A Renal Cell Carcinoma with Biallelic Somatic TSC2 Mutation: Clinical Study and Literature Review

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OBJECTIVE To elucidate the effect of the biallelic somatic *TSC2* mutations, identified in one adolescent patient, in renal cell carcinoma (RCC).

METHODS Mutation analyses, immunohistochemistry and real-time polymerase chain reaction (PCR) were conducted.

RESULTS Two novel somatic mutations of *TSC2* in unilateral and solitary RCC samples from a 14-year-old female were identified. The pathological features suggest the tumor as a clear-cell renal cell carcinoma. In addition, immunohistochemistry revealed elevated levels of phosphorylated S6K1. Results from *in vitro* cellular experiments suggest that the mutant TSC2 proteins were quickly degraded and they failed to repress the phosphorylation of S6K1 and STAT3, which leads to constitutive activation of mTORC1 pathway and ultimately cause the development of RCC.

CONCLUSION Detecting *TSC2* mutation in patients with early RCC onset would be beneficial and mTOR inhibitor could be a therapeutic option for *TSC2* mutation-induced RCC. UROLOGY 133: 96–102, 2019. © 2019 Elsevier Inc.

Renal cell carcinoma (RCC) represents about 90% of renal malignancies, which is the 13th most common malignancy among all cancers. Approximately 2%-3% of RCC occurs in patients with hereditary diseases including von Hippel-Lindau (VHL) syndrome, hereditary papillary RCC, and Birt-Hogg-Dube' syndrome.[1] In addition, 2%-4% of tuberous sclerosis (TSC) patients suffer from RCC, which is higher than the estimated incidence among the general population. Multiple lines of evidence suggest that loss of function of either *TSC1* or *TSC2* could result in RCC.[2-4]

The *TSC1* and *TSC2* genes are located on 9q34 and 16q13.3, respectively.[5] The protein complex composed of hamartin and tuberin, which is translated from *TSC1* and *TSC2* respectively, possesses GTPase activity responsible for turning GTP-Rheb to GDP-Rheb. Therefore, both *TSC1* and *TSC2* were also known as cancer suppressors partially due to their inhibitory role on mammalian target of rapamycin complex 1 (mTORC1). Mechanistically, loss-of-function mutations in *TSC1* and/or *TSC2* result in inactivation of the hamartin-tuberin complex and subsequently inactivate GTPase and ultimately lead to constitutive activation of the mTORC1 pathway.[3] TSC is an autosomal dominant disease and almost all organs in these patients were affected[5] characterized by facial angiofibroma, seizures, and cognitive disability. TSC also can cause renal lesions including RCC.[6] Since the first reported RCC in a patient with TSC in 1986,[7] numerous cases of RCC have been reported in familial TSC with germ line mutations in either *TSC1* or *TSC2*. However, cases of RCC due to a somatic mutation of *TSC2* are relatively rare. Here we present a case of clear cell RCC (ccRCC) in an adolescent with 2 novel biallelic somatic mutations in *TSC2*.

MATERIAL AND METHODS

Patient Presentation

A 14-year-old female was referred to the Southwest Hospital on July 23, 2017, due to the finding of a mass in the upper pole of

Conflicts of interest: *none*

Funding Support: *This work did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.*

Financial Disclosure: *The Institutional Review Board of Daping Hospital of Third Military Medical University waived the IRB approval for the study; moreover, for the use of medical records and related images, the written informed consent was obtained from the patients before the publication of the study. The authors have nothing to disclose.*

