

Tuberin and hamartin are aberrantly expressed and linked to clinical outcome in human breast cancer: The role of promoter methylation of *TSC* genes [☆]

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Abstract

Purpose: The tuberous sclerosis (TSC) genes *TSC1* and *TSC2* encode the protein products hamartin and tuberin, respectively, and are putative tumour suppressor genes. Germ-line mutation of either TSC gene leads to the development of the heritable disorder TSC. This disorder is characterized by the development of hamartomas in many organs and is associated with the proliferative lung disease, lymphangioleiomyomatosis, the brain tumour giant cell astrocytoma and occasionally with renal cell carcinoma. However, the TSC genes have not been studied in breast cancer. The current study investigated the expression of the *TSC* gene products and the potential mechanisms of their aberrancy in human breast cancer cells and tissues.

Experimental design and results: Using immunohistochemical analysis, both hamartin and tuberin were found to be strongly stained in normal mammary epithelial cells and weakly in stromal cells. In invasive tumour tissues, however, the staining of both proteins were to be markedly reduced ($P < 0.01$). At message level, although normal and tumour tissues expressed both *TSC* products, the transcript levels of tuberin was significantly lower in tumour tissues compared with normal tissues ($P < 0.05$). There was no statistical difference between node negative and node positive tumours with both hamartin and tuberin. Tumours from patients who developed recurrence and died from breast cancer had significantly low levels of tuberin compared with those who remained disease free ($P = 0.03$ and 0.05 , respectively). Likewise, hamartin levels were significantly lower in patients with metastasis, recurrence and mortality, when compared with those remained disease free ($P = 0.001$, 0.041 and 0.003 , respectively). Using methylation specific PCR, the *TSC1* promoter was found to be heavily methylated in ZR751, MDA MB 435, and BT549, but not in MCF-7 which expressed highly level of hamartin. *TSC1* promoter methylation was also seen in most breast tumours, but only in a limited number of normal tissues. The methylation of *TSC2* promoter appears to be less frequent. MDA MB 468, MDA MB 483, MDA MB 435S and weakly MDA MB 435 were found to have methylated *TSC2* promoter. In breast tissues, however, a very small number of samples were found to have methylation of the *TSC2* promoter.

Conclusion: *TSC1* genes are aberrantly expressed in human breast cancer cell lines and breast tumour tissues and their promoters are seen to be methylated in breast tumour tissues. The expression of *TSC1* is associated with an unfavourable clinical outcome in patients with breast cancer.

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1. Introduction

Hamartin and tuberin are the products of the tuberous sclerosis complex genes, *TSC1* and *TSC2*. They were identified initially by positional cloning strategies in patients with the inherited disorder tuberous sclerosis (TSC) [1,2]. TSC is characterized by the development of hamartomatous tumours involving multiple organs, notably the kidneys, brain and skin. Female patients with TSC also have a massively increased risk of developing the proliferative lung, lymph node and kidney disease, lymphangioleiomyomatosis (LAM) that involves proliferation of atypical smooth muscle cells that apparently metastasize from renal primaries [3].

Tuberin and hamartin act as tumour suppressors. Loss of heterozygosity or intragenic second-hit mutations have been characterized in a wide variety of TSC-associated hamartomas and cancers and bi-allelic somatic mutations have been identified in sporadic LAM [3,4]. Germ-line *TSC1* and *TSC2* mutations in experimental rodent models are also associated with the occurrence of tumours in various organs including renal cystadenomas and carcinomas that exhibit LOH at the corresponding locus [5–11]. However, very few studies have investigated the *TSC1* and *TSC2* genes in relation to sporadic cancers. Sporadic astrocytomas and ependymomas have been shown to exhibit reduced tuberin RNA and protein expression [12], but systematic genomic studies of sporadic primary brain tumours and renal cell carcinomas have not revealed evidence for biallelic inactivation of either gene.

The cellular mechanisms through which hamartin and tuberin normally act to suppress tumourigenesis have been subject to intensive investigation in recent years. The proteins interact directly [13] and a variety of non-truncating mutations that disrupt their binding are TSC-causing [14]. Hamartin stabilizes tuberin by preventing its ubiquitination [15] and the complex regulates activity of p70 S6 kinase via the PI3K/Akt/mTOR pathway. *TSC1/TSC2* thereby exerts translational control of protein synthesis and cell growth [16,17]. Hamartin and tuberin deficient cells also show increased proliferation and reduced expression of the cyclin dependent kinase (CDK) inhibitor p27 [18,19]. In addition to these roles in cell growth and proliferation, *TSC1* and *TSC2* may play more direct roles in cell adhesion. Hamartin interacts with the ezrin–radixin–moesin family of cytoskeletal proteins and activates the small GTPase Rho [20,21] that regulates cell adhesion by mechanisms including activation of focal adhesion complexes, while tuberin appears to play an as yet ill-defined

role in E-cadherin mediated cell adhesion and the β -catenin pathway [22].

