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Original Paper

Analysis of Aberrant Transcription of *TSG101* in Hepatocellular Carcinomas

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A variety of studies suggest that tumour suppressor loci on chromosome 11p are important in various forms of human neoplasia. Recently, a gene located at the chromosome 11p 15.1–15.2 region called *TSG101* was discovered and proposed as a candidate tumour suppressor gene in breast cancers. We evaluated the *TSG101* gene in a panel of liver cancer cell lines and paired tumours and non-malignant tissues. In this study, four of the seven (57%) cell lines, eight of the 18 (44%) tumours and four of the 18 (22%) non-malignant liver tissues exhibited aberrant *TSG101* transcripts by nested reverse transcription–polymerase chain reaction (RT–PCR) analysis. However, a normal-sized transcript without sequence abnormalities verified by single-stranded conformation polymorphism (SSCP) analysis was expressed at robust levels in all the cell lines and most of the tissue samples tested. In addition, Southern blot analysis could identify no genomic abnormalities of the gene. Our results suggest either that the *TSG101* gene may not be involved in hepatocarcinogenesis or that it plays a role in the development and/or progress of hepatocellular carcinomas through an unusual mechanism.

Key words: *TSG101*, hepatocellular carcinoma (HCC), RT–PCR
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INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is one of the most common human malignancies in the world, especially in areas such as China and sub-Saharan Africa [1, 2]. The precise aetiology of HCC is not yet clear, but it is well known that HCC is frequently associated with a background of chronic liver disease [3]. Predisposing factors include hepatitis B virus or hepatitis C virus infection and aflatoxin B1 exposure [1, 2, 4]. Hepatocarcinogenesis is considered a multifactorial and multistep process [5, 6].

Loss of heterozygosity (LOH) in multiple regions of the genome is frequently observed in human solid tumours. Areas with a high rate of loss of genetic material could harbour putative tumour suppressor genes. In HCC, allele losses have been documented on chromosomes 1, 4, 5, 8, 10, 11, 13, 16, 17 and 22, but none of these losses has yet led to the identification of any specific tumour suppressor gene for HCC [7].

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Recently, the *TSG101* gene, a candidate tumour suppressor gene, was identified at human chromosome 11, subbands p15.1–15.2 and mutations of this gene in human breast cancer have frequently been identified [8, 9]. Since LOH at 11p15 has been reported in HCC [10–13], in the current study, we used a molecular genetic approach to determine the frequency and extent of alterations of this gene in a panel of HCC cell lines and primary tissues.

MATERIALS AND METHODS

Cell lines and patients

A panel of six human HCC cell lines (Hep 3B, HA22T, HCC36, Tong, SK-Hep-1 and HuH-7), one human hepatoblastoma cell line (HuH-6) [14–17] and 18 HCC tissues and their corresponding non-malignant liver tissues obtained from patients who had surgery at the Taipei Municipal Jen-Ai Hospital were included in this study. Informed consent was obtained from all patients. Tumours were dissected to eliminate normal tissue contamination. All the specimens were frozen immediately after surgical resection and stored in liquid nitrogen before testing. All malignant and

non-malignant tissue specimens were confirmed by pathological examination.

The clinicopathological features of each patient were reviewed and recorded. There were 12 men and 6 women. Their mean age at resection was 60.3 years. Except for 1 patient who was positive for both serum hepatitis B virus surface antigen (HBsAg) and anti-hepatitis C virus antibody (anti-HCV antibody), serum HBsAg was positive in 10 of the 18 patients (56%), and serum anti-HCV antibody was positive in the remaining 7 patients (39%). 12 of the 18 patients (67%) had cirrhosis and the other 6 patients had chronic hepatitis (33%).

RNA extraction, reverse transcription–polymerase chain reaction (RT–PCR) and sequence analysis

The transcripts of the *TSG101* gene were examined using nested RT–PCR. RNA was purified from cell lines, tumours and non-malignant liver tissues, and cDNA was generated from RNA as described previously [18]. Nested PCR amplifications were carried out using primers, flanking the full coding sequence of the *TSG101* cDNA (Table 1). Details of the procedures have been previously described [9]. All the reactions were performed at least twice and for verification of the integrity of the RNA samples, control RT–PCR amplifications using primers specific for the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene were performed [19]. The procedures for sequencing the aberrant PCR products have been described previously [18].