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Submitted: June 13, 2019, accepted (with revisions): August 10, 2019

https://doi.org/10.1016/j.urology.2019.08.016
0090-4295

her left kidney identified during an ultrasound test. Kidney tumor was later diagnosed based on the abdominal computed tomography (CT) scan. The laboratory tests did not reveal any other notable abnormalities. A robot-assisted nephron-sparing enucleation was conducted on July 26, 2017, with a da Vinci surgical system and a 3.0 × 3.0 cm mass was excised. No sign of recurrence nor metastasis was seen in repeat CT scans during the reexamination conducted 9 months after the surgery. Based on the clinical manifestation,[8] mutation analyses were performed to determine whether there are germ line or somatic mutations responsible for the ccRCC. We collected both peripheral blood and tumor samples for mutation analyses. Mutations in several potential oncogenesis-associated genes (supplement Table 1) were screened using ER-seq (Gene+, China). To confirm the *TSC2* mutation, polymerase chain reaction (PCR) was conducted on the patient's DNA with primers specifically designed for *TSC* gene amplification, the PCR products were sub-cloned into a pMD18-T vector (D103A, TAKARA, Shiga, Japan), and finally Sanger sequencing was conducted. The informed consent was obtained from the patient and their parents, and all procedures performed in this study were in accordance with the standards of institutional research committee, and approved by the Research Ethics Committee of Daping Hospital, Third Military Medical University.

Immunohistochemistry

Immunohistochemistry was conducted as described.[9] After deparaffinization and rehydration, slices were retrieved and incubated with primary antibodies against TSC2 (Santa Cruz), VHL (OriGene, MD), phosphorylated S6K1(p-S6K1) (Cell Signaling Technology), HIF1α (Abcam), HIF2α (OriGene, MD), EPO (Santa Cruz), SDHB (Proteintech) and VEGF (OriGene, MD) followed by incubation with HRP-labeled goat anti-mouse/rabbit IgG. In addition, the samples from a patient with VHL syndrome were used as a positive control.

Cell Culture

Rat uterine leiomyoma-derived *Tsc2*-null cells, kindly provided by Dr. Hongbing Zhang of Peking Union Medical College, were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco) at 37°C with 5% CO_2.

Transient Transfection

Since the two mutations identified in this patient were not reported previously, the function of them was elusive. To identify the function, plasmids containing either *TSC2* mutations or wild type *TSC2* were constructed (Mutant 1: c.1039-1049delAAGAAGTATAG in exon 11, Mutant 2: c.2721insT in exon 24) and transiently transfected into *TSC2*-null cells with Lipofectamine 3000 reagent (Invitrogen).

Western Blotting

Lysates were collected from cells transfected with different plasmids and separated on SDS-PAGE followed by western blotting assay described previously[10] using the primary antibodies against TSC2 (Abcam), S6K1 (Abcam), p-S6K1 (T389+T412) (Abcam USA), STAT3 (Abcam USA), phosphorylated STAT3 (p-STAT3) (Cell signaling) and β-actin (Cell signaling).

Real-time PCR

According to the manufacturer's protocol, RNA and DNA were isolated from cells with Ultrapure RNA Kit (CWBio) and TIANamp Genomic DNA Kit (TIANGEN), respectively. One microgram RNA was subjected to reverse transcription with Hiscript III RT SuperMix for qPCR (Vazyme), following the manufacturer's protocol. As previously described,[11] real-time PCR was performed with the LightCycler®96 System (Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) using AceQ qPCR SYBR Green Master Mix (Vazyme). The primers of *TSC2* were described below: 5'-CGAGTCAAACAAGCCAATC-3' (forward) and 5'-ATCGTGCCAGCAGTAGGTG-3' (reverse). PCR data were analyzed using Graph Pad Prism (Graph Pad Software, San Diego, California).

Literature Review

Literature published between 1966 and June 1 2019, were searched in the Medline database. TSC AND kidney neoplasms (MeSH Terms) AND carcinoma renal cell (MeSH Terms) were used for searching related cases and articles. All RCC cases with *TSC* mutations were included in the review if they meet the following criteria: (1) RCC with *TSC* mutation; (2) pathological examination was applied to verify the diagnosis; and (3) gene mutations were analyzed and confirmed.

