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Mutation Spectrum of the 9q34 Tuberous Sclerosis Gene *TSC1* in Transitional Cell Carcinoma of the Bladder

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ABSTRACT

Deletions of the long arm of chromosome 9 are the most common genetic alteration in transitional cell carcinoma (TCC) of the bladder. Several regions of deletion on 9q have been mapped by loss of heterozygosity (LOH) analysis, one of which encompasses one of the two loci for tuberous sclerosis, *TSC1*, at 9q34. Tuberous sclerosis complex (TSC) is an autosomal dominant condition in which affected individuals develop benign tumors (hamartomas) in many organs. There is a small increase in risk of renal cell carcinoma (<2%), but the hamartomas are of stromal origin and patients do not develop an excess of epithelial malignancies. However, during a search for candidate bladder tumor suppressor genes within the 9q34 region of LOH, we previously found a small number of mutations of *TSC1*, raising the possibility that this represents a bladder tumor suppressor. Here, we have carried out mutation analysis of 62 bladder tumors and 33 bladder tumor-derived cell lines to establish the frequency and spectrum of *TSC1* mutations in TCC. Twelve percent of samples contained mutations. We found 10 somatic mutations, 9 of which are novel mutations not found previously in TSC cases. Two of these were missense mutations, a type of change only rarely observed in the germ line in TSC. We also identified a bladder tumor patient carrying a germ-line mutation but with no symptoms of TSC. The tumor in this case and in two other cases with somatic mutations retained the wild-type allele. Thus 3 cases with mutation retained heterozygosity for *TSC1* despite our selection of tumors mostly with 9q LOH (>80%) for the study. This may indicate that haploinsufficiency for *TSC1* can contribute to the development of bladder cancer and, if so, that the LOH of *TSC1* observed in >50% of TCCs is biologically significant.

INTRODUCTION

TSC[4] is an autosomal dominant disease with an incidence of 1 in 10,000. In affected individuals, hamartomas develop in many organs. These are benign growths that include cortical tubers, subependymal nodules, and subependymal giant cell astrocytomas in the brain, renal angiolipomas, hypomelanotic macules in the skin, facial angiofibromas, periungual fibromas, cardiac rhabdomyomas, and pulmonary lymphangiomyomatosis (1). The disease is caused by mutations in one of two genes, *TSC1* on 9q34 (MIM605284; Ref. 2) or *TSC2* on 16p13 (MIM 191092; Ref. 3). Population studies indicate that approximately two-thirds of cases are sporadic, and one-third are familial with high penetrance (4). Large-scale germ-line mutation analyses have shown that in sporadic cases, there is a preponderance of *TSC2* mutations and that such patients have a more severe phenotype (5). Both are large genes (*TSC2*, 41 coding exons; *TSC1*, 21 coding exons), and many mutations, spread throughout the genes, have been described in TSC patients. To date, 154 *TSC1* and 292 *TSC2* mutations have been reported, but as none of these account for >5% of all patients, this renders genotype-phenotype correlations impossible.

Both genes act as tumor suppressor genes and genetic studies of hamartoma tissues have demonstrated loss of the wild-type allele in many lesions (6–8). However, LOH appears to be less common in brain lesions than in kidney lesions (8), a finding confirmed in microdissected tissues (9), indicating that haploinsufficiency may be important in the pathogenesis of hamartomas in certain tissues in TSC.

TSC is not a cancer prone syndrome. Patients with TSC have a small increase in the risk of developing renal cell carcinoma (<2% of patients) but do not have increased risk of other epithelial cancers. Indeed, there is some doubt that the renal cell carcinomas observed are of epithelial origin (10). It would not be expected therefore that the TSC genes would be involved in the genesis of sporadic human cancers, particularly those of epithelial origin. However, during a search for candidate bladder tumor suppressor genes in a common region of LOH on 9q34, we previously identified *TSC1* mutations in a few cases of TCC (11), indicating that *TSC1* may act as a bladder tumor suppressor. Here, we have extended our previous study to include more tumors and have used F-SSCP on a capillary sequencer to achieve high sensitivity mutation scanning. We describe 10 somatic mutations in bladder tumors and tumor-derived cell lines, 9 of which represent novel *TSC1* mutations not previously described in TSC patients. We also show that in some cases mutations appear heterozygous, indicating possible haploinsufficiency. These results indicate that *TSC1* mutation may play a role in the development of many sporadic bladder tumors.