Single-stranded conformation polymorphism (SSCP) analysis

Since point mutation of the non-deleted allele of the *TSG101* gene has been reported in breast cancer cases [9], we designed and performed the non-isotope SSCP analysis to screen for possible mutation of the non-deleted allele of the gene in the HCC cell lines and tissues. Details of the method have been previously described [20]. Briefly, six sets of the primers (Au + Ad, Bu + Bd, Cu + Cd, Du + Dd, Eu + Ed and Fu + Fd) were designed to cover the full coding region of the *TSG101* cDNA (Table 1). The PCR amplification was performed with each set of the primers for 35 cycles in a Perkin Elmer Cetus DNA thermal cycler (Foster City, California, U.S.A.), in which each cycle remained 1 min at 94°C, 1 min at 56°C and 1 min at 72°C. The PCR samples were mixed with loading dye (95% formamide and 5% 6×loading dye; Promega, Madison, Wisconsin, U.S.A.), and then denatured at 95°C for 10 min and loaded in parallel on a 12% non-denaturing acrylamide gel (49:1, 5% glycerol in 0.5×tris–borate–EDTA buffer (TBE). The acrylamide mini-gel (8.0 cm×8.0 cm×1.5 mm) was then run under 3 W for 4–4.5 h at 4°C on the Novex Xcell Mini-Cell Instruction (Novex, San Diego, California, U.S.A.). The gels were stained with 0.5 μg/ml ethidium bromide in 0.5×TBE for 20 min, destained with distilled water for 15 min, and then visualised and photographed under ultraviolet light.

Southern hybridisation

High molecular weight DNAs from liver cancer cell lines and tumours and non-malignant liver tissues were extracted using standard methods. Between 5 and 10 μg of DNA was digested with restriction enzymes, *Hin*dIII, *Eco*RI or *Bam*HI according to the manufacturer's instructions. The digested DNAs were electrophoresed on 0.8% agarose gels, blotted to Nylon membranes, and fixed by ultraviolet cross-linking. Filters were hybridised with a 1177 bp cDNA probe containing exons 1–6 of the *TSG101* gene. The probe was labelled with [α-^{32}P]dCTP by random priming (DECA primer™ II DNA labelling kit, Ambion, Austin, Texas, U.S.A.). Both prehybridisation and hybridisation were performed at 65°C in 5×saline–sodium phosphate–ethylene diamine tetraacetic acid (EDTA), 5×Denhardt's solution, 1% sodium dodecyl sulphate (SDS) and 0.1 mg/ml salmon sperm DNA. The hybridised filters were washed in 2×standard sodium citrate buffer (SSC), 0.1% SDS for 15 min, in 0.1% SSC, 0.1% SDS for 15 min at room temperature and twice for 15 min at 65°C. After being washed, the membranes were exposed to X-ray film to obtain an autoradiographic image of the results.

Table 1. *Oligonucleotide primers for the study of the* TSG101 *gene*

Primers	Sequences	cDNA location*
First RT–PCR		
P1u	5′-AGCCCAGCAGCGGCTGACCCTCT-3′	nt 35–57
P1d	5′-CTTATTCTGGGCACCTACTGAT-3′	nt 1342–1321
Second RT–PCR		
P2u	5′-CGGTGTCGGAGAGCCAGCTCAAG-3′	nt 95–117
P2d	5′-CTCAACCTCCAGCTGGTATC-3′	nt 1290–1271
For SSCP or sequencing		
Au	Same as P2u	nt 95–117
Ad	5′-AGTAGCCATAGGCATATTTGG-3′	nt 320–301
Bu	5′-GCCTTATAGAGGTAATACATAC-3′	nt 273–294
Bd	5′-ATAGGATGCCGAAATAGGAC-3′	nt 540–521
Cu	5′-CTTCCTTATCTACATGAATGG-3′	nt 421–441
Cd	5′-CACCAGGTGGGTAAGGACAG-3′	nt 670–651
Du	5′-GATACCCTCCCAATCCCAGTG-3′	nt 620–640
Dd	5′-TTCGTTTCAAGGCATTGAGC-3′	nt 865–846
Eu	5′-ATCTCTGCGGTCAGTGACA-3′	nt 781–799
Ed	5′-AGGGAGCTGTGGGAATGATA-3′	nt 1060–1041
Fu	5′-GGAAAAAATGGAAAATCAGTCTG-3′	nt 996–1018
Fd	Same as P2d	nt 1290–1271

*According to the nucleotide sequence of *TSG101* cDNA (GenBank accession number U82130). nt, nucleotide; RT–PCR, reverse transcription–polymerase chain reaction; SSCP, single-stranded conformation polymorphism.