RESULTS

Biallelic Somatic Mutations were Identified in a Patient With RCC

A diagnosis of T1N0M0 kidney tumor was made based on the heterogeneous enhancement of the mass in the abdominal CT scan (Fig. 1A). Tumor samples from a patient with VHL syndrome showed specific ccRCC morphology characterized by transparent and empty cytoplasm, well-defined cell borders and thin-walled vessels. In the tumor samples of our patient with *TSC2* mutation, round and regular nuclei, cytoplasm with granular eosinophilic material around the nucleus and thin-walled vessels were shown in hematoxylin-eosin staining, suggesting a ccRCC, Furhman Grade 3 (Fig. 1C). In addition, immunohistochemical staining results of the tumor samples with *TSC2* mutation also showed positive CK, Vimentin, CD10, RCC staining, and negative CA9, CK7 and TFE-3 staining (data not shown). Two pathologists confirmed these results independently.

Considering the early onset age and clinical characteristics, hereditary RCC should be identified. Susceptibility genes highly associated with RCC oncogenesis (supplement Table 1 and supplement Table 2) were examined in both peripheral blood and tumor samples. No germ line mutations were observed, while 2 novel somatic mutations in *TSC2* were identified in the tumor, but not the peripheral blood. These biallelic frameshift mutations were not found in the Catalogue of Somatic Mutations in Cancer (http://cancer.sanger.ac.uk). Therefore we conclude that we identified a novel 11-base deletion of 1039_1049 (c.1039-1049del AAGAAGTATAG) in exon 11 which leads to a frameshift (p.K347Efs*36) and a novel 1-base insertion at base 2721 (c.2721insT) in exon 24 which also leads to a frameshift (p.V909Cfs*6) (Fig. 1B). Of note, neither somatic nor germ line mutation was found in the other oncogenesis genes examined (data are not shown).

Higher p-S6K1 Expression in Tumor Cells With Mutant *TSC2*

It appears that the frameshift mutations in either exon 11 or 24 of *TSC2* would result in truncated TSC2 protein which might be loss-of-function. We have also noticed that in the tumor cells

