Molecular Characterization and Putative Pathogenic Pathways of Tuberous Sclerosis Complex–Associated Renal Cell Carcinoma

Abstract
Tuberous sclerosis complex–associated renal cell carcinoma (TSC-RCC) has distinct clinical and histopathologic features and is considered a specific subtype of RCC. The genetic alterations of TSC1 or TSC2 are responsible for the development of TSC. In this study, we assessed the mTOR pathway activation and aimed to evaluate molecular characteristics and pathogenic pathways of TSC-RCC. Two cases of TSC-RCC, one from a 31-year-old female and the other from an 8-year-old male, were assessed. The mTOR pathway activation was determined by immunohistochemistry. The mutational spectrum of both TSC-RCCs was evaluated by whole exome sequencing (WES), and pathogenic pathways were analyzed. Differentially expressed genes were analyzed by NanoString Technologies nCounter platform. The mTOR pathway activation and the germline mutations of TSC2 were identified in both TSC-RCC cases. The WES revealed several cancer gene alterations. In Case 1, genetic alterations of CHD8, CRISPLD1, EPB41L4A, GNA11, NOTCH3, PBRM1, PTPRU, RGS12, SETBP1, SMARCA4, STMN1, and ZNRF3 were identified. In Case 2, genetic alterations of IWS1 and TSC2 were identified. Further, putative pathogenic pathways included chromatin remodeling, G protein–coupled receptor, Notch signaling, Wnt/β-catenin, PP2A and the microtubule dynamics pathway in Case 1, and mRNA processing and the PI3K/AKT/mTOR pathway in Case 2. Additionally, the ALK and CRLF2 mRNA expression was upregulated and CDH1, MAP3K1, RUNX1, SETBP1, and TSC1 mRNA expression was downregulated in both TSC-RCCs. We present mTOR pathway activation and molecular characteristics with pathogenic pathways in TSC-RCCs, which will advance our understanding of the pathogenesis of TSC-RCC.

Introduction
Renal cell carcinoma (RCC) is one of the most fatal genitourinary tumors and accounts for approximately 90% of renal cancers [1]. Histopathologic features and molecular studies have identified and classified various subtypes of RCC [2, 3]. These subtypes include clear cell RCC (CCRCC), papillary RCC (PRCC), chromophobe RCC (ChRCC), MiT family translocation RCC, and clear cell papillary RCC. Additionally, syndrome-associated hereditary renal cell tumors, including Von Hippel–Lindau syndrome, hereditary papillary RCC,
and tuberous sclerosis, have been identified. The National Cancer Data Base revealed 5-year survival rates of 80.9% in stage I, 73.7% in stage II, 53.3% in stage III and 8.2% in stage IV kidney cancer patients [4]. Various aspects of the treatment for advanced RCC and metastatic RCC have been studied, and target therapy and immunotherapy have shown considerable improvement in patient survival [5–7].

Tuberous sclerosis complex–associated RCC (TSC-RCC) is an emerging subtype of RCC [8, 9]. TSC showed autosomal dominant inheritance and was characterized by multisystem disorders, including epilepsy, developmental delay, angiofibromas, hypomelanotic macules, cortical dysplasias, lymphangiomyomatosis, and angiomylipoma (AML) [10, 11]. The disease is caused by alterations in TSC1 or TSC2 genes, which encode hamartin and tuberin, respectively [10]. Studies of TSC-RCC from multiple institutions have revealed the clinical and histopathologic features of TSC-RCC [8, 9]. Clinically, TSC-RCC is characterized by an association with TSC, female predominance, early age of onset, and indolent clinical outcomes. Histopathologically, TSC-RCC has been classified according to several distinct morphologies, including renal angiomyoadenomatous tumor (RAT)–like, TSC-associated papillary RCC, chromophobe-like or hybrid oncocytic/chromophobe tumor (HOCT)–like, eosinophilic/macrocystic, and unclassified RCC.

Cancer genomics have greatly expanded our knowledge of cancer biology. In kidney cancers, CCRCC, PRCC, and ChRCC were analyzed, and important genomic events and pathways were elucidated [12–14]. Moreover, actionable targets for RCC have been identified, and patients with those alterations have been enrolled in clinical trials [15, 16]. As precision medicine has been initiated in earnest, unveiling the genomic landscape of cancer has progressed, resulting in promising improvements in treatment modalities and patient prognoses [17, 18]. Additionally, studies of rare but specific genetic alterations, such as those in TSC-RCC, will advance our understanding of cancer biology and the discovery of novel cancer-related genes.

In this study, we assessed the activation of the mTOR pathway and the genetic alterations in two cases of TSC-RCC by immunohistochemistry and whole exome sequencing (WES). Additionally, we analyzed mRNA expression of cancer genes. We aimed to evaluate the mutation spectrum of both patients and search for the genetic basis of the pathogenesis and actionable targets of TSC-RCC.

Material and Methods

Patient Selection and Clinicopathologic Review

We retrospectively reviewed all RCCs surgically removed by radical or partial nephrectomy between January 1, 2009, and December 31, 2014, at Seoul National University Hospital. We reviewed the medical records to identify TSC patients and identified two cases of TSC-RCC. Each TSC-RCC was evaluated with regard to the clinical and histopathologic features, such as history of epilepsy, RCC histologic type, WHO/ISUP grade [19], and the stage of the tumor [4]. Disease progression was determined based on the clinical and radiographic findings and the patients’ medical records. This study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. H-1602-040-739).

