Tuberous sclerosis complex (TSC) gene involvement in sporadic tumours

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Abstract
In tuberous sclerosis patients, inactivation of the tuberous sclerosis complex tumour-suppressor genes TSC1 and TSC2 contributes to the development of a wide range of hamartomatous lesions. These patients do not, however, show an increased risk of the common adult solid cancers. Recent evidence that the TSC genes play a role in the phosphoinositide 3-kinase pathway, a pathway whose dysregulation is implicated in a wide range of human malignancies, raises the possibility that their inactivation could contribute to the development of some sporadic cancers. To date the only evidence for this comes from the finding of mutations of TSC1 in bladder cancer. The mutation spectrum of TSC1 in bladder cancer and functional evidence from TSC1-gene-replacement studies in bladder tumour cells will be presented. The literature on genetic changes in several other sporadic epithelial cancers reveals relatively common deletions in the region of the TSC genes. In ovarian and gall bladder carcinoma and non-small-cell carcinoma of the lung, deletions in both 16p13 and 9q34 are found at significant frequency. Mutation analyses in such tumours are now merited.

Introduction
The tuberous sclerosis complex (TSC) genes TSC1 and TSC2 act as tumour-suppressor genes. In TSC patients, both genes conform to the classical two-hit mechanism for tumour-suppressor gene inactivation in which germline mutation of one allele and somatic inactivation of the second allele by deletion leads to complete loss of gene function in a significant proportion of hamartomas [1]. However, in contrast to some other familial hamartoma syndromes, TSC does not confer predisposition to any of the common adult malignancies (OMIM #191100). The lesions that develop in patients do not contain epithelial cells and although some patients develop renal tumours that resemble renal cell carcinoma, there is doubt as to whether these are true epithelial malignancies [2]. Thus it might be considered unlikely that these genes can participate in the development of common sporadic cancers, particularly those of epithelial origin.

However, recent results suggest that TSC1 and TSC2 may play a direct role in the development of some malignancies. Mutations in TSC1 have been identified in sporadic tumours of the bladder and initial functional assays in urothelial cells indicate important phenotypic consequences of modulation of hamartin expression. It is also clear from studies of a range of biological systems from fruit flies to humans that these genes are involved in pathways or biochemical activities known to play a role in a large range of sporadic human cancers and this may indicate a more widespread involvement, either directly or indirectly.

We will summarize what is known about involvement of TSC1 in bladder cancer, discuss the possible role of TSC1 and TSC2 in other human malignancies and comment on the absence of increased risk of malignancy in TSC patients.

Involvement of TSC1 in carcinoma of the bladder
Loss of heterozygosity (LOH) for markers on chromosome 9 is one of the most frequent genetic events detected in transitional cell carcinoma (TCC) of the bladder. More than 50% of tumours of all grades and stages show LOH, which often includes all markers studied on both chromosomal arms [3,4]. This indicates likely loss of an entire parental homologue during tumour development. Studies from several laboratories have identified subsets of tumours with defined regions of LOH and these have pinpointed critical regions where relevant tumour-suppressor genes may be located. These include a single region on chromosome 9p containing the known tumour-suppressor genes CDKN2A and CDKN2B [5,6] and at least three regions on chromosome 9q, one of which, at 9q34, contains TSC1 [4,7].

In a preliminary mutation screen of all coding exons of TSC1 in 36 bladder tumours and 15 TCC-derived cell lines, we identified four inactivating mutations in tumours and one in a cell line [8]. This confirmed the inactivation of both alleles of TSC1 in some cases of TCC, although the frequency of mutation was much lower than the observed frequency of LOH on chromosome 9q34. Possible reasons for such a discrepancy between LOH and mutation frequencies are that haplo-insufficiency of TSC1 can contribute to

Key words: bladder, sporadic tumour, TSC1.