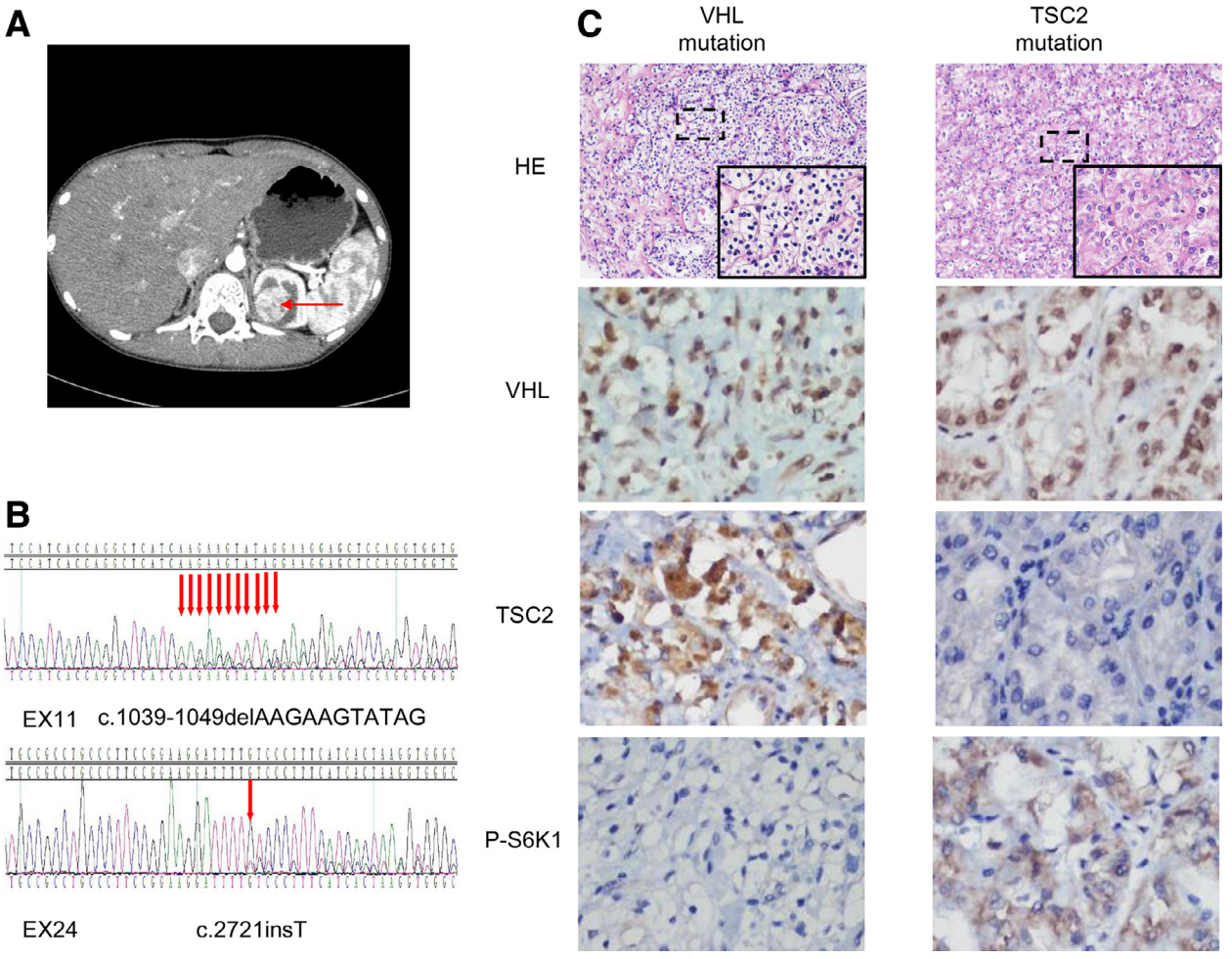

FIGURE 1. Characterization of *TSC2* mutation-induced RCC. (**A**) Cross-section of the CT scan revealed an RCC in the left kidney of the patient with an arrow indicating the location of the tumor. (**B**) Sanger sequencing identified the *TSC2* mutations with arrows indicating the mutation sites. (**C**) Hematoxylin-eosin staining (100× and 400×) and immunohistochemical staining (400×) of VHL, TSC2, and p-S6K1 in the RCC samples from this patient with *TSC2* mutations and another patient with VHL syndrome. (Color version available online.)

with *TSC2* mutation the TSC2 staining is negative. On the contrary, the TSC2 staining in samples of another patient with VHL syndrome is positive. Next, we examined the levels of downstream of TSC2 (p-S6K1) in tumor samples. The results substantiated that the mutant TSC2 proteins were loss-of-function evidenced by positive staining of p-S6K1 in the patient with *TSC2* mutations but negative staining with VHL syndrome. The VHL staining in both samples is positive with similar intensities (Fig. 1C). In addition, the papillary RCC associated-gene *SDHB* is not only expressed but with comparable intensities in these samples (data not shown).

The Effect of *TSC2* Mutations on the Levels of p-S6K1 and p-STAT3 in vitro

To elucidate the relationship between the above-mentioned *TSC2* mutations and RCC, we first tried to express either wild type or mutant TSC2 in *TSC2*-null cells by transient transfection of a plasmid expressing corresponding TSCs. The plasmids expressing either wild type or mutant TSC2 were successfully delivered to the cells and transcribed into their corresponding mRNAs evidenced by positive results from PCR (Fig. 2B,C). However, only the wild type, but not the mutant, TSC2 were detectable by western blot assays (Fig. 2A). Since the results from PCR indicate that the transfected plasmids were successfully transcribed, we speculate that the mutant and presumably misfolded TSC2s is quickly degraded in the cells. Consequently, phosphorylation of STAT3 and S6K1 in *TSC2*-null cells were repressed only when they were transfected with the plasmid expressing the wild type, but not the mutant, TSC2. Of note, the levels of STAT3, S6K1, and β-actin were comparable among in these experiments. These results altogether suggest that the mutant TSC2 might be quickly degraded and consequently lose repression of the downstream pathways, which ultimately leads to RCC oncogenesis.[2-4]