RESULTS

To study abnormalities of the *TSG101* gene, we first examined the expressions of the *TSG101* transcripts in seven liver cancer cell lines and 18 paired tumours and non-malignant liver tissues by nested RT–PCR amplification. Using primers flanking the *TSG101* coding region, in addition to the normal-sized *TSG101* transcript, bands of smaller size were detected in four of the seven (57%) liver cancer cell lines, eight of the 18 (44%) tumours and four of the 18 (22%) non-malignant liver tissues (Figure 1). Sequence analysis confirmed that these smaller size RT–PCR products represented aberrant *TSG101* transcripts. A detailed description of these aberrant transcripts is summarised in Figure 2 and Tables 2 and 3. In brief, six different aberrant transcripts with loss of different segments of the normal *TSG101* transcript were observed: products lacking nucleotides 154–1054, 278–1270, 264–862, 520–1270, 150–1157 and a product lacking nucleotides 134–738 and an insertion of a 37 bp sequence (Figure 3). Among them, the abnormal transcript lacking nucleotides 154–1054 was identified most frequently. All the deletions encompassed all or part of the segment encoding the coiled coil domain that interacts with stathmin, also known as oncoprotein 18 [9, 21, 22]. Therefore, they may not be able to encode a functional TSG101 protein. In one cell line (HuH-7) and one cancerous liver tissue (case 2) two abnormal bands were identified by RT–PCR amplification (Figure 1), and sequence analysis revealed that they represent aberrant *TSG101* transcripts with different intragenic deletions.

4 cases (cases 2, 3, 4 and 7) revealed aberrant transcripts in both tumours and non-malignant liver tissues. 3 of these had the same abnormal pattern of aberrant transcripts between tumours and non-malignant tissues (deletion of nucleotides 154–1054). However, in the other case (case 3), the pattern was different between the paired samples (Table 3). Although it seems that there is no correlation between the existence of the aberrant transcripts and the background condition of the liver (cirrhosis, hepatitis B or C virus infection), we only had limited cases, so no firm conclusion can be drawn.

The normal-sized *TSG101* transcripts were present at robust levels in all the cell lines and all but three of the tumours and three of the non-malignant tissues examined. SSCP analysis revealed no band shifting or missing, indicating no mutations within the coding region of the non-deleted alleles of the gene (data not shown).

Table 2. Aberrant TSG101 *transcripts observed in liver cancer cell lines*

	TSG101 transcripts	
Cell lines	Normal	Aberrant*
HCC36	+	–
Hep 3B	+	nt 154–1054
SK-Hep-1	+	–
HuH-7	+	(a) nt 154–1054 (b) nt 150–1157
Tong	+	nt 154–1054
HA22T	+	nt 154–1054
HuH-6	+	–

*The positions of the first and last nucleotides of the deletions are shown according to the nucleotide sequence of *TSG101* cDNA (GenBank accession number U82130). nt, nucleotide.

