Mutational Analysis of the von Hippel Lindau Gene in Clear Cell Renal Carcinomas from Tuberous Sclerosis Complex Patients

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Tuberous sclerosis complex (TSC) is an autosomal-dominant disorder characterized by seizures, mental retardation, autism, and tumors of multiple organs. Renal disease in TSC includes angiomyolipomas, cysts, and renal cell carcinomas. It is known that somatic mutations in the von Hippel Lindau (*VHL*) tumor suppressor gene occur in most clear cell renal carcinomas. To determine whether TSC-associated clear cell carcinomas also contain *VHL* mutations, we analyzed six tumors for loss of heterozygosity in the *VHL* gene region of chromosome 3p and for mutations in the *VHL* gene. Four of the patients were women between the ages of 34 and 68 years, and two were males under the age of 21 years. The loss of heterozygosity analysis was performed using polymorphic microsatellite markers, and the mutational analysis was performed using direct sequencing. Chromosome 3p loss of heterozygosity was not detected, and no *VHL* mutations were identified. These findings suggest that mutations in the *TSC1* and *TSC2* genes lead to clear cell renal carcinogenesis via an alternate pathway not involving *VHL* mutations.

KEY WORDS: Angiomyolipoma, Loss of heterozygosity, Pair 3, Renal cell carcinoma, Tuberous sclerosis, von Hippel-Lindau disease.

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Tuberous sclerosis (TSC) is a tumor suppressor gene syndrome characterized by seizures, mental retardation, autism, and tumors in the brain, retina, kidney, heart, and skin (1). The birth incidence of TSC is estimated to be 1 in 11,000 (2). TSC is transmitted with an autosomal-dominant pattern of inheritance with 95% penetrance. Sixty percent of TSC patients have apparent new mutations with no prior family history of the disease (3). TSC affects nearly every major organ system. The most common causes of death in TSC patients are renal disease, brain tumors, and lung disease (4).

Renal disease in TSC includes cysts, angiomyolipomas, and renal cell carcinoma. Angiomyolipomas are benign tumors with vascular, smooth muscle, and lipomatous components (5). Renal cell carcinoma has been reported in young children, as well as adults, with TSC and has an average age at onset of about 33 years (6–9). Renal cell carcinoma in TSC is pathologically heterogeneous, including clear cell, papillary, and chromophobe types (7, 8). This heterogeneity contrasts with renal cell carcinoma in von Hippel Lindau (VHL) disease or hereditary papillary renal carcinoma (HPRC), which are associated with clear cell and papillary tumors, respectively. Clear cell renal carcinoma appears to be the most common histologic subtype in TSC patients (10).

Mutations in the *VHL* gene occur in most sporadic clear cell renal carcinomas (11–13). In this study, we analyzed six clear cell renal carcinomas for mutations in the *VHL* gene and for loss of heterozygosity in the *VHL* gene region of chromosome 3p. We reasoned that if *VHL* mutations were found, it would indicate that mutations in *VHL* are involved in clear cell renal carcinogenesis in TSC patients. If mutations were not found, it would strongly suggest the presence of an alternate pathway for clear cell renal carcinogenesis involving the *TSC1* and *TSC2* genes.

METHODS

Patients and Tumor Specimens

All patients had clinical diagnoses of TSC, as defined by the 1998 Tuberous Sclerosis Complex Consensus Conference (14). Their underlying *TSC1* or *TSC2* mutations were not known. All underwent nephrectomy because of lesions suspicious for renal cell carcinoma, with the exception of Patient 354. Patient 354 has dialysis-dependent end-stage renal failure and underwent nephrectomy because of painful polycystic kidneys. His renal cell carcinoma was an incidental finding.

Pathologic Analysis

Paraffin sections of each tumor were stained with hematoxylin and eosin for pathologic review. Immunohistochemistry was performed using AE1/AE3 cytokeratin antibodies and HMB-45 antibody (both from Biogenex, San Ramon, CA). Endogenous peroxidase activity was quenched by a 30-minute incubation in 0.3% hydrogen peroxide. Slides were blocked in normal goat serum. After incubation with the specific antibodies and with the secondary biotinylated goat anti-mouse antibody (Biogenex), the signal was visualized by incubation with streptavidin-peroxidase and diaminobenzidine (Biogenex).

