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Transforming Growth Factor-ß (TGFß) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells. In general, advanced breast cancers are refractory to TGFß-mediated growth inhibition. The TGFß type I and -II receptors (TßR-I and -II) are the primary transducer of TGFß's growth inhibitory effect. It is our working hypothesis that TGFß-resistance is caused by molecular lesions in the TßR genes.

We first asked whether or not molecular lesions of the TGFß receptor genes are involved in the origin and/or progression of breast cancer. Our findings indicate that a specific somatic mutation within the kinase domain of the TßR-I receptor (S387Y) occurs in primary breast cancers, and, more commonly, in lymph node metastases. This is the first example of a missense mutation in this gene in human malignancy. Secondly, we demonstrated that the S387Y mutation has a negative impact on the ability of the TßR-I receptor receptor to transduce the TGFß signal. Finally, we have shown that allelic losses of both the TßR-I and -II genes occur in subpopulations of cells contained within primary breast carcinomas, suggesting that TGFß-refractory clones are selected for during breast cancer progression.

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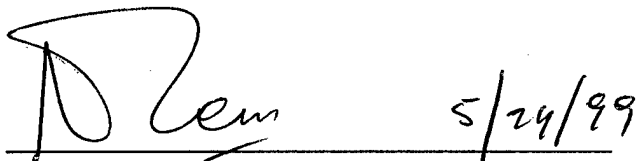

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INTRODUCTION

Transforming Growth Factor-ß (TGFß) is the most potent known inhibitor of cell cycle progression of normal mammary epithelial cells; in addition, it causes cells to deposit increased amounts of extracellular matrix, which affects cell-cell and cell-substrate interactions. In general, advanced breast cancers are refractory to TGFß-mediated growth inhibition, while the TGFß they secrete apparently serves to enhance invasion into surrounding structures and perhaps their metastatic potential. The effects of TGFß on cell cycle progression are transduced by two cell surface receptors, TGFß type I (TßR-I) and -II receptors (TßR-II), and relayed from the membrane to the cell nucleus by three recently discovered members of the MAD family of proteins, Smad2, -3, and -4. It is our working hypothesis that TGFß-resistance can, in principle, be caused by molecular lesions in any of these five genes, that such lesions are likely to occur during the development or progression of human breast cancer, and that they may impact on prognosis or treatment response.

This project addresses three of the fundamental research issues raised by the USAMRMC Breast Cancer Research Program. The first question is whether or not molecular lesions of the genes involved in the TGFß signaling pathway contribute to the origin and/or progression of breast cancer. We expected changes in these genes to be relatively late events, perhaps characteristic of metastatic cancer. Secondly, we proposed to determine how molecular lesions in the TGFß receptor and/or Smad genes affect receptor function, and how they might play a role in the development and/or progression of breast cancer. Thirdly, we intended to examine the question whether genetic lesions in TGFß receptor and/or Smad genes are able to predict the outcome of patients with breast cancer. Because the anti-tumor effects of anti-estrogens such as tamoxifen are thought to be mediated by the auto- and paracrine induction of TGFß, we wished to test the hypothesis that resistance of hormone-receptor positive cancers to tamoxifen is the result of inactivation of TGFß pathway genes.

BODY

The Statement of Work in our original proposal included the following tasks/timeline:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

a. Identification of genetic alterations of TGFß-receptor genes in invasive breast cancer specimens. - Months 1-24

b. Identification of genetic alterations of TGFß-receptor gene in sets of pre-invasive, primary invasive and metastatic (lymph node positive) breast cancers in order to determine the stage of tumor development at which these mutations occur. Months 12-36.

Task 2. Determination of the functional consequences of TGFß-receptor mutations

Cloning of TGFß-receptor mutants into mammalian expression vectors and transfection into TGFß-sensitive and -resistant human mammary epithelial cells to determine whether the mutations are dominant or recessive, and correlation of the site of mutations within the molecule with the way they affect the cellular phenotype. - Months 12-36

Task 3. To determine the potential clinical significance of genetic alterations of the TßR genes in breast cancer

Test the hypothesis that genetic alterations of TGFß-receptor genes predict for resistance to anti-estrogen therapy in patients with estrogen-receptor positive tumors. Months 36-48.

This report concerns progress achieved on Tasks 1 and 2, which we will describe separately:

Task 1. Screening for mutations in TGFß receptor genes in breast cancer

Our initial studies of genes involved in TGFß signaling focused on the TßR-II gene. Using a chemical mismatch cleavage (CCM) assay, we were the first to identify missense mutations within the TßR-II serine-threonine kinase domain in human cancer cell lines [Garrigue-Antar, 1995 #717]. These findings raised two important questions: (1) Do such structural alterations of the TßR genes also occur in primary tumors (particularly breast cancers) *in vivo*? and, if so (2) How do mutations in the TßR genes affect receptor function?

Selection of breast cancers for genomic analysis. In collaboration with our breast pathologist, Dr. Daryl Carter, we selected a series of 36 primary stage I and -II breast carcinoma specimens for which both frozen and paraffin-embedded material is available. In 12 of these cases, we also had lymph node metastatic lesions available for analysis. Uring the past year, we have completed the molecular structure of the TßR-I and -II genes in this series. The final results are presented here:

Tissue specimens and nucleic acid extraction: Breast carcinoma specimens were provided by the Program for Critical Technologies in Breast Oncology at Yale after hisopathological review by one of us (D.C.). Genomic DNA was extracted from tumor and normal tissues as previously described (18). Isolating genomic DNA from a single 5 µm microdissected paraffin-embedded tumor section using InstaGene matrix (Bio-Rad, Hercules, CA) typically yielded 200 µl of DNA template solution. Total cellular RNA was extracted from three or more 50 µm serial thick frozen sections using TriZOL® reagent (GIBCO-BRL).

Genotyping of TGFß signaling intermediates: The TßR-II gene was analyzed by chemical mismatch cleavage as previously described (19), or by conventional PCR-SSCP (For primers used to amplify TßR-II exons, see (1)). The TßR-I gene was analyzed by "cold" PCR-SSCP (2). In this case, each 20-µl PCR reaction contained 500 nM of unlabeled primers. Following an initial 3 minute denaturation at 95°C, PCR was performed for 35 cycles of 95°C for 30 seconds, 55°C for 40 seconds, 72° for 30 seconds followed by a 5 min final extension at 72°C. For PCR amplification of the GC-rich exon 1 we used the Advantage-GC genomic polymerase mix (Clontech Palo Alto, CA) according to the instructions supplied by the manufacturer. The 9 exons of the TßR-I gene were amplified using the following flanking intronic forward and reverse primers: Exon 1: 5'-gaggcgaggtttgctggggtgaggca-3' and 5'-catgtttgagaaagagcaggagcgag-3'; exon2: 5'-ctacacaatctttctctttttcc-3' and 5'-gtttttcttgtagtatctagg-3'; exon 3: 5'-gtttatttcactcgaggcc-3' and 5'-ggagaaacaattatgttac-3'; exon 4: 5'-gattgtgttgagtactattta-3' and 5'-ggaaaagcaaatgttacagac-3'; exon 5: 5'-gcccaaccgaaatgttaattc-3' and 5'-ggtagaactgcttatagaat-3'; exon 6: 5'-gcagtcatgtttaatttttgattc-3' and 5'-gaacgcgtattaaatatagttg-3'; exon 7: 5'-tgtctgaaaggaggttcatcc-3' and 5'-gaacaacttctgctcatgacg-3'; exon 8: 5'-gccttgcattagctgaataaat-3' and 5'-gcttactaagcagaagcag-3'; exon 9: 5'-ggaaaatggtgcatgcatta-3' and 5'-gagttcaggcaaagctgtag-3'. For SSCP analysis, 5 µl aliquots of amplified PCR product were mixed with 15 µl loading buffer (12.5 µl 10x TBE buffer, 2 µl of 15% Ficoll, 0.1% bromophenol blue & xylene cyanol, 0.5 µl methyl mercury hydroxide), denatured by heating at 80°C for 3 minutes, and quenched on ice. The single stranded DNA fragments were then resolved using precast 20% TBE acrylamide gels on a Novex Xcell II Thermoflow apparatus (Novex, San Diego, CA) with the gel temperature precisely maintained at 10°C throughout the run. Bands were visualized by staining the gel in a 1:10,000 dilution of SYBR™ Green II (Molecular Probes, Inc., Eugene, OR) for 20-30 minutes and using an Eagle Eye charged coupled device camera equipped with a SYBR™ Green band pass filter (Stratagene) for photographic documentation.

Suspect bands were excised from the gels with a razor blade and reamplified. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), and

subjected to DNA sequencing using a thermocycling sequencing kit (Epicentre® Technologies, Madison, WI) with either a forward or reverse primer end-labelled with [γ-^{32}P]-ATP. Reaction products were denatured at 70°C for 3 minutes, resolved on 7% (w/v) denaturing polyacrylamide gels at 50°C and visualized by exposing dried gels to X-ray film overnight at 20°C. The presence of any sequence alteration was always confirmed by repeated PCR-SSCP and DNA sequencing using an independent aliquot of tumor-derived genomic DNA as template. Whether any mutations were somatic in nature or present in the germline was determined by analyzing genomic DNA isolated from non-cancer tissue of the same patient.

TßR Gene Expression in Primary Breast Cancer: In order to test the hypothesis that breast carcinomas *in vivo* are refractory to TGFß, we analyzed the molecular characteristics of the two cell surface receptor genes, TßR-I and -II. TßR expression was determined using a reverse transcription-PCR assay in 14 frozen surgical breast cancer specimens from which we were able to extract good quality RNA. Each of these samples expressed both TßR-I and -II mRNA transcripts (data not shown). This is in contrast to our previous studies in esophageal- and small cell lung cancers, in which loss of TßR-II mRNA was found in 25% and 100% of cases, respectively .

Structural Analysis of the TßR-II Gene in Primary Breast Cancer: The entire open reading frame of TßR-II was screened for the presence of mutations by chemical mismatch cleavage or by PCR-SSCP followed by DNA sequencing. No DNA sequence alterations were encountered in a total of 30 cases examined. Thus, the TßR-II gene is normally expressed in primary human breast cancer, and mutations of this gene are probably rare. This result is perhaps not surprising in light of previous studies of other cancer types: Missense and/or nonsense mutations in the TßR-II gene have only been found sporadically in colorectal- and head-&-neck cancers and in cutaneous T-cell lymphomas. The only exceptions are tumors that are associated with DNA mismatch repair deficiencies which frequently display TßR-II nonsense mutations.

Structural Analysis of the TßR-I Gene in Primary Breast Cancer: In order to determine whether mutations in the TßR-I gene might be found in human breast cancer, we screened each of the 9 exons of the TßR-I gene by PCR-SSCP in 31 primary breast carcinoma specimens and in 12 associated lymph node metastases. Areas of tumor tissue were isolated from paraffin sections by microdissection and the remaining surrounding breast tissue was used to extract germline genomic DNA. Individual exons were amplified by PCR and the products screened for the presence of novel single-strand conformation polymorphisms. Suspect bands were re-amplified and subjected to direct DNA sequencing. Individual sequence abnormalities were confirmed by repeating the entire procedure using a second aliquot of genomic DNA.

Our most important finding was a C to A transversion at nucleotide 1160 in exon 7 of TßR-I, which predicts for a serine to tyrosine substitution at codon 387 (**Figure 1**). This was the only mutation encountered in the entire series and was present in 7 of the 43 specimens (16%, 95% CI: 7-31%) (**Figure 1A**). This is the first report of a mutation in the TßR-I gene in any type of human malignancy. Moreover, this mutation may be specifically associated with breast cancer as we have not found it in any cervical carcinomas nor in head-&-neck cancer cell lines (V.F. Vellucci and M. Reiss, unpublished data). In addition, Pasche et al. (3) recently reported the absence of TßR-I mutations in acute myeloid leukemias.