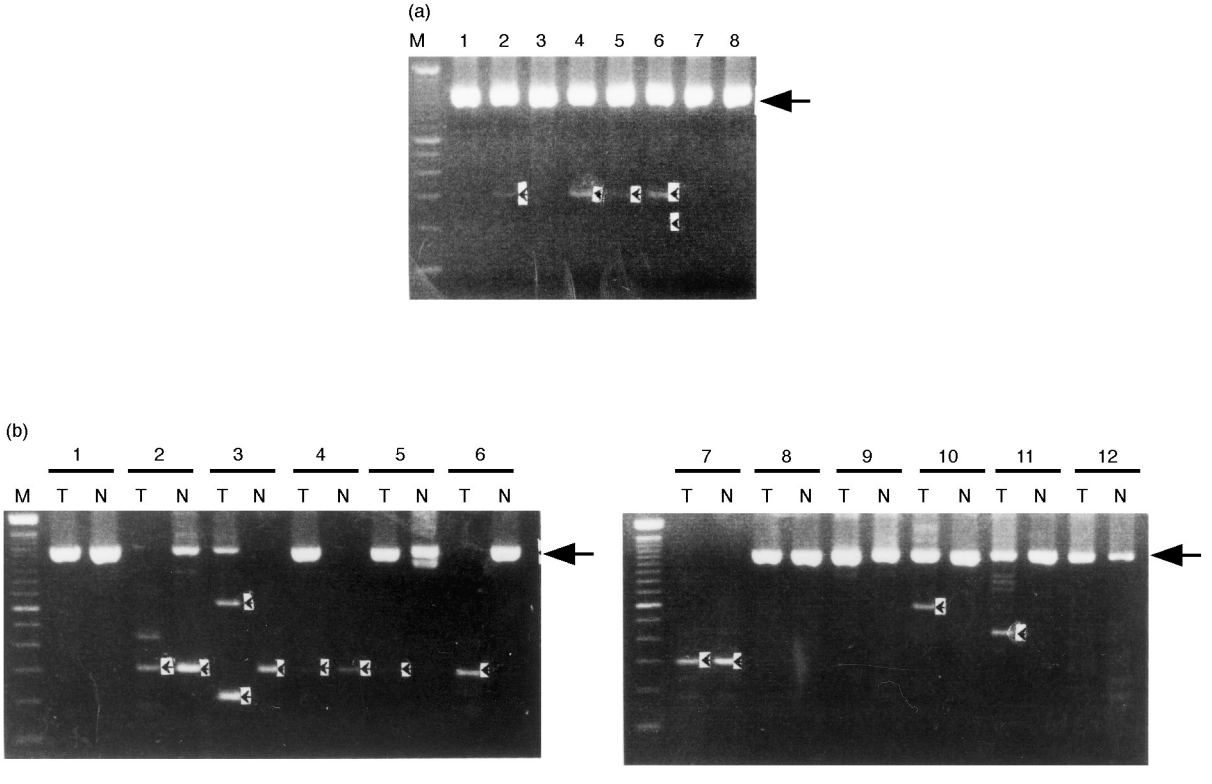

Figure 1. Nested reverse transcription–polymerase chain reaction (RT–PCR) amplification of *TSG101* from (a) liver cancer cell lines; and (b) representative cases of matched primary liver cancerous (T) and non-malignant (N) tissues. Large arrows: normal-sized *TSG101* cDNA (1195 bp); small arrows: aberrant transcripts; M, 100 bp DNA ladder markers; 1, HCC36; 2, Hep 3; 3, SK-Hep-1; 4, HuH-7; 5, Tong; 6, HA22T; 7, HuH-6; 8, peripheral mononuclear cells from a normal healthy person.

