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Original Paper

Truncated *TSG101* Transcripts are Present in Peripheral Blood from Both Familial Breast Cancer Patients and Controls

C.-F. Xu, J. Greenman and E. Solomon

Division of Medical and Molecular Genetics, UMDS, 8th Floor, Guy's Tower, Guy's Hospital, St Thomas' Street, London SE1 9RT, U.K.

***TSG101* is a recently identified putative tumour suppressor gene which has been implicated in human breast cancer. To address whether germline disruption of *TSG101* predisposes individuals to this disease, we analysed genomic DNA and mRNA isolated from peripheral blood from 20 familial breast cancer cases. No evidence of large intragenic insertions/deletions or point mutations in *TSG101* was found by Southern blot analysis and sequence analysis of the entire coding region. However, in 11 of 20 samples, 'aberrant' transcripts were detected. Sequence analysis suggested that these variants were generated by the use of different cryptic splicing sites. Such alternative/aberrant splicing events were not restricted to cancer patients, but were also detected in peripheral blood of non-cancer patients and in normal tissues.** © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: tumour suppressor gene, *TSG101*, breast cancer, alternative splicing
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INTRODUCTION

INVESTIGATION OF families with a strong history of breast cancer led to the identification of the breast cancer susceptibility genes, *BRCA1* and *BRCA2* [1, 2]. However, recent studies have demonstrated that up to 30% of breast cancer families cannot be accounted for by mutations in either of these genes, suggesting the existence of additional *BRCA* genes (reviewed in [3]). *TSG101* is a recently isolated putative tumour suppressor gene on chromosome 11p15. Truncated *TSG101* transcripts and intragenic deletions have been detected in sporadic breast tumours [4, 5], suggesting that abnormality of *TSG101* may be implicated in human breast cancer. Subsequent studies, however, have shown that these truncated transcripts may be the result of alternative or aberrant splicing events [5–7]. Nevertheless, Lee and Feinberg [5] reported that truncated *TSG101* transcripts were found more commonly in tumours compared with normal breast tissues. None of these studies have addressed whether the abnormality of *TSG101* is implicated in familial breast cancer. In this study, we analysed *TSG101* in 20 familial breast cancer patients. We found no evidence of genetic abnormalities of this gene in these patients, whilst detected 'aberrant' *TSG101* transcripts in familial breast cancer patients which were also present in controls.

Correspondence to C.-F. Xu.
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MATERIALS AND METHODS

Samples

This study included 20 patients with familial breast cancer attending the cancer genetics clinic at Guy's hospital. Based on our experience, these families were unlikely to have mutations in either *BRCA1* or *BRCA2*. Indeed, single strand conformation polymorphism (SSCP) and heteroduplex analysis of 10 affected individuals from these families showed no evidence of mutations in *BRCA1*. None of these families have individuals with ovarian or male breast cancer. All probands were under 45 years of age when the diagnosis of breast cancer was made and had at least one first degree relative with the disease. Blood samples were taken after informed consent was obtained.

Controls were blood samples from patients with haemophilia A ($n = 10$) and Duchenne muscular dystrophy (DMD, $n = 9$) who attended Guy's hospital for genetic testing. Poly A^+ RNAs from normal human tissues, i.e. placenta, mammary gland, testis, thymus and brain, were purchased from Clontech (Palo Alto, California, U.S.A.).

Preparation of DNA and RNA

Total genomic DNA and RNA from breast cancer patients and controls was extracted from white blood cells contained

within 10 ml of peripheral blood, using a standard salting out procedure [8] and a modified guanidinium thiocyanate method [9], respectively.

Reverse transcription–polymerase chain reaction (RT–PCR) and cDNA sequencing

Reverse transcription was carried out on approximately 200 ng of poly A^+ RNA or 1 μg of total RNA with oligo(dT) primer using a standard protocol [10]. The cDNA was diluted with water to a total volume of 120 μl and 5 μl of the diluted cDNA templates were used in the first round PCR reactions. PCRs were carried out in a volume of 50 μl with conditions reported by Li and colleagues [4]; 10 μl of the second round PCR products were analysed on 1% agarose gels.

