Inhibiting Mutations in the Transforming Growth Factor β Type 2 Receptor in Recurrent Human Breast Cancer

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Abstract

Members of the transforming growth factor β (TGF-β) family are potent inhibitors of the growth of many epithelial cell types. Transmembrane signaling by TGF-β occurs via a complex of the serine/threonine kinases TGF-β type 1 receptor and TGF-β type 2 receptor (TGFBR2), and inactivating mutations in the latter have been detected in some primary tumors and in several types of tumor-derived cell lines. The most common mutations that have been identified in TGFBR2 are frameshifts in a repetitive polyadenine region in replication error-positive colorectal carcinomas that result in a truncated protein and absence of receptor expression at the cell surface. A number of point mutations in the highly conserved serine/threonine kinase domain of TGFBR2 have also been reported, some of which have been correlated with either loss of trans-phosphorylation of TGF-β type 1 receptor or constitutive activation of trans-phosphorylation. No TGFBR2 mutations have been reported in human breast tumors, but anomalous expression of TGF-β in breast carcinomas suggests that TGF-β signaling may be defective. We have therefore systematically examined unmatched sets of 17 primary and 17 recurrent breast tumor samples for mutations in TGFBR2, restricted to those regions of the gene in which mutations have previously been reported. None of the previously reported mutations was detected, but four novel mutations (V387M, N435S, V447A, and L452M) were found in the kinase domain in recurrent tumors. No mutations were detected in primary tumors. TGF-β signaling was significantly inhibited by each of the N435S, V447A, and L452M mutations.

Introduction

TGF-β is a ubiquitously expressed paracrine polypeptide, of which three highly homologous forms (TGF-β1, TGF-β2, and TGF-β3) have been detected in humans and other mammals. Each isoform of this cytokine inhibits the growth of a wide range of normal cells (1) and also inhibits the growth of some cancer cell lines, including most but not all human breast tumor cell lines (2). However, resistance to growth inhibition by TGF-β has been demonstrated in a wide variety of human epithelial and lymphoid malignant cell lines (2). Two of the TGF-β receptors that mediate the actions of members of the TGF-β family are transmembrane serine/threonine kinases. TGF-β binds directly to TGFBR2, a constitutively active kinase, and is then recognized by TGFBR1, which is directly phosphorylated and activated by TGFBR2 (1, 3). TGFBR2 maps close to or within one of the interstitial deletions that occur in 30–50% of head and neck, breast, and small cell lung cancers. Truncation, deletion, or decreased expression of TGFBR2 has been detected in a variety of primary tumors and tumor cell lines, and mutations occur in the serine/threonine kinase domain (Fig. 1) in hereditary nonpolyposis colorectal carcinoma and in sporadic colorectal carcinoma (3). Point mutations of highly conserved sites in the TGFBR2 kinase domain causing defective autophosphorylation or constitutive kinase activation of TGFBR1 have been identified (4). Mutations in TGFBR2, causing the absence of receptor expression at the cell surface, have been detected in a subset of colon cancer cell lines exhibiting RERs (RER+) caused by defective mismatch repair (5, 6). The principal mutation is a frameshift within a 10-bp pA repeat in the TGFBR2 coding region (codons 125–128; Ref. 7). The inactivation of TGFBR2 in a wide range of malignant cell types, together with the evidence for anomalous TGF-β signaling in some but not all human breast cancers (8), raises the question of whether mutations in TGFBR2 may be associated with the development of breast carcinoma. We have therefore screened regions of TGFBR2 in DNA samples from primary human breast tumors and from recurrent tumors for mutations in the pA and kinase domains of the receptor.

Materials and Methods

Patient Treatment. Unmatched sets of 17 primary and 17 recurrent breast tumor samples were examined for mutations in TGFBR2. Tamoxifen treatment of patients with recurrent tumors was used as adjuvant therapy rather than as treatment of recurrent disease. Tamoxifen resistance was defined as the development of a histologically proven tumor of increasing size while tamoxifen treatment continued, before salvage surgery. All of the 17 recurrent tumors were derived from infiltrating ductal tumors and were locoregional recurrences or relapses (breast/chest wall, lymph nodes, or skin).

