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Aberrant Splicing but not Mutations of *TSG101* in Human Breast Cancer[1]

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Abstract

The 11p15 gene *TSG101* was recently reported to undergo frequent large intragenic deletions in human breast cancer. Here we show that that is generally not the case, but the gene shows aberrant splicing, based on the following observations: identical products were observed in matching normal and fetal tissues; deleted cDNA sequence revealed canonical splicing donor and acceptor site sequences; and genomic Southern blots showed no intragenic deletions in all 72 tumors studied. Nevertheless, relaxation of RNA splicing fidelity may be an oncodevelopmental marker in cancer and may play a general role in other genes and tumors.

Introduction

The mouse *TSG101* gene was initially isolated by random homozygous knock out, selecting for inactivated tumor suppressor genes using a NIH3T3 cell transformation assay (1). The human homologue of the *TSG101* gene was then isolated and mapped to 11p15 (2). We had earlier discovered frequent LOH[3] of 11p15 in Wilms' tumor (3), and this observation has been extended to many other cancers, including breast, lung, bladder, testicular, adrenal cortical carcinoma, hepatoblastoma, and ovarian cancer (4–13). We also demonstrated directly the existence of a tumor suppressor gene in this region, using STFs from 11p15, localizing this gene more precisely to a region between the markers *D11S988* and *D11S1318* (14). A second 11p15 tumor suppressor gene may also lie more centromerically between the markers *D11S12* and *L163* (14).[4] Identifying mutations of genes in this region has been a major effort of many laboratories.

Recently, Li *et al.* (2) reported the presence of large intragenic deletions of the *TSG101* gene in 7 of 15 primary human breast cancers, based on the presence of truncated transcripts observed by RT-PCR and Southern hybridization of PCR products of genomic DNA. Remarkably, these deletions involved in many cases the same precise nucleotides, and in some cases, associated intragenic insertions in DNA of the same tumors. They also mapped *TSG101* near the *SAA* gene on 11p15.1-p15.2 (2). We have analyzed *TSG101* in 72 primary breast cancers, at the level of the cDNA by RT-PCR, and at the level of genomic DNA by Southern hybridization. We have also sequenced the full-length cDNA in 10 tumors.

Materials and Methods

Isolation of DNA and RNA from Tissues. Breast cancers and their matched normal tissues were obtained from the Cooperative Human Tissue Network, and normal fetal tissues were from the University of Washington Fetal Tissue Bank. The tissues were stored at −135°C until use. Breast cancers were stages 2–4. The tissues were pulverized in liquid nitrogen and suspended in TE9 [0.5 M Tris-HCl (pH 9.0), 20 mM EDTA (pH 8.0), and 10 mM NaCl]. Proteinase K (0.2 mg/ml) and 1% SDS were added to lyse the cells and digest the proteins at 50°C for overnight. To isolate RNA, tissues were cut into small pieces and homogenized in 4 ml of RNAzol[B] (Tel-Test, Inc.). RNAs were stored at −70°C.

RT-PCR and Sequencing of cDNA. Two μg of RNA were mixed with 50 ng of primer 1 in 35 μl of diethyl pyrocarbonate water, and denatured at 70°C for 10 min; the reverse transcription reaction was carried out in 50 μl solution containing 1× buffer [10 mM Tris (pH 8.0), 50 mM KCl, and 1.5 mM MgCl], 0.2 mM deoxynucleotide triphosphate, 1 unit/μl RNase inhibitor, and 3 units AMV reverse transcriptase. The reaction mixture was incubated at 42°C for 1 h, and cDNA was stored at −20°C. Nested RT-PCR reactions were carried out using the condition of Li *et al.* (2). The RT-PCR products were analyzed in 1.2% agarose gels. DNA fragments were cut out and purified with Qiaex II (Qiagen). DNAs were directly sequenced using an ABI377 automatic sequencer. To quantify the PCR products, we labeled primer 4 with T4 polynucleotide kinase and used 5′ end-labeled primer 4 in the PCR reaction. Agarose gels were dried and analyzed by a PhosphorImager (Molecular Dynamics). The primers used for PCR and sequencing were as follows: primer 1, ATTTAGCAGTCCCAACATTCAGCACAAA; primer 2, CGGGTGTCGGAGAGCCAGCTCAAGAAA; primer 3, CCTCCAGCTGGTATCAGAGAAGTCGT; primer 4, AGCCAGCTCAAGAAAATGGTGTCCAAG; primer 5, TCACTGAGACCGGCAGTCTTTCTTGCTT; primer 6, TTGTCACTGACCGCAGAG; primer 7, ATAGGATGCCGAAATAGG; and primer 8, CCATTCATGTAGATAAGG.