RT–PCR products were separated on agarose gels and purified using SPIN-X® centrifuge tube filters (Costar, Cambridge, Maryland, U.S.A.) or MicroSpin™ S-400 HR columns (Pharmacia Biotech, St. Albans, U.K.). Purified templates were sequenced using an AmpliTaq® Dye Terminator Cycle Sequencing kit and analysed on a 377 sequencer (ABI, Foster City, California, U.S.A.).

Southern blot analysis

Ten micrograms of genomic DNA was digested with 40 units of *Eco*RI restriction enzyme at 37°C overnight, separated on 0.8% agarose gels and transferred to Hybond™-N^+ nylon membranes (Amersham Life Science, Amersham, Buckinghamshire, U.K.) by alkali blotting. Blots were hybridised with a full length *TSG101* cDNA probe at 65°C in 5×SSPE (750 mM Nace, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.4), 5×Denhardt's solution, 0.5% sodium dodecyl sulphate (SDS) and 0.01% yeast RNA. Filters were washed to 0.2×SSC (30 mM Nace, 3 mM sodium citrate, pH 7.0), 0.1% SDS at 65°C and exposed to X-ray films.

RESULTS

'Aberrant' transcripts, but no mutations or rearrangement of the TSG101 *gene in familial breast cancer*

Following Southern blot analysis of genomic DNA isolated from the peripheral blood of 20 familial breast cancer patients, five ECORI fragments (23, 7.4, 6.1, 5.3, 4.5 kb) were detected in all the samples studied, with no evidence of aberrant bands in any of the samples (data not shown). Based on the size of the *Eco*RI fragments that hybridised to the *TSG101* cDNA probe, it was estimated that intragenic insertions/deletions greater than 0.1–1.0 kb could be readily detected by this analysis. The results suggest that large gene rearrangements of *TSG101* are not a frequent cause of familial breast cancer.

To determine whether subtle genetic changes of *TSG101*, such as small insertions/deletions or point mutations, were present in these patients, RNAs were prepared from the peripheral blood and examined by nested RT–PCR using primers encompassing the entire coding region [4]. While all 20 samples had a normal-sized RT–PCR product (1.1 kb), nine