MATERIALS AND METHODS

Tumor Tissues. Tissue samples obtained at cystoscopy from 62 primary transitional cell carcinomas were used for DNA extraction. A venous blood sample from each patient provided a source of constitutional DNA. Some of these samples were used in our previous study (11). Tissues were stored at −80°C or in liquid nitrogen until use. High molecular weight DNA was extracted as described previously (12). Tumor grade and stage were classified according to the tumor-node-metastasis system and WHO criteria, respectively (13, 14). For 59 of the tumors, information was available on grade (13 G1; 22 G2; and 24 G3), and for 53 tumors, information was available on stage (11 pTa; 15 pT1; 27 ≥ pT2). Fifty tumors were known to have 9q LOH.

Cell Lines. Thirty-three bladder tumor-derived cell lines were used. These were RT4, RT112, T24, SD, HT1376, HT1197, SW1710, DSH1, VMCUBII, VMCUBIII, UMUC3, J82, 253J, 5637, JO'N, SCaBER, KU19–19, HCV29, TCCSUP, BC-3C, 647V, BFTC905, BFTC909, and a series of 10 recently established cell lines (15) kindly provided by Dr. Catherine Reznikoff. An SV40 large T-immortalized cell line SV-HUC was also provided by Dr. Reznikoff.

Mutation Analysis. Mutation screening was by F-SSCP. This achieves higher resolution detection of single strand mobility shifts than can be detected using ^{32}P-labeled products run on large format sequencing gels as used in our previous study (11). Primers with FAM or HEX labels were designed for all 23 exons of *TSC1* (this includes the two noncoding exons 1 and 2) using Primer Design software (Applied Biosystems, Warrington, United Kingdom) and GenBank 1529700 as reference sequence (primer sequences available on request). Sequences were amplified in 10-μl reactions using Hotstart reaction mix (Qiagen, Crawley, United Kingdom), 2 pmol of each primer, 10 ng of template DNA, and the following cycle parameters: 96°C for 10 min followed by 35 cycles of 96°C for 10 s; 65°C for 30 s; and 72°C for 30 s. Samples were then incubated at 72°C for 4 min and chilled to 4°C.

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[4] The abbreviations used are: TSC, tuberous sclerosis complex; LOH, loss of heterozygosity; TCC, transitional cell carcinoma; F-SSCP, fluorescent single strand conformation polymorphism.

Products were analyzed on an ABI3100 genetic analyzer. PCR products were diluted with water by a factor of between 1 in 10 and 1 in 40 depending on the yield of the reaction. One to 2 μl of the diluted product were mixed with 0.5 μl of ROX-500 size standards (Applied Biosystems) and 10.5 μl of HiDi Formamide (Applied Biosystems). Samples were denatured at 95°C for 2 min and snap cooled on ice. F-SCCP analysis was on a 3100 Genetic analyzer (Applied Biosystems) at 18°C and 30°C using 5% Genescan polymer with 10% glycerol and 1× Tris-borate EDTA. Data were analyzed using Genescan 3.7.1 and Genotyper 2.5 software (Applied Biosystems). Mutation detection was by visual inspection of electropherograms by two observers. We used NM_000368 as reference sequence for *TSC1* mRNA and numbered nucleotides using the A of the initiating codon as 1.

DNA Sequencing. Products that showed mobility shifts under at least one of the F-SSCP conditions were sequenced after reamplification with unlabelled primers. Unincorporated primers and deoxynucleotides were removed from the PCR products using shrimp alkaline phosphatase and exonuclease I (Amersham Biosciences, Chalfont St. Giles, United Kingdom). Sequencing reactions were carried out using the PCR primers and ABI Prism BigDye Terminator Sequencing Kit version 2 (Applied Biosystems). Data analysis was carried out using sequence analysis 3.0 software (Applied Biosystems) and by visual inspection of the electropherograms.

Western Blot Analysis. Tumor cell lines were analyzed for expression of hamartin protein. Cells were lysed during logarithmic growth phase in 60 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and protease inhibitor mixture (Sigma). DTT and bromphenol blue (100 mM and 0.025%, respectively) were added, and lysates were boiled for 5 min. Protein lysates (20 μg/track) were resolved by electrophoresis in 6% polyacrylamide gels and transferred to a nitrocellulose membrane (Hybond C; Amersham Biosciences). Protein loading was visualized by Ponceau Red Staining and equal loading confirmed. Blots were blocked with 4% nonfat dried milk and incubated for 1 h with affinity purified rabbit antiserum raised against hamartin amino acids 748–957 (16) at 1:10,000, incubated with horseradish peroxidase-conjugated antirabbit immunoglobulin (Southern Biotechnology Associates) and washed. Antibody binding was visualized by chemiluminescence (ECL; Amersham Biosciences).