Southern Hybridization. Five μg of genomic DNA were digested with 10 units of restriction enzyme at 37°C overnight. Digested DNAs were resolved on 0.8% agarose gel, transferred to Hybond-N$^+$ filters, and fixed by UV cross-linking. Filters were hybridized with the human *TSG101* cDNA probe prepared by the random priming method (15). Hybridizations were carried out at 65°C overnight in Church-Gilbert buffer [0.5 M sodium phosphate (pH 7.2), 7% SDS, and 1 mM EDTA].

Results

No Intragenic Deletion of *TSG101* in Human Breast Cancer. The *TSG101* gene was recently mapped to 11p15.1-p15.2 and was reported to contain large (7.4–16.4 kb) intragenic DNA deletions in 7 of 15 primary human breast cancers (2). We first mapped the *TSG101* gene using somatic hybrid cells and STFs containing a functional 11p15 tumor suppressor gene(s) (14). *TSG101* mapped centromeric to tumor-suppressing STFs (data not shown). It also mapped at least 6 Mb centromeric to *D11S860*, which is located in 11p15.5 within these STFs and shows the highest frequency of 11p LOH in breast cancer (5).[4] Thus, the *TSG101* gene mapped outside both regions known to contain a tumor suppressor gene.

To assess the frequency of intragenic DNA deletions of *TSG101* gene in human breast cancer, we performed genomic Southern blots on 72 primary tumors probed with *TSG101* cDNA. Genomic DNA was digested with *Bgl*II, which generates 8.5- and 6.5-kb fragments (Fig. 1*a*), and *Pst*I, which generates 13-, 4.5-, and 2.0-kb fragments (Fig. 1*b*). There were no alterations of DNA in any breast cancers (Fig. 1 and Table 1). The digestion pattern was identical for all tumors, matched normal tissues, and unrelated normal tissues. We repeated genomic Southern hybridizations with additional restriction enzyme digests: *Pvu*II (8.5, 5.5, 4.0, 2.5, and 2.0 kb); *Eco*RI (23, 6.5,

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[3] The abbreviations used are: LOH, loss of heterozygosity; STF, subchromosomal transferable fragment; RT-PCR, reverse transcription-PCR.

[4] Unpublished results.