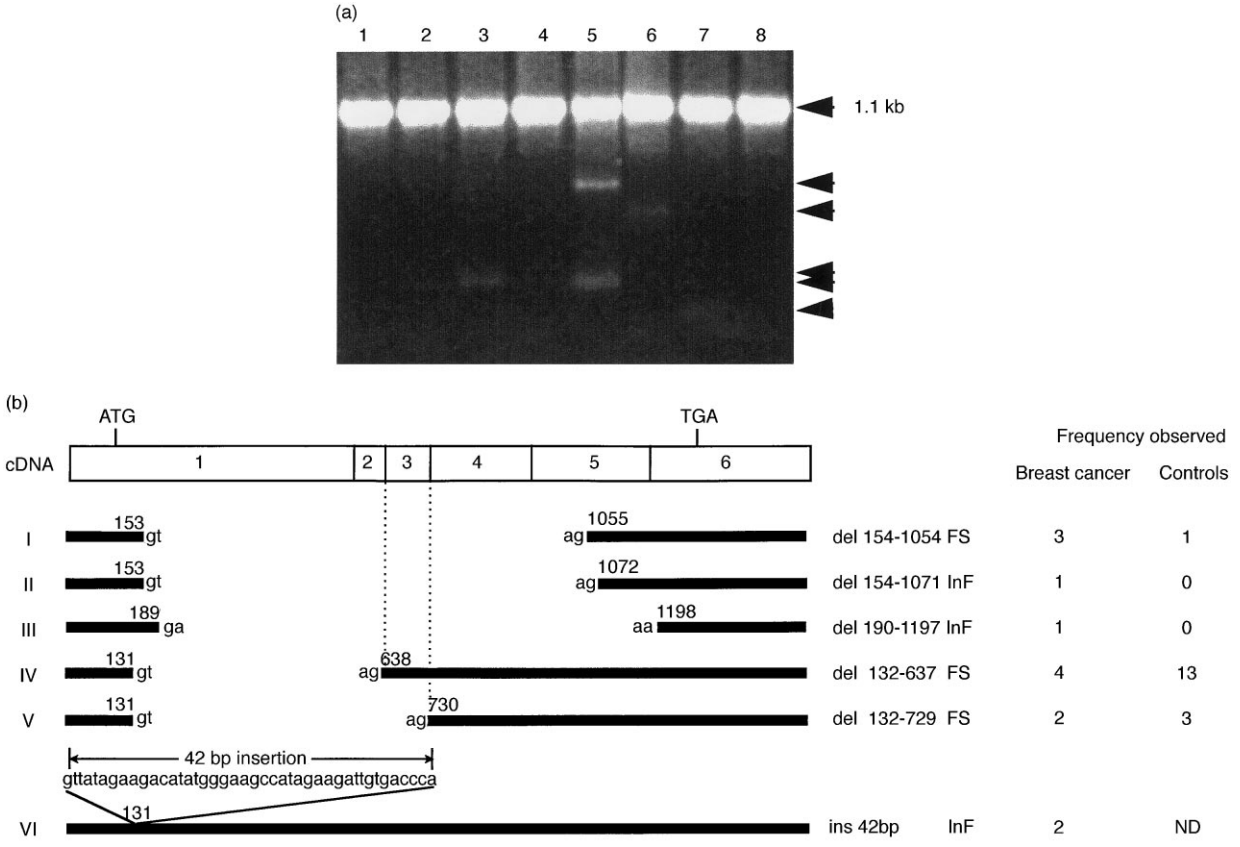

Figure 1. Detection of *TSG101* transcripts in familial breast cancer. (a) Agarose gel analysis of the reverse transcription–polymerase chain reaction products. The normal (1.1 kb) and truncated transcripts are indicated by arrows. 8 of the 20 samples studied are shown. (b) Schematic representation of various *TSG101* transcripts. The white box represents the normal *TSG101* transcript and the exon boundaries defined by Li and colleagues [4]. The solid bars represent variant transcripts detected in familial breast cancer patients (types I–VI). The numbers above the bars indicate the nucleotide positions where the insertion/deletions occurred. FS, frameshift deletion; InF, in frame insertion/deletion.

samples showed additional smaller transcripts, some of which are presented in Figure 1(a). Each of the RT–PCR products from all 20 samples was then sequenced completely. Sequence analysis confirmed the presence of the 1.1 kb product previously shown by Li and colleagues to represent the wild-type transcript [4]. In two samples, an additional larger transcript resulting from a 42 bp insertion in the 1.1 kb product was also identified (Figure 1b, type VI). Because of the relatively small increase in size, this variant was not readily distinguished from the wild-type on agarose gels. This insertion transcript has not been described in any of the previous studies [4–7] . No other changes were found in the full length cDNA products. Sequence analysis of the various smaller transcripts showed that they represent internal deletions of the full length cDNA encompassing two or more exons (types I–V, Figure 1b). The most frequently occurring variant transcript was type IV, being detected in 4 individuals (Figure 1b).

Detection of truncated TSG101 *transcripts in blood samples derived from non-cancer patients and in normal tissues*

To address whether the observed variant *TSG101* transcripts were cancer patient specific, RNA was prepared from 19 controls with no personal or family history of cancer. Variant *TSG101* transcripts were detected in 16 of the 19 samples, some of which are shown in Figure 2(a). It was estimated from their size, and subsequently confirmed by sequence analysis, that at least three of the truncated transcripts observed in breast cancer patients were found in controls (type I, IV and V; Figure 2a). The most frequently observed variant transcript was also type IV, occurring in 13 of 19 samples (Figure 1b).