Literature Review

Among the 59 articles retrieved by searching the Medline database published between (1969 and 2017), 33 were included in this review. In 1986, Graves et al reported the first case of TSC associated RCC.[7] Since then, dozens of RCC cases associated with TSC have been reported although gene sequencing for the specific mutations was not conducted. In 1995, Sampson et al

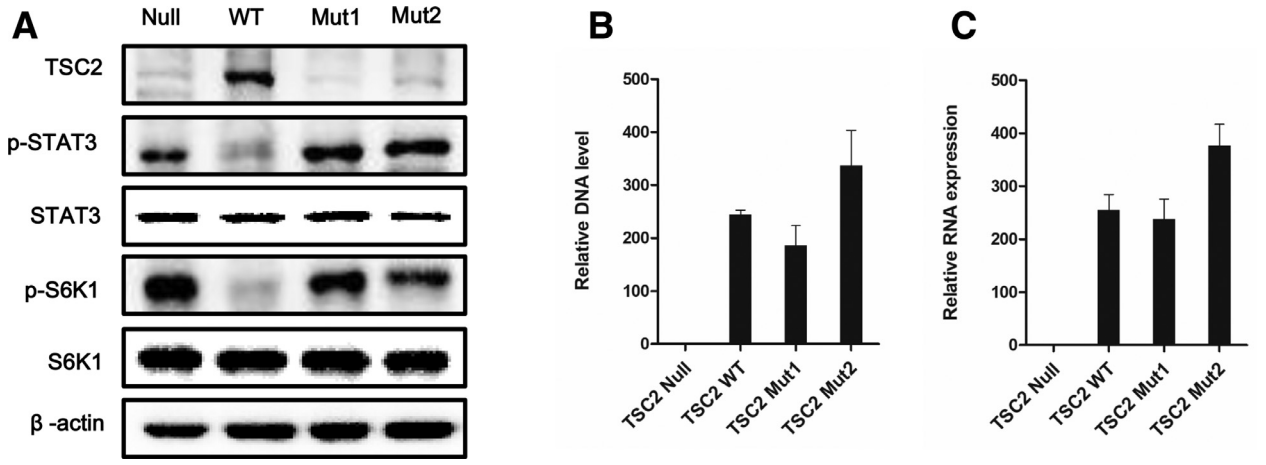

FIGURE 2. The efficiency of transfection, the expression, and the effects of the wild type and mutant *TSC2*. (**A**) The level of TSC2, as well as STAT3, p-STAT3, S6K1 and p-S6K1 in *TSC2*-null cells transfected with plasmids expressing wild type or mutant *TSC2*. (**B** and **C**) The efficiency of transfections (**B**) and transcription of *TSC2* (**C**) when the cells were transfected with plasmids expressing wild type or mutant *TSC2*.

reported RCC accompanied with a *TSC1* mutation in 2 sisters of the same family who also suffered from TSC.[12] In 1996, Bjornsson et al reported a case of RCC in a patient with TSC due to a loss-of-heterozygosity (LOH) in *TSC2*.[13] In 2011, Kucejova et al identified a somatic mutation in *TSC1* in 3 cases of TSC-associated ccRCC.[14] Not until 2015, have the first 2 cases of TSC-associated RCC with a somatic mutation in *TSC2* been reported by Tyburczy et al. Also in the same article, the second-hit theory was introduced to explain *TSC2* inactivation.[15] Samer Alsidawi et al described a patient with *TSC2*-associated metastatic RCC and showed the efficacy of mTOR inhibition in therapy.[16] Seven patients with eosinophilic solid and cystic RCC were reported with somatic biallelic loss of *TSC* gene.[17] So far, only 26 cases of RCC with *TSC* mutation have been reported and the characteristics of these patients were summarized in Table 1.