Abbreviations used: TSC, tuberous sclerosis complex; LOH, loss of heterozygosity; TCC, transitional cell carcinoma; P16INK4A, phosphatase and tensin homologue on chromosome 10; CD, Cowden disease; B–Z, Banayan–Zonana syndrome; PI3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin.

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form dense multilayered colonies in monolayer culture. Stable TSC1-transfectant clones formed much flatter colonies (Figures 1a and 1b) and these were more readily removed and disaggregated during passaging. The effect of re-expression of hamartin on cell migration was assessed by wounding of confluent monolayers. Wound healing in all RT4 cells was slow, with wounds made by a 200–1000 μl micropipette tip taking 7–8 days to heal. However, in all cases, hamartin-expressing cells closed the wounds faster than vector and untransfected controls (Figures 1c and 1d). During the course of wound healing, hamartin-expressing cells showed much flatter and extended cell margins than controls. Thus re-expression of hamartin appeared to induce a migration phenotype in these epithelial cells. Given the known role of signalling via the phosphoinositide 3-kinase (PI3K) pathway in cell motility, this observation was counterintuitive and more detailed analysis, of cell division, cell motility and the activity and distribution of several key proteins, is now in progress. In contrast to findings in other cell types [10], we found no significant effect on cell proliferation and cell migration is shown in Figure 1. Parental RT4 cells

We identified three TCC cell lines with mutation in TSC1. One of these, RT4, a cell line established from a G1–G2 muscle invasive primary papillary TCC [9], has been used for preliminary functional studies. This cell line has lost one TSC1 allele and has a single base deletion in the retained allele causing a frameshift, which if translated would result in a truncated protein product of 627 amino acids. Gene-replacement experiments have been carried out and several phenotypic assays have been done on stable hamartin-expressing transfectants. These indicate significant effects on cell morphology, cell migration and tumorigenicity in nude mice (N. Hornigold, E. Pitt and M.A. Knowles, unpublished work).

The effect of re-expression of hamartin on cell morphology and cell migration is shown in Figure 1. Parental RT4 cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>Exon</th>
<th>Nucleotide change</th>
<th>Effect</th>
<th>Tumour grade/stage</th>
</tr>
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<tr>
<td>T1</td>
<td>4</td>
<td>203A &gt; G</td>
<td>H68R</td>
<td>G2T2</td>
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<tr>
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<td>6</td>
<td>4731 &gt; G</td>
<td>F158C</td>
<td>G1T1a</td>
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<td>7</td>
<td>525insT</td>
<td>Frameshift</td>
<td>NI</td>
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<td>IVS7 − 1G &gt; A</td>
<td>Splicing</td>
<td>NI</td>
</tr>
<tr>
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<td>11</td>
<td>1041G &gt; A</td>
<td>W347X</td>
<td>G3T2</td>
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<tr>
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<tr>
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<td>2074C &gt; T</td>
<td>R692X</td>
<td>G2–3T3</td>
</tr>
</tbody>
</table>

Table 1 | Mutations of TSC1 found in bladder tumours

Data from [8,43] and M.A. Knowles and D. Cuthbert-Heavens, unpublished work.
Figure 1  Changes in cell morphology, cell migration and morphology of reconstituted epithelia following re-expression of hamartin in the bladder tumour cell line RT4

(a) RT4 parental cells in monlayer culture. (b) Stable TSC1 transfectant clone in monolayer culture. (c) Appearance of wound in confluent epithelial monolayer of RT4 control cells 7 days after wounding. (d) TSC1 transfectants following wounding as in (c). (e) Reconstituted epithelium formed from RT4 control cells 28 days after seeding on to organ-cultured normal urogenital stroma. (f) Epithelium reconstituted from TSC1-transfected cells as in (e).

take rate and tumour size was found. Tumours formed at all 10 sites inoculated with control cells, but at only 9/20 sites inoculated with TSC1 transfectants (45%). In those animals where tumours did form from TSC1 transfectants, they were only very small nodules (Figure 2).

