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

Qing Zhong, Chi-Fen Chen, Yumay Chen, Phang-Lang Chen, and Wen-Hwa Lee

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 to 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 ['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 of 15 uncultured primary breast carcinomas and matched normal breast tissue from the breast cancer patients (2). If the same antibodies, 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 homozgyously 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., Cincinnati, OH). cDNA was prepared from 1 μg of RNA with the reverse transcription system (Promega, Madison, WI), which includes the manufacturer’s instructions. IPs were done using 0.05 of the total reaction volume.

Received 7/15/97; accepted 8/15/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by NIH Grants CA58318 and P50-CA58183 and by the Alice McDermott Endowment Fund. Q. Z. and C.-F. C. contributed equally to this work.

2 To whom requests for reprints should be addressed, at Department of Molecular Medicine and Institute of Biotechnology, University of Texas Health Science Center, 15335 Lambda Drive, San Antonio, TX 78245. Phone: (210) 567-7353; Fax: (210) 567-7377.

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

4225
Metabolic Labeling and IP Analysis. About $1 \times 10^7$ 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 1:10000 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-Immunon, Inc., Pittsburgh, PA). Ektachrome 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 (Hpall to Hpall) comprising amino acid residues 167–374 was fused in frame with GST to produce GST-TSG101 fusion protein using a modified pGEX vector. 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. 1C, the antisera alone or after competition with excess GST protein specifically recognized a 46-kDa protein and other internally initiated proteins (Lane 1 and 4). Re-IP of the first immunoprecipitates with the same antisera after dissociation detected the similar in vitro translated proteins (Lanes 1 and 4), indicating that the antisera prepared can specifically recognize the proteins translated from TSG101 cDNA.

Identification of a 46-kDa Protein in Human Cells by the Anti-TSG101 Antiserum. To identify cellular TSG101 protein in human cells, [35S]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.

Fig. 2. Identification of a cellular protein by anti-TSG101 polyclonal antibody. Lysates of $1 \times 10^7$ 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.
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. 3A). 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.

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. 4A). A 1.15-kb band characteristic of amplification of the TSG101 mRNA was seen in all of the cell lines. A negative control using H2O 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 BglII or HindIII 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. [35S]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. 4B). 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...
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, 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.

Acknowledgments

We thank Stanley Cohen for his valuable suggestion, Andrew Farmer for critical reading of the manuscript, and Diane Jones for antibody preparation.

References

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

Qing Zhong, Chi-Fen Chen, Yumay Chen, et al.


Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/19/4225

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.