RESULTS AND DISCUSSION

Sixty two bladder tumor samples and 33 cell lines were screened by F-SSCP for mutations in all exons of *TSC1*. Fifty of the tumors had been found previously to have LOH at 9q34 (data not shown). Band shifts were recorded in 14 fragments and were sequenced. Of the sequence alterations detected, 4 represented known polymorphisms (Table 1). For the remaining variants, we sequenced the relevant fragment from the blood sample of the patient to determine whether these alterations were somatic in origin. Five sequence variants were novel polymorphisms seen also in the blood DNA samples (Table 1). In total, 7 mutations were detected only in the tumor sample. Three mutations were found in tumor cell lines (Table 2). Fig. 1 shows examples of the sequences of these mutations.

We cannot exclude the possibility that the mutations found in the three cell lines represent germ-line mutations because nontumor DNA was not available from these patients. However, the original descriptions of the establishment of the cell lines do not mention any specific clinical features in the patients that might indicate possible TSC. All of these mutations in cell lines were homozygous, indicating that even if they had been present in the germ line, loss of the wild-type allele had occurred in the tumor. Western blotting with an antibody raised against amino acids 748–957 of hamartin (16) identified a band of M_r ~130,000 in all bladder tumor cell lines tested apart from the 3 with *TSC1* mutations, all of which have mutations that are predicted to result in truncation of the protein NH_2-terminal to the epitope recognized by the antibody (Fig. 2). These lines will be useful for future functional studies of hamartin.

Table 1 *TSC1 polymorphisms identified in this study*

Nucleotide change	Effect	Reported
489A>T	S163S	Novel
965T>C	M322T	Known[a]
1047A>G	P349P	Novel
IVS11-33G>A	Intronic	Novel
1726T>C	L576L	Known[b]
2646C>T	A882A	Known[a]
2829C>T	A943A	Known[a]
3303G>A	E1101E	Novel
3324C>T	G1108G	Novel

[a] http://expmed.bwh.harvard.edu/ts/TSC1-222.htm.
[b] Ref. 17.

Table 2 *TSC1 mutations identified in transitional carcinoma tumor samples and cell lines*

Sample	Exon	Change	Effect	Tumor grade/stage
1	4	203A>G	H58R	G2T2
2	6	473T>G	F158C	G1Ta
3	Intron 7	IVS7-1G>A	Splicing	NI
4	11	1041G>A	W347X	G3T2
5	12	1250C>T	T417I[a]	G3T2
6	15	1727_1748del22insG	L576C 577-583del	G2T1
7	15	1958_1959delTA	Frameshift	G2T1
8	Intron 20	IVS20+1G>A	Splicing	G2T2
HCV29[b]	4	163C>T	Q55X	NI
RT4	15	1669delC	Frameshift	G1-2T2
97-1	17	2074C>T	R692X	G2-3T3

[a] This variant, which has been described in TSC patients, was also found in the germ line of the bladder cancer patient.
[b] TCC-derived cell lines.

One of the mutations identified in a tumor, T417I, has been previously described in the germ line of TSC patients (17), and this variant was also identified in the normal DNA of the individual from whom the bladder tumor sample had been obtained. No obvious features of TSC had been recorded in this patient or other family members. Interestingly, the tumor sample did not show 9q34 LOH, and both the mutant and wild-type allele of *TSC1* were clearly detected in the tumor (tumor 5, Fig. 1*a*). It is well known that the phenotype of *TSC1* disease is milder than that of *TSC2* disease (18), and this may explain why this patient had no obvious or recorded symptoms of TSC. However, the possible contribution of the mutation to development of the bladder tumor is not clear, particularly because this was not associated with somatic loss of the wild-type allele. If *TSC1* is haploinsufficient in the bladder as in some hamartomas, then this may be a biologically significant event.

