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Advances in Brief

Identification of Cellular TSG101 Protein in Multiple Human Breast Cancer Cell Lines[1]

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Abstract

***tsg101* was identified as a tumor susceptibility gene by homozygous functional inactivation of allelic loci in mouse 3T3 fibroblasts. The human homologue was mapped at chromosome 11p15.1–2 and found to have intragenic deletion in 7 of 15 breast cancer specimens. To further confirm the relevance of defects in this gene to breast cancer, antibodies specific for the putative gene product were prepared and used to identify cellular TSG101 protein. The antibodies recognized a 46-kDa protein in human retinoblastoma WERI-27 cells labeled with [^{35}S]methionine. This protein was not detected with preimmune sera. In cell fractionation studies, the 46-kDa protein cofractionating with glutathione *S*-transferase was found mainly in the cytoplasm. Similarly, when cells were immunostained with anti-TSG101 antibodies, fluorescence was localized in the cytoplasm of most of the cells. A full-size 46-kDa TSG101 protein was detected in a panel of 10 breast cancer cell lines and 2 normal breast epithelial cell lines with the same antibodies. Consistently, the full-length *TSG101* mRNA was also detected in these breast cells using reverse transcription-PCR. These results indicate that homozygous intragenic deletion of *TSG101* is rare in breast cancer cells.**

Introduction

TSG101 is a recently discovered tumor suppressor gene. The gene was cloned based on a novel strategy that uses regulated antisense RNA initiated within a retrovirus-based gene search vector to identify previously unknown autosomal genes whose inactivation is associated with a defined phenotype (1). In this case, functional knockout of *tsg101* in mouse fibroblasts leads to transformation and the ability to form metastatic tumors in nude mice. The cellular transformation and tumorigenesis that result from inactivation of *tsg101* are reversible by deleting the transactivator gene required for the production of antisense transcripts complementary to *tsg101* mRNA (1). These results suggested that *tsg101* may act as a tumor suppressor. Sequence analysis of mouse *tsg101* cDNA indicates that the gene encoded a 43-kDa protein containing a proline-rich domain and a leucine heptad repeat (coiled-coil) domain (1).

The human homologue *TSG101* was mapped to chromosome 11, bands 15.1–15.2 (2), a region proposed to contain tumor suppressor genes (3–6). Interestingly, analysis by RT-PCR[3] of 15 uncultured primary breast carcinomas and matched normal breast tissue from the same patients detected intragenic deletions that disrupted sequences encoding the coiled-coil domain of the protein in 7 of 15 cases (2). Analysis of genomic DNA confirmed the presence of deletions in at least one allele of all six of the seven patients showing mutations in *TSG101* transcripts. No *TSG101* defects were found in matched normal breast tissue from the breast cancer patients (2). If the above finding is substantiated, *TSG101* would be the key gene involved in breast cancer in general. So far, there are several tumor suppressor genes known to be involved in breast cancer. Both *BRCA1* and *BRCA2* are rarely mutated in sporadic breast cancer (7, 8). Mutation of *RB* has been found in 15% of cases, and *p53* is mutated about 20–30% of late-stage breast cancer (9). The lack of a threshold tumor suppressor gene in breast cancer etiology is puzzling. However, a high level of heterogeneity of breast cancer may provide a partial explanation for the lack of a single key gene in this pathway. Thus, the finding of the role of *TSG101* in breast cancer described above is both exciting and important.

The consequence of the intragenic deletion found in breast cancer cells will be truncation of the gene product, including both mRNA and protein. Based on this premise, we prepared antibodies specifically recognizing TSG101 protein and screened a panel of 10 breast cancer cell lines. We found that the full-length TSG101 protein was detected in all of the tumor and normal cells studied. Consistently, 15 of 15 breast cancer cell lines contain full-length *TSG101* mRNA. These results suggest that *TSG101* is rarely deleted homozygously in breast cancer cells.