DISCUSSION

Approximately 2%-3% of RCC were considered as hereditary diseases, including VHL syndrome, hereditary papillary RCC and Birt-Hogg-Dubé syndrome commonly.[1] In addition, about 2%-4% of TSC patients also suffer from RCC.[2-4] It is well-established that germ line mutations in *TSC1/2* can cause RCC.[13,18-20] Somatic inactivation of *TSC1/2* either due to heterozygous loss of *TSC1* or mutations in *TSC1* and *TSC2* occurs in 1%-2% of adult RCC patients.[21] However, biallelic somatic inactivation of *TSC2* associated RCC in an adolescent is rare. Here we reported RCC in a female adolescent with 2 novel somatic inactivating mutations in *TSC2*. Additionally, results from *in vitro* experiments demonstrated that the mutant TCS2s were incapable of repressing its downstream pathways.

The classic 2-hit theory is a widely accepted mechanism in the oncogenesis of patients with TSC.[3,6,22] Tyburczy et al found that patients with TSC carrying germ line mutations in *TSC1* or *TSC2*. The second hit of the remaining allele was found in RCC.[15] Potter et al reported 2 somatic *TSC2* mutations in tumor samples from a 6-year-old female with RCC and methylmalonic acidemia.[21] Our findings in this report also strongly support the 2-hit theory. Of note, Tyburczy et al conducted next-generation sequencing on DNAs purified from blood/saliva and some skin biopsy samples of 53 TSC patients. They found that the majority (26 of 45, 58%) of the patients in this cohort have mosaic TSC mutations.[23] Although 2 distinct TSC2 mutations have been identified in the tumor of the patient reported in this article, we cannot exclude the possibility that the tumor tissue is made of cells expressing the 2 TSC2 mutations mosaically. Together with TSC1, TSC2 forms a complex essential for GTPase-activating and by hydrolyzing guanosine triphosphate (GTP) in Ras-homolog enriched in brain (RHEB) to inactivate mTORC1 pathway.[24] Therefore, loss-of-function of *TSC2* results in accumulation of GTP-RHEB and constitutive activation of mTORC1 which subsequently upregulates p-S6K1 and p-STAT3. Each of these factors plays important roles in the oncogenesis of RCC.[25,26] In the current case, we found that up-regulated p-S6K1 was accompanied by the mutations of *TSC2* in immunohistochemical staining of the tumor sample. P-STAT3 and p-S6K1, the downstream targets of mTORC1, were significantly elevated in *TSC2*-null cells transfected with plasmids expressing the *TSC2* mutants. These results suggest that *TSC2* mutation-caused mTORC1 pathway over-activation could be the underlying molecular mechanism for the development of ccRCC. Although plasmids expressing either wide type or mutant *TSC2* were successfully delivered and transcribed in *TSC2*-null cells (Fig. 2B,C), the mutant TSC2s were undetectable in western blots. We hypothesized that the mutant TSC2s are misfolded and quickly degraded.[27] However, this needs to be confirmed experimentally in future studies.

Renal lesions are the most frequent manifestation in patients with TSC with 70%-80% having angiomyolipomas, 20% with renal cysts although RCC is much less