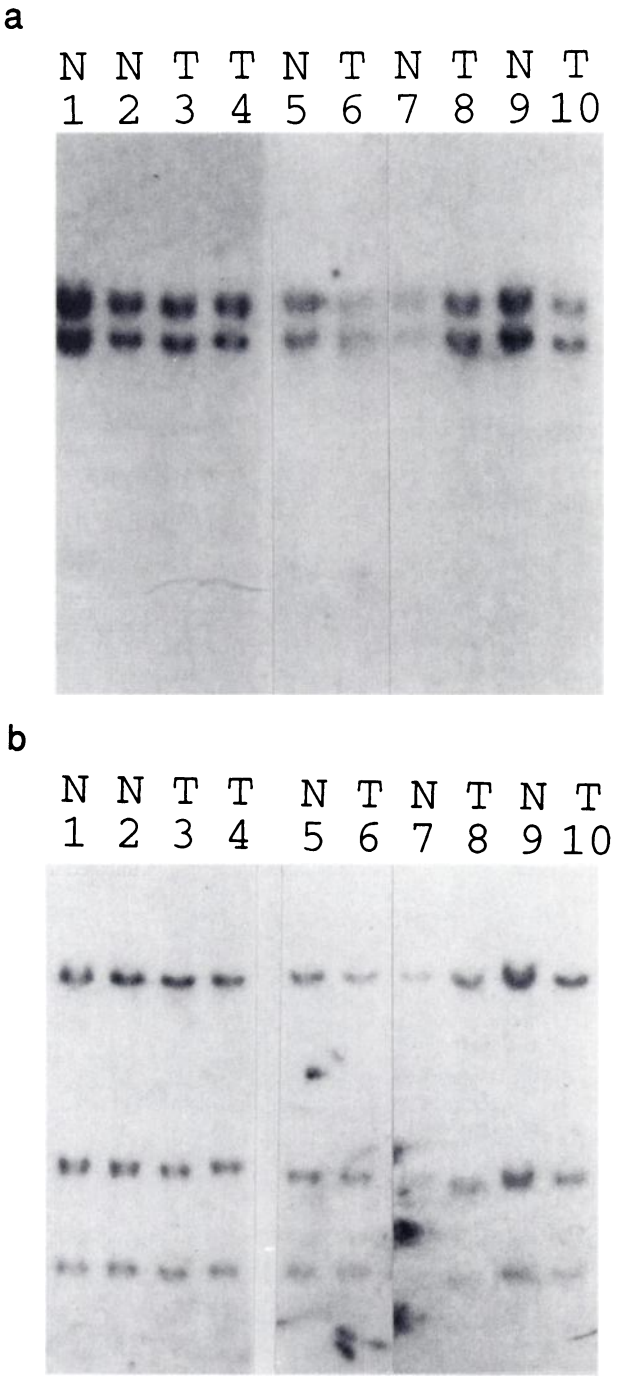

Fig. 1. Lack of intragenic deletion of the *TSG101* gene in human breast cancers. Genomic DNAs were digested with (*a*) *Bgl*II or (*b*) *Pst*I, and the filters were probed with the full-length cDNA of *TSG101*. *N*, normal breast tissue DNA; *T*, human primary breast cancer DNA. *Lanes 1* and *2*, two unrelated normal DNAs; *Lane 3*, tumor 1; *Lane 4*, tumor 3; *Lanes 5* and *6*, normal and tumor of case 2; *Lanes 7* and *8*, normal and tumor of case 5; *Lanes 9* and *10*, normal and tumor of case 6, which did not show a truncated transcript. The *Bgl*II fragments are 8.5 and 6.5 kb, and the *Pst*I fragments are 13, 4.5, and 2.0 kb.

6.0, and 4.5 kb); *Bam*HI (23 kb); and *Sal*I (25 kb). There were no alterations in any tumors (Table 1). For 22 of the breast cancers, matched normal breast tissues were available. DNA samples from the normal tissues showed identical patterns to those of the tumors, as did 10 additional unrelated normal tissues (Fig. 1 and Table 1). The lack of any alteration of DNA fragment on direct genomic Southern blots in any of the 72 breast cancer DNA samples, including those with truncated transcripts, indicated that the truncated transcripts were not caused by intragenic deletions of sufficient size to account for them.

Truncated Transcripts of *TSG101* Caused by Aberrant Splicing. Because there were no intragenic DNA deletions of *TSG101* gene in all 72 breast cancer DNAs analyzed by genomic Southern hybridization, we examined whether there were truncated transcripts as reported by Li *et al.* (2). Five of 12 breast cancers contained truncated transcripts using RT-PCR (*Lanes 2, 4, 6, 8,* and *10* in Fig. 2*a,* corresponding to tumor numbers 1–5). Sequence analysis also confirmed that the truncated transcripts contained internal deletions in the cDNA. Tumor 1 displayed four truncated transcripts in addition to the full-length transcript (Fig. 2*a, Lane 2*), the largest of which we termed type A, which deleted nucleotides from 154 to 1054 (Fig. 3*a* and Table 2). The second largest deletion, termed type B, deleted nucleotides from 132 to 729 (Fig. 3*a* and Table 2). The third largest deletion (type C) deleted nucleotides from 132 to 637 (Fig. 3*a* and Table 2). The smallest deletion, type D, removed nucleotides from 132 to 446 (Fig. 3*a* and Table 2). However, Southern blot analysis of tumor 1 clearly demonstrated no alteration of genomic DNA that could account for the various sized transcripts (Fig. 1, *Lane 3*).