The other potentially inactivating mutations identified comprised 2 missense mutations (H68R and F158C), 3 nonsense mutations (Q55X, W347X, and R692X), 2 splicing mutations, 2 small deletions, and 1 missense mutation (L576C) combined with a larger deletion. Of these, R692X has been previously described in TSC patients. Fig. 3 illustrates the *TSC1* mutation spectrum in TSC patients and bladder tumors described in this study and a mutation found in a tumor (525 insT) in a previous study (19). In TSC, mutations are widely distributed within the gene, and the bladder tumor mutations follow a similar pattern with no hot spots.

In TSC, ~40% of germ-line mutations are single nucleotide substitutions, of which, 63% are nonsense mutations and 23% are splicing mutations. Here, we found single nucleotide changes in 7 of 10 cases with somatic mutation. Of these, the 3 nonsense and 2 splicing mutations are predicted to be loss of function mutations. Only 7 of 131 mutations of *TSC1* recorded in the Human Gene Mutation Database[5] are missense mutations. The majority of these germ-line missense mutations have subsequently been found to be rare polymorphisms. Therefore, our finding of 3 missense somatic mutations (tumors 1, 2, and 6) may indicate a difference in mutation spectrum from TSC. In all cases, the alteration was clearly a tumor-specific somatic mutation

[5] Internet address: http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html.