Despite this exciting progress, the tumour suppressor roles of tuberin and hamartin have been investigated only in solid tumours in organs that are frequently affected as part of the tuberous sclerosis phenotype, mainly renal and brain tumours. As part of an ongoing study of tumour suppressor gene expression in human breast cancers, we recently investigated the expression of *TSC1* and *TSC2* in a series of human breast cancers for which 10 year outcome data were available. The data from these studies indicate that hamartin and tuberin could be valuable prognostic markers for breast cancer and, since therapeutic agents exist that modulate *TSC1/2* signalling, the relationship between hamartin and tuberin expression and the behaviour of breast cancer cells demands investigation. Here, we report the aberrant expression of both tuberin and hamartin, which appears to be linked to the promoter methylation of the *TSC* genes. In addition, the study demonstrated a relationship between the aberrant expressed TSC products and clinical outcome.

2. Materials and methods

2.1. Materials

RNA extraction kit and RT kit were obtained from AbGene Ltd., Surrey, England, UK. PCR primers were designed using Beacon Designer (CA, USA) and synthesised by Invitrogen Ltd. (Paisley, Northern Ireland, UK). Molecular biology grade agarose and DNA ladder were from Invitrogen. Master mix for routine PCR and quantitative PCR was from AbGene. Rabbit anti-human tuberin, anti-human hamartin, and an universal staining kit purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA, USA), and Vector Laboratories (Nottingham, England, UK), respectively.

2.2. Samples collection

Breast cancer cell lines MCF-7, ZR751, MCF10A, MDA MB 435, MDA MB 468, MDA MB 483, MDA MB 435S, BT474, BT549, and MDA MB 231, and human fibroblast MRC-5 were purchased from the European Collection of Animal Cell Cultures (ECACC, Salisbury, England). Human umbilical vein endothelial cells (HUVEC) were purchased from TCS Biologicals (Oxford, England). Breast cancer tissues ($n = 120$) and ‘normal’ background tissues, obtained from surgically

removed tissues away from the tumour and histologically verified to be free from cancer cells by a consultant pathologist ($n = 32$), were collected immediately after surgery and stored in the deep freezer until use. Patients were routinely followed clinically after surgery. The median followup period was 120 months. The presence of tumour cells in the collected tissues was verified by a consultant pathologist (ADJ), who examined H&E stained frozen sections. Clinical information of the cohort has been previously described [23] and is given in Table 1.

Tissues from patients with breast cancer who had undergone mastectomy were collected immediately after surgery and stored at -80°C . Details of histology were obtained from pathology reports. Patients were routinely followed up on a regular basis and details stored in a database.

2.3. Tissue processing, RNA extraction and cDNA synthesis

Frozen sections of tissues were cut at a thickness of 5–10 μm and were kept for immunohistochemistry and routine histology. A further 15–20 sections were mixed and homogenized using a hand-held homogenizer, in ice cold RNA extraction solution. The concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was carried using a RT kit with an anchored oligo-dt primer supplied by AbGene, using 1 μg total RNA in 96-well plate. The quality of cDNA was verified using β -actin primers.

2.4. Quantitative analysis of the transcripts of tuberin and hamartin

The level of tuberin and hamartin transcripts from the above-prepared cDNA was determined using a