It seems implausible that four cDNA deletions plus a full-length transcript could be generated from intragenic DNA deletions of two chromosomes. We considered that the most likely explanation for the generation of multiple truncated transcripts without apparent DNA deletion was aberrant RNA splicing. Analysis of additional tumors also supported this view. Tumor 2 shows two truncated transcripts in addition to the full-length transcript (Fig. 2*a, Lane 4*). Sequencing analysis of cDNAs from this tumor showed that they contained the type A and type D deletions (Fig. 3*a* and Table 2). Genomic Southern analysis of tumor 2 DNA also showed no deletion that could account for the altered transcript size (Fig. 1, *Lane 6*; matched normal DNA in Fig. 1, *Lane 5*). Tumor 3 showed four truncated transcripts in addition to the full-length transcript (Fig. 2*a, Lane 6*). The second largest deletion represented an additional truncated transcript, termed type F. Sequencing analysis confirmed the presence of types A, B, and D in tumor 3 (Fig. 3*a* and Table 2). Genomic Southern analysis of tumor 3 similarly showed no intragenic deletion (Fig. 1, *Lane 4*). Tumor 4 also showed a truncated transcript as well as the full-length transcript (Fig. 2*a, Lane 8*). The shortened cDNA was a new type E, which deleted nucleotides from 285 to 1054 (Fig. 3*a* and Table 2). Tumor 5 showed two truncated transcripts, types A and D, which were confirmed by sequencing analysis (Fig. 3*a* and Table 2). Genomic Southern analysis of tumor 5 and its matched normal DNA also exhibited no deletion (Fig. 1, *Lanes 7* and *8*). It is clear that although all of these tumors had multiple truncated transcripts, there were no intragenic DNA deletions that could account for them.

Sequence analysis of these transcripts at the junctions of the deleted sequence supported the idea that these truncated transcripts were generated by the aberrant RNA splicing, rather than intragenic DNA deletions. The most common truncated transcript was type A, the deleted sequences of which contained GT at the 5′ end and AG at the 3′ end (Fig. 3*b*), which are canonical splicing donor and acceptor site sequences. Thus, $G_{154}T_{155}$ in exon 1 and $A_{1053}G_{1054}$ in exon 5 were apparently recognized by the RNA splicing machinery. Similarly, the

Table 1 *Genomic Southern analysis of TSG101 gene in human breast cancers*

Seventy-two tumors were analyzed, of which 22 of 72 had matched normal samples. Twenty-one of 72 were tested by at least two different enzyme digests. DNA fragment sizes are in kilobases. There is an *Eco*RI polymorphic site giving an 8.0-kb band in 4 of 33 samples, which was also present in matched normal samples.

Enzymes	Tumors	Matched normals
*Pvu*II (8.5/5.5/4.0/2.5/2.0)	11	11
*Eco*RI (23/6.4/6.0/4.5)	33	8
*Bgl*II (8.5/6.5)	38	6
*Pst*I (13/4.5/2.0)	7	6
*Bam*HI (23)	11	9
*Sal*I (25)	3	2

