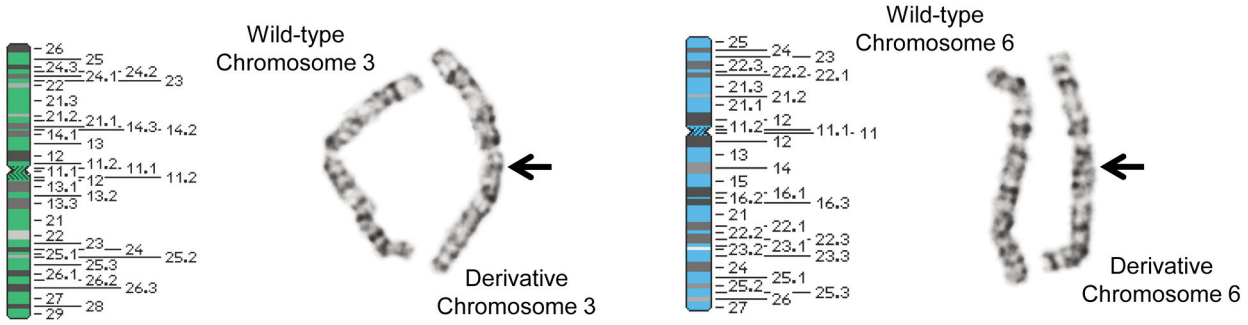

Figure 3. *G-banded karyogram showing translocation of chromosomes 3q and 6q. Partial lymphocyte-derived karyotypes of normal chromosomes 3 and 6 and the translocation derivatives der(3) and der(6). Arrows indicate breakpoints.*

suppressor. In addition to *VHL*, BAP1 gene alteration is a frequently detected gene alteration in ccRCC. Although, the functional loss of *VHL* is one of reasons for the pathogenesis of ccRCC, little is on about how *VHL* mutations are generated. The different mutations occurring in somatic rather than germline cells may explain somatic mutations caused by environmental effects including radiation effects and interaction of DNA with alkylating agents (23). Further research should be conducted to clarify these mechanisms.

Belzutifan, a potent, selective small-molecule inhibitor of HIF-2α, has been recently approved for the treatment of adult patients with VHL disease associated with RCC, central nervous system hemangioblastoma or pancreatic neuroendocrine tumors that do not require immediate surgery (24). In another study, belzutifan showed promising anti-cancer activity in ccRCC (25). As chromosome 3 translocation followed by a loss of the derivative chromosome containing 3p is attributable to tumorigenesis of ccRCC through HIFs activation, HIF inhibitors, including belzutifan, could help these patients. HIFs up-regulate transcription of hypoxia-responsive genes promoting tumor growth (26). Therefore, targeting the pathways of hypoxia-responsive genes using tyrosine kinase inhibitors in combination with HIF inhibitors could be a promising treatment strategy (26, 27).

Although multifocal RCC is rare and bilateral RCC is known to be characteristic of hereditary RCC, without the patient's father and brother (who had RCC) undergoing genomic testing, hereditary RCC cannot be proven in the present patient. Nevertheless, the patient's family history of RCC and the results of the genomic analysis described above suggest the possibility of hereditary ccRCC.

In summary, we presented a case of ccRCC with t(3;6)(q12;q14) followed by differential *VHL* mutations suggesting a three-step model for the development of ccRCC by CGP testing and karyotype analysis. To date, a karyotype analysis or whole genome sequencing have been adequate to detect chromosome translocation; however, these tests are not suitable in many clinical scenarios. In such cases, a CGP analysis may be useful to identify candidates who should undergo further genomic analysis to detect chromosomal translocation-related multifocal ccRCC.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. The data are not publicly available due to privacy or ethical restrictions.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

Conception and design: KMi, SI and TD. Data analysis and interpretation: KMi, SSa, IS, KK, SK, KH, SSu, KMa, TY and JY. Data curation: IS, SSu, SSa, KY and HN. Clinical evaluations and treatment: SY. Article writing: KMi. All Authors read and approved the final manuscript.

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