Mutation Spectrum of the 9q34 Tuberous Sclerosis Gene TSCI 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, TSCI, 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 TSCI, 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 TSCI 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 TSCI despite our selection of tumors mostly with 9q LOH (>80%) for the study. This may indicate that haploinsufficiency for TSCI can contribute to the development of bladder cancer and, if so, that the LOH of TSCI observed in >50% of TCCs is biologically significant.

INTRODUCTION

TSC4 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 angiomyolipomas, hypomelanotic macules in the skin, facial angiofibromas, perianal fibromas, cardiac rhabdomyomas, and pulmonary lymphangiomyomatosis (1). The disease is caused by mutations in one of two genes, TSCI on 9q34 (MIM 605284; Ref. 2) or TSC2 on 16p13 (MIM 191092; Ref. 3). Population studies indicate that approximately 10,000 cases of TSC are diagnosed each year worldwide (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 TSCI mutations in a few cases of TCC (11), indicating that TSCI 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 TSCI mutations not previously described in TSC patients. We also show that in some cases mutations appear heterozygous, indicating possible haploinsufficiency. These results indicate that TSCI 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, DSHI, VMUCBII, VMUCBIII, UMUC3, J82, 253J, 5637, J0N, 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 32P-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, and sequencing was performed on all exons except exon 1 and 2. A large T-immortalized cell line SV-HUC was also provided by Dr. Reznikoff.

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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.
Patients 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 mRNAs 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-SCCP 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 (Amerham 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 incubated with 4% nonfat dried milk and incubated for 1 h with affinity purified rabbit antiserum raised against amino acids 748–957 of hamartin (16) identified a band of M, ~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 NH2-terminal to the epitope recognized by the antibody (Fig. 2). These lines will be useful for future functional studies of hamartin.

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. 1a). It is well known that the phenotype of TSC 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 Database5 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.

### Table 1 TSC1 polymorphisms identified in this study

<table>
<thead>
<tr>
<th>Nucleotide change</th>
<th>Effect</th>
<th>Reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>489A&gt;T</td>
<td>S163S</td>
<td>Novel</td>
</tr>
<tr>
<td>965T&gt;C</td>
<td>M322T</td>
<td>Known6</td>
</tr>
<tr>
<td>1047A&gt;G</td>
<td>P349P</td>
<td>Novel</td>
</tr>
<tr>
<td>IVS11-33G&gt;A</td>
<td>Intronic</td>
<td>Novel</td>
</tr>
<tr>
<td>1726C&gt;T</td>
<td>L576L</td>
<td>Known6</td>
</tr>
<tr>
<td>2646C&gt;T</td>
<td>A882A</td>
<td>Known6</td>
</tr>
<tr>
<td>2829C&gt;T</td>
<td>A943A</td>
<td>Known6</td>
</tr>
<tr>
<td>3303G&gt;A</td>
<td>E1101E</td>
<td>Novel</td>
</tr>
<tr>
<td>3324C&gt;T</td>
<td>G1108G</td>
<td>Novel</td>
</tr>
</tbody>
</table>

5 Internet address: http://archive.cancer.gov/ncicb/images/hgmd0.html.
6 Ref. 17.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exon</th>
<th>Change</th>
<th>Effect</th>
<th>Tumor grade/stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>403A&gt;G</td>
<td>H58R</td>
<td>G2T2</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>473T&gt;G</td>
<td>F158C</td>
<td>G1T1</td>
</tr>
<tr>
<td>3</td>
<td>Intron 7</td>
<td>IVS7-1G&gt;A</td>
<td>Splicing</td>
<td>NI</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>1041G&gt;A</td>
<td>W347X</td>
<td>G3T2</td>
</tr>
<tr>
<td>5</td>
<td>12</td>
<td>1250C&gt;T</td>
<td>T417I</td>
<td>G3T2</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>1727,1748del22insG</td>
<td>L576C 577-583del</td>
<td>G2T1</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>1958,1959delTA</td>
<td>Frameshift</td>
<td>G2T1</td>
</tr>
<tr>
<td>8</td>
<td>Intron 20</td>
<td>IVS20+1G&gt;A</td>
<td>Splicing</td>
<td>G2T2</td>
</tr>
<tr>
<td>9</td>
<td>HCV29b</td>
<td>4</td>
<td>163C&gt;T</td>
<td>Q55X</td>
</tr>
<tr>
<td>10</td>
<td>RT4</td>
<td>15</td>
<td>1669delC</td>
<td>Frameshift</td>
</tr>
<tr>
<td>11</td>
<td>97-1</td>
<td>17</td>
<td>2074C&gt;T</td>
<td>R692X</td>
</tr>
</tbody>
</table>

b TCC-derived cell lines.
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.

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 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. 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.

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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.

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, 9T-1; Lane 4, RT4.
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 \textit{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 \textit{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 \textit{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 \textit{TSC1} is haploinsufficient in the bladder, LOH of 9q34 alone may be sufficient for phenotypic effect. A corollary of this is that mutation of \textit{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.

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 (T1 or T2) lesions. \chi^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 \textit{TSC1} occurs as a late event in tumors where \textit{TSC1} haploinsufficiency contributed during earlier stages in tumor development.

The products of both TSC genes, tuberin and hamartin, are known to form a complex \textit{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 \textit{TSC1} and \textit{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 \textit{TSC2} but in a previous allelotype study of TCC did not find frequent LOH on 16p (24). The tumor suppressor gene \textit{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 glacial 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 in 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 this conclusion is therefore warranted.

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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