Immunohistochemistry

We performed immunohistochemistry on a representative slide from TSC-RCC cases (Supplementary Table 1). For the differential diagnosis, we assessed pan-cytokeratin, HMB-45, Melan A, CD10, CK7, and c-kit immunoreactivity. To evaluate mTOR pathway activation, we assessed phospho-mTOR immunoreactivity. Immunohistochemical staining was performed using autostainers for each antibody. The binding of the primary antibody was detected using a detection kit according to the manufacturer’s instructions.

WES and Bioinformatics Analysis [12–14, 20–37]

Normal and cancer tissue were obtained from formalin-fixed, paraffin-embedded tissue block in both TSC-RCCs. DNA was extracted, and the quality of DNA was checked. WES was performed using HiSeq 2500 sequencing system (Illumina). Sequencing libraries were prepared, and adapter ligated DNA was amplified. Sequence was mapped to NCBI b37 human reference genome sequence, and BAM files were realigned. Germline and somatic mutation calling was performed. The alterations of cancer-related genes were identified. Detailed methods are described in Supplementary methods (Supplementary Figure 1, Supplementary Tables 2 and 3).

Copy Number Variation and Loss of Heterozygosity Analysis [22, 38–41]

Copy number variations (CNVs) and large loss of heterozygosities (LOHs) were assessed in WES data. In each case, read count was evaluated and normalized using paired normal and cancer sequencing data. The reads per kilobase million (read count/exon length/total read count × 109) ratio was assessed for detecting amplification or deletion. VAF was evaluated for assessing the CNVs in megabase scale. Detailed methods are described in Supplementary methods.

Validation of Cancer Genes Alterations

We performed Sanger sequencing for the validation of cancer genes in TSC-RCC cases. Additionally, we performed droplet digital PCR for cancer genes in TSC-RCC Case 1 due to low tumor purity (inflammatory cell infiltration) and obscuring factors (abscess). Detailed methods are described in supplementary methods (Supplementary Tables 4 and 5).

Pathway Analysis

For the analysis of pathogenic pathways for each TSC-RCC, we selected cancer genes according to the following criteria: depth (≥10×), VAF (≥20%), score (≥3), no strand bias, altered function (≥2 prediction method), tumor suppressor genes (TSGs), or oncogenes (OGs) [31]. We used KEGG pathway maps (http://www.genome.jp/kegg/pathway.html) [36, 37] and Ingenuity pathway analysis (http://www.ingenuity.com/products/pipe) [42] for pathway analysis. Additionally, we used STRING (http://string-db.org) [43] for assessment of protein-protein interaction networks.

mRNA Expression Analysis

Total RNA was extracted using an eCube RNA Mini Kit (Philekorea Technology, Seoul, Korea). RNA yield and purity were assessed using a DS 11 Spectrophotometer (Denovix Inc., DE, USA). Total RNA (300 ng) was added to the sample preparation reaction in the available 5 μl volume. RNA quality was verified using a Fragment Analyzer (Advanced Analytical Technologies, IA, USA). The digital multiplexed nanoString nCounter human mRNA expression assay (nanoString Technologies) was performed. The mRNA data analysis was performed using the nSolver software analysis. The mRNA profiling data were normalized using housekeeping genes.

Comparison with Reported Data and Public Database

We analyzed the sequencing results with the COSMIC, TCGA, and eBioPortal database [12, 13, 33–35]. Genetic alterations were compared...
to common RCCs (CCRCC, PRCC, and ChRCC) sequencing data [12–14]. Also, we compared our results to genetic data of TSC-associated papillary RCC [44] and molecular characteristics of eosinophilic/macrocystic RCC [45, 46]. Potential actionable targets were evaluated by matching molecular targets of FDA-approved drugs [47] and Tumor Alterations Relevant for GEnomics-driven Therapy database (http://www.broadinstitute.org/cancer/cga/target).

**Results**

**Clinical Features of TSC-RCC Patients**

The Case 1 patient was a 31-year-old female with a history of seizures from a young age who was clinically diagnosed with TSC for her facial angiofibromas and subependymal nodules. The patient had multiple renal masses, and one of them was diagnosed as AML. Upon follow-up, she visited the hospital for abdominal pain, and a 15.4 × 10.0–cm mass was detected in the right kidney. The Case 2 patient was an 8-year-old male with a history of seizures from 12 months who was suspected to have TSC due to his facial angiofibromas. On workup, multiple subependymal nodules and cortical tubers were identified. Additionally, a 5.2 × 7.1 × 6.0–cm mass was detected in the left kidney. Radical nephrectomy was performed on both patients.