Our second major finding is the highly significant association between the S387Y mutant and axillary lymph node metastases (**Figure 1A**): While we encountered this mutation in only 2 of 31 (6%, 95% CI: 1-21) primary breast cancer specimens, it was present in 5 of the 12 (41%, 95% CI: 15-72) lymph node metastases (Fisher's Exact Test, p=0.012). The dramatically increased frequency of this mutation in lymph node metastases indicates that inactivation of the TGFß pathway may represent a late event in breast cancer progression. The fact that most breast carcinoma cell lines are refractory to TGFß is also consistent with this idea, as most of these cell

lines were initially derived from metastatic cells isolated from malignant pleural effusions or ascites (34). Moreover, in animal models of skin carcinogenesis, TGFß resistant tumor cell clones also do not emerge until the tumors have become highly aggressive and metastatic (35). In contrast, in colorectal cancers associated with DNA mismatch repair deficiencies, the acquisition of TßR-II gene mutations appears to coincide with the transition from pre-invasive adenoma to invasive carcinomas. Thus, the stage of tumor development at which the TGFß signaling pathway becomes inactivated appears may vary depending on the tumor type and on the underlying molecular genetic events that drive the carcinogenetic process.

As the S387Y mutation was not detected in germline DNA of the same individuals (**Figure 1B**), we can practically exclude the possibility that this sequence alteration represents a normal polymorphism. On the other hand, besides the mutant band, a wild type band could be detected in each of the tumor specimens (**Figure 1B**). Although these findings suggest that the tumors may have retained a wild type allele, it is impossible to exclude the possibility that this wild type band was the result of the almost inevitable contamination of the specimens with at least some normal cells. However, even loss of function of one of the two TßR-I alleles may be sufficient to confer a significant a selective advantage. Such a dosage effect occurs, for example, in transgenic animals that express a dominant-negative TßR-II gene in conjunction with two endogenous wild type alleles, and in knock-out mice that carry only a single TGFß1 gene allele (4, 5). In both of these situations, the animals are significantly more susceptible to tumor formation.

Besides the S387Y somatic missense mutation, we also detected a variant allele of the TßR-I gene with an in-frame deletion of 3 of 9 repeating GGC trinucleotides within exon 1. Thirteen of 24 evaluable cases with BC were heterozygous carriers of this del$(GGC)_3$ TßR-I variant (54%, 95% C.I. 33-74%). This deletion results in the loss of 3 of the 9 alanine residues that constitute the hydrophobic core of the putative TßR-I signal. Comparative hydrophobicity plots of wild type and the del $(GGC)_3$ TßR-I variant clearly show that the deletion shortens the hydrophobic core of the signal peptide . These findings suggested that this deletion may well have functional consequences for the receptor protein, particularly its ability to be targeted to the cell membrane.

In order to determine whether there might be an association between the carrier state of the del$(GGC)_3$ TßR-I variant and the development of breast cancer, we determined the frequency of the del$(GGC)_3$ allele in a cohort of germline DNA samples from 43 independently and randomly selected individuals. Only one of these individuals was heterozygous for the del$(GGC)_3$ variant of TßR-I (2%, 95% CI: 0-12%). This translates into a highly significant increased relative risk of developing BC in carriers over control (Fisher's Exact test: $p<0.0001$)(Relative risk: 3.18; 95% C.I.: 2.32-4.36). These findings strongly argue in favor of the hypothesis that the del$(GGC)_3$ variant of TßR-I confers an increased cancer risk, presumably by decreasing the sensitivity of normal breast epithelial cells to TGFß.

Case-control Study: In order to test the validity of these results, we have taken advantage of the recently completed Yale Environment and Breast Disease Study. In this prospective case-control study, Dr. Tongzhang Zheng has been testing the hypothesis that exposure to organochloride pesticides increases the risk of BC. Close to 400 cases and 200 control women were enrolled between January 1994 and August 1997. All cases had histologically confirmed diagnoses of primary BC (TNM stages 0-III). Standardized structured questionnaires were used to ascertain demographic factors, menstrual and reproductive history, past medical history and family history of cancer, occupation, household pesticide use, use of hair dyes, alcohol and tobacco, and dietary history. In addition, blood clots were stored frozen to be used for future studies of genetic polymorphisms. The epidemiological data that have been collected and the availability of genomic DNA from all cases and controls represented an invaluable opportunity for us to rigorously test the idea that the del$(GGC)_3$ TßR-I gene variant may represent a novel and common breast cancer susceptibility gene.

Cases (n=98) were selected from among previously ascertained subjects who participated in the Yale Environment and Breast Disease Study. All cases had histologically confirmed primary BC (stages 0-III). Age-matched controls (n=92) were selected from among the women in the same study who did not have a diagnosis of BC. Eleven cases (11%, 95% CI: 6-19%) and 14 of the controls (15%, 95% CI: 9-24%) were heterozygous carriers of the del$(GGC)_3$ TßR-I gene variant. These results indicate that there was no significant association between the del$(GGC)_3$ TßR-I gene variant carrier state and breast cancer (Fisher's Exact test, p=0.52).

In summary, in this initial series of primary breast cancers, we identified one particular structural alterations of the TßR-I gene that appears to be uniquely associated with breast carcinomas, and is found more frequently in axillary lymph node metastases than in primary tumors. In order to confirm these findings, Dr. Daryl Carter provided us with an additional 24 cases of axillary lymph node metastases from breast carcinoma. Tumor tissue was microdissected, and genomic DNA extracted as described above. Exon 7 of the TßR-I gene was analyzed by PCR-SSCP. In one single case, we detected and confirmed the presence of the identical S387Y mutation found in the initial series. Thus, these results further support our hypothesis that mutations of the TßR-I gene represent relatively late events in breast cancer progression.

Detection of TßR-I and -II Gene Losses by FISH: For cells to loose all responsiveness to TGFß, both alleles of any one of the signaling intermediate genes need to be inactivated (6). In analogy with other tumor suppressor genes, this is likely to be a two-step process involving loss of one allele and inactivation of the second allele by intragenic mutation [Knudson Jr., 1985 #296]. Allelic deletions are often identified by using PCR-based assays for the detection of polymorphic DNA sequences. This approach has several drawbacks: First, it requires the availability of paired tumor- and germline DNA samples. Secondly, such assays are informative only if the individuals are heterozygous for the marker used. Finally, and most importantly, the test will only yield a positive result if the majority of tumor cells has undergone loss of heterozygosity (LOH). Thus, PCR-based approaches will fail to detect allelic losses if they are present in only a minority of the tumor cells.

Fluorescent *in situ* hybridization (FISH) is a particularly attractive alternative method for detecting LOH because it does not require access to normal tissue from the same individual and can be used to detect changes in gene copy numbers in individual cells. Moreover, FISH has been used effectively to detect allelic losses in interphase nuclei in tissue sections or touch preparations of tumor samples (7-15).

The main purpose of this study was to determine whether the genes that encode the two TGFß receptors (TßR-I and TßR-II) undergo allelic deletions during breast cancer development and progression. We approached this question by examining interphase nuclei in breast cancer specimens by FISH. A total of 18 primary cancer specimens were examined. These included 15 invasive ductal cancers, 2 invasive lobular carcinomas, and 1 intracystic papillary cancer. Interphase nuclei were hybridized with BAC clones containing the complete genomic sequences of either TßR-I or TßR-II. Specimens were co-hybridized with centromeric probes for the corresponding chromosomes (chromosome 9 for TßR-I, chromosome 3 for TßR-II).

An example of a touch preparation co-hybridized with TßR-I (green fluorescence) and a chromosome 9 probe (red fluorescence) is shown in **Figure 2A**. We determined the number of chromosomal copies and the number of TßR alleles in each individual nucleus, and at least 150 nuclei were scored on each slide. **Figure 2B** displays the results obtained for cells that contained 2 copies of chromosome 9 and 3, respectively, in the case of tumor 2T. Individual nuclei that lacked one or both alleles of TßR-I or -II could easily be identified.

The results for all 18 cases of primary breast cancer have been depicted graphically in

Figure 3. In most cases, we could identify subpopulations of nuclei in which the number of TßR-specific signals was less than 2. However, the hybridization efficiency of locus-specific DNA probes is probably lower than that obtained with the repeat-sequence probes used to identify centromeres, because the signals are smaller and less intense than centromeric signals. In order to estimate the proportion of false-negative TßR gene signals, we examined touch preparations of 4 different normal axillary lymph nodes that had been obtained at the time of breast surgery and were processed in a manner identical to the tumor samples. The average fraction of nodal lymphocytes with <2 TßR-specific signals was 19% (95% CI: 9-29) for TßR-I and 21% (95% CI: 3-38) for TßR-II. Using the upper boundaries of the 95% confidence intervals as threshold values (29% for TßR-I and 38% for TßR-II), we concluded that tumor cell subpopulations with bona fide TßR-I deletions were present in 2 of 6 (33%), and TßR-II deletions in 6 of 10 (60%) touch preparations (**Figure 3**). In all cases, approximately half of the losses involved both copies of the TßR-I or -II gene.

The frequency of presumed TßR losses seemed to be consistently greater in the cases in which we examined nuclei isolated from frozen sections than in the touch preparations (**Figure 3**). Although we did not have access to control nuclei from frozen sections of normal tissue to set a threshold, it is likely that we overestimated the frequencies of TßR allelic loss in the frozen tumor nuclei (**Figure 3**).

Our results indicate that many of the primary invasive carcinoma specimen contained subpopulations of cells that had undergone allelic losses of either the TßR-I or the TßR-II gene. These findings raised the question at which stage of tumor development these losses had occurred. To answer this question, we will have to examine a series of cases that span the spectrum of pre-invasive to metastatic breast cancer. However, our data provide some preliminary insight. For example, in the single case of non-invasive intracystic papillary carcinoma (6T), we found no evidence of significant allelic loss of either of the two receptor genes. We also examined a single metastatic lesion. This chest wall recurrence demonstrated extensive aneuploidy of both chromosomes 3 and 9 with increasing allelic loss of the TBR genes with increasing chromosome copy number. However, in spite of the high frequency of chromosomal gains and losses, losses of both TßR genes in this case were of the same order of magnitude as those seen in the primary tumors. Thus, these findings suggest that losses of genes that encode TGFß signaling intermediates may occur progressively as breast cancers evolve from pre-invasive to invasive to metastatic lesion.

Task 2. Determination of the functional consequences of TGFß-receptor mutations

In order to test whether the serine to tyrosine substitution at position 387 found in primary and metastatic breast cancer specimens disrupts receptor function, we introduced this mutation into a full-length wild type TßR-I cDNA. We studied the effects of the mutation on receptor function in transient transfection assays using the TßR-I-deficient R-1B (L17) mink lung epithelial cell line. As shown in **Figure 4A**, expression of wild type TßR-I in R-1B (L17) cells resulted in an approximately 50% reduction in cyclin A promoter activity compared to cells transfected with an inert control vector. In contrast, pCAL2 activity was repressed by less than 30% in cells transfected with the S387Y receptor mutant (**Figure 4A**). In cells transfected with wild type TßR-I, pSBE4-dependent luciferase activity was increased approximately 15-fold over controls, while the increase observed in cells transfected with the receptor mutant was only approximately 10-fold (**Figure 4B**). As shown in **Figure 4C**, the S387Y mutation appears to induce a shift in the TGFß dose-response relationship: wild type TßR-I expressing cells responded maximally to 50 pM TGFß, whereas S387Y expressing cells required at least 100 pM TGFß for maximal response. Repression of the cyclin A promoter activity (pCAL2) correlates extremely well with the ability of cells to respond to TGFß-mediated cell cycle arrest, and activation of the Smad DNA-binding element (SBE) in pSBE4 reflects TGFß-induced gene transcription (16, 17).To rule out that the observed differences in reporter gene activity were due to variations in levels of expression of wild type and mutant TßR-Is in transfected cells, cell lysates were subjected to Western immunoblotting

using anti-HA monoclonal antibody (**Figure 4D**). Discreet 55 kDa bands of equal intensity corresponding to the TßR-I receptor were detected in extracts from both wild type- and mutant TßR-I-transfected cells. Thus, the S387Y mutation did not affect receptor protein expression. In summary, cells expressing the S387Y mutant were significantly less sensitive to the effects of TGFß on cell cycle regulation as well as transcriptional responses than cells expressing the wild type receptor.