Table 1. Published cases of RCC in patients with mutation in TSC1 or TSC2

ID	Sex	Age at Onset	Somatic/Germline Mutation in TSC	Loss or Mutation	Mutation Site	Protein	Histology	Authors and Year
1	F	38	Germ line	LOH in TSC1	NA	NA	Clear cell renal cell carcinoma	Bjornsson et al (1996)[13]
2	M	23	Germ line	LOH in TSC1	NA	NA	Anaplastic and clear cell renal cell carcinoma	Bjornsson et al (1996)[13]
3	F	34	Germ line	LOH in TSC2	NA	NA	Anaplastic and clear cell renal cell carcinoma	Bjornsson et al (1996)[13]
4	M	7	Germ line	Mutation in TSC2	c.1832G>A	p.R611Q	Renal cell carcinoma	Pressey et al (2010)[18]
5	NA	NA	Somatic	Mutation in TSC1	c.1546C>T	p.Q516X	Clear cell renal cell carcinoma	Kucejova et al (2011)[14]
6	NA	NA	Somatic	Mutation in TSC1	IVS211-2A>T	Sp	Clear cell renal cell carcinoma	Kucejova et al (2011)[14]
7	NA	NA	Somatic	Mutation in TSC1	c.1342C>T	p.P448S	Clear cell renal cell carcinoma	Kucejova et al (2011)[14]
8	F	3-month	Germ line	Mutation in TSC2	NA	NA	Papillary renal cell carcinoma	Kubo et al (2011)[19]
9	M	17-month	Germ line	Mutation in TSC2	NA	NA	Renal cell carcinoma	Kubo et al (2011)[19]
10	F	24	Germ line and somatic	Mutation in TSC2	c.2714G>A (G); c.4051G>T (S);	p.R905Q(G); p.E1351 (S)	Papillary renal cell carcinoma	Tyburczy et al (2015)[15]
				Mutation in TSC2	c.2714G>A (G); c.4178_4179delCT (S)	p.R905Q (G);	Papillary renal cell carcinoma	Tyburczy et al (2015)[15]
				Mutation in TSC2	c.2714G>A (G); c.3094C>T (S)	p.R905Q (G); p.R1032 (S)	Papillary renal cell carcinoma	Tyburczy et al (2015)[15]
				Mutation in TSC2	c.2714G>A (G); c.5138G>A (S)	p.R905Q (G); p.R1713H (S)	Papillary renal cell carcinoma	Tyburczy et al (2015)[15]
				LOH in TSC2	c.2714G>A (G)	p.R905Q(G)	Papillary renal cell carcinoma	Tyburczy et al (2015)[15]
11	M	36	Germ line and somatic	Mutation in TSC2	c.2714G>A (G); c.2355+1G>T (S)	p.R905Q (G)	Papillary renal cell carcinoma	Tyburczy et al (2015)[15]
12	F	14	Germ line	Mutation in TSC1	c.659T>A	NA	Renal cell carcinoma	Peron et al (2016)[20]
13	F	39	Germ line	Mutation in TSC1	c.458T>A	NA	Chromophobe renal cell carcinoma	Peron et al (2016)[20]
14	F	41	Germ line	Mutation in TSC1	c.458T>A	NA	Chromophobe renal cell carcinoma	Peron et al (2016)[20]
15	F	16	Germ line	Mutation in TSC1	c.547_571del25	NA	Clear cell renal cell carcinoma	Peron et al (2016)[20]
16	M	NA	Germ line	Mutation in TSC1	c.2681_2682delAT	NA	Renal oncocytoma	Peron et al (2016)[20]
17	F	25	Germ line	Mutation in TSC2	c.1852delC	NA	Clear cell renal cell carcinoma	Peron et al (2016)[20]
18	F	6	Somatic	Mutation in TSC2	c.246G>A; c.3370_3382del	p.W82X; p.A1124fs	Renal cell carcinoma	Potter et al (2017)[21]
19	M	47	Germ line	Mutation in TSC2	c.4859A>G c.4949A>G	p.H1620R p.Y1650C	Papillary RCC	Alsidawi et al (2018)[16]
20	NA	NA	Somatic	Mutation in TSC2	NA	p.L741fs p.K533Splice	Eosinophilic solid and cystic RCC	Mehra et al (2018)[17]