**Histopathologic Features of TSC-RCC Cases**

The TSC-RCC Case 1 consisted of a 16.5 × 10.5 × 7.9–cm solid mass with hemorrhage and necrosis (Figure 1, upper panel). The tumor showed sheet-like growth pattern and was composed of discohesive large atypical cells with ample, light eosinophilic cytoplasm and vesicular nucleus with prominent nucleolus. The emperipolysis, neutrophilic infiltration, and abscess as well as angiolymphatic invasion were identified. WHO/ISUP grade was 4 and pTNM stage was II (pT2bN0M0). The TSC-RCC Case 2 consisted of a 7.3 × 5.3 × 4.0–cm solid mass with focal cystic change (Figure 1, lower panel). The tumor showed trabecular growth with atypical cells with plump, eosinophilic, and granular cytoplasm. The tumor cells revealed vesicular and wrinkled nuclei with small nucleoli. The cysts showed hobnail pattern of cyst lining cells with plump eosinophilic granular cytoplasm. Additionally, the tumor revealed hyalinized stroma. The angiolymphatic invasion was identified. WHO/ISUP grade was 2 and pTNM stage was II (pT2aN0M0). Both cases had multiple renal AMLs. The Case 2 patient had multiple variable-sized renal cysts lined by epithelial cells with plump eosinophilic cytoplasm, which was reported as a histologic feature of epithelial cysts in TSC [48] or cuboidal cells. Immunohistochemically, both cases were negative for myomelanocytic markers (HMB-45, Melan A) and positive for epithelial marker (pan-cytokeratin), suggesting RCC rather than epitheloid AML. Case 1 can be classified as unclassified RCC and Case 2 as RCC with eosinophilic/macrocystic feature. Additionally, phospho-mTOR was positive in TSC-RCCs but not in adjacent unaffected renal parenchyma.
Germline Mutations of TSC1 or TSC2 Genes in TSC-RCC Cases

We regarded identical mutations on both normal and cancer tissues as germline mutations. In Case 1, a TSC2 c.4707C > A (p. Tyr1569*) mutation was identified, and in Case 2, a TSC2 c.2548+2 T > G mutation was seen (Table 1 and Supplementary Figure 2), which were stop gained effect and a splice donor variant, respectively.

Somatic Mutations and Alterations of Cancer-Related Genes in TSC-RCC Cases

The somatic mutations of SNV or small indels were analyzed (Supplementary Figure 3, Supplementary Tables 6 and 7). In Case 1, a total of 589 mutations (567 SNVs, 1 insertion, and 21 deletions) were identified. In Case 2, a total of 258 mutations (257 SNVs and 1 deletions) were identified. Further, alterations of cancer-related genes were assessed. In Case 1, 72 cancer-related genes were identified (Figure 2A and Supplementary Table 8). There were 69 SNVs and 3 deletions, including 58 missense mutations, 6 nonsense mutations, 5 splice site SNVs, 2 in frame deletions and 1 frame shift deletion. In Case 2, we identified 32 altered cancer-related genes. These included 23 missense mutations, 5 nonsense mutations, 3 splice site SNVs, and 1 frame shift deletion.

Genetic Alterations of Cancer Genes in TSC-RCC Cases

To assess the pathogenic basis of each TSC-RCC case, we analyzed alterations of cancer genes (Table 2). In Case 1, we identified three potentially actionable targets. These included CHD8, PBRM1, and SMARCA4. In Case 2, a potential actionable target was identified. It was TSC2, and mTOR inhibitors can be considered, respectively. In Case 2, there was one potentially actionable target. It was TSC2, and mTOR inhibitors can be considered for targeted therapy.

Potential Actionable Targets in TSC-RCC Cases

In Case 1, we identified three potentially actionable targets. These included GNA11, NOTCH3, and ZNRF3 for which MAPK pathway inhibitors, gamma secretase inhibitors, and porcupine inhibitors can be considered, respectively. In Case 2, there was one potentially actionable target. It was TSC2, and mTOR inhibitors can be considered for targeted therapy.

Differentially Expressed Genes in TSC-RCC Cases

We assessed 770 genes to identify differentially expressed genes (DEGs) (Supplementary Table 11). Case 1 includes 20 upregulated and 33 downregulated genes with a two-fold change, and Case 2 has 202 upregulated and 308 downregulated genes with a two-fold change (Supplementary Table 12). Among those, the ALK and CRLF2 mRNA expression was upregulated and CDH1, MAP3K1, RUNX1, SETBP1, and TSC1 mRNA expression was downregulated in both TSC-RCCs. Cancer-related pathways with DEGs are presented in Supplementary Figures 8 and 9.

Figure 2. Somatic mutational spectrum of cancer-related genes in TSC-RCC cases. (A) TSC-RCC Case 1 and (B) TSC-RCC Case 2. (C) Circos plot inset legend.
The histopathologic features of TSC-RCC have been elucidated [8, 9]. One study classified them as TSC-associated papillary RCC (52%, 24 cases), HOCT (33%, 15 cases), and unclassified RCC (15%, 7 cases) [9]. The authors emphasized the uniformly deficient expression of SDHB in TSC-associated papillary RCC. Another study classified TSC-RCC as RAT-like (30%, 17 cases), chromophobe-like (59%, 34 cases), and eosinophilic/macrocytic RCC (11%, 6 cases) [8]. Though those studies classified different histopathologic subtypes, TSC-associated papillary RCC and RAT-like RCC have similar

| Case | Gene | rsID | Reference | Alternate Variant | Effect | Impact | Feature Type | HGVS.c | HGVS.p | 1000G Maf | 1000G ASN | VAF (%) | Polyphen2 | SIFT | Validation |
|------|------|------|-----------|------------------|--------|--------|--------------|--------|--------|----------|-----------|---------|----------|------|-----------|
| 1    | TSC2 |      |           | C    G         | SNV    | Stop gained | HIGH Transcript | c.4707C > A | p.Tyr1569* | NA       | NA       | 45.35   | .        | .        | +      |
| 2    | TSC2 |      |           | T    G         | SNV    | Splice donor variant | HIGH Transcript | c.2578+2 T > G | NA       | NA       | 52.00   | .        | .        | +      |

Abbreviations: NA, not available; VAF, variant allele frequency.