Tumor Samples. Sections (10 μm) were cut from paraffin-embedded blocks of breast tumor tissue. One section of each block was stained with H&E to determine regions with the highest tumor cell density. Corresponding regions were then microdissected from aniline blue-stained sections, placed in 100 μl of extraction buffer [2× Taq DNA polymerase buffer (Pharmacia); 3 mM MgCl2, 0.9% NP40, 0.9% Tween 20, and 40 μg of proteinase K], incubated at 55°C overnight, boiled for 8 min, chilled, and centrifuged for 10 min at maximum speed (10,000 × g).

Extraction of Normal Human Peripheral Blood Lymphocyte DNA. Human peripheral blood lymphocytes from a normal individual (TRH) were isolated by centrifugation of a 1:3 mixture of blood and a 0.9% saline solution through a Ficoll-Triosil gradient (10 parts 32.8% Triosil plus 24 parts 9% Ficoll; density, 1.076) at room temperature for 20 min at 400 × g. The interfacial layer containing the lymphocyte and monocyte fraction was removed, 4–5× the volume of PBS [137 mM NaCl, 2.7 mM KCl, 1.5 mM KH2PO4, and 8.0 mM Na2HPO4 (pH 7.4)] was added, and the mixture was centrifuged at 400 × g for 6 min. The pellet was chilled (15–30 min) before the addition of 0.4 ml of ice-cold cell lysis buffer [0.15 mM NaCl, 10 mM Tris (pH 7.4), 1 μM MgCl2, 0.02% NP40, 1 mM DTT, and 10 μM RNasin (Promega)], resuspension, and centrifugation (5 min, 12,000 × g) to pellet the
nuclei. DNA was isolated from the pellets using a QIAamp Blood Kit (Qiagen) according to the manufacturer’s instructions and eluted with 2× 200 µl of Tris-HCl (10 mM, pH 9.0; preheated to 70°C).

**PCR.** A 73-bp fragment of exon 3 that contained the pA tract (nucleotides 665–737; GenBank accession number M85079) was amplified using forward primer TA10-F1 (5′-CTTATCTGGAGATGCTGC-3′) and reverse primer TA10-R1 (5′-GAAGAGTCTCAGCAAGC-3′) following the method of Myeroff et al. (7) and Parsons et al. (5). Fifty-μl PCR reactions containing 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0 at room temperature), 0.2 mM deoxynucleotide triphosphates, and 2.5 units of Taq DNA polymerase (Pharmacia), approximately 160 ng of genomic DNA or an equivalent amount of plasmid DNA (0.45 µg), 130 pg of 32P-labeled TA10-F1 were set up. The DNA was amplified for 35 cycles as follows: 94°C- 30 s, 80°C- hold (hot start); 94°C, 3 min, denaturation 95°C- 1 min, extension 70°C- 1 min, 20°C- refrigerate/hold. A 205-bp fragment containing the 3′ end of exon 4 of TGFBR2 was amplified using the above-mentioned buffer and the forward and reverse primers 5′-AGAGGGCAGCCTCTTTGG-3′ and 5′-TAAAGGGGTAATTGAGCTC-3′.

**Methylation.** A 73-bp fragment of exon 3 that contained the pA tract (nucleotides 665–737; GenBank accession number M85079) was amplified using forward primer TA10-F1 (5′-CTTATCTGGAGATGCTGC-3′) and reverse primer TA10-R1 (5′-GAAGAGTCTCAGCAAGC-3′) following the method of Myeroff et al. (7) and Parsons et al. (5). Fifty-μl PCR reactions containing 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 9.0 at room temperature), 0.2 mM deoxynucleotide triphosphates, and 2.5 units of Taq DNA polymerase (Pharmacia), approximately 160 ng of genomic DNA or an equivalent amount of plasmid DNA (0.45 µg), 130 pg of 32P-labeled TA10-F1 were set up. The DNA was amplified for 35 cycles as follows: 94°C- 30 s, 80°C- hold (hot start); 94°C, 3 min, denaturation 95°C- 1 min, extension 70°C- 1 min, 20°C- refrigerate/hold. A 205-bp fragment containing the 3′ end of exon 4 of TGFBR2 was amplified using the above-mentioned buffer and the forward and reverse primers 5′-AGAGGGCAGCCTCTTTGG-3′ and 5′-TAAAGGGGTAATTGAGCTC-3′.