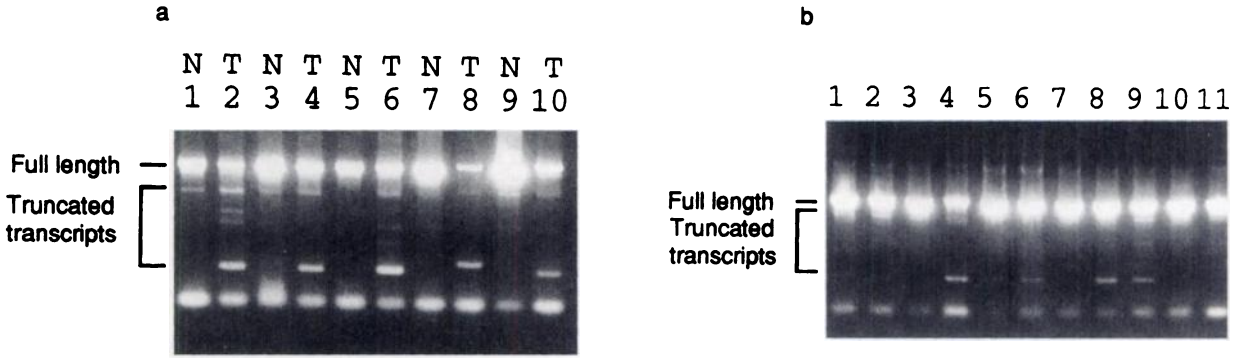

Fig. 2. RT-PCR Analysis of aberrant transcripts of *TSG101*. *a*, RT-PCR analysis of five breast cancer RNAs and their matched normal RNAs. *N* and *T*, normal and paired tumor RNAs, respectively. *Lanes 2, 4, 6, 8,* and *10* are from tumors 1–5, respectively; *Lanes 1, 3, 5, 7,* and *9* are from their matched normal tissues, respectively. *b*, RT-PCR analysis of aberrant transcripts in various tissues of two fetuses. *Lanes 1–6* are from heart, gut, trachea, lung, tongue, and skin of fetus 1; *Lanes 7–11* are from heart, lung, kidney, testes, and brain of fetus 2.

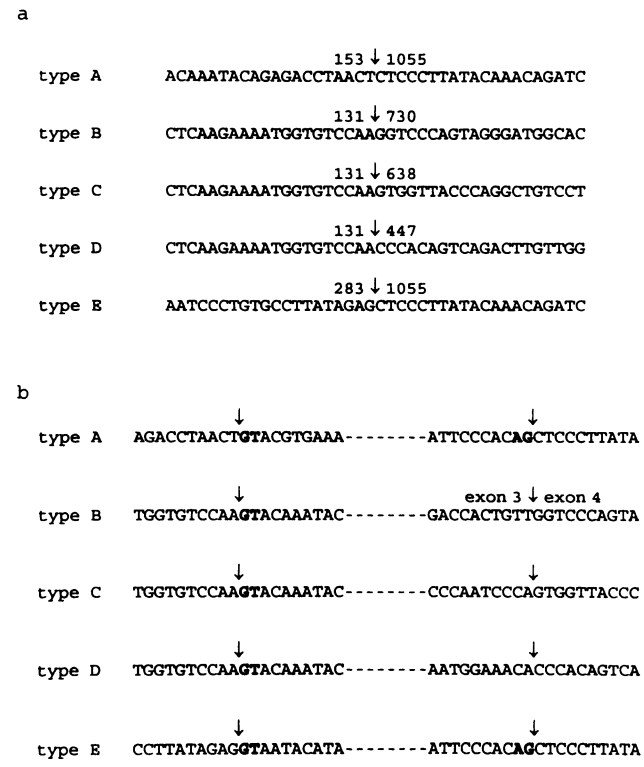

Fig. 3. Sequencing Analysis of Aberrant RNA Splicing. *a*, 20 nucleotides on both sides of each deletion type are displayed. *Arrows*, boundaries of deletions. The numbers on the *left* and *right* sides of the *arrow* mark the two nucleotides flanking the deleted region, and these flanking nucleotides remain within the truncated transcripts. See Table 2 for details. *b*, *arrows*, splicing junctions. Sequences between the arrows are spliced out. *Dashed lines*, nucleotides within the deleted sequences. *Boldface* highlights canonical splicing donor and acceptor sites. Although there is not a canonical splice acceptor site for the type B truncated transcript, it occurs at the boundary of exons 3 and 4.