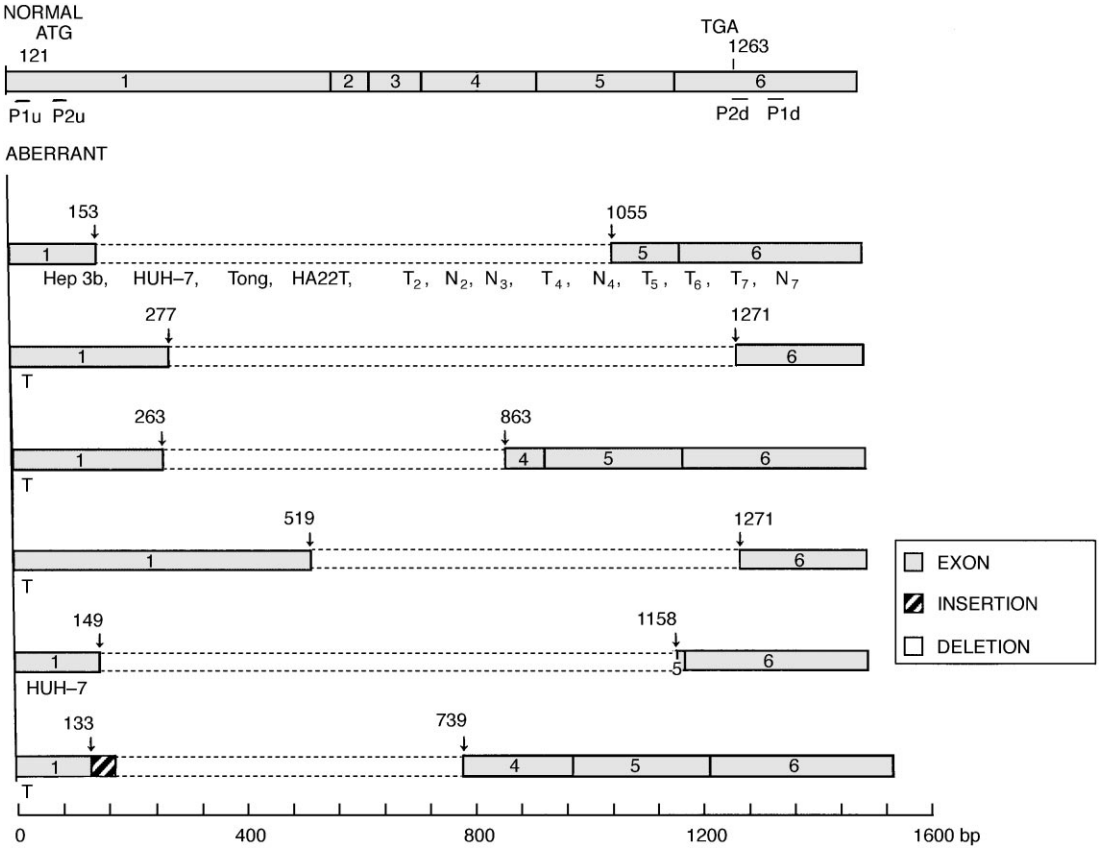

Figure 2. A schematic representation of the aberrant transcripts detected in the liver cancer cell lines and paired cancerous (T) and non-malignant (N) primary liver tissue specimens. The coding exons of the *TSG101* gene indicated by numbers 1–6 are shown in grey and a small segment of the 37 bp insertion is indicated by lines. Broken lines indicate the segment of deletion. Cases revealing aberrant transcripts are shown below each aberrant transcript figure. The locations of primers P1u, P1d, P2u and P2d used for nested reverse transcription–polymerase chain reaction (RT–PCR) amplification of *TSG101* are indicated. Arrows and nucleotide numbers indicate abnormal junctions between the deletions. The nucleotide numbers are shown according to the nucleotide sequence of *TSG101* cDNA, GenBank accession number U82130.

Table 3. *Aberrant* TSG101 *transcripts observed in hepatocellular carcinoma tissues*

						Abnormal transcript (cDNA sequence)*	
Case	Age	Sex	HBsAg	HCV	Cir	Non-tumour	Tumour
1	77	M	+	–	–	–	–
2	67	M	–	+	–	nt 154–1054 loss	nt 154–1054 loss
3	74	F	–	+	+	nt 154–1054 loss	(a) nt 278–1270 loss (b) nt 134–738 loss and insertion 37 bp
4	37	M	+	–	–	nt 154–1054 loss	nt 154–1054 loss
5	53	M	+	–	+	–	nt 154–1054 loss
6	57	M	+	–	–	–	nt 154–1054 loss
7	76	F	–	+	+	nt 154–1054 loss	nt 154–1054 loss
8	51	M	+	–	+	–	–
9	62	M	+	–	+	–	–
10	60	F	+	–	+	–	nt 264–862 loss
11	20	M	+	–	–	–	nt 520–1270 loss
12	47	F	+	–	–	–	–
13	71	F	–	+	+	–	–
14	50	M	–	+	+	–	–
15	62	M	+	+	+	–	–
16	67	F	–	+	+	–	–
17	72	M	+	–	+	–	–
18	83	M	–	+	+	–	–

In addition to the aberrant transcripts indicated above, a normal-sized transcript was present in most of the tumours and non-malignant tissues examined except in the tumours for cases 2, 6 and 7 and non-malignant tissues of cases 3, 4 and 7. *The positions of the first and last nucleotides of the deletions are shown according to the nucleotide sequence of *TSG101* cDNA (GenBank accession number U82130). HBsAg, hepatitis B virus surface antigen; HCV, anti-hepatitis C virus antibody; Cir, liver cirrhosis; M, male; F, female; nt, nucleotide.