Molecular Genetic Analysis and Detection of Allelic Loss

DNA was extracted from unstained, paraffin-embedded tumor tissue in 50 mM of KCl, 10 mM of Tris (pH 8.3), 1.5 mM of $MgCl_2$, 100 μg/mL of bovine serum albumin, 0.45% Tween 20, 0.45% NP-40, and 100 μg/mL of proteinase K. After overnight incubation at 65° C, the proteinase K was heat inactivated at 95° C for 10 minutes. A 2-μL aliquot of DNA was used in a 20-μL polymerase chain reaction (PCR). The three coding exons of the *VHL* gene were examined by direct sequencing in both the forward and reverse directions, using primers that have been previously reported (15). *VHL* mutational analysis from paraffin tissue has been previously validated (16).

Loss of heterozygosity analysis was performed in tumors and paired normal kidney from patients 353, 618, 622, and 628 using the chromosome 3p marker D3S1478 (Research Genetics, Huntsville, AL). Loss of heterozygosity analysis on chromosome 3p was previously reported for the other two patients (7, 8). A 2.5-μL aliquot of DNA was used in a 10-μL PCR containing 10% glycerol. The PCR amplification consisted of 95° C for 5 minutes, 40 cycles of 95° C for 30 seconds, 55° C for 30 seconds, 72° C for 45 seconds, and a final extension of 72° C for 10 minutes. PCR was performed with [^{32}P]dGTP in the reaction mix. TaqStart antibody (Clontech, Palo Alto, CA) was used in PCR to enhance the specificity. The PCR products for the loss of heterozygosity analysis were resolved by denaturing 8 M urea polyacrylamide gel electrophoresis (Gibco, Grand Island, NY) and visualized by autoradiography.

RESULTS

The patients ranged in age from 11 to 68 years (Table 1). The two younger patients were male, under the age of 21 years. The four older patients were women ranging in age from 34 to 68 years (Table 1). Two of the tumors were included in previous reports of TSC-associated renal cell carcinomas (Patients 120 and 354; 7, 8).

The tumors ranged in maximum dimension from 1.8 to 13 cm. Four of the tumors were Stage I, one was Stage II, and one was Stage III. In four cases, the resected specimen also contained angiomyolipomas, and in five cases, the resected specimen also contained epithelial cysts. Each tumor was centrally reviewed by one of the authors (TA-S) and confirmed to be a clear cell carcinoma and not an epithelioid variant of angiomyolipoma (17; Fig. 1). All of the tumors were positive for cytokeratin and negative for HMB-45. Three tumors were stained for vimentin. The tumors from patients 618 and 628

TABLE 1. Clinical, Pathologic, and Genetic Characteristics of Tuberous Sclerosis Complex–Associated Clear Cell Renal Carcinomas

Patient No.	Sex	VHL Mutation	3p LOH	Size (cm)[a]	Stage	Grade	Angiomyolipomas[b]	Cysts[b]	Recurrence[c] (Time of Follow-Up)
120	F	No	No	2.5	III	G4	No	Yes	No, 1 yr
353	F	No	No	13	II	G2	Yes	No	Not available
354	M	No	No	3.5	I	G4	Yes	Yes	No, 2 yr
618	F	No	No	1.8	I	G1	Yes	Yes	No, 1 yr
622	F	No	No	4	I	G3	No	Yes	No, 1 yr
628	M	No	No	3	I	G2	Yes	Yes	No, 2 yr

[a] The maximum dimension of the tumor.
[b] The presence or absence of angiomyolipomas and cysts in the resected specimen.
[c] There was no known disease recurrence or metastasis at the indicated follow-up times for the patients for whom follow-up information was available.