type E truncated transcript contained GT at the 5′ end and AG at the 3′ end of the deleted sequences (Fig. 3*b*). In this case, $G_{284}T_{285}$ in exon 1 and the same $A_{1053}G_{1054}$ in exon 5 were apparently recognized as splicing sites. The type B truncated transcript was particularly interesting. It used $G_{132}T_{133}$ in exon 1 as the splicing donor and an intrinsic AG in intron 3 as the splicing acceptor site (Fig. 3*b*). Because the junction in type A occurred within the interior of exons and the type A truncated transcript was observed in four different tumors, such precise deletion was very unlikely to be caused by somatic DNA deletion events in different tumors. This again supported the view that these truncated transcripts were generated by aberrant RNA splicing.

Aberrant Splicing in Normal Breast Tissue and Fetal Development. Two observations indicated that the aberrant RNA splicing was not unique to cancer: (*a*) the type D truncated transcript was also present in three of five matched normal samples of these tumors (Fig. 2, *Lanes 1, 3,* and *9*; Table 2), indicating that such splicing can also occur in normal tissue; and (*b*) we found the same aberrant splicing patterns in several fetal tissues from two independent fetuses (Fig. 1*b*). We detected the type A truncated transcript in the heart, gut, lung, tongue, and skin of fetus 1 (Fig. 2*b*, *Lanes 1, 2, 4, 5,* and *6*; Table 2) and both lung and kidney of fetus 2 (Fig. 2*b*, *Lanes 8* and *9*; Table 2). In addition, the gut of fetus 1 contained a type D truncated transcript (Fig. 2*b*, *Lane 2*, and Table 2), the kidney of fetus 2 contained type B and D truncated transcripts (Fig. 2*b*, *Lane 9*), and the brain of fetus 2 contained the type D truncated transcript (Fig. 2*b*, *Lane 11*, and Table 2). The presence of these truncated transcripts in normal fetuses unrelated to tumors clearly demonstrated that these truncated transcripts were not generated specifically in tumors by an intragenic DNA deletion mechanism.

Table 2 *Truncated transcripts of TSG101 in breast cancers and normal tissues*

Cases	RT-PCR[a]	Type of truncated transcripts[b]	Deletions
1T	1	A	154–1054
	2	B	132–729
	3	C	132–637
	4	D	132–446
1N	4	D	132–446
2T	1	A	154–1054
	2	D	132–446
2N	2	D	132–446
3T	1	A	154–1054
	2	F	
	3	B	132–729
	4	D	132–446
4T	1	E	285–1054
5T	1	A	154–1054
	2	D	132–446
5N	2	D	132–446
Fetus 1			
Heart	1	A	154–1054
Gut	1	A	154–1054
	2	D	132–446
Lung	1	A	154–1054
Tongue	1	A	154–1054
Skin	1	A	154–1054
Fetus 2			
Lung	1	A	154–1054
Kidney	1	A	154–1054
	2	B	132–729
	3	D	132–446
Brain	1	D	132–446

[a] The RT-PCR products were labeled from small to large fragment sizes as 1–4. The fragment in the matched normal tissue (N) was assigned to the same number as the corresponding fragment in the tumor (T).

[b] Many of the RT-PCR fragments were sequenced. Some were deduced based on the size. Also see Fig. 3 for the sequences flanking the splicing junction.