real-time quantitative PCR, based on the Amplifluor™ technology, modified from a method previously reported [23,24]. Briefly, pairs of PCR primers were similarly designed using the Beacon Designer software (version 2, CA, USA), but to one of the primers, an additional sequence, known as the Z sequence (5'actgaacctgaccgtaca/3') which is complementary to the universal Z probe (Intergen Inc., England, UK), was added. Sequences of the respective primers were: hamartin, 5'agaccacccatcttttggac/3' and 5'actgaacctgaccgtacaatctcaattcaaacacactg/3', tuberin, 5'ttggtaaattcaatagctg/3' and 5'actgaacctgaccgtacagcactgaccctatgtccac/3', and β -actin: 5'atgatatcgccgcctcg/3' and 5'cgctcggttaggatctca/3'. A Taqman detection kit for β -actin was purchased from Perkin–Elmer. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence, 10 pmol of FAM-tagged probe (Intergen Inc.), and cDNA from approximate 50 ng RNA. The reaction was carried out using IcylerIQ™ (Bio-Rad) which was equipped with an optical unit that allows real-time detection of 96 reactions, using the following condition: 94 °C for 12 min, 50 cycles of 94 °C for 15 s, 55 °C for 40 s and 72 °C for 20 s. The levels of the transcripts were generated from a standard that was simultaneously amplified with the samples. Cyto-keratin-19 (CK19) was used to normalize cellularity during the analysis and primers for CK19 were 5'-caggccgaggtaactgac-3' and 5'-actgaacctgaccgtacacttctgc cagtgtcttc-3', respectively [25]. Data are shown here as either the number of transcripts, or as hamartin (or tuberin): CK19 ratio.

3. Biosulfite modification and methylation specific PCR for *TSC1/2* promoters [26]

Genomic DNA from multiple sections from frozen tissue and from cells was extracted, using a standard DNA extraction protocol. Approximately 1 μg DNA was denatured in NaOH (final concentration 0.2 M) for 10 min at 37 °C, followed by addition of 10 mM freshly prepared hydroquinone and 3 M freshly prepared 3 M sodium bisulfite (pH 5). The mixture was incubated at 50 °C overnight. DNA was purified using the Wizard DNA purification kit (from Promega). Eluted DNA from the resin was added NaOH (final concentration 0.3 M), followed by standard ethanol precipitation. Primers for methylated regions are 5'ctataaataaacaactaaaaaaaacga/3', and 5'cgaaggcggtgtgttttaggac/3', 5'ggtaaatagacggagtttattatcg/3' and 5'ttctctcgctgaaaacgctactaa/3', for hamartin and tuberin promoters, respectively. Primers for unmethylated sequence are 5'actataaataaacaactaaaaaaaacaa/3', and 5'ttgaagggtgtgtgttttaggat/3', 5'ggtaaatagatggatttattattgt/3' and cattctcactaaaaacactataactaa, for

Table 1
Clinical and pathological information of the patients

Node status		Node negative		Node positive	
n=		65		55	
Grade		Grade 1		Grade 2	
n=		23		41	
Histology	Ductal	Lobular	Others	Medullary	Tubular
					Mucinous
n=	88	14	2	2	4
TNM staging		TNM 1	TNM 2	TNM 3	TNM 4
n=		69	40	7	4
Clinical outcome	Disease free	With metastasis	With local recur.	Died of breast cancer	Died of unrelated diseases
n=	81	7	5	20	7

hamartin and tuberin promoters, respectively. Products from MS-PCR were separated on 12% PAGE gel.

3.1. Immunohistochemical staining of tuberin and hamartin [23,24]

Frozen sections of breast tumour and background tissue were cut at a thickness of 6 μ m using a cryostat. The sections were mounted on super frost plus microscope slides, air dried and then fixed in a mixture of 50% acetone and 50% methanol. The sections were then placed in “Optimax” wash buffer for 5–10 min to rehydrate. Sections were incubated for 20 min in a 0.6% BSA blocking solution and probed with the primary antibody (1:200 dilution) for 1 h at room temperature. Following extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody (Multilink Swine anti- goat/mouse/rabbit immunoglobulin, Dako Inc.). Following washings, Avidin Biotin Complex (Vector Laboratories) was then applied to the sections followed by extensive washings. Diamino benzidine chromogen (Vector Labs) was then added to the sections which were incubated in the dark for 5 min. Sections were then counter stained in Gill’s Haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip. Staining intensity from digitized images was determined using density analysis package of Optimas software (Optimas 6).