Materials and Methods

Generation of Polyclonal Antibody. The GST fusion system was used for preparation of GST fusion proteins as antigen (10). A cDNA fragment of *TSG101* encoding amino acid residues 167–374 was fused in frame with GST. Expression of the fusion protein was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM to an exponentially growing bacterial culture at 25°C. After 6 h of incubation, bacteria were collected and lysed as described previously (11). Fusion proteins were purified with glutathione-agarose beads. Antisera were raised in female BALB/c mice injected s.c. with 100 μg of GST-TSG101 bound to glutathione beads in 75 μl of sterile PBS (0.9% saline). Mice were boosted with 100 μg of GST-TSG101 beads after 2 weeks and again after 2 months.

***In Vitro* Transcription and Translation.** The *in vitro* translated TSG101 protein was prepared from the cDNA using the TNT coupled reticulocyte-lysate system (Promega, Madison, WI) according to the manufacturer's instructions. IPs were done using 0.05 of the total reaction volume.

RT-PCR Analysis. Total RNA was isolated using TRI REAGENT protocol (Molecular Research Center, Inc., Cincinatti, OH). cDNA was prepared from 1 μg of RNA with the reverse transcription system (Promega) in a 20-μl reaction. PCR was carried out in a 50-μl reaction containing 2 μg of cDNA, 0.4 μg of each primer, 10 mM Tris (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, and 1 unit of Amplitaq (Perkin-Elmer, Branchburg, NJ). Specific primers for *TSG101* were 5′-CCCGAATTCAGCTCAAGAAAATGGTGTCCAAGTAC-3′ and 5′-CCCGAATTCCAGCTGGTATCAGAGAAGTCAGTAG-3′. Primers for Gβ-like protein were 5′-GACCAACTATGGAATTCCACGTGCTC-3′ and 5′-TCGTTGAGATCCCATAACATGGCCTG-3′. PCR amplifications were carried out in a PTC-100 thermal cycler at 94°C for 1 min (for denaturing), at 65°C for 1 min and 20 s (for annealing), and at 72°C for 2 min (for extension). The PCR products were resolved in a 1.2% agarose gel and stained with ethidium bromide.

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[3] The abbreviations used are: RT-PCR, reverse transcription-PCR; GST, glutathione *S*-transferase; IP, immunoprecipitation.

Metabolic Labeling and IP Analysis. About 1×10^7 cells were labeled with [^{35}S]methionine (100 μCi/ml) for 90 min, and cell lysates were prepared for IP. IP with the polyclonal antiserum against TSG101 was done according to the standard protocols (12), using the antiserum at a dilution of 1:1000. After IP, proteins were separated by 9% SDS-PAGE and detected by autoradiography. For Western analysis, the immunoprecipitates were transferred to Immobilon-P membranes (Milipore, Bedford, MA) and probed with the antiserum at a dilution of 1:1000 according to our standard procedure (13). Double IPs were done as described previously (14). For the detection of p84 by Western analysis, monoclonal antibody 5E10 was used as primary antibody, as described previously (13), and cellular GST protein was precipitated by glutathione-agarose beads, separated by 12% SDS-PAGE, and detected by Commassie Blue staining.

Cell Fractionation. Cells were separated into membrane, nuclear, and cytoplasmic fractions following the procedures published previously (15). All three fractions were then assayed for TSG101 protein, p84, and GST as described above.

Immunostaining. The procedure for indirect immunofluorescence staining was adapted as described previously (16). Briefly, cells grown on coverslips in tissue culture dishes were washed in PBS and fixed for 30 min in 4% formaldehyde in PBS with 0.5% Triton X-100. After treating with 0.05% saponin in water for 30 min and extensive washing with PBS, cells were blocked in PBS containing 10% normal goat serum. A 1-h incubation with suitable antibody diluted in 10% goat serum was followed by five washes, then by another 1-h incubation with fluorochrome-conjugated secondary antibody. The antigen was then visualized with goat antimouse antibody conjugated to FITC. After washing extensively in PBS with 0.5% NP40, cells were further stained with 4′, 6-diamidino-2-phenylindole and mounted in Permafluor (Lipshaw-Immunonon, Inc., Pittsburgh, PA). Ekachrome P1600 film was used when pictures were taken from a standard fluorescence microscope (Axiophot photomicroscope; Zeiss).