The exact mechanism whereby the S387Y mutation diminishes TGFß signaling remains to be determined. According to the canonical domain subdivisions found in all protein kinases, the serine residue at position 387 in TßR-I is located in the linker region between subdomains VIII and IX which typically form the peptide recognition domain of protein kinases (**Figure 5A, B**). The structure of subdomains VIII and IX are highly conserved among the family of type I TGFß-, activin- and bone morphogenic protein (BMP) receptor serine-threonine kinases (**Figure 5B**). The fact that these receptors share highly homologous substrates (Smads) further suggests that this region participates in substrate recognition. Alternatively, it may well affect the homodimerization of TßR-I molecules, or perhaps the interactions between TßR-I and –II molecules when they form heterotetrameric complexes during receptor activation.

The primary substrate of TßR-I, Smad2, is phosphorylated on two serine residues located within the consensus sequence RCSS(465)MS(467) at the C-terminus of the protein. Although the C-terminal tail of Smad2 is not absolutely required for its physical interaction with TßR-I, structure-function studies indicate that it clearly plays a complimentary role in enzyme-substrate recognition and partly determines specificity between TGFß and BMP signaling. Comparison between the crystal structures of activated protein kinase A in complex with an inhibitory peptide and that of the TßR-I kinase indicates that the S387Y residue does not fall precisely within the canonical substrate binding site as defined in the protein kinase A-inhibitory peptide structure. However, this does not exclude the possibility that the substitution of a tyrosine with its larger side chain for the serine at position 387 in TßR-I interferes with productive substrate recognition, particularly as the interface between a Smad and TßR-I is probably much larger than the protein kinase A-inhibitory peptide interface (18). Furthermore, based on the Chou and Fassman algorithm, one would predict that the S387Y mutation alters the secondary structure of the TßR-I kinase by introducing two ß-sheets flanking the loop that connects the E and F α-helices of the catalytic core (**Figure 5C**). It is worth noting that two other TGFß type I receptors, TSR-1 and TskL7, contain different polar residues at position 387 (threonine and glutamine, respectively) (**Figure 5B**). Interestingly, neither of these two receptors is able to elicit the same cellular responses as TßR-I, perhaps because they are unable to interact with Smad2 or –3.

Finally, the functional importance of this region is also illustrated by the fact that several syndromes have been associated with mutations within subdomains VIII or IX in other protein kinases. For example, two different arginine-to-tryptophan and methionine-to-arginine mutations in the TSR-1 gene have been described in hereditary haemorrhagic telangiectasia type 2 (**Figure 5D**). Moreover, amino acid substitutions at highly conserved glutamate and aspartate residues in the catalytic subunit of phosphorylase B kinase result in loss of enzyme activity, glycogenosis and liver cirrhosis. In addition, Wang et al. recently described a case of head-&-neck cancer with a tyrosine-to-cysteine mutation within subdomain IX of the TßR-II serine-threonine kinase. Although the effects of this mutation on receptor function were not reported in this case, it is likely that it affects enzyme activity as well.

In summary, we have identified a single missense mutation of the TßR-I gene that occurs with relatively high frequency in invasive ductal breast cancer and that has a significant negative impact on receptor signaling. This is the first reported missense mutation in this gene reported in any human malignant neoplasm and provides further support for the idea that inactivation of the TGFß signaling pathway can play an important role in human carcinogenesis. Furthermore, the high frequency of the S387Y mutation in lymph node metastases suggests that inactivation of this signaling pathway may be particularly associated with the metastatic phenotype.

Our main goal for the coming year is to further define at which stage of breast cancer progression the TGFß pathway becomes inactivated. To address this question, we have entered into a collaboration with Dr. David Rimm, Chief of the Cytology Laboratory at Yale-New Haven Hospital. Since 1995, Dr. Rimm has been banking all cytology specimens obtained from metastatic tumor lesions at Yale-New Haven Hospital. Between 1995 and 1998, 85 cases of metastatic carcinoma have been collected. Of these, 16 were obtained from metastatic breast cancer deposits mainly in lungs and liver. We are in the process of analyzing the genomic structure of the TßR-I and -II genes in the tumor cells present in these specimens.

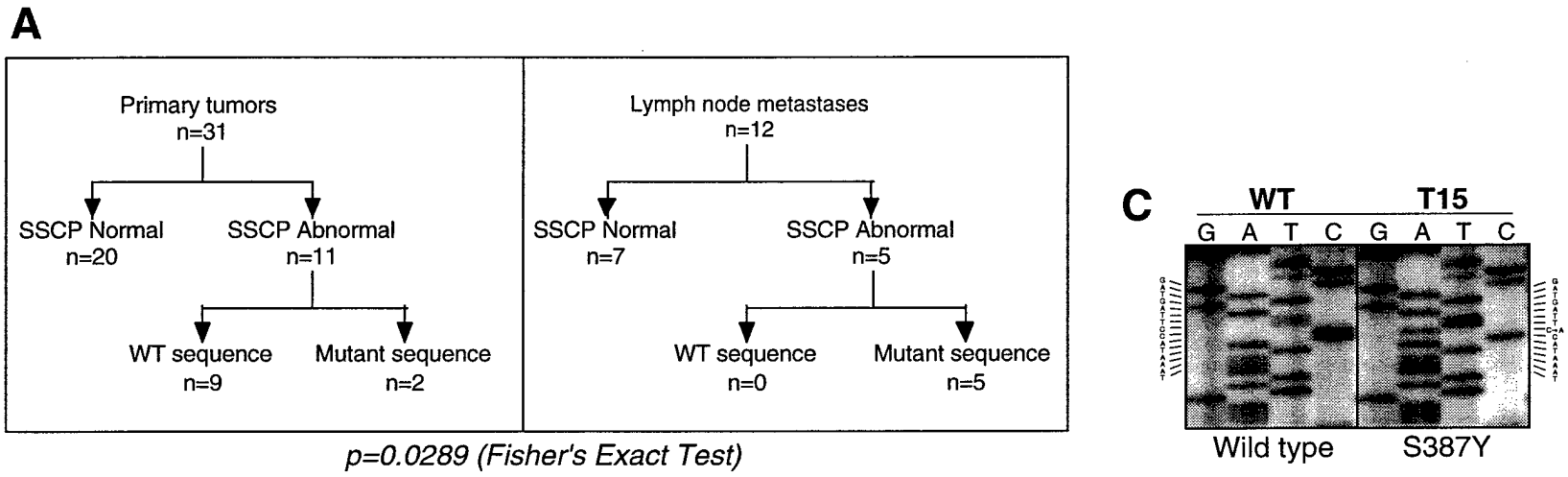
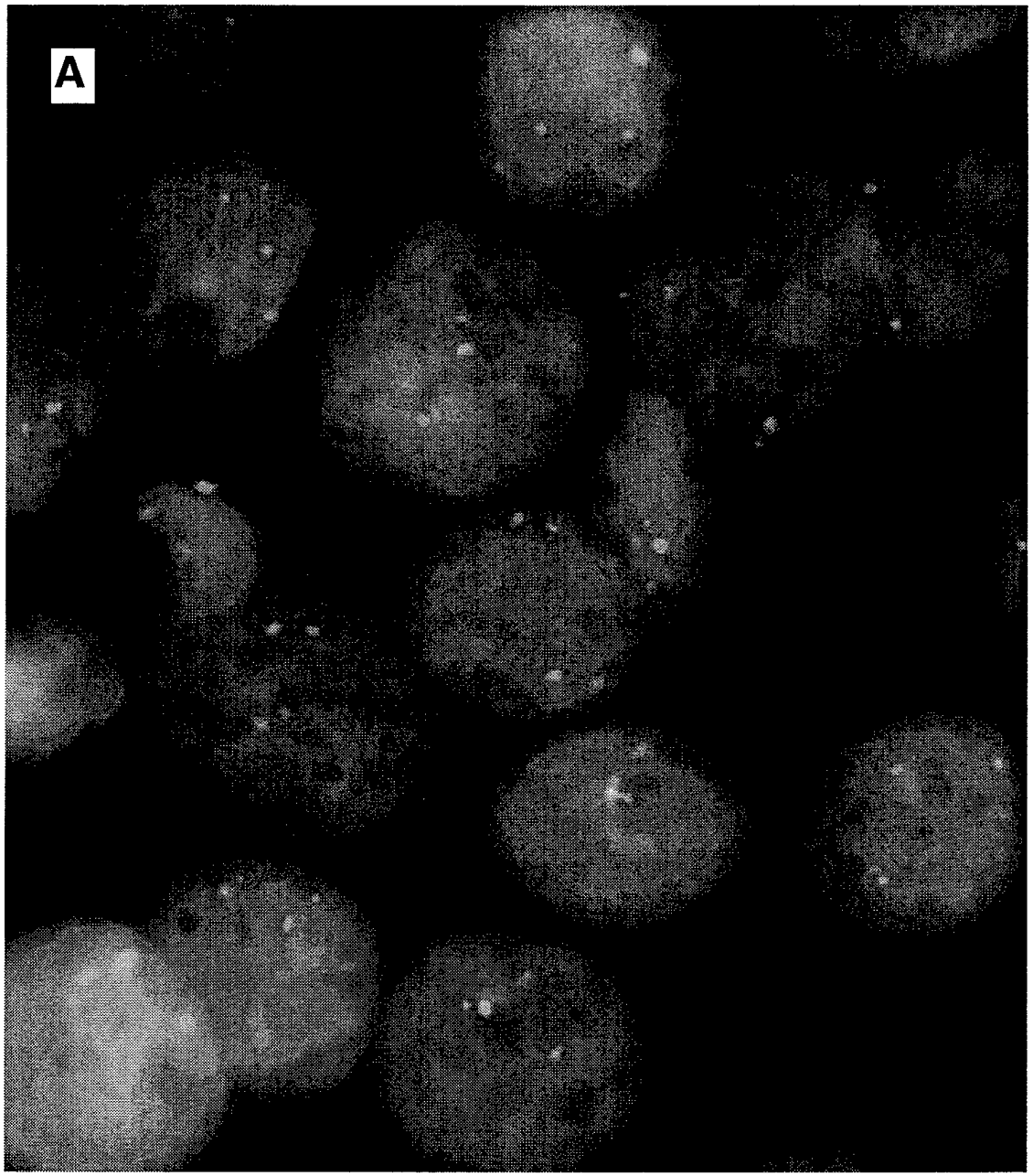

A
Primary tumors
n=31
SSCP Normal
n=20
SSCP Abnormal
n=11
WT sequence
n=9
Mutant sequence
n=2
Lymph node metastases
n=12
SSCP Normal
n=7
SSCP Abnormal
n=5
WT sequence
n=0
Mutant sequence
n=5
p=0.0289 (Fisher's Exact Test)
C
WT
T15
G A T C G A T C
Wild type
S387Y
B
CON
T15
T26
N15
M11
M18
M25
M33
M34
M36
WT
S387Y