After a nonradioactive 50-µl PCR amplification for 30 or 40 cycles, samples were separated on 2% agarose gels and purified using a Qiagen gel extraction kit. DNA was sequenced using the Sanger dyeoxy chain termination method at the DNA Sequencing Facility of the Department of Biochemistry, University of Cambridge.

**Plasmid Constructs and in Vitro Transcription.** Full-length wild-type TGFBR2 cDNA from H23FF (9) was cloned into pALTER-1 (Promega). Site-directed mutagenesis was carried out using a Promega Altered Sites II kit to generate the variant forms TGFBR2 (1304 A→G; N435S), TGFBR2 (1340 T→C; V447A), and TGFBR2 (1354 C→A; L452M). Fragments (~3100 bp) containing the full coding sequence for wild-type and mutant forms of TGFBR2 were excised from pALTER-1 plasmids (plasmid DNA isolated using a Quantum Prep kit (Bio-Rad)) by digestion with BamHI and EcoRI, and a dominant negative TGFBR2 was released by EcoRI and Pml1. The inserts were cloned into pCS2+ (10, 11) for RNA injections into Xenopus laevis embryos. Receptor constructs were verified by restriction enzyme digestion and confirmed by sequencing. Constructs were linearized by NotI and transcribed in vitro using a MESSANGER MACHINE kit (Ambion).

**Animal Cap Assays.** Embryos were obtained from X. laevis adult frogs by hormone-induced egg laying followed by in vitro fertilization and staged by the method of Nieuwkoop and Faber (12). Ten nl of 0.2 ng/µl RNA were injected into the animal pole of each blastomere of the two-cell stage (13). Animal caps were dissected from stage 8.5–9 embryos (4.5–5.5 h after injection) and placed in 2 ml of 0.7× modified Ringer’s solution plus 0.1% BSA and human recombinant TGF-β1 (0, 50, 250, or 500 µM; R&D Systems) in a multiwell dish coated with agarose. Caps were analyzed by light microscopy 18 h after injection (see the Fig. 3 legend).

**Results and Discussion**

Thirty-four tumor samples (Table 1) were analyzed [17 from primary human breast carcinomas (samples 1P to 17P) and 17 from recurrent tumors (samples 1R to 17R)]. We systematically screened all of the regions of the gene in which mutations have previously been reported (Fig. 1). To detect mutations in the serine/threonine kinase encoding domain of TGFBR2, we screened the 3′ region of exon 4 (nucleotides 1100–1254) and the whole of exons 5 and 7 by PCR-SSCP analysis and/or sequencing for all 34 samples. No mutations were detected in exon 7. We also screened a 73-bp fragment of exon 4.

![Fig. 1. Structure of TGFBR2. Previously identified mutations are shown above the schematic, and those detected in breast carcinoma in this study are shown below the schematic. hatched box, signal sequence.](Image 199x640 to 561x741)