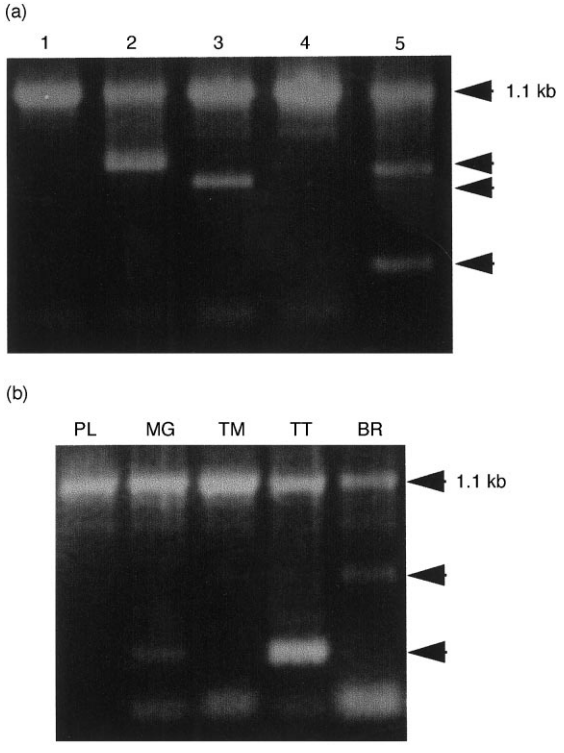

Figure 2. *TSG101* transcripts detected in controls. Examples of reverse transcription–polymerase chain reaction products from (a) peripheral blood of non-cancer patient and (b) normal human tissues. Arrows indicate various *TSG101* transcripts. PL, placenta; MG, mammary gland; TM, thymus; TT, testis; BR, brain.

We further analysed *TSG101* transcripts in normal tissues, i.e. human placenta, mammary gland, thymus, testis and brain. Truncated *TSG101* transcripts (type I or V) were observed in all tissues except placenta (Figure 2b).

DISCUSSION

TSG101 is a recently identified tumour suppressor gene, in which truncated transcripts and intragenic deletions have been detected in sporadic breast cancer [4, 5, 7]. This study aimed to determine whether or not abnormalities in *TSG101* are involved in the development of familiar breast cancer. Genomic DNA and mRNA from peripheral blood of 20 breast cancer patients with a family history of this disease, but unlikely to have *BRCA1/2* mutations, were analysed. Although no intragenic deletions/insertions or point mutations were observed, truncated *TSG101* transcripts were detected. These variant transcripts were also found in peripheral blood from non-cancer patients, as well as in normal tissues and are, therefore, not restricted to cancer patients. Sequence analysis of these variant transcripts showed that they represent internal deletions/insertion of the *TSG101* mRNA.

Six different variant transcripts were detected in this study, among which type I and type V transcripts were also detected by Li and colleagues in breast carcinomas [4]. Based on results from Southern hybridisation of PCR products from genomic DNA, Li and colleagues [4] proposed that these 'aberrant' transcripts resulted from large intragenic deletions of *TSG101*. Our data, however, are at variance with this conclusion. All the truncated transcripts observed contained deletions extending over at least two exons. Had these been due to genomic deletions, they should have been readily detected by Southern blot analysis. However, no abnormal bands were seen, indicating that these variant transcripts were not caused by gene rearrangement.

We next explored whether or not the variant *TSG101* transcripts were generated through alternative splicing, by examining nucleotide sequences at the insertion/deletions junctions. As shown in Figure 1(b), type I and type II deletions started at the same cryptic splicing donor site (GT) within the reported exon 1 [4] and ended at different cryptic splicing acceptor sites (AG) in exon 5. Similarly, type IV and type V deletions began at the same GT site in exon 1 and finished at exon 2/3 and 3/4 junctions using intrinsic AG sites, in introns 2 and 3, respectively, for their splicing acceptor sites. Therefore, it is plausible to suggest that these aberrant transcripts are caused by alternative/aberrant splicing using either genuine or cryptic splice sites.

The fusion junction of type III transcript did not contain the consensus sequence for a splicing site. Although this variant may represent an artefact of RT–PCR, such unusual transcripts have been reported previously [5, 7]. The underlying mechanism by which these aberrant transcripts might have been generated is unknown.

Type VI transcripts contain a 42 bp insertion, which shares no sequence homology to any other genes in the database. The junction at which point the insertion occurs coincides with the 5′ junction of the type IV and type V transcripts. This indicates that the original reported exon 1 of *TSG101* [4] may in fact contain two or more exons and that the observed insertion may represent, either an aberrantly spliced exon using a cryptic splicing site, or an alternative new exon. Furthermore, Steiner and associates recently suggested the

presence of a previously unreported intron located within exon 1 [6]. This is consistent with our results.

Our results suggest that *TSG101* is unlikely to be involved in the development of familial breast cancer. Whether abnormalities in this gene are truly implicated in sporadic breast cancer remains to be seen. Indeed, subsequent studies have failed to confirm the presence of genomic defects in *TSG101* in a large number of primary breast cancer cases [5–7] and have indicated that the truncated transcripts were also the result of alternative or aberrant splicing events.

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