Statistical analysis was carried out using Mann–Whitney *U* test and the Kruskal–Wallis test. Survival analysis was carried out using Kaplan–Meier survival curve and Cox Proportion hazardous analysis using SPSS package

4. Results

4.1. Expression of tuberin and hamartin in mammary tissues and cells

We first assessed the presence of gene products of *TSC1/2* in breast cancer tissues and cell lines. Fig. 1(a) has demonstrated that hamartin mRNA was present in all the tissues and cell lines tested. In some tumour samples, the signals appeared to be weaker. However, the signal for tuberin varied, with most normal tissues display tuberin mRNA, and tumour tissues at relatively lower levels. It is interesting to note that a highly aggressive MDA MB 231 cell and fibroblast MRC5 did not have tuberin mRNA. Real-time quantitative PCR had shown that, of the same paired samples as presented in Figs. 1(b) and (c), levels of hamartin transcripts were marginally lower in tumour tissues, compared with the matched normal tissues (Figs. 1(b) and (c)), although the difference was not statistically significant ($P > 0.05$). However, levels of tuberin were much lower in

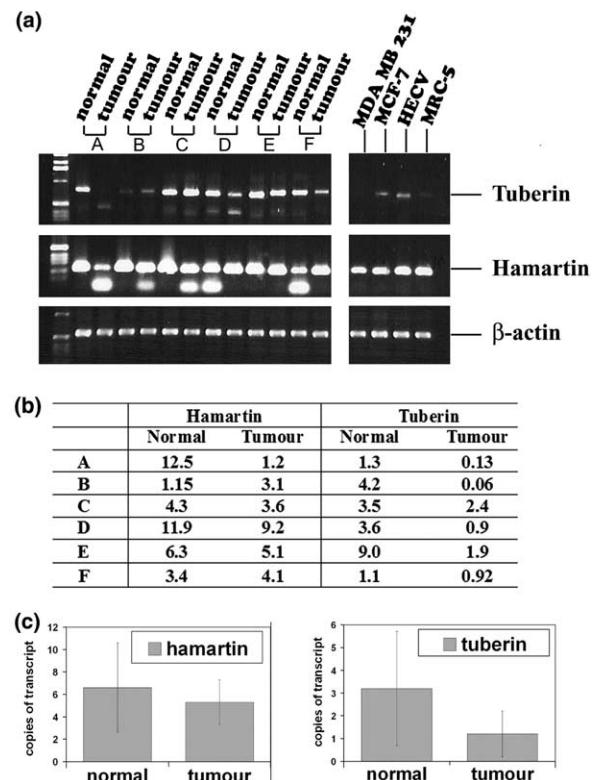


Fig. 1. Expression of tuberin and hamartin in human breast tissues and cell lines (normal and tumour-each pair are tissues from the same patient): (a) conventional RT-PCR; (b) levels of transcript for respective patients as shown in A; (c) quantitative PCR showing the levels of transcript from the same pairs as given in A.

tumour tissues, compared with control. This was, again, not statistically significant ($P = 0.087$), largely due to small sample numbers.

We then applied the the quantitative analysis of the transcripts to the entire cohort and have revealed that while levels of hamartin remained similar between normal ($n = 32$) and tumour tissues ($n = 120$), tumour tissues displayed a significantly lower level of tuberin than normal tissues ($P < 0.05$) (Fig. 2). A similar observation was obtained when the transcripts were normalized by CK19 (Fig. 2 insets, shown are hamartin and tuberin:CK19 ratio).

4.2. Distribution of tuberin and hamartin in mammary tissues

Both hamartin and tuberin are primarily seen in normal mammary epithelial cells (Figs. 3 and 4 left panel). A weak staining of hamartin and tuberin was seen in stromal cells. In invasive tumour tissues, both proteins appear to be markedly reduced (Figs. 3 and 4 right panel). In case of DCIS (ductal carcinoma *in situ*), the staining appears to be stronger than invasive tumours, but visibly weaker than the normal tissues.

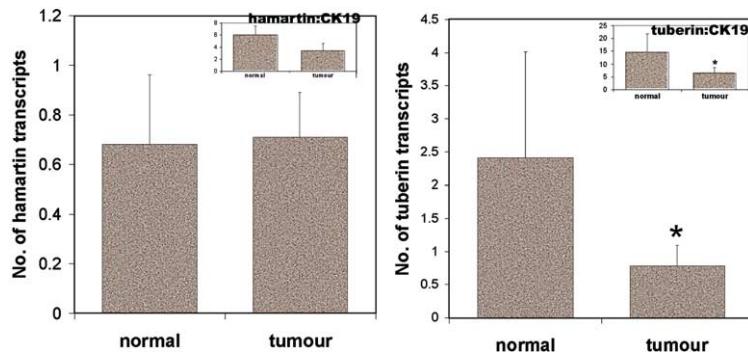


Fig. 2. Levels of transcripts for hamartin and tuberin in normal ($n = 32$) and tumour ($n = 120$) tissues (mean \pm SD). Shown are the number of transcripts from approximate 50 ng RNA. * $P < 0.05$ vs. normal. Insets: Hamartin (or tuberin):CK19 ratio.