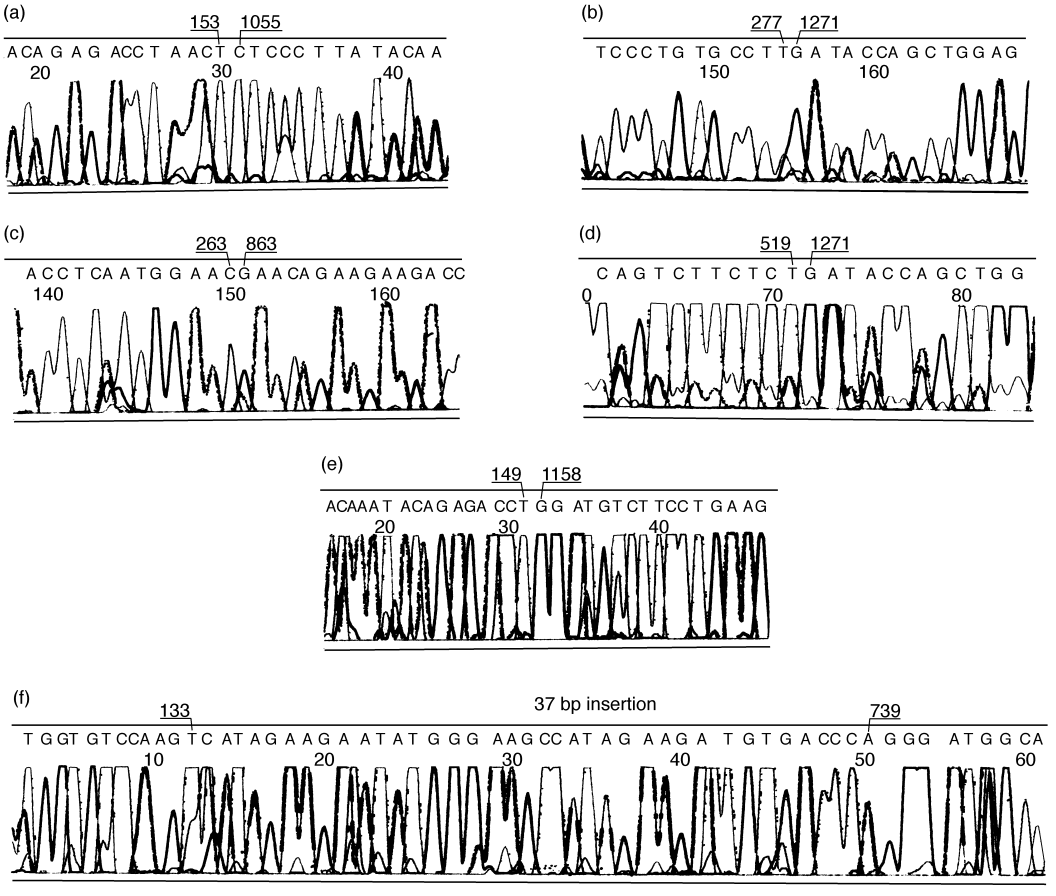

Figure 3. Sequence at the *TSG101* cDNA deletion junction in representative cases of human primary hepatocellular carcinoma and liver cancer cell lines. (a) Hep 3B; (b) T_3; (c) T_{10}; (d) T_{11}; (e) HuH-7; and (f) T_3. Numbers above each figure indicate abnormal junctions between the deletions.

We analysed the sequences at the aberrant splicing junctions and found that there were four types of junctional sequence (Figure 4). Type 1 had a splicing donor or acceptor site-like sequence at the deletion junction, such as deletion from cDNA 154 to 1054. Type 2 had a homologous sequence at the deletion junction, such as deletion from cDNA 520 to 1270, deletion from cDNA 264 to 862, deletion from cDNA 150 to 1157. Type 3 had a homologous sequence near the deletion junction, such as deletion from cDNA 270 to 1270. Type 4 had a deletion and an insertion at the deletion area, such as deletion from cDNA 134 to 738 and an insertion of 37 bp at the deletion area.

To characterise the genomic alterations of the *TSG101* gene, we performed Southern blot analysis of DNA from liver tumour cell lines and paired tumours and non-malignant liver tissues using *TSG101* cDNA as probes. Despite aberrant transcripts of the *TSG101* gene being detected at a high frequency, no alterations of the DNA, digested by *Hin*dIII, *Eco*RI or *Bam*HI in any cell line or tissue sample could be identified. Only a possible polymorphism in two cell lines (SK-Hep-1 and HA22T) was found (Figure 5). Therefore, it seems that the *TSG101* gene does not undergo large rearrangements in a significant proportion of HCC tissues and liver cancer cell lines.