* Sanger sequencing and droplet digital PCR were performed in normal and tumor tissues from both cases.

---

**Discussion**

The histopathologic features of TSC-RCC have been elucidated [8, 9]. One study classified them as TSC-associated papillary RCC (52%, 24 cases), HOCT (33%, 15 cases), and unclassified RCC (15%, 7 cases) [9]. The authors emphasized the uniformly deficient expression of SDHB in TSC-associated papillary RCC. Another study classified TSC-RCC as RAT-like (30%, 17 cases), chromophobe-like (59%, 34 cases), and eosinophilic/macrocytic RCC (11%, 6 cases) [8]. Though those studies classified different histopathologic subtypes, TSC-associated papillary RCC and RAT-like RCC have similar...
histologic features and could be categorized as RCC with (angio) leiomyomatous stroma [3]. Also, HOCT and chromophobe-like RCC could be regarded as one subtype.

The mTOR activation has been thought to be one of the pathogenic alterations in TSC-RCC because alterations of TSC1 or TSC2 genes were responsible for the development of TSC. However, the pathogenic role of mTOR activation on TSC-RCC pathogenesis has not been studied. We identified mTOR activation by phospho-mTOR immunohistochemistry. Our results suggest that the mTOR pathway is activated and responsible for the pathogenesis and serves as a rationale for the possible use of mTOR inhibitor in our two patients with TSC-RCC.

We performed WES and reported genetic alterations in TSC-RCC, especially first results of unclassified and eosinophilic/macrocytic RCC. There were genetic analysis of 5 TSC-associated papillary RCC cases from 1 patient and molecular karyotyping of 15 eosinophilic/macrocytic RCC cases without clinical evidence of TSC [44-46]. In TSC-associated papillary RCC cases, there were second-hit mutations (3 SNVs, 1 indel, and 1 LOH) in TSC2, and somatic mutations of PROS1, NFPE2R, TLL2, and RASA1 were identified [44]. In sporadic cases of eosinophilic/macrocytic RCC, copy number gain of 16p-q, 7p-q, 13q, 19p, 1p, and 10q; copy number loss of Xp, 22q, 19p, 19q, and Xq; and LOH in 16p, Xq, 11p, and 9q were identified [45, 46].

In our cases, the germline mutations of TSC2 were identified, a TSC2 nonsense mutation in Case 1 and a TSC2 splice donor variant mutation in Case 2. For the development of tumors related to TSC, biallelic TSC2 inactivation is needed. However, we could not find additional TSC2 mutations or LOH in Case 1. There is the possibility that other types of mutations (large indel or epigenetic alterations), not detected in WES, may exist in the Case 1 patient or TSC2 inactivation was not responsible for mTOR activation, and tumor progression as histopathologic feature of Case 1 was truly unclassifiable. In Case 2, there was additional TSC2 somatic mutation.

The somatic mutation analysis showed different genetic mutation profiles from common RCCs. In Case 1, all genetic alterations, except KMT2C (in PRCC) and PBRM1 (in CCRCC), were found with a less than 5% frequency with those in common RCCs. In Case 2, there were no common genes, except the TG gene, which showed 3.08% frequency with those in ChRCC. These genetic features support the idea that TSC-RCC should be classified as a distinct entity. The USP34 and NDE1 alteration in TSC-associated papillary RCC was found in Case 1 and 2, respectively.

We aimed to elucidate the pathogenic basis of TSC-RCC. In Case 1, we selected 12 cancer genes and identified 6 pathways. The CHD8 gene acts as a TSG and is associated with the chromatin remodeling pathway and affects cell survival and cell proliferation. CHD8 c.2368C > T, p.R790C mutation was previously reported in malignant melanoma and is considered pathogenic based upon FATHMM [33]. The pathogenic role on cancer of CRISPLD1 and EPB41L4A genes was not studied well. CRISPLD1 c.1363C > T, p.R455* was in LCCl domain and has not been previously reported. Also, EPB41L4A c.1618C > T, p.R540C mutation has not been previously reported. The GNA11 gene is an OG and is a component of GPCR pathway and involved in cell proliferation, invasion, and differentiation. GNA11 c.604C > T, p.R202W mutation located in G-alpha domain has not been previously reported. The NOTCH3 gene acts as OG and belongs to Notch signaling pathway and is involved in stem-like properties, cell differentiation, and proliferation. NOTCH3 c.1194C > T, p.G398G mutation in EGF_CA domain has previously been reported in urothelial