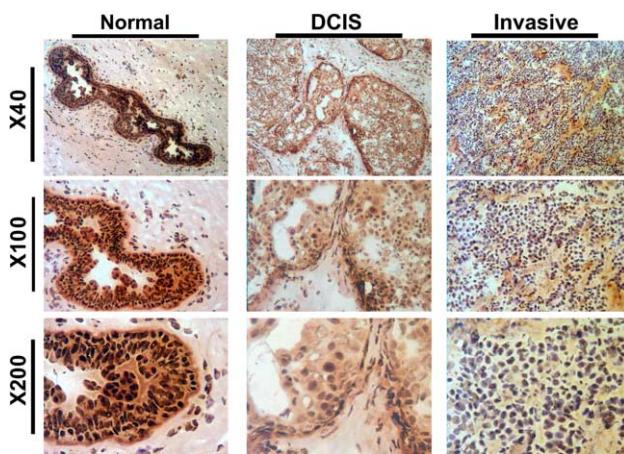


Fig. 3. Immunohistochemical staining of hamartin in normal (left), DCIS (middle), and invasive (right) carcinoma. Shown are magnification at 40 \times (top), 100 \times (middle) and 200 \times (bottom panel). The hamartin transcript for the normal tissue (from patients 138) was at 1.07 copies and hamartin:CK19 was 0.53. The hamartin transcript for the DCIS tissue (patient 118) was 2.33 copies and hamartin:CK19 was 0.15. The hamartin transcript for the invasive cancer (patient 123) was 0.0132 copies and hamartin:CK19 was approx. 0.001.

4.3. Reduction of tuberin and hamartin transcripts was associated with nodal involvement and association with prognostic indices

Node positive tumours had a marginally lower level of hamartin transcript, but had significantly lower levels of tuberin transcript, compared with node negative tumours (Fig. 5). The ratio between hamartin or tuberin and CK19 showed a similar trend (Fig. 5 insets). The levels of the transcripts were further analysed against the Nottingham Prognostic Index [NPI = (0.2 \times size) + grade + Nodal status], where NPI < 3.4, 3.4–5.4 and >5.4 represented good (15 year survival rate 80%) (NPI1), moderate (15 year survival 42%) (NPI2) and poor prognosis (15 year survival 13%) (NPI3), respectively. There was a progressive and significant reduction of tuberin from groups with good prognosis to poor prognosis (Fig. 6). There was also a significant reduction

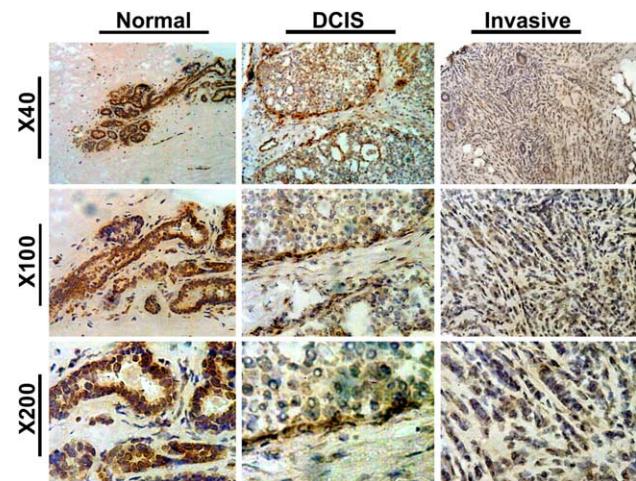


Fig. 4. Immunohistochemical staining of tuberin (left), DCIS (middle), and invasive (right) carcinoma. Shown are magnification at 40 \times (top), 100 \times (middle) and 200 \times (bottom panel). The tuberin transcript for the normal tissue (from patients 110) was 0.382 and tuberin:CK19 was 0.294. The transcript for the DCIS (patient 118) was 0.164 and tuberin:CK19 was 0.184. The tuberin transcript for the invasive cancer (patient 123) was 0.0286 and tuberin:CK19 was 0.0023.

of tuberin:CK19 ratio as shown in Fig. 6 inset. However, significant reduction of hamartin transcript was only seen in patients with a poor prognosis.