There did appear to be a genuine increase in the number of classes of aberrant transcripts in tumors compared to normal breast tissue. Except for type D, none of the other truncated transcripts was detected in the matched normal breast tissue samples (Fig. 2*a* and Table 2). Comparison of normal and tumor of case 1 revealed the presence of a comparable amount of type D truncated transcript, whereas truncated transcripts A, B, and C were exclusively the products of the tumor. Similarly, we detected comparable amounts of type D truncated transcript in both normal and tumor of cases 2 and 5, whereas other types of truncated transcripts were detected only in tumors and fetuses (Fig. 2*a* and Table 2). Using 5′-end labeled primers in the PCR, we quantified the amount of truncated transcripts by PhosphorImager analysis. The types A, B, C, and D truncated transcripts accounted for 63, 7, 6, and 9% of RT-PCR products in tumor 1, respectively. In the matched normal tissue, the type D truncated transcript accounted for 8% of the RT-PCR product, whereas types A, B, and C did not appear. Similarly, the truncated transcripts accounted for 85, 63, 59, and 33% (sum of all of the aberrant transcripts) in tumors 2–5, respectively. Matched normal tissue from three of these cases again showed truncated transcript D, accounting for 3–4% of mRNA. The normal fetal tissues showed numbers of truncated transcripts comparable to that of the tumors, but at lower relative levels of expression (types A, B, and D, at 1–5% of total RT-PCR products) in heart, gut, lung, tongue, skin of fetus 1, and lung, kidney, and brain of fetus 2. Therefore, relaxation of splicing fidelity is associated with both tumorigenesis and fetal development.

No Point Mutation of *TSG101* Detected in Breast Cancers. We have shown that there were no intragenic DNA deletions of *TSG101* in breast cancer and that truncated transcripts are caused by aberrant RNA splicing. To determine whether somatic alterations of *TSG101* play any common role in breast cancers, we looked for evidence of subtle changes, such as point mutation or small insertion/deletions of the gene. We amplified the entire coding region of the *TSG101* gene from 10 breast cancers and sequenced all 10 cDNAs. No point mutations or small insertions/deletions were detected (data not shown). We concluded that there were neither point mutations nor intragenic deletions of *TSG101* in the breast cancers studied here.

Discussion

In summary, we have found frequent truncated transcripts of TSG101 in human breast cancer but no intragenic deletions that would account for them in 72 breast cancers studied. We have shown that the truncated transcripts can occur in both matched normal breast tissues and normal fetal tissues as well as breast cancer, and that these truncated transcripts are caused by aberrant RNA splicing. Finally, no mutations were found over the entire coding sequence in 10 tumors.

These results have three important implications:

(*a*) *TSG101* is unlikely to show frequent somatic mutation in human breast cancer, although we cannot exclude rare mutations of the gene. Furthermore, we mapped *TSG101* outside the two known regions of tumor suppressor activity by genetic complementation as well as by LOH studies. Finally, we found no deletions on Southern hybridization of genomic DNA that could account for the shortened cDNA products. We could also not detect such deletions after PCR of DNA using the identical conditions of Li *et al.* (Ref. 2 and data not shown). Thus, *TSG101* is not the 11p15 tumor suppressor gene commonly involved in breast cancer.

(*b*) Aberrant RNA splicing of *TSG101* clearly underlies the abnormal transcripts seen, as evidenced by the presence of identical products in matched normal tissues and normal fetal samples, the presence of four truncated transcripts in individual specimens, the presence of the full-length transcript in all tumor samples, the absence of appropriately sized deletions on genomic Southern blots, and the presence of canonical splicing donor and acceptor site sequences within the deleted DNA.

(*c*) This aberrant splicing does appear to increase in tumors, at least in the number of aberrant transcripts that are seen, and it can also occur in fetal tissues. Thus, relaxation of RNA splicing fidelity appears to be a novel form of oncodevelopmental marker for cancer. Normal embryonic cells possess phenotypic markers common to many cancer cells (*e.g.*, α-fetoprotein, carcinoembryonic antigen, and fetal isoenzyme patterns). Because of these observations, many observers, beginning with Laennec, have proposed that some steps in cancer involve the abnormal expression of normal cellular genes (16, 17). Thus, relaxation of RNA splicing fidelity may contribute generally to abnormal gene expression in cancer. We have recently found that *Staf50* also shows aberrant splicing products in Wilms' tumor and breast cancer.[5] Nevertheless, it is important to consider that aberrant splicing rather than somatic mutation may be present when altered transcripts are observed in tumors.

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[5] Unpublished observations.