| Case | Score | Gene | Driver Role | Variant Classification | Variant Type | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Variant | Reference | Variant | Vari
The SMARCA4 c.3067G:p.G17R mutation was previously reported in prostate adenocarcinoma; malignant melanoma [36, 37]. The carcinoma of urinary bladder, hepatocellular carcinoma, and cutaneous malignant melanoma [36, 37]. The PBRM1 gene is a TSG and is a component of chromatin remodeling pathway and is involved in cell cycle progression, invasiveness, and stemness. PBRM1 c.49G>T, p.G17R mutation was previously reported in prostate adenocarcinoma [34, 35]. The PTPRU gene acts as a TSG and affects Wnt/β-catenin pathway and is involved in cell proliferation, focal adhesion, and invasiveness. PTPRU c.1412G>A, p.R471H mutation was not previously reported. The RGS12 gene is associated with GPCR pathway; however, the pathogenic role on cancer was not established well. RGS12 c.4073C>T, p.R1358L mutation was in RGS12 usC domain and was not previously reported. The SETBP1 gene is an OG and affects PP2A pathway and cell proliferation, apoptosis, and cell migration. SETBP1 c.2572G>A, p.E858K mutation was previously reported in esophageal carcinoma, cutaneous malignant melanoma, esophagus-stomach cancer, and hematopoietic neoplasm [33–35]. The FATHMM prediction was pathogenic [33]. The SMARCA4 is a TSG and is a component of the chromatin remodeling pathway and is involved in cell cycle progression, invasiveness, and stemness. SMARCA4 c.3067G>A, p.E1023K mutation was in SNF2_N domain and previously reported in colorectal adenocarcinoma and lung cancer [33–35]. The STMN1 gene acts as an OG and affects microtubule dynamics and is involved in cell cycle progression, invasiveness, and metastasis. STMN1 c.235G>A, p.E79K was in Stathmin domain and has not been reported previously. The ZNRF3 acts as a TSG and affects Wnt/β-catenin pathway and is involved in cell proliferation, apoptosis, and invasiveness. ZNRF3 c.1361G>A, p.R454H has not been previously reported.

In Case 2, we identified two cancer genes and pathways. The IWS1 gene acts as an OG and involved in mRNA processing and tumor growth, migration, and invasiveness. IWS1 c.2048A>G, p.N683S mutation was in Med26 domain and was not previously reported. The TSC2 gene is thought to be a TSG and is involved in PI3K/ AKT/mTOR pathway and affects cell proliferation, metabolism, and cell survival. TSC2 c.1372C>T, p.R458* was in DUF3384 domain and was previously reported in sporadic pulmonary lymphangioleiomyomatosis [33]. The FATHMM prediction was pathogenic.

It is difficult to determine whether the genetic alterations are pathogenic or not. The “20/20 rule” can be a helpful criterion for identifying driver genes [30]; however, it cannot tell us whether any mutations that are not well documented are pathogenic. One study evaluated mutational signature patterns and reported >200 each TSGs and OGs [31]. However, an aneuploidy pattern without pathogenic features can lead to misinterpretation. The oncogenic IWS1 gene is predicted as TSG in that study. Mutational features of well-known genes are relatively well documented. For instance, APC