DISCUSSION

Initially, by a novel strategy that enables the isolation of previously unknown genes encoding selectable recessive phenotypes, Li and Cohen identified a mouse gene (*Tsg101*) whose functional knockout in mouse fibroblasts leads to cellular transformation and the ability to form metastatic tumours in nude mice [8]. Sequence analysis revealed that the mouse *Tsg101* cDNA contains a proline-rich domain and DNA-binding motifs characteristic of transcription factors. In addition, the TSG101 protein encodes a coiled coil domain that interacts with stathmin [21, 22], a cytoplasmic phosphoprotein previously implicated in tumorigenesis [21, 23–25]. Subsequently, the human homologue, *TSG101*, was isolated and mapped to chromosome 11, bands p15.1–p15.2 [9]. Using a PCR-based strategy, large intragenic deletions involving 50–95% of the genomic locus of the gene were identified in seven of 15 uncultured primary human breast carcinomas [9]. In addition, a point mutation in a breast tumour sample in which only one allele sustained a deletion was reported. Therefore, the *TSG101* gene was considered a possible tumour-suppressor gene implicated in breast carcinogenesis.

LOH at 11p15 has been identified in a variety of human malignancies, including HCC [10–13, 26–32]. Therefore, in order to elucidate the role of *TSG101* in hepatocarcinogenesis, seven liver cancer cell lines and 18 paired tumours and non-malignant liver tissues were studied. Consistent with the results of breast cancers studied by Li and colleagues [9], aberrant transcriptions with different intragenic deletion of the *TSG101* gene were frequently identified in liver cancer cell lines (57%) and HCC malignant tissues (44%). In addition, four (two with cirrhosis and two with chronic hepatitis)

of the 18 (22%) paired non-malignant liver tissues also revealed aberrant transcriptions of the gene, possibly indicating that the abnormalities of *TSG101* might occur early in the process of hepatocarcinogenesis.

However, according to Knudson's two-hit model for inherited and sporadic forms of retinoblastoma, tumour suppressor genes can usually be inactivated through deletion of one allele by various mechanisms, and inactivation of another allele by mutation, loss or other mechanisms [33, 34]. In the current study, although it could be assumed that the findings of aberrant RT–PCR products are indicators of the existence of an altered allele [35], in most of the cell lines and tissue samples which showed abnormal transcripts, only one abnormal transcript was observed in each case. In addition, the normal-sized transcript was present in all the cell lines and most of the tissue samples, and no mutation could be identified by SSCP analysis. Li and colleagues argued that these full-length RT–PCR products might be contamination from non-neoplastic cells [9]. However, because the normal RT–PCR products were always at a robust level, and the normal products were observed in all seven liver tumour cell lines, we believe that these normal products were derived from neoplastic cells. Therefore, the *TSG101* gene may not act as a classical tumour suppressor gene in hepatocarcinogenesis. Whether the aberrant transcripts that eliminate most of the coiled coil domain but maintain a normal NH2-terminus of the protein inactivate the wild-type *TSG101* by a dominant-negative mechanism or whether there are non-mutational mechanisms causing inactivation of *TSG101* functions in HCCs deserve further study.

By Southern blotting of PCR-amplified genomic DNA from cancerous breast tissues, Li and colleagues confirmed that the aberrant transcripts of the *TSG101* gene detected by nested RT–PCR resulted from genomic deletions [9]. In the current study using nested RT–PCR, we also demonstrated similar aberrant transcription of the *TSG101* gene in a significant number of liver cancer cell lines and HCC tissue. However, although large intragenic deletions should be readily detectable by Southern blotting of the restriction enzyme digested genomic DNA samples, we failed to show any rearrangement in the *TSG101* genomic locus in the cell lines and tissues showing aberrant transcripts. Recently, Steiner and associates and Lee and Feinberg simultaneously reported an absence of rearrangements in the *TSG101* genomic locus in