Figure 1


A

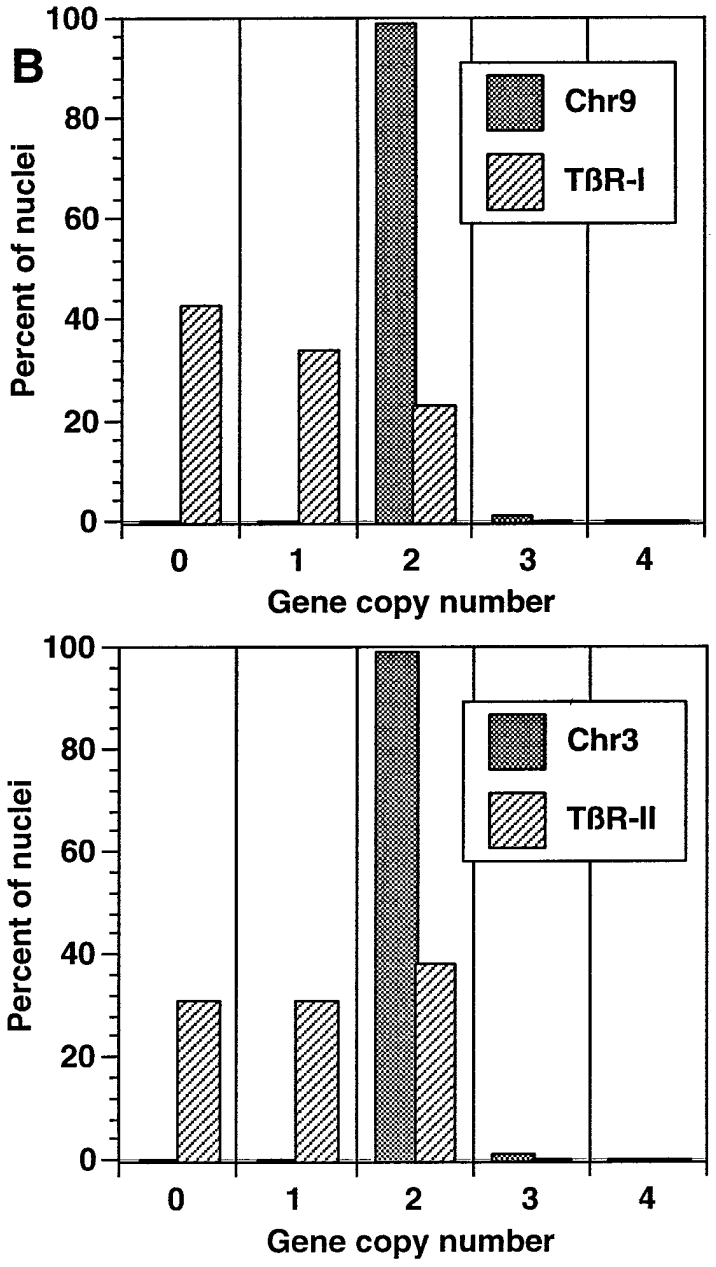

B
Percent of nuclei
100
80
60
40
20
0
0
1
2
3
4
Gene copy number
Chr9
TßR-I
Percent of nuclei
100
80
60
40
20
0
0
1
2
3
4
Gene copy number
Chr3
TßR-II

Figure 2

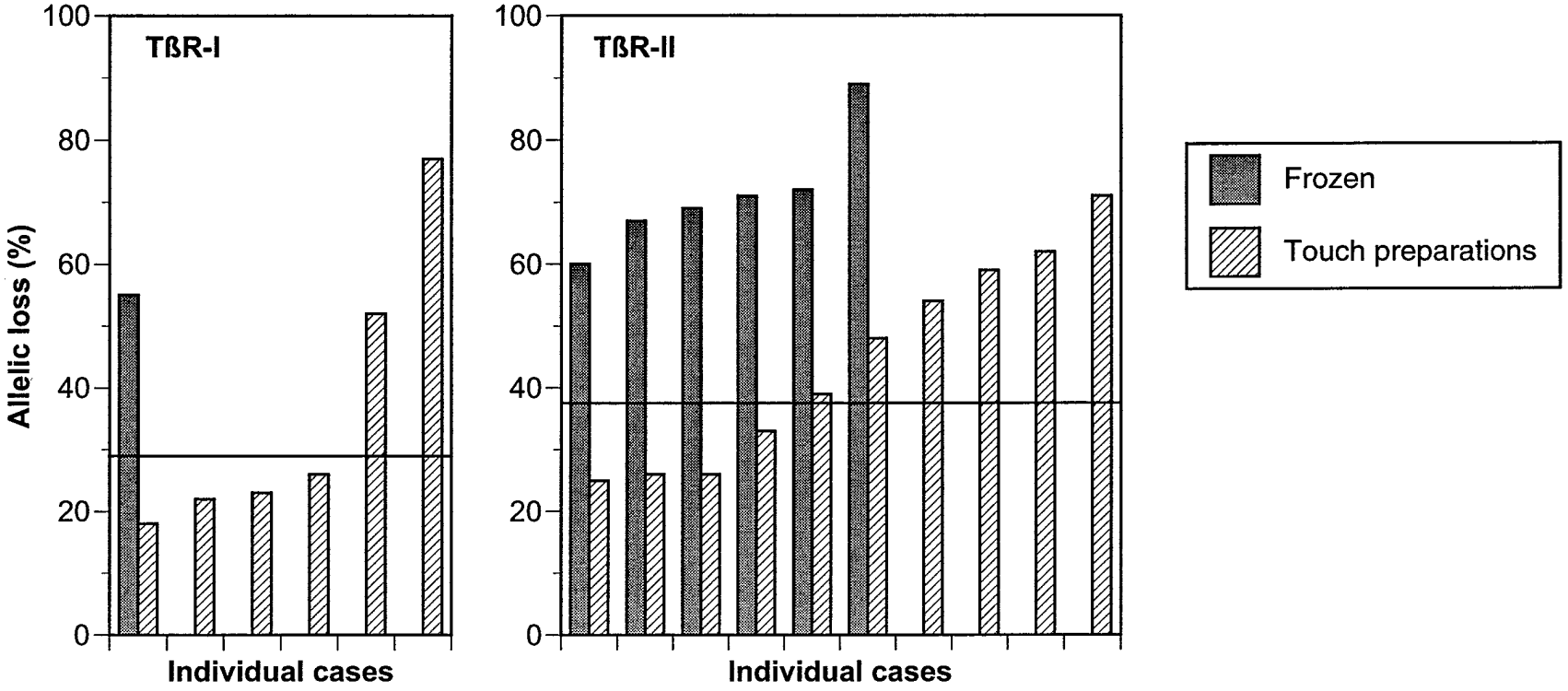

TßR-I
TßR-II
Allelic loss (%)
100
80
60
40
20
0
Individual cases
Frozen
Touch preparations

Figure 3

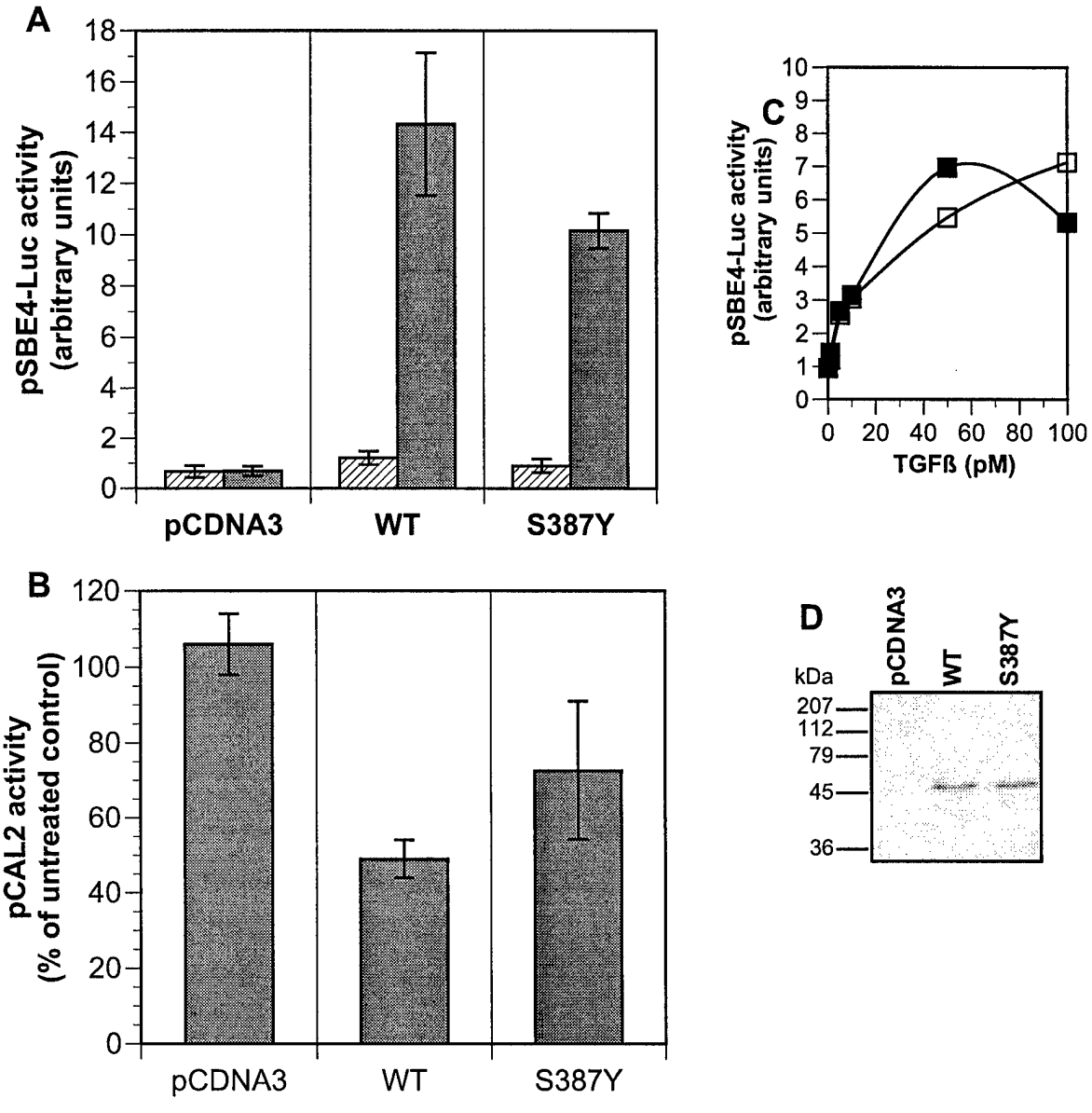
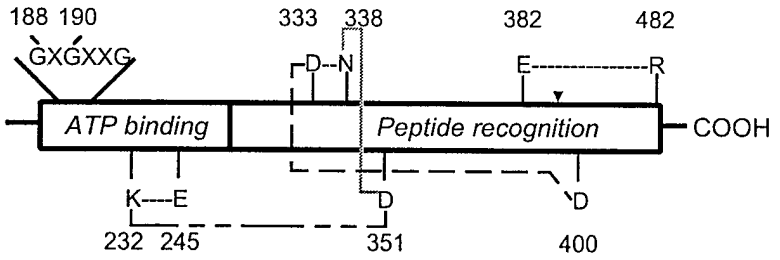

A
pSBE4-Luc activity (arbitrary units)
18
16
14
12
10
8
6
4
2
0
pCDNA3
WT
S387Y
B
pCAL2 activity (% of untreated control)
120
100
80
60
40
20
0
pCDNA3
WT
S387Y
C
pSBE4-Luc activity (arbitrary units)
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
TGFß (pM)
D
kDa
pCDNA3
WT
S387Y
207
112
79
45
36