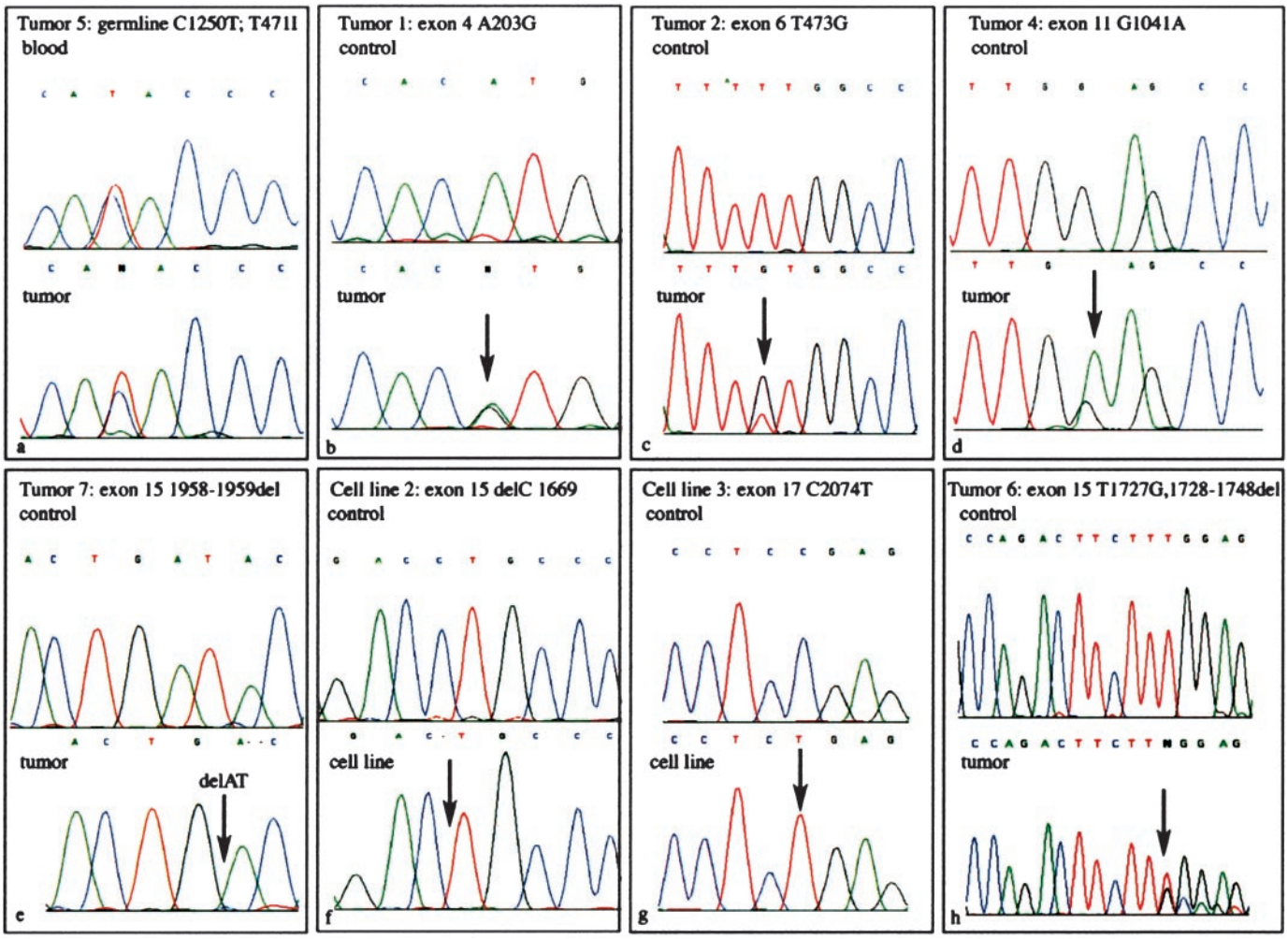

Fig. 1. Sequence alterations identified in bladder tumors. *a*, mutation found in the germ line of a bladder cancer patient (top) with retention of both mutant and wild-type alleles in the tumor (bottom). *b–e*, mutations found in bladder tumor tissues. Tumors *c*, *d*, and *e* are judged to be homozygous for the mutation. The small wild-type peak seen in *c* and *d* comes from contaminating normal DNA in the tissue sample. Sample *b* shows heterozygosity for the mutation in the tumor, which was associated with retention of heterozygosity for markers on 9q. *f* and *g*, homozygous mutations found in cell lines. *h*, heterozygous missense mutation with 21 nt deletion in tumor 6.

not present in the germ line of the patient, indicating that these do not represent rare polymorphisms but must have occurred and been selected during tumor development. For the two simple missense changes (tumors 1 and 2), a comparison of the amino acid sequences of *TSC1* orthologues listed in GenBank and fragments of the sequence of other species retrieved using TBLASTN searching of the EST database is shown in Fig. 4. The amino acids affected by these novel missense mutations (H68R and F158C) are identical in all species for which homologous sequences could be found, indicating a likely effect on protein function if altered. The change of histidine 68 to arginine might be considered relatively conservative (BLOSUM62 score: 0), but until the mutation is assessed in functional assays, no conclusions can be drawn. F158C, is a nonconservative change (BLOSUM62 score: −2) where the change to a cysteine residue may allow the formation of an aberrant disulphide link within the protein.

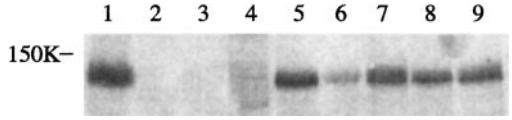

Fig. 2. Expression of hamartin in bladder tumor cell lines. Western blot analysis of total cell lysates from: *Lanes 1*, *5–9*, cell lines with wild-type *TSC1*; *Lane 2*, HCV29; *Lane 3*, 97-1; *Lane 4*, RT4.

The finding of each of these missense mutations as a clonal somatic event in the patient's tumor indicates that they are likely to be disease causing.

Given the possible role of the germ-line mutation T417I (BLOSUM62 score: −1) in the development of this patient's bladder tumor, we also examined this amino acid. Interestingly, this residue is not identical in all species and yet has been associated with TSC (17), albeit not in the case studied here. Possibly, this threonine residue is an important phosphorylation site in the protein, supported by the finding that the in two species, Drosophila and fugu in which the residue is not identical, there is a serine in this position. As for the other missense mutations, functional assessment of this mutant protein is now required.

The 2 nucleotide changes that resulted in an amino acid substitution were subjected to analysis using Splice Site Prediction by Neural Network.[6] Neither altered the predicted splicing pattern. Recently, sequence motifs within exons that affect splicing, Exonic Splicing Enhancers, have been described (20). None of the base changes were found within such motifs. Because no RNA was available from these tumors, reverse transcription-PCR could not be carried out to confirm these predictions.

[6] Internet address: http://www.fruitfly.org/seq_tools/splice.html.