Table 2 summarises the levels of the transcript in relation to tumour type and tumour grade. There was no significant difference between hamartin and tuberin in different tumour grade.

4.4. Very low levels of hamartin and tuberin is associated with recurrence and mortality

Over a 10 year follow-up, patients were divided into those who remained disease free, those developed metastasis, local recurrence, and those who died of breast cancer (excluding death from unrelated cause). Significantly low levels of hamartin and tuberin were seen in patients who developed local recurrence and who died of breast cancer. Patients who developed metastasis had also

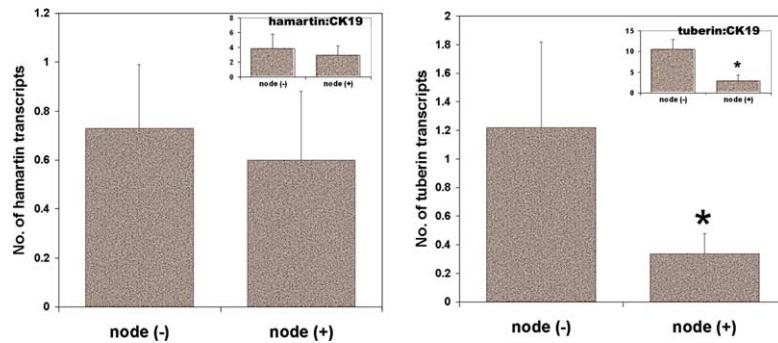


Fig. 5. Levels of expression of hamartin (left) and tuberin (right) transcripts in node positive ($n = 55$) and negative ($n = 65$) breast tumours. * $P < 0.05$ vs. node negative tumours. Shown are means \pm SD of the number of transcripts from approximate 50 ng RNA. Insets: Hamartin (or tuberin):CK19 ratio.

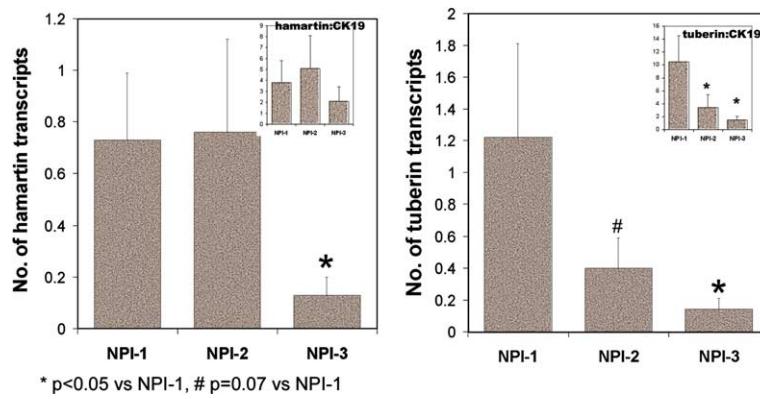


Fig. 6. Expression of hamartin (left) and tuberin (right) in relation to the prognostic index (Nottingham Prognostic Index, NPI-1: indices < 3.4 with good prognosis, NPI2: indices 3.4–5.4 with moderate prognosis, and NPI3: indices >5.4 with poor prognosis). *# $P < 0.05$ vs. NPI-1 tumours. Shown are means \pm SD of the number of transcript from approximate 50 ng RNA. Insets: Hamartin (or tuberin):CK19 ratio.

significantly low levels of hamartin and low levels of tuberin (Fig. 7). When the transcript was normalized by CK19, the similar trend was seen with both hamartin and tuberin (Fig. 7 insets).

Using Kaplan–Meier's survival analysis, patients with high levels of tuberin had a marginally longer survival (137.5 (125.4–149.7) months) than those with low levels (131.4 (120.4–142.5) months), $P = 0.192$. A similar trend was seen with hamartin in that patients with high hamartin had a longer survival (144.5 (133.4–155.8) months) than those with low levels (123.2 (109.5–136.9) months), $P = 0.168$. In ER negative tumours,

no difference was seen between survival of patients with high or low tuberin and hamartin. However, in ER positive tumours, high levels of tuberin were associated with longer survival (123.0 month) than low tuberin (79.0 months), $P = 0.068$.

4.5. Methylation of *TSC1* and *TSC2* promoters in breast cancer cells and breast tumour tissues

TSC1 promoter in ZR751, MDA MB 435, MDA MB 435S, MDA MB 468, BT549 were found to be heavily methylated. Interestingly, *TSC1* promoter in fibroblast cell line MRC5 was also methylated (Fig. 8(a)). The methylation was also seen in most of the breast tumour samples, and only in a portion normal tissues (Fig. 8(b)).