Figure 4

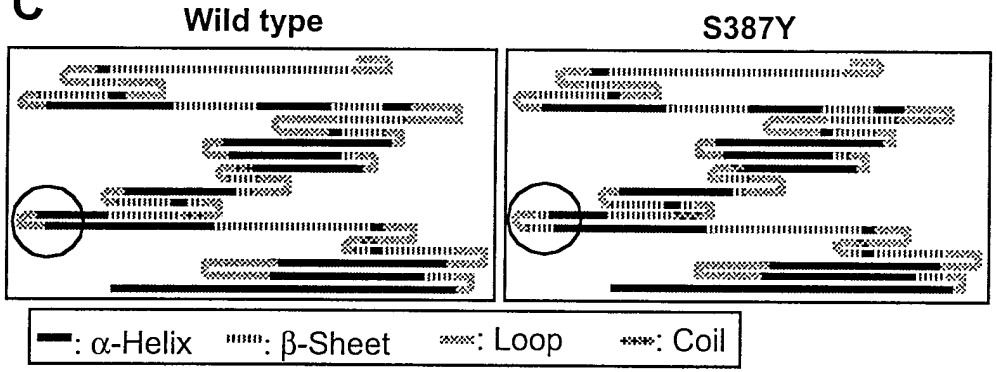
A



B

```
                    VIII                    IX
TßR-I     NHRVGTKRYMAPEVLDDSIN-MKHFESFKRADIYAMGL
ActR-IA   NPRVGTKRYMAPEVLDETIQ-VDCFDSYKRVDIWAFGL
ActR-IB   NQRVGTKRYMAPEVLDETIN-MKHFDSFKCADIYALGL
TskL7     NPRVGTKRYMAPEVLDETIQ-VDCFDSYKRVDIWAFGL
TSR-I     NPRVGTKRYMAPEVLDEQIR-TDCFESYKWTDIWAFGL
BMPR-IA   NTRVGTKRYMAPEVLDESLN-KNHFQPYIMADIYSFGL
BMPR-IB   NTRVGTKRYMPPEVLDESLN-RNHFQSYIMADMYSFGL
```

C



D

```
                    VIII                    IX
TßR-I     --NHRVGTKRYMAPEVLDDSIN-MKHFESFKRADIYAMGL
          ----------------------Y----------------------

TßR-II    --SGQVGTARYMAPEVLESRMN-LENAESFKQTDVYSMAL
          ------------------------------------C-----

TSR-1     --NPRVGTKRYMAPEVLDETIQ-VDCFDSYKRVDIWAFGL
          ---------W------------------------------
          ----------R------------------------------

PHKG2     KLRELCGTPGYLAPEILKCSMDETHPGYGK-EVDLWACGV
          ------E-----------------------------------
          ----------------------------------N------
```

Figure 5

KEY RESEARCH ACCOMPLISHMENTS

- Identification of somatic missense loss-of-function mutations of the TßR-I gene in human breast cancer
- Identification of allelic losses of TßR genes in human breast cancer

REPORTABLE OUTCOMES

Chen, T., Carter, D., Garrigue-Antar, L., and **Reiss, M.** Transforming Growth Factor-ß type I receptor kinase mutant associated with metastatic breast cancer. Cancer Res. 1998. 58:4805-4810.

Gollerkeri, A., Bray-Ward, P., Flynn, S.D., and Reiss, M. Allelic losses of Transforming Growth Factor-ß receptor genes in primary breast cancer detected by fluorescence in situ hybridization. Lab. Invest. 1999. In Press

CONCLUSIONS

1. We have identified two specific structural alterations of the TßR-I gene that are particularly common in breast cancer. One of these is a somatic loss-of-function mutation that is associated with metastatic breast cancer.

2. Allelic losses of both the TßR-I and -II genes occur with high frequency in subpopulations of cells in primary breast carcinomas.

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APPENDICES

Reprints

[CANCER RESEARCH 58, 4805–4810, November 1, 1998]

Advances in Brief

Transforming Growth Factor β Type I Receptor Kinase Mutant Associated with Metastatic Breast Cancer[1]

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Abstract

Malignant breast carcinoma cell lines are frequently refractory to transforming growth factor β (TGF-β)-mediated cell cycle arrest. To identify molecular mechanisms of TGF-β resistance, we have conducted a comprehensive structural analysis of the TGF-β receptor types I (*TβR-I*) and II (*TβR-II*) genes in primary human breast carcinomas and associated axillary lymph node metastases. No evidence for loss of expression (n = 14) or structural alterations of the *TβR-II* gene (n = 30) were identified. However, 2 of 31 primary carcinomas and 5 of 12 lymph node metastases carried a C to A transversion mutation resulting in a serine to tyrosine substitution at codon 387 (S387Y) of the *TβR-I* receptor gene. This *TβR-I* mutant has a diminished ability to mediate TGF-β-dependent effects on gene expression as compared with wild-type *TβR-I*. S387Y is the first reported mutation in the *TβR-I* gene in human cancer that was primarily associated with lymph node metastases in the present series.

Introduction

TGF-β[4] is a M_r 25,000 dimeric polypeptide that is the most potent known inhibitor of normal human mammary epithelial cell replication *in vitro* (1). *In vivo*, TGF-β seems to regulate the normal development of ductal and lobular epithelium in the mammary gland (2, 3). Moreover, in the adult mammary gland, TGF-β probably mediates the massive cell death and restructuring that takes place in the mammary gland during postlactational involution (4).

Besides these physiological functions, there is considerable evidence that TGF-β plays an important role in mammary carcinogenesis (reviewed in Ref. 5). First of all, TGF-β is able to protect against mammary tumor formation *in vivo*. For example, transgenic mice that produce a constitutively active form of TGF-β1 are relatively resistant to carcinogen-induced mammary tumor formation (6), Conversely, heterozygous TGF-β1 knockout mice that express lower than normal levels of TGF-β1 have an increased propensity for tumor development (7). The same holds true for mice that express a dominant-negative *TβR-II* mutant gene or that have a targeted deletion of the *TβR-II* gene (8, 9). Thus, either a relative lack of TGF-β or inactivation of the TGF-β signaling pathway results in loss of tumor suppression and promotes carcinogenesis. Secondly, many mammary carcinomas seem to be composed of TGF-β-insensitive cells. Thus, virally transformed tumorigenic mammary epithelial cell lines as well as most of the cell lines derived from invasive human breast carcinomas are resistant to the antiproliferative effects of TGF-β *in vitro* and do not respond to treatment with TGF-β *in vivo* (5). These observations have raised the question of what is the molecular basis for TGF-β resistance in breast cancer.

The TGF-β signal is transduced by a pair of transmembrane serine-threonine kinase receptors (10). TGF-β binds primarily to TβR-II receptor homodimers, which then form heterotetrameric complexes with two TβR-I molecules. As a consequence, the TβR-II kinase phosphorylates TβR-I thereby activating its serine-threonine kinase. In response to TGF-β binding, the two cytosolic proteins, Smad2 and Smad3, become transiently associated with and phosphorylated by the TβR-I kinase. Following their activation, Smad2 and -3 form heteromeric complexes with a third homologue, Smad4. These complexes are translocated to the nucleus, bind to DNA in a sequence-specific manner, and regulate gene transcription (10). The resulting repression of cyclins and induction of cyclin-dependent kinases and cdc25A phosphatase lead to G_1 phase cell cycle arrest.

A number of breast carcinoma cell lines have been described that fail to express either the *TβR-II* or the *Smad4* gene and are refractory to TGF-β (11–14). In two of these lines, intragenic mutations of the *Smad4* gene were noted in conjunction with loss of the second allele (13, 14). On the basis of these observations, one would predict that the TGF-β signaling pathway would be disrupted in primary breast carcinomas *in vivo* as well. However, Riggins *et al.* (15) failed to identify any structural alterations of the *Smad1*, *-3*, *-5*, or *-6* genes in over 20 breast cancer cell lines. Moreover, other investigators have found the *Smad2* and *-4* genes to be intact in substantial numbers of primary breast carcinoma specimens (16, 17). Thus, we are faced with the apparent paradox that most breast carcinoma cell lines are refractory to TGF-β *in vitro*, whereas the inactivation of the *Smad* genes in breast carcinoma specimens seems to occur quite infrequently. These findings suggest that the *TβR* genes may be the primary targets for genetic inactivation in this disease.

To address this possibility, we have investigated the *TβR-I* and *-II* genes in a panel of primary breast carcinomas and associated axillary lymph node metastases. We have identified a particular somatic missense mutation within the catalytic core of the TβR-I serine-threonine kinase that disrupts the signaling function of the receptor. This is the first inactivating mutation of the *TβR-I* gene described in human cancer. Moreover, our findings indicate that inactivation of the TGF-β signaling pathway in sporadic breast carcinoma is probably a relatively late event because the mutation was found predominantly in metastatic lesions. This may partly explain the fact that previous studies have failed to uncover molecular evidence for TGF-β pathway inactivation because they have focused exclusively on primary tumor specimens.

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[4] The abbreviations used are: TGF-β, transforming growth factor β; TβR-I, type I TGF-β receptor; TβR-II, type II TGF-β receptor; SSCP, single-strand conformation polymorphism; TBE, 89 mM Tris, 89 mM borate, 50 mM EDTA (pH 8.0); BMP, bone morphogenic protein; CMV, cytomegalovirus.

Materials and Methods

Tissue Specimens and Nucleic Acid Extraction. Breast carcinoma specimens were provided by the Program for Critical Technologies in Breast Oncology at Yale after hisopathological review by one of us (D. C.). Genomic DNA was extracted from tumor and normal tissues as described previously (18). Isolating genomic DNA from a single 5-μm microdissected paraffin-embedded tumor section using InstaGene matrix (Bio-Rad, Hercules, CA) typically yielded 200 μl of DNA template solution. Total cellular RNA was extracted from three or more 50-μm serial thick frozen sections using TriZOL reagent (Life Technologies).

Genotyping of TGF-β Signaling Intermediates. The *TβR-II* gene was analyzed by chemical mismatch cleavage as described previously (19) or by conventional PCR-SSCP. (For primers used to amplify TβR-II exons, see Ref. 20.) The *TβR-I* gene was analyzed by "cold" PCR-SSCP (21). In this case, each 20-μl PCR contained 500 nM of unlabeled primers. After an initial 3-min denaturation at 95°C, PCR was performed for 35 cycles of 95°C for 30 s, 55°C for 40 s, 72° for 30 s followed by a 5-min final extension at 72°C. For PCR amplification of the GC-rich exon 1 we used the Advantage-GC genomic polymerase mix (Clontech, Palo Alto, CA) according to the instructions supplied by the manufacturer. The 9 exons of the *TβR-I* gene were amplified using the following flanking intronic forward and reverse primers: (*a*) exon 1: 5′-gaggcgaggtttgctggggtgaggca-3′ and 5′-catgtttgagaaagagcaggagcgag-3′; (*b*) exon 2: 5′-ctacacaatctttctcttttttcc-3′ and 5′-gtttttcttgtagtatctagg-3′; (*c*) exon 3: 5′-gtttatttcactcgaggcc-3′ and 5′-ggagaaacaattatgttac-3′; (*d*) exon 4: 5′-gattgt-gttgagtactattta-3′ and 5′-ggaaaagcaaatgttacagac-3′; (*e*) exon 5: 5′-gcccaac-cgaaatgttaattc-3′ and 5′-ggtagaactgcttatagaat-3′; (*f*) exon 6: 5′-gcagtcatgtttta-atttttgattc-3′ and 5′-gaacgcgtattaaatatagttg-3′; (*g*) exon 7: 5′-tgtctgaaaggaggttcatcc-3′ and 5′-gaacaacttctgctcatgacg-3′; (*h*) exon 8: 5′-gccttgcattagctgaataaat-3′ and 5′-gcttactaagcagaagcag-3′; and (*i*) exon 9: 5′-ggaaaatggtgcatgcatta-3′ and 5′-gagttcaggcaaagctgtag-3′. For SSCP analysis, 5-μl aliquots of amplified PCR product were mixed with 15 μl of loading buffer (12.5 μl of 10× TBE buffer, 2 μl of 15% Ficoll, 0.1% bromphenol blue and xylene cyanol, and 0.5 μl methyl mercury hydroxide), denatured by heating at 80°C for 3 min, and quenched on ice. The single-stranded DNA fragments were then resolved using precast 20% TBE acrylamide gels on a Novex Xcell II Thermoflow apparatus (Novex, San Diego, CA) with the gel temperature maintained precisely at 10°C throughout the run. Bands were visualized by staining the gel in a 1:10,000 dilution of SYBR Green II (Molecular Probes, Inc., Eugene, OR) for 20–30 min and using an Eagle Eye charged coupled device camera equipped with a SYBR Green band pass filter (Stratagene) for photographic documentation.