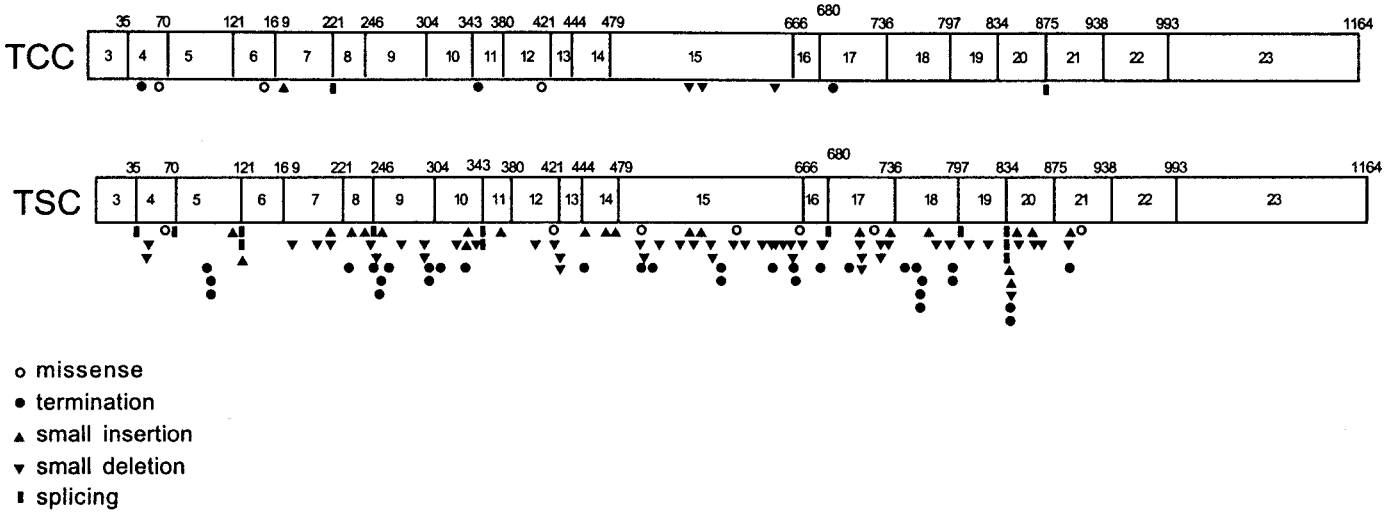

Fig. 3. Spectrum of TSC1 mutations identified in TCC and of germ-line mutations identified in TSC patients. The TCC mutation in exon 7 was described in Ref. 19.

It is possible that the mutation spectrum may differ in sporadic epithelial cancers from those found in the germ line. Some mutations that are tolerated in a somatic cell may be lethal in the germ line and never seen. Because *TSC1* has few polymorphisms, it has been suggested that this could indicate a high requirement for conservation and that any missense mutations may exert a dominant negative embryonic lethal effect, thus excluding them from the germ line (21). These constraints may not apply in somatic cells. Cell type specificity may also play a role.

In selecting tumors for this study, we chose predominantly those in which we had previously detected LOH on 9q. Our expectation was that both alleles of any target gene on 9q would need to be inactivated and that tumors with LOH would likely harbor small inactivating mutations on the retained allele. Interestingly, in addition to the tumor from the patient with the germ-line T417I mutation, we found two other tumors with retention of heterozygosity for markers on 9q that contained mutations in *TSC1*. These were tumors 1 and 6. The sequences are shown in Fig. 1 (*b* and *h*) and clearly contain both wild-type and mutant alleles. This suggests that *TSC1* may be haploinsufficient in the bladder. An alternative possibility is that these mutations are dominant negative mutations. As knowledge of specific functional roles of different regions of the gene is scanty, functional assays will be required to test this. If *TSC1* is haploinsufficient in the bladder, LOH of 9q34 alone may be sufficient for phenotypic effect. A corollary of this is that mutation of *TSC1* may be found more frequently than we initially expected in tumors without LOH. More extensive mutation screening of such tumors must now be carried out.