**Table 1 Characteristics of breast tumor samples and mutations detected in TGFBR2**

<table>
<thead>
<tr>
<th>Tumor no.</th>
<th>Primary</th>
<th>Recurrent</th>
<th>Exon</th>
<th>Nucleotide no.</th>
<th>Base change</th>
<th>Codon</th>
<th>Amino acid change</th>
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<tbody>
<tr>
<td>1P-17P</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1R-3R</td>
<td>+</td>
<td>+</td>
<td></td>
<td>5</td>
<td>1304</td>
<td>A→G</td>
<td>435</td>
</tr>
<tr>
<td>4R</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>1340</td>
<td>T→C</td>
<td>447</td>
<td>Val→Ala</td>
</tr>
<tr>
<td>5R</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>1304</td>
<td>A→G</td>
<td>435</td>
<td>Asn→Ser</td>
</tr>
<tr>
<td>6R</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>1354</td>
<td>C→A</td>
<td>452</td>
<td>Leu→Met</td>
</tr>
<tr>
<td>7R</td>
<td>+</td>
<td>+</td>
<td>5</td>
<td>1304</td>
<td>A→G</td>
<td>435</td>
<td>Asn→Ser</td>
</tr>
<tr>
<td>8R-11R</td>
<td>+</td>
<td>+</td>
<td></td>
<td>1159</td>
<td>G→A</td>
<td>387</td>
<td>Val→Met</td>
</tr>
<tr>
<td>12R</td>
<td>+</td>
<td>+</td>
<td>4</td>
<td>1167</td>
<td>C→T</td>
<td>389</td>
<td>Asn silent</td>
</tr>
<tr>
<td>13R</td>
<td>+</td>
<td>+</td>
<td>4</td>
<td>1167</td>
<td>C→T</td>
<td>389</td>
<td>Asn silent</td>
</tr>
<tr>
<td>Normal DNA</td>
<td>+</td>
<td>+</td>
<td>4</td>
<td></td>
<td></td>
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</table>
mutations in this region, suggesting that frameshift mutations in the pA tract of TGFBR2 are unlikely to contribute significantly to the development of primary breast carcinoma or to tumor recurrence.

For exon 5, the wild-type SSCP patterns comprised four bands (Fig. 2), suggesting that this sequence of the normal allele can assume several stable conformations. More complex SSCP patterns were observed with tumors 4R, 5R, and 7R (Fig. 2), and sequencing indicated that each of these recurrent tumors carried two mutations (Table 1). These mutations were confirmed by sequencing the negative strand. The nucleotide substitutions in TGFBR2 comprised two transitions [nucleotides A1304G and T1340C; numbering from the reverse strand. The nucleotide substitutions in indicated that each of these recurrent tumors carried two mutations observed with tumors 4R, 5R, and 7R (Fig. 2), and sequencing confirmed that these SSCP patterns arose from the presence of normal TGFBR2. Thus, the mutations identified were not rare polymorphisms. The SSCP patterns for tumors 4R and 7R were very similar, consistent with these tumors carrying the same pair of mutations (Table 1). A mutation was also identified in exon 4 in tumor 12R (nucleotide G1159A; Table 1) and confirmed by sequencing as described above.

Striking features of the data are that two distinct mutations were identified in tumors 4R, 5R, and 7R (Table 1) and that 4R and 7R shared the same pair of mutations. It is possible that pairs of mutations in TGFBR2 arose through the isolation of more than one clone, each carrying a different mutation. Alternatively, the mutations in tumors 4R and 7R may have been present in different alleles or may have arisen as distinct mutations in the same allele. Either possibility would be consistent with the data showing wild-type sequence together with the mutations at nucleotides 1304 and 1340 in tumors 4R and 7R, or the samples may contain DNA derived from normal as well as tumor cells. In the sequences for tumor 5R, both normal and mutated nucleotides are present at position 1354, but only the mutated form is detectable at nucleotide 1304, suggesting loss of heterozygosity at this locus. There is persuasive evidence that mutational heterogeneity occurs within primary tumors (14) and also that multiple mutations in one gene can occur within a tumor, most notably in hereditary nonpolyposis colon cancer, in which up to six mutations in APC and up to four mutations in P53 have been detected in the same tumor (15). The accumulation of mutations within single genes was ascribed to loss of function of the mismatch repair gene MSH2, giving rise to a RER phenotype (RER

\[ \text{N435S, V447A, L452M, and Uninjected} \]

\[ \text{TGFBR2 AND BREAST CANCER} \]