Results and Discussion

Anti-TSG101 Antiserum Specifically Immunoprecipitated the *in Vitro* Translated TSG101 Protein. *TSG101* cDNA fragment (*Hpa*II to *Hpa*II) comprising amino acid residues 167–374 was fused in frame with GST to produce GST-TSG101 fusion protein using a modified pGEX vector.[4] The bacterially expressed fusion protein was purified with glutathione beads and used as antigen to generate mouse polyclonal antiserum (see Fig. 1, *A* and *B*). As shown in Fig. 1*C*, the antiserum alone or after competition with excess GST protein specifically recognized a 46-kDa protein and other internally initiated proteins from the *in vitro* translation/transcription system using full-length *TSG101* cDNA as template. (Fig. 1*C*, *Lanes 2* and *3*). However, the preimmune sera and the antisera competed with excess of the original fusion protein failed to detect the *in vitro* translated proteins (*Lanes 1* and *4*). Re-IP of the first immunoprecipitates with the same antisera after dissociation detected the similar *in vitro* translated proteins (*Lane 5*), indicating that the antisera prepared can specifically recognize the proteins translated from *TSG101* cDNA.

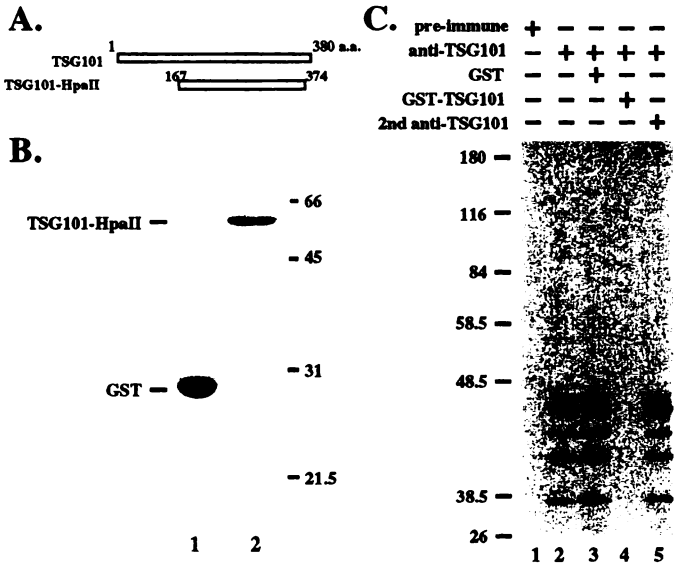

Fig. 1. *A*, schematic showing the cDNA clones used to construct a GST-TSG101 fusion protein. *B*, expression of GST-TSG101 fusion protein in *Escherichia coli*. *C*, anti-TSG101 antiserum specifically immunoprecipitated the *in vitro* translated TSG101 protein. *In vitro* translated TSG101 (0.05 total product) was immunoprecipitated with either preimmune serum (*Lane 1*), anti-TSG101 polyclonal antiserum (*Lane 2*), anti-TSG101 after preincubation with GST (*Lane 3*), or anti-TSG101 after preincubation with the original GST-TSG101 antigen (*Lane 4*) or double immunoprecipitated with anti-TSG101 (*Lane 5*).