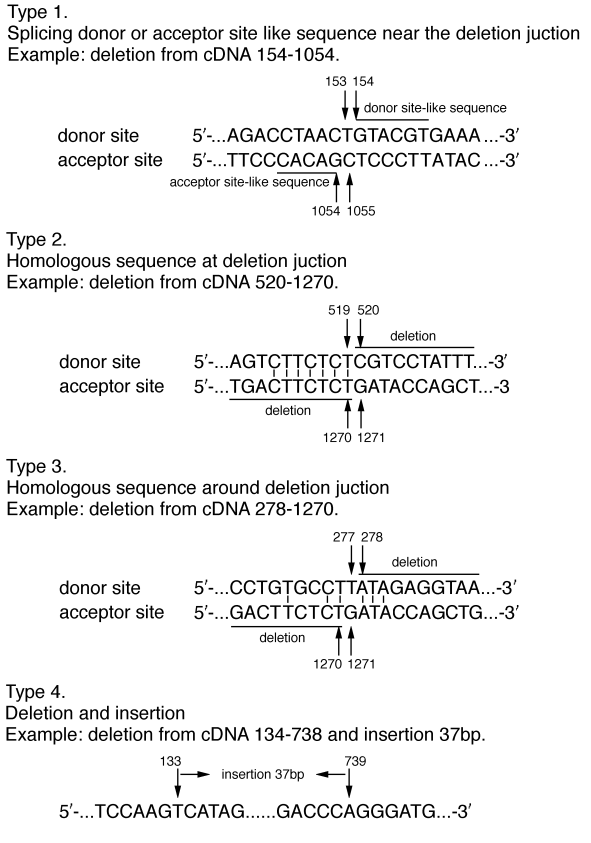

Figure 4. Sequencing analysis of the deletion junction for aberrant RNA splicing. There were four types of aberrant transcripts. Type 1 has a splicing donor or acceptor site-like sequence at the deletion junction region. Type 2 has a homologous sequence at the deletion junction region. Type 3 has a homologous sequence around the deletion junction region. Type 4 has both deletion and insertion.

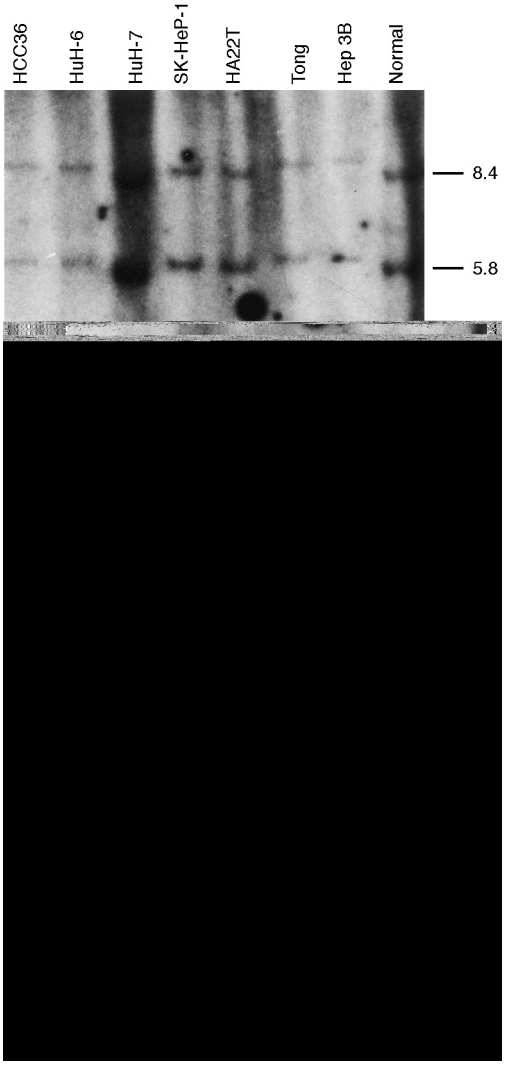

Figure 5. The results of Southern blot analysis of seven liver cancer cell lines. Normal, DNA from a normal healthy person as a control. No intragenic deletion of the *TSG101* gene was identified. Genomic DNAs were digested with *Hin*dIII and the filter was probed with the full-length cDNA of *TSG101*. Irrespective of the presence of aberrant transcript (HA22T) or not (SK-Hep-1), as indicated by arrows there was an extra band detected in both cell lines. Therefore, the band represented polymorphism only.

human breast cancers and questioned the role of the *TSG101* gene in breast carcinogenesis [36, 37]. In fact, as indicated by Thiagalingam and colleagues, nested RT–PCR often results in over-representation of shorter, alternatively spliced transcripts in addition to or instead of the normal product [38]. In the current study, abnormal nested RT–PCR products were the only positive results. Therefore, we believe that the *TSG101* gene did not undergo large rearrangements in the cases studied and the presence of aberrant transcripts might only reflect relaxation of RNA splicing fidelity as indicated by Lee and Feinberg [37].

In conclusion, these data indicate that abnormalities of the *TSG101* gene transcripts occur quite frequently in liver cancer cell lines, tumours and non-malignant liver tissues. However, a normal-sized transcript without sequence abnormalities was always present and Southern blot analysis could identify no genomic abnormalities of the gene. Therefore, either the *TSG101* gene might not be involved in hepatocarcinogenesis or it may play a role in the development and/or progress of HCCs through an unusual mechanism.

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