Continued

Table 1. Continued

ID	Sex	Age at Onset	Somatic/Germline Mutation in TSC	Loss or Mutation	Mutation Site	Protein	Histology	Authors and Year
21	NA	NA	Somatic	LOH in TSC2	NA	p.C519fs	Eosinophilic solid and cystic RCC	Mehra et al (2018)[17]
22	NA	NA	Somatic	LOH in TSC2	NA	p.Y349fs	Eosinophilic solid and cystic RCC	Mehra et al (2018)[17]
23	NA	NA	Somatic	Mutation in TSC2	NA	p.L773* p.N1250fs	Eosinophilic solid and cystic RCC	Mehra et al (2018)[17]
24	NA	NA	Somatic	Nonesense	NA	p.S9*	Eosinophilic solid and cystic RCC	Mehra et al (2018)[17]
25	NA	NA	Somatic	LOH in TSC1	NA	p.Y176fs	Eosinophilic solid and cystic RCC	Mehra et al (2018)[17]
26	NA	NA	Somatic	Mutation in TSC1	NA	p.Q653* p.Q696fs	Eosinophilic solid and cystic RCC	Mehra et al (2018)[17]

Abbreviations: F, Female; G, Germ line; LOH, Loss of heterozygosity; M, male; NA, Not Available; S, Somatic; TSC, Tuberous Sclerosis Complex.
* stop condon.

common.[5] In RCC patients with familial TSC, tumors often occur bilaterally with a tendency of early-onset. The average onset of RCC in patients with TSC is 28-year-old, 25 years earlier than that in the general population.[28] The onset of RCC in the current case is 14, which is not only much earlier than that of the general population but also the average onset age in patients with TSC. Contrary to the general distribution pattern in most RCC patients with TSC, the tumor is unilateral (the left kidney) and solitary.

RCC in TSC generally shows histological heterogeneity[29] with different subtypes of RCC including ccRCC, papillary RCC, chromophobe carcinoma, and oncocytomas.[6] Among the 46 renal neoplasms from 19 TSC patients, Yang et al identified 3 subtypes: 52% are TSC-associated papillary RCC with a deficiency in succinate dehydrogenase subunit B (SDHB); 33% showed morphological features similar to a hybrid oncocytic/chromophobe tumor (HOCT), and 15% remained unclassifiable.[4] However, Guo et al reported that among 57 RCCs from 18 patients with TSC, 59% of the tumor was similar to chromophobe RCC; 30% resemble "renal angiomyoadenomatous tumor" or "RCC with smooth muscle stroma"; 11% of the tumors exhibited a granular eosinophilic-macrocystic morphology.[30] These findings suggest that TSC-associated RCC has distinctive pathological features. In the current case, a ccRCC subtype characterized by the regular nucleus, thin-walled vessels, positive CK, Vimentin, CD10, RCC staining and negative CA9, CK7, TFE-3 staining was confirmed by 2 independent pathologists.

Inhibitors of the mTOR signaling pathway are FDA-approved therapeutic reagents for adult patients with relapsed or metastatic RCC.[21] Multiple lines of evidence suggest that gene mutations in the mTOR pathway (including *TSC1*, *TSC2*, and *mTOR*) respond well to mTOR inhibition.[30] Based on the elevated level of p-S6K1 and p-STAT3, we speculate that the mTOR pathway in the current case could be super active. Although no signs of recurrence nor metastatic in this patient were seen so far, we believe that mTOR inhibitor could be administered once the tumor recurs or the patient is under situations unsuitable for surgery. In summary, we reported a rare case of ccRCC with novel biallelic somatic mutations in *TSC2*. This provides new insight into the mechanism of RCC oncogenesis. In addition, genetic testing for a *TSC2* mutation in patients with early RCC onset could be beneficial in both diagnosis and therapy. Finally, mTOR inhibitor could be a therapeutic option for *TSC2* mutation-induced RCC.

Acknowledgment. The authors are grateful to the patient and her family.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.urology.2019.08.016.

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