| Case | Gene | Driver | Role | Pathway | Biologic Function | Tumors |
|------|------|--------|------|---------|------------------|--------|
| 1    | CHD8 | -      | TSG > OG | Chromatin remodeling | Cell survival; cell proliferation | Hematopoietic malignancy, gastric cancer, colorectal cancer, prostate cancer, breast cancer |
|      | CRISP5L    | -      | -    | -       | -                | -      |
|      | EP4111A4   | -      | -    | -       | -                | -      |
|      | GNA11      | +      | OG   | GPCR pathway | Cell proliferation; cell survival; invasion; apoptosis; differentiation; migration | Melanoma, mesothelioma, endometrial cancer, esophageal cancer, breast cancer, ovarian (mucinous) cancer |
|      | NOTCH3     | -      | OG > TSG | Notch signaling pathway | Stem-like property; differentiation; cell proliferation; cell motility; invasiveness; metastasis; cell adhesion; epithelial mesenchymal transition; apoptosis; cellular senescence | Breast cancer, T-cell acute lymphoblastic leukemia, B-cell acute lymphoblastic leukemia, ovarian cancer, lung cancer, oral squamous cell carcinoma, pancreatic ductal adenocarcinoma, colorectal carcinoma, skin cancer, melanoma, hepatocellular carcinoma, thyroid cancer, cholangiocarcinoma, renal cell carcinoma, gastric cancer, esophageal cancer, laryngeal cancer, glioblastoma, endometrial cancer, EBV-associated nasopharyngeal cancer, cervical squamous cell carcinoma, chondrosarcoma, Ewing sarcoma family of tumors |
|      | PBRM1      | +      | TSG  | Chromatin remodeling | Cell cycle progression; invasiveness; stemness; differentiation | Clear cell renal cell carcinoma, breast cancer, bladder cancer, cholangiocarcinoma, mesothelioma, gallbladder cancer, prostate cancer, thymic carcinoma, gastric cancer |
|      | PTPRU       | -      | TSG > OG | Wnt/β-catenin pathway | Cell proliferation, focal adhesion; cell motility; invasiveness | Non–small cell lung carcinoma, small cell lung carcinoma, colon cancer, endometrial cancer, stomach cancer, glioma, melanoma |
|      | RGS12       | -      | -    | GPCR pathway | -                | -      |
|      | SETBP1      | +      | OG   | PP2A pathway | Cell proliferation; apoptosis; cell survival; cell migration; differentiation | Small cell carcinoma of the ovary, hypercalcemic type, non–small cell lung carcinoma, amplyllia and pancreatic ductal adenocarcinoma, endometrioid adenocarcinoma, colorectal cancer, rhodoid tumor, thoric sarcoma, Wilm tumor, neuroendocrine carcinoma, Burkitt lymphoma, oligodendroglioma, gastric cancer, thymic carcinoma, clear cell renal cell carcinoma, mantle cell lymphoma, cervical cancer, medulloblastoma |
|      | SMARCA4     | +      | TSG  | Chromatin remodeling | Cell cycle progression; invasiveness; stemness; differentiation | Gastric cancer, breast cancer, non–small cell lung carcinoma, gallbladder cancer, cutaneous squamous cell carcinoma, oral squamous cell carcinoma, colorectal cancer, osteosarcoma, melanoma, bladder cancer, ovarian cancer, high grade pelvic serous carcinoma, prostate cancer, hepatocellular carcinoma, endometrial cancer, acute myelogenous leukemia, lymphoma, neuroblastoma, mesothelioma, HPV-positive osteopharyngeal carcinoma, hypopharyngeal squamous cell carcinoma, nasopharyngeal carcinoma, laryngeal squamous cell carcinoma, small cell lung carcinoma, myelodysplastic syndrome, glioma |
|      | STMN1       | -      | OG   | Microtubule dynamics | Cell cycle progression; invasiveness; metastasis | Colorectal cancer, gastric cancer, lung cancer, papillary thyroid carcinoma, osteosarcoma, adrenocortical carcinoma |
|      | ZNRF3       | -      | TSG  | Wnt/β-catenin pathway | Cell proliferation; apoptosis; cell cycle progression; invasiveness | Lung cancer, colon cancer, hepatocellular carcinoma |
| 2    | IWS1        | -      | OG (+ TSG) | mRNA processing | Tumor growth; migration; invasiveness | Lymphangioleiomyomatosis, renal angiomyolipoma, head and neck squamous cell carcinoma, renal cell carcinoma, hamartoma, cortical tuber, subependymal giant cell astrocytoma, angiofibroma |
|      | TSC2        | -      | TSG  | PI3K/AKT/mTOR pathway | Cell proliferation; cell growth; metabolism; angiogenesis; cell survival; cell mobilization | Breast cancer, T-cell acute lymphoblastic leukemia, B-cell acute lymphoblastic leukemia, ovarian cancer, lung cancer, oral squamous cell carcinoma, pancreatic ductal adenocarcinoma, colorectal carcinoma, skin cancer, melanoma, hepatocellular carcinoma, thyroid cancer, cholangiocarcinoma, renal cell carcinoma, gastric cancer, esophageal cancer, laryngeal cancer, glioblastoma, endometrial cancer, EBV-associated nasopharyngeal cancer, cervical squamous cell carcinoma, chondrosarcoma, Ewing sarcoma family of tumors |

Abbreviations: OG, oncogene; TSG, tumor suppressor gene.
mutations within N-terminal 1600 amino acids are pathogenic, and those within C-terminal 1200 amino acids are not. We identified APC mutations in Case 2, and the mutation was in C-terminal side. The β-catenin immunohistochemistry revealed no aberrant nuclear expression, consistent with the absence of any pathogenic effect (data not shown). Features of many other driver genes are still unknown. More specific functional studies with base editing and more representative biologic system studies will provide the proper rationale for patient selection and target therapy.

We analyzed mRNA expression on 770 genes. Among the cancer genes from TSC-RCC cases, we evaluated GNAI1, NOTCH3, PBRM1, SETBP1, SMARCA4, and STMN1 mRNA expression. NOTCH3 was upregulated and SETBP1 was downregulated with a two-fold change in Case 1. Among cancers, the NOTCH3 mRNA expression was upregulated rather than downregulated [33]. The NOTCH3 mRNA expression with our mutation was 26 percentile in hepatocellular carcinoma and 56 percentile in melanoma [34, 35]. The SETBP1 mRNA expression was upregulated rather than downregulated in cancers [33]. The SETBP1 mRNA expression with our mutation was 60 percentile in esophageal carcinoma and 97 percentile in melanoma.

Additionally, in both TSC-RCCs, the mRNA expression of ALK and CRLF2 genes was upregulated, and CDH1, MAP3K1, RUNX1, SETBP1, and TSC1 genes were downregulated. The mRNA expression profile would be useful for the molecular classification rather than individual characterization because mRNA expression can vary in identical cancer types. mRNA expression alone is not sufficient to account for its pathogenic role. Expression can be influenced by genetic mutation, upstream molecules, and interaction with other signaling pathways. Additionally, mRNA expression is not well correlated with protein expression, and upregulation or downregulation does not indicate activation or inactivation. mRNA expression was evaluated in a few genes, and pathogenic pathways cannot be discovered based upon protein-protein interactions. The mRNA expression of previously mentioned genes was evaluated using public data (Supplementary Table 13) [33].