The methylation of *TSC2* promoter appears to be less frequent. MDA MB 468, MDA MB 483, MDA MB 435S and weakly MDA MB 435 and MRC5 were found to have methylated *TSC2* promoter (Fig. 8(a)). In breast tissues, however, a very small number of samples was found to have methylation (Fig. 8(b)).

Table 2
Levels of expression of tuberin and hamartin in different grade (A) and histological types (B)

A:	Grade 1	Grade 2	Grade 3	P value
Hamartin	0.77 \pm 0.37	0.57 \pm 0.21	0.78 \pm 0.33	>0.05
Tuberin	0.81 \pm 0.41	1.38 \pm 0.86	0.37 \pm 0.14	>0.05
B:	Ductal	Lubular		P value
Hamartin	0.77 \pm 0.23	0.54 \pm 0.27		>0.05

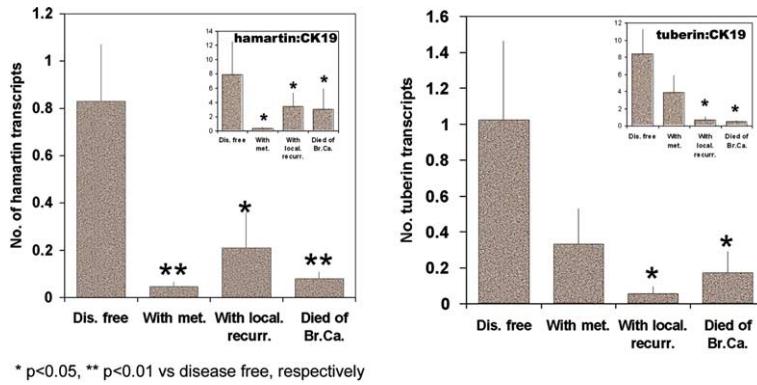


Fig. 7. Levels of expression of hamartin (left) and tuberin (right) are correlated with clinical outcomes, following a 6-year follow up. Disease free: patients who remained Dis free ($n = 87$); with met: patients with distant metastasis ($n = 6$); with Local Recurr: patients with local recurrence ($n = 5$), died of breast cancer ($n = 16$); patients who died of breast cancer (excluding those who died of unrelated diseases). * $P < 0.05$, ** $P < 0.01$ vs. disease free. Shown are means \pm SD of the number of transcript from approximate 50 ng RNA. Insets: Hamartin (or tuberin):CK19 ratio.

5. Discussion

The current study has reported that in a non-hereditary sporadic solid tumour, human breast cancer, the expression of the *TSC* gene product is aberrant. This aberrant expression is linked to the clinical outcome in the patients. We proposed that one of the potential mechanisms of the expression abnormality is via promoter hypermethylation.

TSC genes have been regarded as tumour suppressor genes, primarily in the tuberous sclerosis condition, as mutations of these genes are associated with the occurrence of certain tumours, namely lymphangioleiomyomatosis, the brain tumour giant cell astrocytoma and

occasionally renal cell carcinoma. However, there have been few studies on the link between these genes and gene products with sporadic human solid tumours. The study has provided evidence that the gene products both *TSC-1* and *TSC-2*, tuberin and hamartin, respectively, are aberrant in human breast tumours. Using analyses of both mRNA and protein, the current study has demonstrated a reduction of both tuberin and hamartin at mRNA and protein levels.

The impact of the reduction of tuberin and hamartin on the development of breast cancer is yet to be fully elucidated. However, in the past decade, a few modes of action have been identified in association with the *TSC* complex. It has been recently shown that tuberin