Suspect bands were excised from the gels with a razor blade and reamplified. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Chatsworth, CA), and subjected to DNA sequencing using a thermocycling sequencing kit (Epicentre Technologies, Madison, WI) with either a forward or reverse primer end-labeled with [γ-^{32}P]-ATP. Reaction products were denatured at 70°C for 3 min, resolved on 7% (w/v) denaturing polyacrylamide gels at 50°C, and visualized by exposing dried gels to X-ray film overnight at 20°C. The presence of any sequence alteration was always confirmed by repeated PCR-SSCP and DNA sequencing using an independent aliquot of tumor-derived genomic DNA as a template. Whether any mutations were somatic in nature or present in the germline was determined by analyzing genomic DNA isolated from noncancer tissue of the same patient.

Vectors Used for Transfection. The pHA-1 mammalian expression vector was constructed by subcloning the full-length human *TβR-I* (ALK-5; Ref. 22) into the expression vector, pCDNA3 (Stratagene), thereby placing it under the transcriptional control of a CMV promoter. To facilitate the detection and quantitation of transfected receptor, the influenza virus HA epitope tag YPY-DVPDYA was introduced at the COOH-terminus of the protein (23). The C to A transversion in codon 387 was introduced into the wild-type *TβR-I* sequence by site-directed mutagenesis as described previously (24).

Reporter Gene Assays. The signaling function of the mutant *TβR-I* receptor was assessed in transient transfection assays into R-1B (L17) cells, a subclone of Mv1Lu mink lung epithelial cells (a generous gift of Dr. J. Massagué, Memorial-Sloan Kettering Cancer Center, New York, NY). This cell line is convenient because it is refractory to TGF-β, fails to express detectable levels of TβR-I, and all of the responses to TGF-β can be restored by reexpressing wild-type TβR-I. Two different firefly luciferase reporter gene constructs were used to assess the different types of responses to TGF-β: (*a*) pCAL2 (a generous gift of Dr. R. Derynck, University of California, San Franscisco, CA), which contains cyclin A gene promoter (25); and (*b*) pSBE4 (a generous gift from Dr. B. Vogelstein, Johns Hopkins University, Baltimore, MD), in which four tandem repeats of a Smad4-specific DNA binding element drive the luciferase cDNA (26).

For transfections, R-1B (L17) cells were plated at 1.4×10^5 cells/well in 6-well cluster dishes in RPMI 1640 (Life Technologies) supplemented with 10% (v/v) fetal bovine serum and allowed to adhere overnight at 37°C. Transfections using up to 1 μg of TβR-I and 2 μg of reporter plasmid DNA were carried out using Lipofectin (Life Technologies) as described previously (24). To control for variations in transfection efficiency, we cotransfected a small amount (0.01 μg) of pRL-CMV, a plasmid expressing a *Renilla* luciferase reporter gene (Promega). Firefly and *Renilla* luciferase activities can be detected separately in the same cell lysates because of their different substrate specificities using the protocol provided by the manufacturer (Promega). Cell lysate was mixed with the appropriate luciferase assay reagent and photon emission was measured using a Series 20 Barthold Luminometer (Turner Designs, Sunnyvale, CA).

Receptor Expression. Cell lysates from transfected cells were prepared using buffer containing 1% [v/v] Triton X-100, 0.1% [w/v] SDS, 150 mM NaCl, 50 mM Tris (pH 7.5), 3 mM sodium azide, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin. After boiling for 10 min. in the presence of sample buffer, aliquots containing equal amounts of total protein were resolved by electrophoresis on a 10% (w/v) SDS-polyacrylamide gel and subjected to Western immunoblotting using rabbit polyclonal antiserum directed against the HA peptide (HA.11, BAbCO, Richmond, CA). Blots were developed using horseradish peroxidase-tagged goat antimouse IgG, and the bands were visualized using DuPont NEN Chemiluminescence Reagent as recommended by the manufacturer.

Results and Discussion

To test the hypothesis that breast carcinomas *in vivo* are refractory to TGF-β, we analyzed the molecular characteristics of the two cell surface receptor genes, *TβR-I* and *TβR-II*. TβR expression was determined using a reverse transcription-PCR assay in 14 frozen surgical breast cancer specimens from which we were able to extract good quality RNA. Each of these samples expressed both *TβR-I* and *TβR-II* mRNA transcripts (data not shown). This is in contrast to our previous studies in esophageal cancers and small cell lung cancers, in which loss of *TβR-II* mRNA was found in 25 and 100% of cases, respectively (27, 28).

The entire open reading frame of *TβR-II* was screened for the presence of mutations by chemical mismatch cleavage or by PCR-SSCP followed by DNA sequencing (19). No DNA sequence alterations were encountered in a total of 30 cases examined. Thus, the *TβR-II* gene is normally expressed in primary human breast cancer, and mutations of this gene are probably rare. This result is perhaps not surprising in light of previous studies of other cancer types: missense and/or nonsense mutations in the *TβR-II* gene have only been found sporadically in colorectal cancers, head-and-neck cancers, and cutaneous T-cell lymphomas (19, 20, 29, 30). The only exceptions are tumors that are associated with DNA mismatch repair deficiencies, which frequently display *TβR-II* nonsense mutations (31).

To determine whether mutations in the *TβR-I* gene might be found in human breast cancer, we screened each of the 9 exons of the *TβR-I* gene by PCR-SSCP in 31 primary breast carcinoma specimens and in 12 associated lymph node metastases. Areas of tumor tissue were isolated from paraffin sections by microdissection, and the remaining surrounding breast tissue was used to extract germline genomic DNA. Individual exons were amplified by PCR and the products screened for the presence of novel SSCPs. Suspect bands were reamplified and subjected to direct DNA sequencing. Individual sequence abnormalities were confirmed by repeating the entire procedure using a second aliquot of genomic DNA.

Our most important finding was a C to A transversion at nucleotide 1160 in exon 7 of *TβR-I*, which predicts for a serine to tyrosine substitution at codon 387 (Fig. 1). This was the only mutation encountered in the entire series and was present in 7 (16%, 95% CI, 7–31) of the 43 specimens (Fig. 1*A*). This is the first report of a mutation in the *TβR-I* gene in any type of human malignancy. Moreover, this mutation may be specifically associated with breast cancer inasmuch as we have not found it in any cervical carcinomas nor in head-and-neck cancer cell lines (32).[5] In addition, Pasche *et al.* (33) recently reported the absence of *TβR-I* mutations in acute myeloid leukemias.

Our second major finding is the highly significant association between the S387Y mutant and axillary lymph node metastases (Fig. 1*A*). Although we encountered this mutation in only 2 (6%, 95% CI, 1–21) of 31 primary breast cancer specimens, it was present in 5 (41%, 95% CI, 15–72) of the 12 lymph node metastases (Fisher's exact test, $P = 0.012$). The dramatically increased frequency of this mutation in lymph node metastases indicates that inactivation of the TGF-β pathway may represent a late event in breast cancer progression. The fact that most breast carcinoma cell lines are refractory to TGF-β is also consistent with this idea, inasmuch as most of these cell lines were initially derived from metastatic cells isolated from malignant pleural effusions or ascites (34). Moreover, in animal models of skin carcinogenesis, TGF-β-resistant tumor cell clones also do not emerge until the tumors have become highly aggressive and metastatic (35). In contrast, in colorectal cancers associated with DNA mismatch repair deficiencies, the acquisition of *TβR-II* gene mutations seems to coincide with the transition from preinvasive adenoma to invasive carcinomas (36, 37). Thus, the stage of tumor development at which the TGF-β signaling pathway becomes inactivated may vary depending on the tumor type and on the underlying molecular genetic events that drive the carcinogenetic process.

As the S387Y mutation was not detected in germline DNA of the same individuals (Fig. 1*B*), we can practically exclude the possibility that this sequence alteration represents a normal polymorphism. On the other hand, besides the mutant band, a wild-type band could be detected in each of the tumor specimens (Fig. 1*B*). Although these findings suggest that the tumors may have retained a wild-type allele, it is impossible to exclude the possibility that this wild-type band was the result of the almost inevitable contamination of the specimens with at least some normal cells. However, even the loss of function of one of the two *TβR-I* alleles may be sufficient to confer a significantly selective advantage. Such a dosage effect occurs, for example, in transgenic animals that express a dominant-negative *TβR-II* gene in conjunction with two endogenous wild-type alleles and in knockout mice that carry only a single *TGF-β1* gene allele (7, 9). In both of these situations, the animals are significantly more susceptible to tumor formation.

To test whether the serine to tyrosine substitution at position 387 disrupts receptor function, we introduced this mutation into a full-length wild-type *TβR-I* cDNA. We studied the effects of the mutation on receptor function in transient transfection assays using the TβR-I-deficient R-1B (L17) mink lung epithelial cell line. As shown in Fig. 2*A*, expression of wild-type *TβR-I* in R-1B (L17) cells resulted in an approximately 50% reduction in cyclin A promoter activity compared with cells transfected with an inert control vector. In contrast, pCAL2 activity was repressed by less than 30% in cells transfected with the S387Y receptor mutant (Fig. 2*A*). In cells transfected with wild-type *TβR-I*, pSBE4-dependent luciferase activity was increased approximately 15-fold over controls, whereas the increase observed in cells

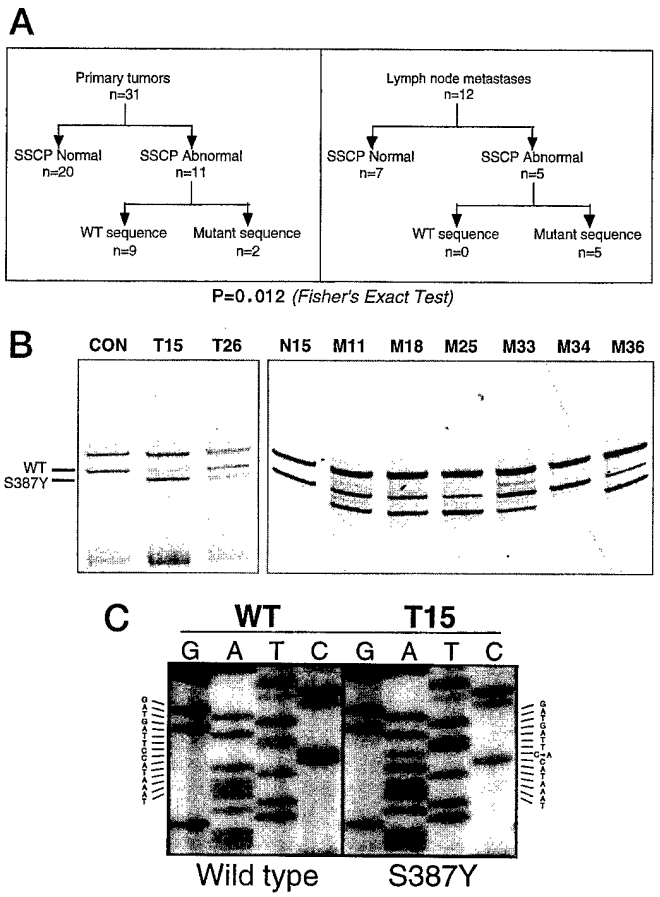

Fig. 1. Analysis of *TβR-I* gene exon 7 in human breast cancer specimens. In *A*, 19 stage I (axillary lymph node metastasis negative) and 12 stage II (axillary lymph node metastasis positive) breast carcinomas were analyzed for the presence of mutations within the *TβR-I* gene by PCR-SSCP and DNA sequencing. In *B*, SSCP analysis suggested the presence of a mutation in exon 7 in approximately one-half of the specimens. In *C*, the presence of a C to A transversion (nucleotide 1160), which predicts for a serine to tyrosine substitution at position 387 was confirmed by DNA sequencing in 7 (16%; 95% CI, 7–31%) of 43 specimens. The mutation was present in 2 (6%; 95% CI, 1–21) of 31 primary breast cancers as compared with 5 (41%; 95% CI, 15–72) of 12 lymph node metastases (Fisher's exact test, $P = 0.012$).