There are some limitations in our study. First, we evaluated just two cases of TSC-RCC due to rarity of this entity. In archival tissue, we could find only two cases of TSC-RCC among 850 RCCs. Second, tumor purity of Case 1 was low, and DNA quality of both cases was relatively low to achieve clear molecular characteristics. Third, we did not perform whole genome sequencing, which could identify large indels, translocations, and fusions. Fourth, we could not perform mRNA sequencing due to poor RNA quality. Alternatively, we analyzed mRNA expression using nanostring. Those limitations should be kept in mind when designing and performing further molecular study.

In summary, we assessed the histopathologic features and genetic alterations in two cases of TSC-RCC. The mTOR activation was assessed by phospho-mTOR immunohistochemistry. WES revealed cancer gene alterations and putative pathogenic pathways that included the chromatin remodeling pathway (CHD8, PBRM1 and SMARCA4), GPCR pathway (GNAI1 and RGS12), Notch signaling pathway (NOTCH3), Wnt/β-catenin pathway (PPTRU and ZNRF3), PP2A pathway (SETBP1), and microtubule dynamics pathway (STMN1) in Case 1 and the mRNA processing (IWS1) and PI3K/AKT/mTOR pathway (TSC2) in Case 2. We evaluated mRNA expression and identified several DEGs, including ALK, CDH1, CRLF2, MAP3K1, RUNX1, SETBP1, and TSC1. Also, we suggest additional therapeutic agents. We hope our results will help advance our understanding of the pathogenesis of TSC-RCC, design molecular cancer studies, and translate cancer research into precision medicine.

Funding
This work was supported by grant 03-2015-0230 from the Seoul National University Hospital Research Fund.

Author contributions
J. H. P, and K. C. M. designed the study. Histopathologic review and acquisition of sample were performed by J. H. P., C. L., and K. C. M. The mTOR immunohistochemical study was performed by M. S. C. Primary bioinformatics analysis was performed by J. H. P. Secondary bioinformatics analysis was performed by K. K. and S. C. Validation of cancer genes alterations was performed by H. L. and H. S. L. The manuscript was prepared by J. H. P. Funding acquisition and supervision were performed by K. C. M. All authors participated in the process of finalizing the manuscript. All authors read and approved the final manuscript.

Competing Interests
The authors declare no conflict of interest.

Acknowledgements
We thank Byeong Chul Ghim at Korea Institute of Toxicology and Byeong-kwon Bae, Jeong-hwa Jang, and Jihye Kim from Bioinformatics Team at DNA Link, Inc., for assistance of primary bioinformatics analysis. We thank Chang-geun Oh and Eun Seo Lim from Next-Generation Sequencing Team at Macrogen, Inc., for assistance of Sanger validation and Circos plot drawing. We thank Sook Lee and Yoo Jung Heo at PhilKorea Technology, Inc., for assistance in mRNA data analysis.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.05.010.

References
[1] Rini BI, Rathmell WK, and Godley P (2008). Renal cell carcinoma. Curr Opin Oncol 20, 300–306.
[2] Srigley JR, Delahunt B, Eble JN, Egevad L, Epstein JI, Grignon D, Hes O, Moch H, Montironi R, and Tickoo SK, et al (2013). The International Society of Urological Pathology (ISUP) Vancouver classification of renal neoplasia. Am J Surg Pathol 37, 1469–1489.
[3] Moch H, Amin MB, Argani P, Cheville J, Delahunt B, Martignoni G, Medeiros LJ, Srigley JR, Tan PH, and Tickoo SK (2016). In: Moch H, Humphrey PA, Ulbright TM, Reuter VE, editors. Renal cell tumours: introduction. World Health Organization classification of tumours of the urinary system and male genital organs. 4th edition. Lyon: IARC Press; 2016. p. 14–17.
[4] Edge SB, Byrd DR, Compton CC, Fritz AG, Greene FL, and Tran T (2009). AJCC Cancer Staging Manual. 7th edition. New York: Springer; 2009 479–489.
[5] Gong J, Gerendash B, Dizman N, Khan A, and Pal SK (2016). Advances in treatment of metastatic renal cell carcinoma. Curr Opin Urol 26, 439–446.
[6] Weinstock M and McDermott D (2015). Targeting PD-1/PD-L1 in the treatment of metastatic renal cell carcinoma. Ther Adv Urol 7, 365–377.
[7] Curtis SA, Cohen JV, and Kluger HM (2016). Evolving immunotherapy approaches for renal cell carcinoma. Curr Opin Rep 18, 57.
[8] Guo J, Tretiakova MS, Troxell ML, Osunkoya AO, Fadare O, Sangoi AR, Shen SS, Lopez-Beltran A, Mehra R, and Heider A, et al (2014). Tuberculosclerosis-associated renal cell carcinoma: a clinicopathologic study of 57 separate carcinomas in 18 patients. Am J Surg Pathol 38, 1457–1467.
[9] Yang P, Cornejo KM, Sadow PM, Cheng L, Wang M, Xiao Y, Jiang Z, Oliva E, Jozwiak S, and Nussbaum RL, et al (2014). Renal cell carcinoma in tuberous sclerosis complex. *Am J Surg Pathol* 38, 895–909.