A problem with gene-replacement studies is that the mammalian expression vectors commonly used, drive high non-physiological levels of expression in the recipient cells, potentially generating artifactual results. Although we used such a vector, the expression levels achieved in RT4 cells were very low, suggesting that there was selection against high levels of expression during clone expansion. Expression was detected only at the RNA level, by reverse transcriptase PCR, and none of three antibodies tested reproducibly detected protein on Western blots, although many other bladder tumour cell lines tested showed detectable levels of hamartin. Thus very low levels of hamartin were responsible for the marked phenotypic alterations recorded.

Apart from the finding of inactivation of TSC1 in bladder tumours, we and others have identified genetic alterations affecting the known tumour-suppressor gene PTEN (phosphatase and tensin homologue deleted on chromosome 10), a negative regulator of PI3K and shown by recent results to lie upstream of TSC1–TSC2 in the PI3K/Akt pathway. A significant number of muscle-invasive bladder tumours show LOH in the region of PTEN and some of these have inactivation of the second allele via small mutations or complete deletion (homozygous deletion) [12,13]. This suggests that inactivation of the PI3K pathway is a critical step in the development and/or progression of many invasive bladder tumours. Studies of TCC to date provide no information on the status of other genes in this pathway including TSC2, nor whether PTEN and TSC1 mutation or haploinsufficiency are mutually exclusive events.

Taken together our results provide compelling evidence that TSC1 plays an important role in tumour development in the bladder. It will now be important to assess the potential significance of loss of function of one copy of the gene. If haplo-insufficient, this could indicate that a very large proportion of bladder tumours may be influenced by reduction in hamartin function, given the finding of chromosome 9q LOH in more than half of TCC of all grades and stages. Since chromosome 9 LOH appears to be a very early genetic event, loss of hamartin function may play an important role in tumour initiation. Also of key importance will be a more comprehensive assessment of the activation status of the PI3K/Akt pathway in the same tumour series.

**Evidence for the involvement of the TSC genes in other cancers**

To date there are no publications describing mutations in either TSC1 or TSC2 in any major epithelial tumour type other than bladder. Possibly this is because no-one has looked. Several studies have assessed TSC1/TSC2 status in tumours related by site or histopathology to lesions typically observed in TSC patients. Most have been carried out by groups involved in TSC research, and some have included
Tumorigenicity in nude mice of RT4 bladder tumour cells and two TSC1-transfectant clones

Cells (5 × 10⁶) were inoculated subcutaneously into nude mice (10 sites each). Total tumour volume (mm³) for each group of mice is shown. ▲, RT4 control; ♦ and ■, TSC1-transfectant clones. Two mice in the control group were killed during the course of the experiment, one for causes unrelated to tumour growth (K1) and one due to large tumour size (K2).

Figure 2

Evidence for deregulation of PI3K signalling in human cancers

There is a large body of information on alterations affecting the PI3K pathway in various human cancers (reviewed by Vivanco and Sawyers [36]). Genetic alterations include amplification of PI3K in ovarian cancer and of AKT2 in breast, ovarian and pancreatic cancers and alterations of PTEN in a large number of tumours, including glioblastoma, ovarian, breast, endometrial, bladder, prostate, liver, melanoma, lung, renal, thyroid and lymphoid malignancies. It is difficult to estimate the total proportion of human cancer in which this pathway is deregulated, but it is likely to be very large since several genes now known to be involved in the pathway have not yet been assessed. These include TSC1 and TSC2. Consequently, much effort is currently focused on ways in which the function of this pathway can be inhibited. Spurred on by the success of small-molecule inhibitors of kinases such as the inhibitor of Bcr-Abl, Gleevec [37], one approach is to design inhibitors of PI3K, Akt and mammalian target of rapamycin (mTOR). Recent work with rapamycin, an inhibitor of mTOR, has shown very encouraging results in a range of early-phase clinical studies (reviewed by Huang and Houghton [38]). Any specific inhibitors of the pathway downstream of the TSC genes, for example mTOR inhibitors, might have clinical application in TSC. In bladder cancer the involvement of the pathway is much greater than those tumours with TSC1 mutation and upstream inhibitors, such as Akt inhibitors, may also be effective in some of these.
**Risk of malignancy in TSC patients**