\[ \text{Fig. 2. SSCP analysis of exon 5 of TGFBR2 in breast tumors. DNA was extracted from breast tumor tissue sections from regions with the highest tumor cell density and from regions of normal morphology within the same section. An abnormal SSCP pattern was shown by tumors 4R, 5R, and 7R. A wild-type SSCP pattern from DNA extracted from the peripheral blood of a healthy individual (left) and from normal tissue adjacent to tumor 5R (right) is shown. Arrowheads indicate the positions of the major, abnormal bands from tumors 4R, 5R, and 7R. Normal tissue adjacent to tumors 4R and 7R also had wild-type TGFBR2 sequence (SSCP pattern not determined). The SSCP band patterns were reproducible from independent PCR amplifications for all samples. Minor differences in the separation of bands were observed in different experiments due to the use of gradient gels.} \]

\[ \text{Fig. 3. Analysis of histology of Xenopus embryo animal caps injected with human TGFBR2 variants after treatment with TGF-β1. A, representative animal caps 18 h after injection of human TGFBR2 RNA (wild type, N435S, V447A, L452M, and uninjected) into 2-cell embryos and treatment with 250 pM TGF-β1.} \]

\[ \text{B} \]

\[ \text{B} \]

\[ \text{B} \]

\[ \text{B} \]
The nucleotide substitutions identified in TGFBR2 (Table 1) each gave rise to a missense mutation (N435S, V447A, and L452M, respectively). None of these mutations in TGFBR2 have been reported previously, although a mutation at codon 452 (L452P) has been detected in a colon cancer cell line (2). All of these mutations in exons 4 and 5 occur in the conserved kinase domain of TGFBR2. The mutations observed were all in samples of recurrent tumors, and three (4R, 5R, and 7R) were in tumors from patients who had become resistant to tamoxifen.

To determine the functional effects of these mutations, TGFBR2 N435S, V447A, and L452M were expressed in Xenopus embryos by injection of equal amounts of mRNA, and the induction of mesoderm formation by TGF-β1 was assayed by cap elongation (Fig. 3; Ref. 13). (We have been unable to achieve the required mutagenesis to make the V387M mRNA, despite attempts using a variety of primers.) Fig. 3B shows that expression of wild-type TGFBR2 rendered caps maximally responsive to TGF-β1. A dominant negative form of TGFBR2 was analyzed in two independent experiments in which caps were totally unresponsive to TGF-β1 at concentrations of up to 500 pM (data not shown). Sensitivity to TGF-β1 was greatly reduced when any of the variant forms of TGFBR2 (N435S, V447A, and L452M) were expressed.

The observations that mutants N435S, V447A, and L452M were defective in TGF-β1 signaling capacity are consistent with the non-conservative amino acid substitutions. For example, the substitution of asparagine by serine may result in abnormal phosphorylation of the receptor. These mutations may therefore compromise either the catalytic activity of TGFBR2 or the interaction between TGFBR2 and TGFBR1 after TGF-β1 binding. Previously detected point mutations in TGFBR2 within the kinase domain have been shown to have functional effects that include defective autophosphorylation and hence inhibition of signal transduction, constitutive activation of trans-phosphorylation of TGFBR1 by TGFBR2 (4, 17), and generation of a dominant negative form (18). These observations suggest that the mutations identified either partially compromise the activation of TGFBR1 or the translocation of TGFBR2 to the membrane.

The results indicate that the three mutations detected in recurrent tumor samples from patients who had become resistant to tamoxifen substantially inhibited TGF-β1 signal transduction. Various studies have linked treatment with tamoxifen to up-regulation of TGF-β in a variety of cell types including human breast cancer cells in vivo (19). Taken together, these data raise the possibility that mutations inhibiting TGF-β signaling are significant in the development of tamoxifen resistance.

Mutational inactivation of TGFBR2 is very common (13%) in RER+ colon carcinoma, and mutations in the downstream signaling proteins SMAD2 and SMAD4 have also been detected (3). Inactivating TGFBR2 mutations also occur in 15% of microsatellite stable cases (20), and it has been estimated that overall loss of function of the TGF-β signaling pathway may be involved in the development of over 80% of colon carcinomas (20). Analogy with the colon carcinoma studies suggests that larger numbers of breast tumor samples should be analyzed for mutations in the entire TGFBR2 gene and in the downstream proteins to establish whether there is an extensive association between resistance to tamoxifen and defects in TGF-β signaling pathways.

Acknowledgments

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References

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