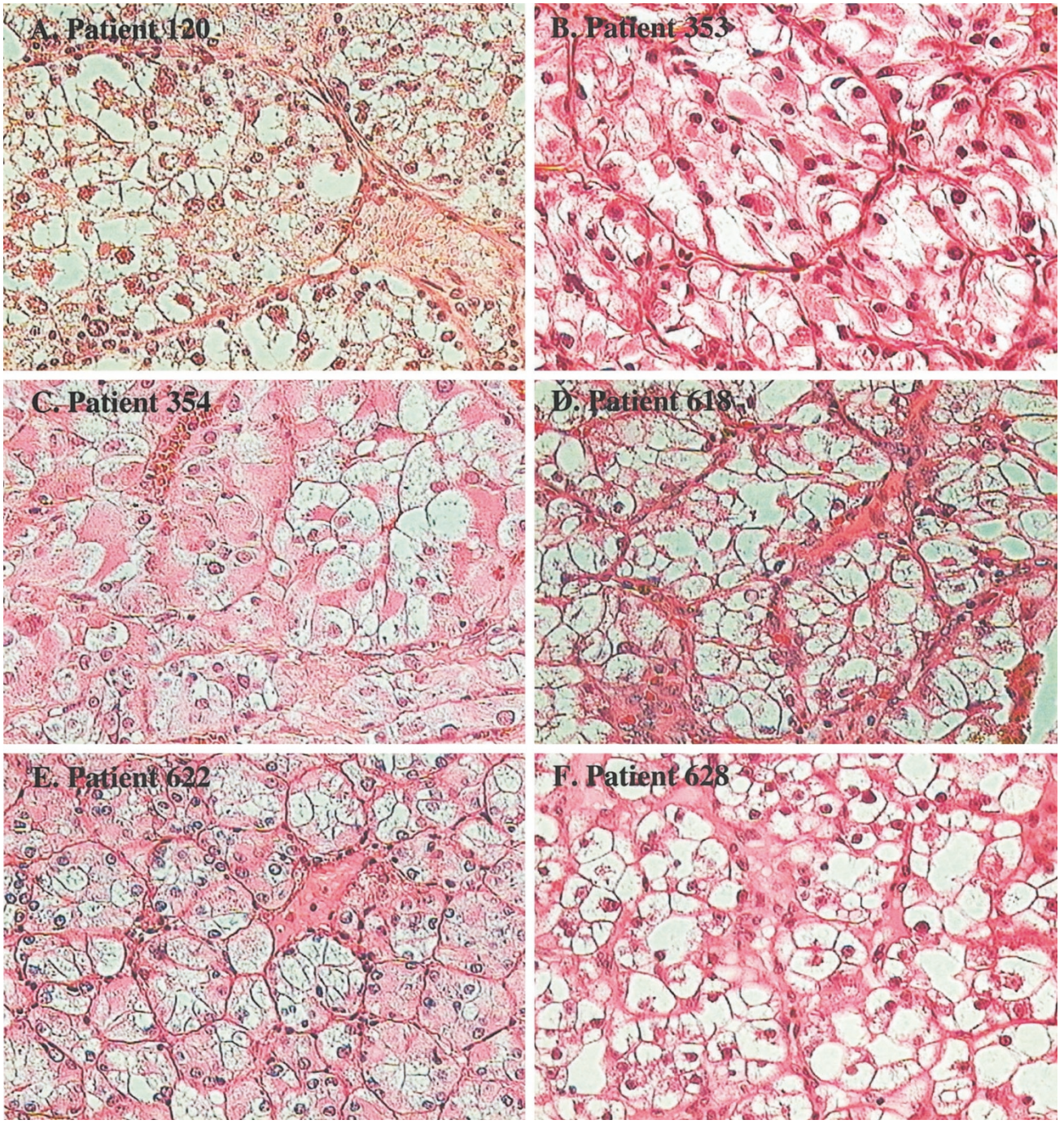

FIGURE 1. Hematoxylin and eosin–stained sections from each tumor. Original magnification, 200×.

were vimentin negative, and the tumor from patient 622 was vimentin positive.

Four of the tumors were analyzed for loss of heterozygosity on chromosome 3p as part of this study (Patients 353, 618, 622, and 628), and two tumors had been previously analyzed and reported (7, 8; Patients 120 and 354). Each of the four patients tested as part of this study was heterozygous at the marker D3S1478. Loss of heterozygosity on chromosome 3p was not found in any of the tumors (Table 1).

The coding region of the *VHL* gene was examined for mutations by PCR amplification, followed by bidirectional DNA sequencing. This was successful for all samples. We did not detect mutations in the *VHL* gene in any of the tumors (Table 1).

DISCUSSION

Renal cell carcinoma is an uncommon manifestation of TSC (18). However, the occurrence of

these cancers in some young children with TSC and in all three rodent models of *TSC2* indicates that these carcinomas are specifically associated with TSC. The literature contains numerous case reports and small case series of TSC-associated renal cell carcinomas (6–8, 10, 19–26). Malignant transformation of angiomyolipomas has also been described (9).