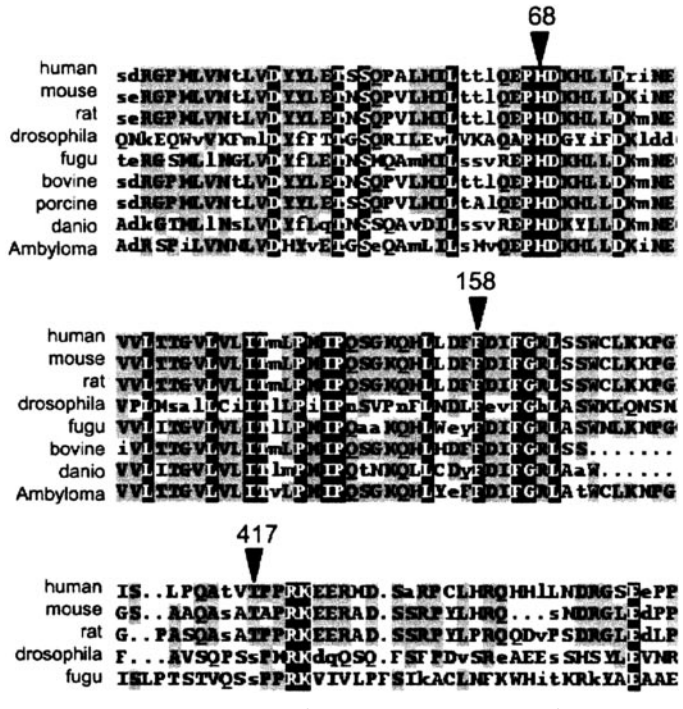

Fig. 4. Cross-species conservation of amino acids 68, 158, and 417 of hamartin. Both H68 and F158, which showed missense mutation in TCC samples, are conserved in all species for which protein sequence information is available.

The overall frequency of mutation in this tumor series was 12%. This is significantly lower than the frequency of 9q34 LOH in the same tumors (81%). It is of interest that all tumors with mutation, apart from one, were invasive (T_1 or T_2) lesions. χ^2 tests for tumor grade or stage and mutation status were not significant ($P = 0.58$ and $P = 0.2$, respectively), and there was no evidence for a trend. However, it will be important to test again for associations when a larger cohort of mutations has been identified. LOH on 9q is equally frequent in tumors of all grades and stages, so any preponderance of mutations in the higher grade and stage tumors may indicate that inactivation of the second allele of *TSC1* occurs as a late event in tumors where *TSC1* haploinsufficiency contributed during earlier stages in tumor development.

The products of both TSC genes, tuberin and hamartin, are known to form a complex *in vivo* and this participates in the phosphatidylinositol 3′-kinase pathway (for review, see Ref. 22). It is postulated that both proteins are also likely to have independent functions, but these are not yet clearly defined. For example, hamartin has been shown to play a role in maintenance of the actin cytoskeleton via interaction with ERM proteins (23). Such independent functions of the two proteins may ultimately help to explain the differences in phenotype of patients with *TSC1* and *TSC2* mutations. Given the known interaction of the proteins and their role in the phosphatidylinositol 3′-kinase pathway, it might be expected that loss of function of either gene could contribute to bladder cancer development. We have not systematically studied *TSC2* but in a previous allelotype study of TCC did not find frequent LOH on 16p (24). The tumor suppressor gene *PTEN*, another negative regulator of signaling via the same pathway (25), is deleted or mutated in some bladder tumors, particularly those of high grade and stage (26, 27), indicating that deregulation of the

pathway via loss of function of either *PTEN* or *TSC1* may be critical for tumor progression. It will be of interest to assess the level of expression of both TSC proteins and PTEN in bladder tumor tissues in the future.

To date, there have been only two reports of mutation analysis of *TSC1* and *TSC2* in sporadic human cancers other than bladder. Both *TSC1* and *TSC2* were assessed in panels of sporadic glial and glioneuronal tumors and in renal cell carcinoma (28, 29) and in neither study were intragenic mutations found. The possible role of these genes in other tumor types is unknown. Several other tumor types show frequent LOH of 9q and/or 16q. Examples include ovarian carcinoma which shows >50% 9q LOH (30–32), carcinoma of the gall bladder with 88% 9q LOH mapped to two discrete regions, one of which between *D9S2133* and *D9S164* contains *TSC1* (33) and nasopharyngeal carcinoma (34). LOH on 16p has been described in several tumor types, including >50% of cases of serous adenocarcinoma of the ovary (32), 60% of gallbladder carcinoma (35), 63% of papillary tumors of the breast (36) and 28% of adenocarcinoma of the lung (37). In nasopharyngeal carcinoma, 40% of tumors show underrepresentation of 16p by comparative genomic hybridization (38). It is noteworthy that in several of these tumor types, namely ovarian carcinoma, gallbladder carcinoma, and nasopharyngeal carcinoma, LOH is found in the regions of both of the TSC genes. Mutation analysis in these tumors is therefore merited.

In conclusion, we have identified somatic mutation of one of the TSC genes, *TSC1*, in a significant number of human bladder tumors and cell lines. The spectrum of mutations was similar to that found in TSC but with a possible excess of missense mutations. Our results raise the interesting possibility that haploinsufficiency of *TSC1* contributes to the development of bladder cancer. Functional studies are now required to explore this more fully.

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