[10] Crino PB, Nathanson KL, and Henske EP (2006). The tuberous sclerosis complex. *N Engl J Med* 355, 1545–1556.

[11] Northrup H and Krueger DA (2013). International Tuberous Sclerosis Complex Consensus Group. Tuberous sclerosis complex diagnostic criteria update: recommendations of the 2012 International Tuberous Sclerosis Complex Consensus Conference. *Pediatr Neurol* 49, 243–254.

[12] Cancer Genome Atlas Research Network (2013). Comprehensive molecular characterization of clear cell renal cell carcinoma. *Nature* 499, 43–49.

[13] Cancer Genome Atlas Research Network (2016). Comprehensive molecular characterization of papillary renal-cell carcinoma. *N Engl J Med* 374, 135–145.

[14] Davis CF, Ricketts CJ, Wang M, Yang L, Cherniack AD, Shen H, Buhay C, Kang H, Kim SC, and Fache CC, et al (2014). The somatic genomic landscape of chromophobe renal cell carcinoma. *Cancer Cell* 26, 319–330.

[15] Manley BJ and Hakimi AA (2016). Molecular profiling of renal cell carcinoma: building a bridge toward clinical impact. *Carr Opin Urol* 26, 383–387.

[16] Monzé RJ, Escudier B, Oudard S, Hutson TE, Porta C, Bracarda S, Grünwald V, Thompson JA, Figlin RA, and Holaender N, et al (2008). Efficacy of everolimus in advanced renal cell carcinoma: a double-blind, randomised, placebo-controlled phase III trial. *Lancet* 372, 449–456.

[17] Collins FS and Varmus H (2015). A new initiative on precision medicine. *N Engl J Med* 372, 28–30.

[18] Jameson JL and Longo DL (2015). Precision medicine—medicine—personalized, problematic, and promising. *N Engl J Med* 372, 2229–2234.

[19] Delahunt B, Cheville JC, Martignoni G, Humphrey PA, Magi-Galluzzi C, and Mendelsohn F (2012). A program for annotating and predicting the effects of protein mutations: application to cancer genomics. *Nucleic Acids Res* 6, 2186–2187.

[20] Robinson JT, Thorvalsdóttir H, Winckler W, Guttman M, Lander ES, Getz G, and Mesirov JP (2011). Integrative genomics viewer. *Nat Biotechnol* 29, 24–26.

[21] Thorvalsdóttir H, Robinson JT, and Mesirov JP (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. *Brief Bioinform* 14, 178–192.

[22] Boeva V, Popova T, Bleakley K, Chiche P, Cappo J, Schleiermacher G, Janoueix-Lerosey I, Delattre O, and Barillot E (2012). Control-FREEC: a tool for assessing copy number and allelic content using next-generation sequencing data. *Bioinformatics* 28, 423–425.

[23] Sathirapongsasuti JF, Lee H, Horst BA, Brunner G, Cochran AJ, Binder S, Quackenbush J, and Nelson SF (2011). Exome sequencing-based copy-number variation and loss of heterozygosity detection: ExomeCNV. *Bioinformatics* 27, 2648–2654.

[24] Krámer A, Green J, Pollard Jr J, and Tugendreich S (2014). Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* 30, 523–530.

[25] Szklarczyk D, Franceschini A, Wyder S, Forslund K, Nef N, Bork P, Jaschke E, Kuhn M, Wyder S, Li N, et al (2011). STRING v10: protein-protein interaction networks, integrated over the tree of life. *Nucleic Acids Res* 40, D448–D452.

[26] Tyburnczy ME, Jozwiak S, Malinowska IA, Chekaluk Y, Pugh TJ, Wu CL, Nussbaum RL, Scepo D, Szิtk and Kutloska K, et al (2015). A shower of second hit events as the cause of multifocal renal cell carcinoma in tuberous sclerosis complex. *Hum Mol Genet* 24, 1836–1842.

[27] Tripkow K, Hes O, Bonert M, Lopez JL, Bonsib SM, Nes L, Comperaz E, Sibony M, Bernier DM, and Martinek P, et al (2016). Eosinophilic solid, and cystic renal cell carcinoma: clinicopathologic study of 16 unique, sporadic neoplasms occurring in women. *Am J Surg Pathol* 40, 60–71.

[28] Tripkow K, Abou-Ouf H, Hes O, Lopez JL, Nes L, Comperaz E, Sibony M, Osunkoya AO, Zhou M, and Golden N, et al (2017). Eosinophilic solid and cystic renal cell carcinoma (ESCC): further morphologic and molecular characterization of ESCC: a distinct entity. *Am J Surg Pathol* 41, 1299–1308.

[29] Meric-Bernstam F, Johnson A, Holla V, Bailey AM, Bruaco L, Chen K, Roubort M, Patel KP, Zeng J, and Kopes J, et al (2015). A decision support framework for genomically informed investigational cancer therapy. *J Natl Cancer Inst* 107, Martignoni G, Pea M, Rocca PC, and Bonetti F (2003). Renal pathology in the tuberous sclerosis complex. *Pathol* 35, 505–512.