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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 harmartin, 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. (Pasley, 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