transfected with the receptor mutant was only approximately 10-fold (Fig. 2*B*). As shown in Fig. 2*C*, the S387Y mutation seems to induce a shift in the TGF-β dose-response relationship: wild-type *TβR-I*-expressing cells responded maximally to 50 pM TGF-β, whereas S387Y-expressing cells required at least 100 pM TGF-β for maximal response. Repression of the cyclin A promoter activity (pCAL2) correlates extremely well with the ability of cells to respond to TGF-β-mediated cell cycle arrest, and activation of the Smad DNA-binding element in pSBE4 reflects TGF-β-induced gene transcription (25, 26). To rule out that the observed differences in reporter gene activity were due to variations in levels of expression of wild-type and mutant *TβR-I* receptors in transfected cells, cell lysates were subjected to Western immunoblotting using anti-HA monoclonal antibody (Fig. 2*D*). Discrete M_r55,000 bands of equal intensity corresponding to the *TβR-I* receptor were detected in extracts from both wild-type and mutant *TβR-I*-transfected cells. Thus, the S387Y mutation did not affect receptor protein expression. In summary, cells expressing the S387Y mutant were significantly less sensitive to the effects of TGF-β on cell cycle regulation as well as transcriptional responses than cells expressing the wild-type receptor.

The exact mechanism whereby the S387Y mutation diminishes TGF-β signaling remains to be determined. According to the canonical domain subdivisions found in all protein kinases (38, 39), the

[5] V. F. Vellucci and M. Reiss, unpublished data.

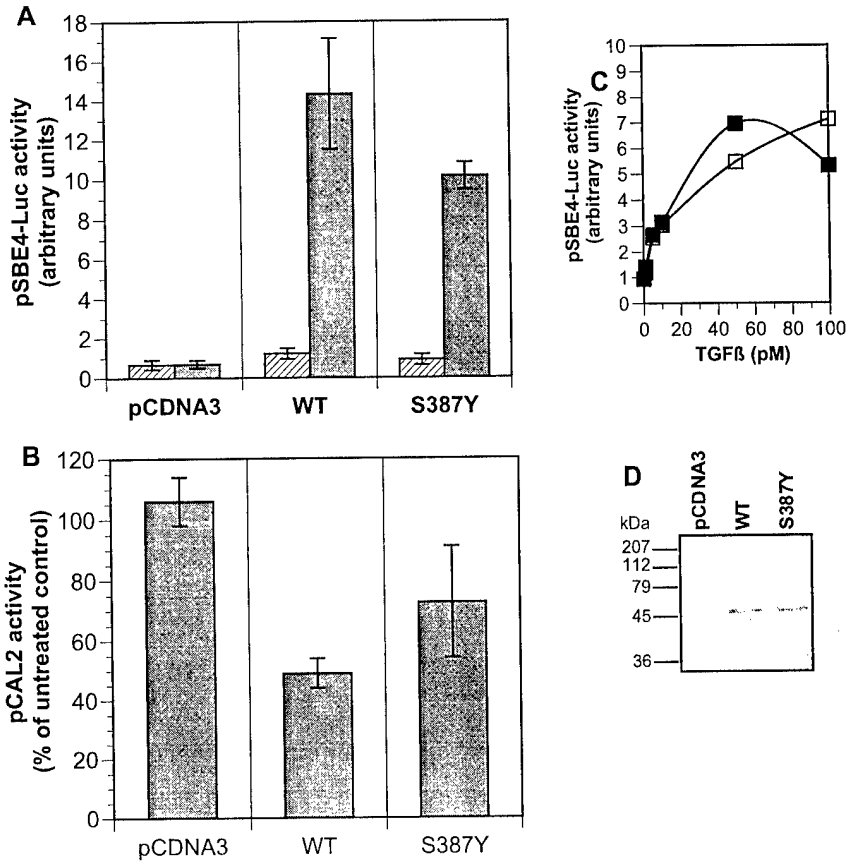

Fig. 2. The effects of transfected wild-type and mutant *TβR-I* receptors on TGF-β-regulated gene expression. R-1B (L17) cells were cotransfected with plasmids expressing either wild-type (*WT*) or mutant (*S387Y*) TβR-I receptor and pCAL2 (*A*) or pSBE4 (*B*) in conjunction with pRL-CMV, and luciferase activities in cell extracts were measured 48 h later as described in "Materials and Methods." Results were normalized for *Renilla* luciferase activity to correct for differences in transfection efficiency between experiments. In *A*, cyclin A promoter activity (*pCAL2*) was inhibited by 50% in cells transfected with the wild-type TβR-I, whereas cells transfected with the S387Y mutant expressed approximately 75% the amount of luciferase activity detected in control vector-transfected control cells. Means ± SE from 4 independent experiments. In *C*, in cells transfected with the TβR-I mutant (*S387Y*), TGF-β-induced SBE4-dependent luciferase activity (*pSBE4*) was increased to a significantly lesser extent than in wild-type TβR-I (*WT*) transfected cells. Means ± SE from four independent experiments. *D*, detection of wild-type (*WT*) and mutant (*S387Y*) TβR-I receptors in transfected R1-B (L17) cells by Western immunoblotting using HA.11 anti-HA antibody. Single discrete M_r 55,000 bands of equal intensity corresponding to the HA-tagged TβR-I receptor were detected in extracts from both wild-type and mutant TβR-I-transfected cells but not in control vector-transfected cells (*pCDNA3*).

serine residue at position 387 in TβR-I is located in the linker region between subdomains VIII and IX, which typically form the peptide recognition domain of protein kinases (38, 39; Fig. 3, *A* and *B*). The structures of subdomains VIII and IX are highly conserved among the family of type I TGF-β, activin, and BMP receptor serine-threonine kinases (Fig. 3*B*). The fact that these receptors share highly homologous substrates (Smads) further suggests that this region participates in substrate recognition. Alternatively, it may well affect the ho-

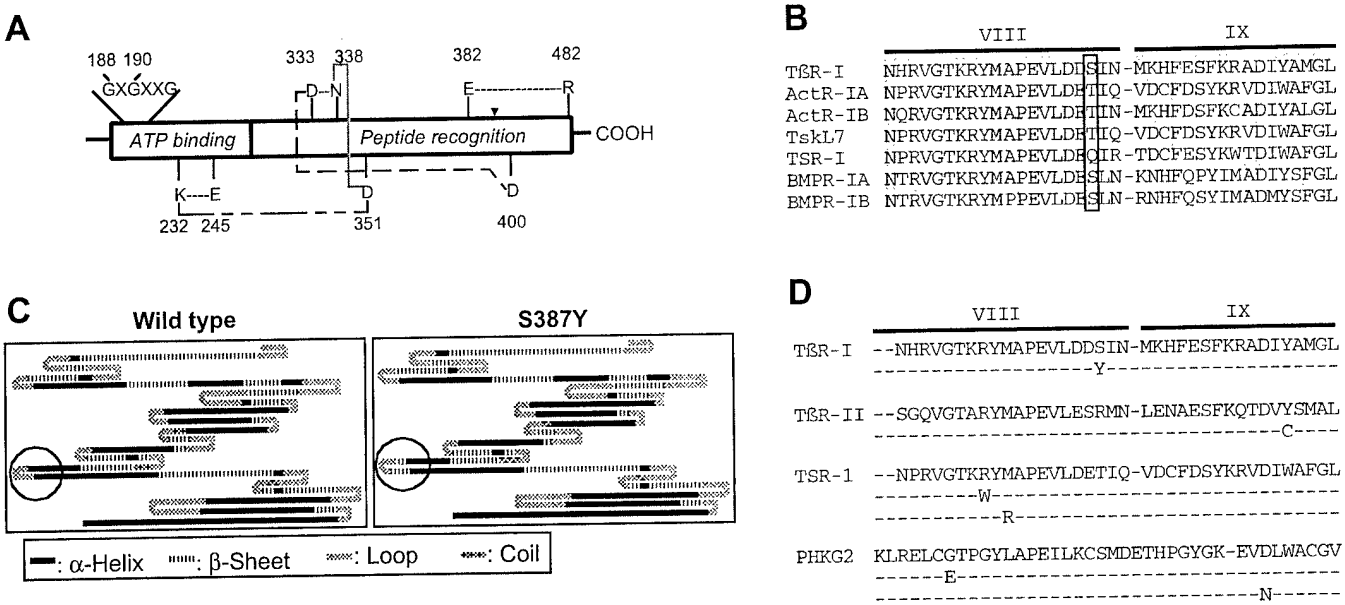

Fig. 3. *A*, structural features of TβR-I serine-threonine protein kinase catalytic domain, including the positions of amino acid residues that are highly conserved throughout the protein kinase superfamily (adapted from Taylor *et al.*; Ref. 38). ▼, position of S387Y mutation. *B*, comparison of amino acid sequence of peptide recognition domains (subdomains VIII and IX) of TGF-β, activin, and BMP type I receptor kinases *C*, predicted secondary structure of the wild-type and S387Y mutant TβR-I proteins based on their amino acid sequence using the algorithms described by Chou and Fasman (43). *D*, comparison of the location of S387Y mutant of TβR-I in breast cancer with mutations in *TSR-1* and *PHKG2* genes associated with hereditary hemorrhagic teleangiectasia type 2 and liver phosphorylase kinase deficiency syndromes, respectively (45–47), as well as in the *TβR-II* gene in head-and-neck cancer (30).

modimerization of TβR-I molecules or perhaps the interactions between TβR-I and -II molecules when they form heterotetrameric complexes during receptor activation.

The primary substrate of TβR-I, Smad2, is phosphorylated on two serine residues located within the consensus sequence $RCSS^{465}$-MS^{467} at the COOH terminus of the protein (40). Although the COOH-terminal tail of Smad2 is not absolutely required for its physical interaction with TβR-I, structure-function studies indicate that it clearly plays a complimentary role in enzyme-substrate recognition and partly determines specificity between TGF-β and BMP signaling (41). Comparison between the crystal structures of activated protein kinase A in complex with an inhibitory peptide and that of the TβR-I kinase indicates that the S387Y residue does not fall precisely within the canonical substrate binding site as defined in the protein kinase A-inhibitory peptide structure (42). However, this does not exclude the possibility that the substitution of a tyrosine with its larger side chain for the serine at position 387 in TβR-I interferes with productive substrate recognition, particularly as the interface between a Smad and TβR-I is probably much larger than the protein kinase A-inhibitory peptide interface.[6] Furthermore, on the basis of the Chou and Fassman algorithm (43), one would predict that the S387Y mutation alters the secondary structure of the TβR-I kinase by introducing two β-sheets flanking the loop that connects the E and F α-helices of the catalytic core (Fig. 3*C*). It is worth noting that two other TGF-β type I receptors, TSR-1 and TskL7, contain different polar residues at position 387 (threonine and glutamine, respectively; Fig. 3*B*). Interestingly, neither of these two receptors is able to elicit the same cellular responses as TβR-I, perhaps because they are unable to interact with Smad2 or -3 (44).