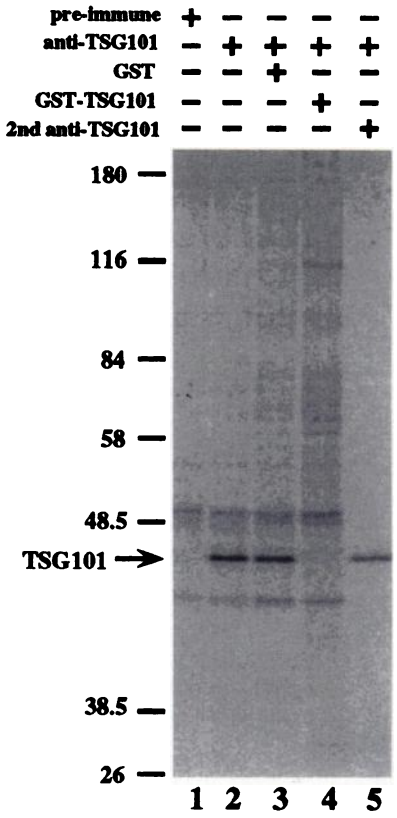

Fig. 2. Identification of a cellular protein by anti-TSG101 polyclonal antibody. Lysates of 1×10^7 WERI-Rb-27 cells labeled with [^{35}S]methionine were immunoprecipitated with preimmune serum (*Lane 1*), anti-TSG101 (*Lane 2*), anti-TSG101 after preincubation with GST (*Lane 3*), or anti-TSG101 after preincubation with GST-TSG101 antigen (*Lane 4*) or double immunoprecipitated with anti-TSG101 to remove coimmunoprecipitating proteins (*Lane 5*). A cellular protein with an apparent molecular mass of about 46 kDa is specifically recognized by the antibody.

Identification of a 46-kDa Protein in Human Cells by the Anti-TSG101 Antiserum. To identify cellular TSG101 protein in human cells, [^{35}S]methionine-labeled WERI-Rb-27 cells were lysed and used for IP with either anti-TSG101 antisera or preimmune serum. The anti-TSG101 antisera specifically immunoprecipitated a 46-kDa protein, whereas the preimmune sera did not. (Fig. 2, *Lane 1* and *2*). The addition of GST-TSG101 fusion protein (*Lane 4*) but not GST alone (*Lane 3*) competed out the cellular 46-kDa protein. Using a similar approach to that described above for the double IP, only the 46-kDa protein was detected. These results suggest that the 46-kDa cellular protein is the gene product of *TSG101*. This result is consistent with the predicted size of protein product of *TSG101* determined from the cloned cDNA sequence.

[4] Unpublished observations.

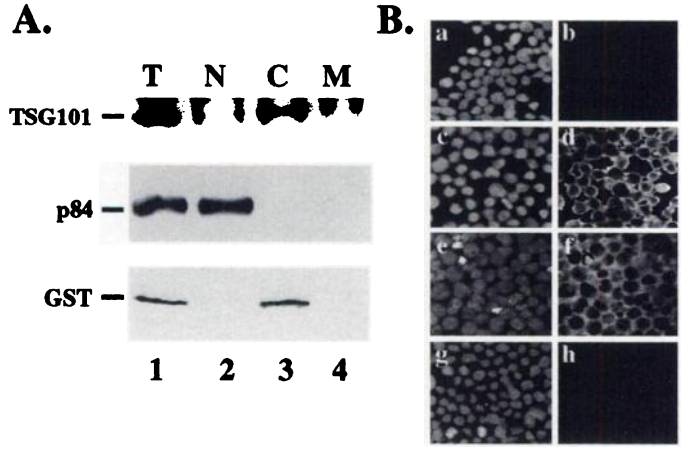

Fig. 3. Subcellular localization of TSG101. *A*, biochemical fractionation of WERI-Rb-27 cells (*T*) into nuclear (*N*), cytoplasmic (*C*), and membrane (*M*) components. Each fraction was immunoprecipitated by anti-TSG101 polyclonal antisera. Two proteins, p84 and cytoplasmic GST, served as markers for nuclear and cytoplasmic fractions, respectively. TSG101 was detected mainly in the cytoplasmic portion that was cofractionated with GST. *B*, indirect immunofluorescence staining. The WERI-Rb-27 cells were stained with 4′, 6-diamidino-2-phenylindole (*a*, *c*, *e*, and *g*) and simultaneously reacted with anti-TSG101 antibodies and then FITC-conjugated secondary antimouse antibody. The majority of the fluorescence was found in the cytoplasmic region but not in the nucleus (*d*). This was also the case when the anti-TSG101 was first competed with GST alone (*f*). However, the preimmune sera (*b*) or immune sera competed with the original fusion protein (*h*) detected no signals in these cells.