Despite the clear evidence that TSC1/TSC2 lie functionally at the heart of a major signalling pathway with known relevance in many human cancers, TSC is not a cancer-prone syndrome. The increased risk is small, with a low percentage of patients developing renal and brain malignancies. How can this be explained?

TSC is not alone in showing apparent discrepancies between the familial disease and the observed role of the causative gene in sporadic tumours. Other familial syndromes provide some relevant information and several suggestions have been made to explain these observations. Key findings are that the tumour spectrum may differ in the familial syndrome and sporadic cancers with involvement of the same gene, that sporadic tumours in the same tissues as in the familial syndrome do not show mutation of the familial gene and that the same mutation may generate a different phenotype in different families. One example involves PTEN, a gene involved in the same pathway as TSC1/TSC2. Germline mutations in PTEN give rise to a range of conditions collectively known as PTEN hamartoma syndrome (PHTS) [39]. This includes several conditions that have been defined as distinct syndromes including Cowden disease (CD), Banayan–Zonana syndrome (B–Z) and Proteus syndrome. In CD, patients commonly develop carcinomas of breast, endometrium and thyroid. However, this does not reflect the wide range of sporadic cancers in which mutation of PTEN has been described which includes glioblastoma (≈44%), endometrial carcinoma (≈50%), some advanced prostate cancers and melanoma (reviewed by Simpson and Parsons [40]). In contrast, an increased risk of neoplasia is not reported in B–Z, despite the finding of identical mutations in some CD and B–Z families. This may suggest that there are important genetic or epigenetic factors that modify phenotypic manifestations and indicates that simple genotype–phenotype predictions are likely to be flawed. Germline mutations of the retinoblastoma tumour-suppressor gene RB1 lead to the development of retinoblastoma in childhood. As for PTEN, many common cancers show somatic bi-allelic inactivation of RB1 and yet these cancers rarely develop in carriers with germline mutation where the most common second malignancy is osteosarcoma.

Several possible explanations may account for these findings. One is that the order in which genetic events are accumulated by a somatic cell may be critical. It is of interest that in the case of sporadic glioblastoma, mutations of PTEN are found only in glioblastoma multiforme, the most aggressive form of glioma, but not in less aggressive putative precursor lesions. Possibly, mutation of PTEN is not compatible with early stages in the development of this type of tumour and this could explain why brain tumours are not a feature of CD.

In the bladder, mutation of TP53 is commonly found only in more aggressive tumours, implying that this event is critical at a late stage in tumour progression. Thus mutations carried in the germline might represent inappropriate first events in tumour development and indeed Li–Fraumeni syndrome patients do not have a high risk of bladder cancer.

A second factor that is likely to have an impact is that epithelial malignancies appear to require multiple heritable changes. For several cancers, studies of age/incidence data indicate that this may be in excess of eight events [41]. Thus even in the presence of a germline mutation and assuming that the affected gene can contribute early in the process, the requirement for multiple other genetic events could mask an increased risk, particularly if the study population is small. In retinoblastoma patients, for example, only a very large study of affected individuals and first-degree relatives revealed a slight increase in risk of bladder cancer [42].

Thus, there are often discrepancies when extrapolation is attempted from familial syndromes to sporadic tumours and vice versa. In the case of TSC, clinical information collected over many years clearly indicates that there is no measurable increase in risk of any of the common epithelial malignancies. Therefore, the finding of mutations in sporadic tumours certainly does not indicate any need for increased surveillance in patients. Rather, it identifies alternative and perhaps scientifically fruitful models in which to study the biological functions of these interesting genes.

**References**


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