Animal models provide additional support for a role of the TSC genes in renal carcinogenesis. The Eker rat, which carries a germline mutation in the rat *TSC2* homologue, develops renal cysts and carcinomas with an autosomal-dominant pattern of inheritance (27, 28). Two groups have developed mice with targeted inactivation of *TSC2* (29, 30). Both mouse models develop renal carcinomas and cysts similar to those of the Eker rat.

In addition to TSC, there are at least two other hereditary forms of renal cell carcinoma in humans for which genes have been identified (31): VHL disease and HPRC. VHL is caused by mutations in the *VHL* gene (32). HPRC is caused by mutations in the *MET* gene (33). VHL patients develop almost exclusively renal carcinomas with clear cell morphology, whereas HPRC patients develop almost exclusively papillary tumors. *VHL* mutations occur in most sporadic clear cell carcinomas (11, 13), but not in other types of renal carcinoma [reviewed in (34)].

In sharp contrast to VHL and HPRC, the renal cell carcinomas in TSC are morphologically heterogeneous, including clear cell, papillary, and chromophobic tumors (7, 8). In this study, we sought to determine whether clear cell renal carcinomas in TSC patients occur independently of mutations in the *VHL* gene. We analyzed paraffin-embedded tissue specimens from six TSC-associated clear cell renal carcinomas for mutations in the *VHL* gene and for loss of heterozygosity in the *VHL* gene region of chromosome 3p. In none of the tumors was loss of heterozygosity in the *VHL* region or mutations in the *VHL* gene detected. This is, to our knowledge, the first mutational analysis of any gene in TSC-associated renal carcinomas.

We initially considered two possible models for the development of renal carcinomas in TSC. In the first model, mutations in the TSC genes increase the risk of different pathologic types of renal cell carcinoma, and the development of a clear cell carcinoma involves additional mutational inactivation of the *VHL* gene. In the second model, mutations in the TSC genes lead to clear cell carcinoma independently of *VHL* mutations. Our data support the second model and strongly suggest that cellular pathways involving the TSC genes provide an alternate pathway to clear cell renal carcinogenesis. Other studies in humans (35–39), rodents (40), and dogs (41) have also suggested the existence of pathways leading to clear cell renal carcinogenesis that do not involve mutations in the *VHL* genes. In contrast to these previous reports, in which the genetic components of the alternate pathways are unknown, all of the patients in our study are known to have TSC.

The fact that TSC patients develop clear cell renal carcinomas indicates that cellular pathways involving the *TSC1* and *TSC2* genes overlap functionally with pathways involving the *VHL* gene. Pathways in which tuberin, the *TSC2* gene product, is believed to participate include vesicular trafficking (42) and cell cycle regulation (43, 44). Hamartin, the *TSC1* gene product, also regulates the cell cycle (45–48) and affects focal adhesion formation via activation of the GTPase Rho (49). To date, no direct connections between the functions of hamartin and tuberin and the function of the VHL protein, pVHL, have been identified. pVHL targets the hypoxia-inducible transcription factor HIF-1 for degradation, and tumor cells lacking pVHL overproduce HIF target genes, including vascular endothelial growth factor [reviewed in (50)]. pVHL is also believed to play a role in cell cycle regulation and cell differentiation (50).

It is not yet known whether the *TSC1* or *TSC2* gene is mutated in sporadic renal cell carcinoma. The *TSC1* gene is on chromosome 9q34, and chromosome 9q loss of heterozygosity occurs in approximately 30% of sporadic renal cell carcinomas (51). Renal cell carcinomas are known to occur in patients with angiomyolipomas who do not have TSC. In a recent study of 36 renal cell carcinomas associated with angiomyolipomas, 25 of which were from patients without TSC, clear cell was the most common type (10). These sporadic angiomyolipoma-associated renal carcinomas are of particular interest for future mutational analyses of the *TSC1* and *TSC2* genes.

Each year, in the United States alone, renal cell carcinoma is diagnosed in 30,000 people, 10,000 of whom will die of metastatic disease. The majority of these patients have clear cell carcinomas. Renal cell carcinoma is among the most chemotherapy and radiotherapy resistant of all human cancers [reviewed in (52)]. Our data indicate that mutations in the *TSC1* or *TSC2* genes lead to clear cell renal carcinoma via an alternate pathway that does not involve *VHL* gene mutations. Elucidating the cellular mechanisms of this alternate pathway may facilitate the identification of novel therapeutic targets for patients with renal cell carcinoma.

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