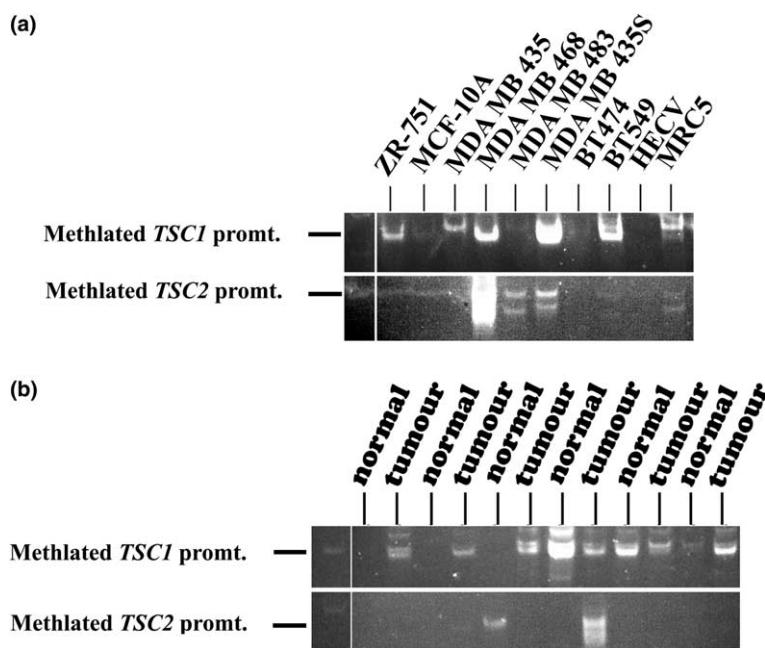


Fig. 8. Methylation of the *TSC1* and *TSC2* promoters in cell lines (a) and breast tissues (b). Breast tissues in b were the same and in the same order as in Fig. 1(a).

interact with p27 and increase the stability of p27 and SMAD, thus regulating cell cycles [18,19,27,28]. TSC-2 is involved in the signalling of mTOR and LKB1 [29]. In addition, TSC-2 has been shown to be essential in the downstream of insulin-PI3K signalling. Mutation of TSC-2 may be responsible for lack of tumourigenic response to IGF and insulin *in vivo* models [30]. In addition, *TSC* mutations are associated with MAPK signalling abnormalities [31]. Lack or mutation of *TSC-1* and *TSC-2* may be associated with the reduction of interferon gamma *in vivo*, which again may contribute to the carcinogenesis in this case [32].

The other potential link between TSC and tumours is the VEGF pathway. It has been recently reported [33–35] that TSC gene products regulate VEGF production through an mTOR signalling pathway. Serum VEGF levels may be a useful clinical biomarker to monitor the progression of TSC-associated lesions and rapamycin or related inhibitors of mTOR may have therapeutic benefit in TSC both by direct tumour cell killing and by inhibiting the development of TSC lesions through impairment of VEGF production. Thus, the weakened expression of tuberin and hamartin as seen in the current study may aid in the production of VEGF, which is frequently activated in human breast cancer [36–38]. The direct correlation between the two groups of molecules would require additional and separate studies, which we are currently developing.

It is noteworthy that the connection between the TSC gene products and breast cancer may be via the ER pathways. It has been shown recently that tuberin interacts with ER- α , via which it antagonized oestrogen-induced cell growth [39]. This observation is partly supported by the current study that in ER- α positive tumours, patients with high levels of tuberin had a longer survival than those with low level, although this was not statistically significant ($P = 0.068$). A larger study with more patients would need to verify this point.

The other important finding from the study is that low levels of both tuberin and hamartin are associated with the aggressive nature of breast tumours, i.e., nodal positive, higher grade, metastasis and mortality. It suggests that these two molecules have a predictive value in assessing the aggressiveness of breast tumours. However, a number of correlations failed to reach statistical difference, i.e., survival time and the correlation with hamartin and tuberin, despite the observation that low levels of the TSC transcripts were associated with shorter survival. One potential possibility is the insufficient number of patients in each of the subgroups in the current study cohort. A larger cohort would certainly assist to answer this question.

The present study has also shown that promoter hypermethylation frequently occurs in human breast cancers, as a high proportion of breast tumours had promoter methylation compared with normal tissues. How-

ever, the promoter methylation in tumour tissues requires further investigation. TSC genes are large and are frequently mutated in TS conditions. Mutations of both genes were not studied in the current study, but will be of significant interest in future studies, given the potential impact of mutations on the function of the TSC complex [40]. We are currently investigating the potential mutation in high risk patients.

In conclusion, the current study has reported aberrant expression of both tuberin and hamartin, products of *TSC-1* and *TSC-2* genes, in human breast tumours. Low levels of these gene products are associated with the aggressive nature of breast tumours and poor clinical outcome. In breast tumour tissues, hypermethylation of the *TSC* promoters are seen. *TSC* and its products thus have relevance to the clinical outcome of patients with breast cancer.

Conflict of interest

None declared.

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