TSG101 Protein Is Localized in the Cytoplasm. To examine the potential function of TSG101 in human cells, the cellular localization of TSG101 was determined, first, by biochemical fractionation. WERI-Rb-27 cells were fractionated into membrane, nuclear, and cytoplasmic fractions following the procedure described previously (14, 15). Two proteins, a known nuclear matrix protein, p84, and cytoplasmic GST, served as markers for nuclear and cytoplasmic fractions, respectively (Fig. 3*A*). TSG101 was detected mainly in the cytoplasmic portion, which cofractionated with GST (Fig. 3*A*). Using an indirect immunofluorescence staining procedure, WERI-Rb-27 cells were stained with anti-TSG101 antibodies and then FITC-conjugated secondary antimouse antibody. The majority of the fluorescence was found in the cytoplasmic region but not in the nucleus (Fig. 3*B*). This is also the case when the anti-TSG101 was first competed with GST alone. However, neither the preimmune sera nor immune sera competed with the original fusion protein detected signals in these cells. These results indicate that cellular TSG101 protein is mainly cytoplasmic. Interestingly, a cytoplasmic phosphoprotein, stathmin, has been reported to interact with coiled-coil domain (17) that later was found to be part of the TSG101 protein (2). This protein is known as oncoprotein 18 and may have a role in cell growth and differentiation (18, 19). The cytoplasmic location of TSG101 is compatible with the observation and provides a spatial window in which this interaction may occur. However, our preliminary data suggested that TSG101 may relocate to the nucleus in some stage of the cell cycle, such as G_2, or bind to chromosomes during M phase (data not shown). The significance of such relocalization remains to be established.

Full-Length TSG101 Protein and mRNA Were Detected in Most Breast Cancer Cell Lines Analyzed. There are several methods for detecting potential mutations of the *TSG101* gene. Direct examination of the given gene product such as mRNA or protein is usually more accurate than genomic DNA blotting analysis. To test whether deletion or mutation of *TSG101* occurs prevalently in breast cancer cells, oligonucleotide primers flanking the TSG101 protein-coding region were used to perform RT-PCR assays. mRNA from 15 breast cancer cell lines including MCF7, T47D, MB468, BT483, MB231, SKBR3, ZR75, MB435, MB175-7, MB361, BT549, MB436, MB453, BT474, and MB134VI and 3 other human cell lines including MCF10A, HBL100, and T24 was prepared and used as a template for RT-PCR. The PCR reaction products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide (Fig. 4*A*). A 1.15-kb band characteristic of amplification of the *TSG101* mRNA was seen in all of the cell lines. A negative control using H_2O instead of RNA in the RT-PCR reaction did not show any amplified product, nor was a product seen after direct PCR amplification of purified genomic DNA from these cell lines. One of the breast cancer cell lines, T47D, apparently expressed much less *TSG101* mRNA when normalized with *Gβ-like mRNA as an internal control. These results suggested that most of the breast cancer cells expressed full-length TSG101* mRNA. Consistent with this result, there is no clear variation in the size of the genomic DNA from these cell lines when they were digested by *Bgl*II or *Hin*dIII and probed with full-length *TSG101* cDNA (data not shown).

To more precisely detect any potential abnormality in *TSG101*, we further examined its protein product in these breast cancer cell lines. [^{35}S]methionine-labeled cells were prepared from 10 breast cancer cell lines, including MCF7, T47D, MB468, BT483, MB231, SKBR3, ZR75, MB435, MB175-7, and MB361, as well as 2 breast epithelial cells, MCF10A and HBL100, for double IP with anti-TSG101 antibodies. The immunoprecipitates were separated by 9% SDS-PAGE and autoradiographed. A 46-kDa protein was detected in all of the breast cell lines (Fig. 4*B*). A known nuclear matrix protein, p84, served as an internal control, because it is expressed uniformly and ubiquitously (13). After normalization to p84, the amount of 46-kDa protein in T47D breast cancer cells was found to be much lower than

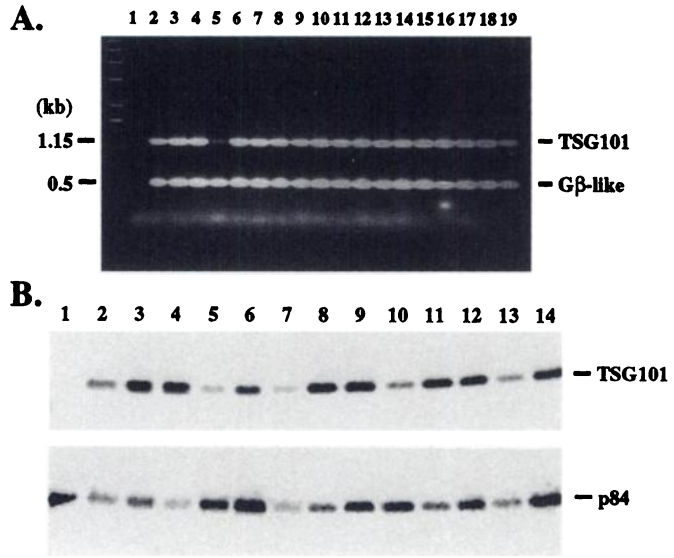

Fig. 4. Detection of full-length TSG101 protein and mRNA in most breast cancer cell lines. *A*, RT-PCR analysis of *TSG101* mRNA from 18 cell lines: *Lane 2*, MCF10A; *Lane 3*, HBL100; *Lane 4*, MCF7; *Lane 5*, T47D; *Lane 6*, MB468; *Lane 7*, BT483; *Lane 8*, MB231; *Lane 9*, SKBR3; *Lane 10*, ZR75; *Lane 11*, MB435; *Lane 12*, MB175-7; *Lane 13*, MB361; *Lane 14*, BT549; *Lane 15*, MB436; *Lane 16*, MB453; *Lane 17*, BT474; *Lane 18*, MB134VI; *Lane 19*, T24. One μg of total RNA from each cell was prepared and used as a template for RT-PCR reactions. The PCR reaction products were electrophoresed on a 1.2% agarose gel and stained with ethidium bromide. *Lane 1*, a negative control using H_2O instead of RNA in the RT-PCR reactions. *Gβ-like* mRNA is expressed constitutively and therefore served as an internal control. *B*, double IP of TSG101 protein with anti-TSG101 serum. A total of 1×10^7 [^{35}S]methionine-labeled cells were prepared from 10 breast cancer cell lines (*Lane 4*, MCF7; *Lane 5*, T47D; *Lane 6*, MB468; *Lane 7*, BT483; *Lane 8*, MB231; *Lane 9*, SKBR3; *Lane 10*, ZR75; *Lane 11*, MB435; *Lane 12*, MB175-7; *Lane 13*, MB361; and *Lane 14*, T24) as well as 2 breast epithelial cells (*Lane 2*, MCF10A; and *Lane 3*, HBL100) for double IP with anti-TSG101 antibodies. *Lane 1*, MCF10A cells immunoprecipitated with preimmune serum as a control. *p84*, a nuclear matrix protein, served as an internal control.

that of the others. These results are consistent with the mRNA study and further suggest that full-length TSG101 protein is present in most of breast cancer cell lines.

The above results are at odds with previously published data (2). If 40% of primary tumors contain intragenic deletion of *TSG101*, it is reasonable to expect that a similar frequency of such a mutation should be in breast cancer cell lines. Although the precise reason remains unclear, it is possible that the apparent intragenic deletion may have been created by artifacts of PCR. If there is a pseudogene of TSG101 as found in mouse genome,[4] genomic DNA blotting or PCR with primers containing coding sequence may generate complicated patterns that are difficult to interpret (2). Particularly, preparation of RNA from precious clinical specimens is hampered by potential contamination with genomic DNA. Using antibodies that specifically detect cellular TSG101 protein excludes such a potential problem. Despite the rare occurrence of deletion in *TSG101* in breast cancer cells, it remains likely that TSG101 may play an important role in cell growth and differentiation, and it may be involved in other type of cancers.

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