Finally, the functional importance of this region is also illustrated by the fact that several syndromes have been associated with mutations within subdomains VIII or IX in other protein kinases. For example, two different arginine to tryptophan and methionine to arginine mutations in the *TSR-1* gene have been described in hereditary hemorrhagic telangiectasia type 2 (Fig. 3*D*; Refs. 45, 46). Moreover, amino acid substitutions at highly conserved glutamate and aspartate residues in the catalytic subunit of phosphorylase B kinase result in the loss of enzyme activity, glycogenosis, and liver cirrhosis (47). In addition, Wang *et al.* (30) recently described a case of head-and-neck cancer with a tyrosine to cysteine mutation within subdomain IX of the TβR-II serine-threonine kinase. Although the effects of this mutation on receptor function were not reported in this case, it is likely that it affects enzyme activity as well.

In summary, we have identified a single missense mutation of the *TβR-I* gene that occurs with relatively high frequency in invasive ductal breast cancer and that has a significant negative impact on receptor signaling. This is the first reported missense mutation in this gene reported in any human malignant neoplasm and provides further support for the idea that inactivation of the TGF-β signaling pathway can play an important role in human carcinogenesis. Furthermore, the high frequency of the S387Y mutation in lymph node metastases suggests that inactivation of this signaling pathway may be particularly associated with the metastatic phenotype.

Acknowledgments

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[6] M. Huse and J. Kuriyan, personal communication.

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ALLELIC LOSS OF TRANSFORMING GROWTH FACTOR-ß RECEPTOR GENES IN PRIMARY BREAST CANCER DETECTED BY FLUORESCENCE *IN-SITU* HYBRIDIZATION

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Running title: TGFß receptors in breast carcinoma

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ABSTRACT

Transforming Growth Factor-ß (TGFß) type I (TßR-I) and type II (TßR-II) receptors are the primary transducers of TGFß-mediated cell cycle arrest in epithelial cells. Resistance of human tumor cell lines to TGFß is thought to result from inactivation of the TGFß signaling pathway. To determine whether the disruption of TGFß signaling in breast cancer is due to the loss of one or both of the TßR genes, we determined the frequency of loss of heterozygosity (LOH) of these two genes in interphase nuclei from primary breast carcinoma specimens using fluorescence *in situ* hybridization. Approximately one third of the cases exhibited allelic losses of the TßR-I gene and 60% of the cases revealed losses of one or both TßR-II alleles. Thus, subpopulations of cells had undergone allelic deletions of the TßR-I or -II genes in the majority of primary invasive breast carcinomas. These findings support the notion that emergence of TGFß-resistant tumor cell clones occurs relatively late during breast cancer progression.

Abbreviations: TGFß: Transforming Growth Factor-ß; HMEC: Human mammary epithelial cells; TßR-I: Type I TGFß receptor; TßR-II: Type II TGFß receptor; FISH: Fluorescence *in situ* hybridization; LOH: Loss of heterozygosity; BAC: Bacterial artificial chromosome; SSC: 150 mM sodium chloride, 15 mM sodium citrate;FITC: fluorescein isothiocyanate; BSA: bovine serum albumen; CI: Confidence interval.

Key words: Transforming growth factor-ß; receptor; breast; carcinoma; allele; fluorescence *in situ* hybridization; interphase

INTRODUCTION

Transforming Growth Factor-ß (TGFß) is a 25 kDa dimeric polypeptide that is the most potent known inhibitor of human mammary epithelial cell replication *in vitro* (Hosobuchi, et al., 1989). In contrast to normal cells, most cell lines derived from invasive human breast carcinomas are resistant to the anti-proliferative effects of TGFß *in vitro* and do not respond to treatment with TGFß *in vivo* (Fynan, et al., 1993). These observations have led to the hypothesis that, at some point during mammary carcinogenesis, TGFß-refractory tumor cell clones emerge and come to dominate the tumor cell population (Reiss, et al., 1997). In order to test this hypothesis, we have examined human carcinomas for direct molecular evidence for inactivation of genes involved in TGFß signaling (Garrigue-Antar, et al., 1995, Garrigue-Antar, et al., 1996, de Jonge, et al., 1997, Chen, et al., 1998, Chen, et al., 1999).

The TGFß signal is transduced by a pair of transmembrane serine-threonine kinase receptors (see (Derynck, et al., 1997, Heldin, et al., 1997) for recent reviews). TGFß binds primarily to TßR-II receptor homodimers, which then form heterotetrameric complexes with two TßR-I molecules. As a consequence, the TßR-II kinase phosphorylates and activates the TßR-I serine-threonine kinase. In turn, two cytosolic proteins, Smad2 and Smad3, become transiently associated with and phosphorylated by the TßR-I kinase. Following their activation, Smad 2 and -3 form heteromeric complexes with a so-called common mediator Smad, Smad4. These complexes are translocated to the nucleus, bind to DNA in a sequence-specific manner and regulate gene transcription (Derynck, et al., 1998). The resulting repression of cyclins and induction of cyclin-dependent kinases and cdc25A phosphatase lead to G_1 phase cell cycle arrest (Heldin, et al., 1997).

A number of TGFß-resistant breast carcinoma cell lines fail to express either TßR-II or Smad4 (Lin, et al., 1992, Kalkhoven, et al., 1995, Schutte, et al., 1996, de Winter, et al., 1997). In two of these lines, one allele of the Smad4 gene was deleted while the second allele had undergone and intragenic mutation (Schutte, et al., 1996, de Winter, et al., 1997). In contrast, Riggins et al. failed to identify any structural alterations of the Smad3 gene in over 20 breast cancer

cell lines [Riggins, 1997 #2181]. Moreover, other investigators have found the Smad2 and –4 genes to be intact in a substantial number of primary breast carcinoma specimens (Eppert, et al., 1996, Yokota, et al., 1997). Our own structural analysis of the TßR-I and -II genes recently revealed a specific TßR-I kinase mutation in 6% of primary breast carcinomas, and in 40% of associated lymph node metastases (Chen, et al., 1998). No sequence alterations of the TßR-II gene were noted (Chen, et al., 1998). Thus, we are faced with the apparent paradox that most breast carcinoma cell lines are refractory to TGFß *in vitro*, while deletion or mutational inactivation of the TßR- and Smad genes in primary breast cancer specimens appears to be an infrequent occurrence. Because most breast carcinoma cell lines were originally derived from metastatic lesions, these findings may indicate that inactivation of the TGFß signaling pathway is an event that occurs late during breast cancer progression.

For cells to loose all responsiveness to TGFß, both alleles of any one of the signaling intermediate genes need to be inactivated (Markowitz, et al., 1996). In analogy with other tumor suppressor genes, this is likely to be a two-step process involving loss of one allele and inactivation of the second allele by intragenic mutation [Knudson Jr., 1985 #296]. Allelic deletions are often identified by using PCR-based assays for the detection of polymorphic DNA sequences. This approach has several drawbacks: First, it requires the availability of paired tumor- and germline DNA samples. Secondly, such assays are informative only if the individuals are heterozygous for the marker used. Finally, and most importantly, the test will only yield a positive result if the majority of tumor cells has undergone loss of heterozygosity (LOH). Thus, PCR-based approaches will fail to detect allelic losses if they are present in only a minority of the tumor cells.

Fluorescent *in situ* hybridization (FISH) is a particularly attractive alternative method for detecting LOH because it does not require access to normal tissue from the same individual and can be used to detect changes in gene copy numbers in individual cells. Moreover, FISH has been used effectively to detect allelic losses in interphase nuclei in tissue sections or touch preparations of tumor samples (Kallioniemi, et al., 1992, Matsumara, et al., 1992, Lee, et al., 1993a, Lee, et al.,

1993b, Persons, et al., 1994, Cher, et al., 1995, Kuchinka, et al., 1995, Kobayashi, et al., 1996, Simpson, et al., 1996).

In this study, we have utilized FISH to determine whether or not allelic losses of the TßR-I and –II genes occur in primary human breast carcinomas. Our results indicate that both genes undergo losses in subpopulations of cells in a large subset of primary invasive carcinomas. These findings support the notion that the emergence of TGFß resistant tumor cell clones occurs relatively late during breast cancer progression.

RESULTS

The main purpose of this study was to determine whether the genes that encode the two TGFß receptors (TßR-I and TßR-II) undergo allelic deletions during breast cancer development and progression. We approached this question by examining interphase nuclei in breast cancer specimens by FISH. A total of 18 primary cancer specimens were examined (**Table 1**). These included 15 invasive ductal cancers, 2 invasive lobular carcinomas, and 1 intracystic papillary cancer. Interphase nuclei were hybridized with BAC clones containing the complete genomic sequences of either TßR-I or TßR-II. Specimens were co-hybridized with centromeric probes for the corresponding chromosomes (chromosome 9 for TßR-I, chromosome 3 for TßR-II).

An example of a touch preparation co-hybridized with TßR-I (green fluorescence) and a chromosome 9 probe (red fluorescence) is shown in **Figure 1A**. We determined the number of chromosomal copies and the number of TßR alleles in each individual nucleus, and at least 150 nuclei were scored on each slide. **Figure 1B** displays the results obtained for cells that contained 2 copies of chromosome 9 and 3, respectively, in the case of tumor 2T. Individual nuclei that lacked one or both alleles of TßR-I or -II could easily be identified.

The results for all 18 cases of primary breast cancer have been summarized in **Table 2** and depicted graphically in **Figure 2**. In most cases, we could identify subpopulations of nuclei in which the number of TßR-specific signals was less than 2. However, the hybridization efficiency of locus-specific DNA probes is probably lower than that obtained with the repeat-sequence probes

used to identify centromeres, because the signals are smaller and less intense than centromeric signals. In order to estimate the proportion of false-negative TßR gene signals, we examined touch preparations of 4 different normal axillary lymph nodes that had been obtained at the time of breast surgery and were processed in a manner identical to the tumor samples. The average fraction of nodal lymphocytes with <2 TßR-specific signals was 19% (95% CI: 9-29) for TßR-I and 21% (95% CI: 3-38) for TßR-II. Using the upper boundaries of the 95% confidence intervals as threshold values (29% for TßR-I and 38% for TßR-II), we concluded that tumor cell subpopulations with bona fide TßR-I deletions were present in 2 of 6 (33%), and TßR-II deletions in 6 of 10 (60%) touch preparations (**Figure 2**). In all cases, approximately half of the losses involved both copies of the TßR-I or -II gene.

The frequency of presumed TßR losses seemed to be consistently greater in the cases in which we examined nuclei isolated from frozen sections than in the touch preparations (**Figure 2**). Although we did not have access to control nuclei from frozen sections of normal tissue to set a threshold, it is likely that we overestimated the frequencies of TßR allelic loss in the frozen tumor nuclei (**Table 2, Figure 2**).

Flow cytometric analysis to assess ploidy of the tumor cell population is routinely performed on breast carcinoma specimens at our institution. As shown in **Table 3**, 10 of the 18 tumors contained discernible aneuploid subpopulations. Not all of the cases that were globally aneuploid included subpopulations with duplication of individual chromosomes 3 and/or 9. Up to 50% (average: 14%) of cells carried more than 2 copies of chromosome 3, and up to 32% (average: 6%) of cells had undergone duplication of chromosome 9. Even though these represented relatively small subpopulations of tumor cells, these nuclei were analyzed separately to determine TßR gene copy numbers. **Figure 3** shows that the frequency of TßR-I or –II gene losses was proportionately greater in cells that were polyploid for the corresponding chromosome.

Our results indicate that many of the primary invasive carcinoma specimen contained subpopulations of cells that had undergone allelic losses of either the TßR-I or the TßR-II gene. These findings raised the question at which stage of tumor development these losses had occurred.

To answer this question, we will have to examine a series of cases that span the spectrum of pre-invasive to metastatic breast cancer. However, our data provide some preliminary insight. For example, in the single case of non-invasive intracystic papillary carcinoma (6T), we found no evidence of significant allelic loss of either of the two receptor genes (**Table 2**). We also examined a single metastatic lesion. This chest wall recurrence demonstrated extensive aneuploidy of both chromosomes 3 and 9 with increasing allelic loss of the TBR genes with increasing chromosome copy number (**Figure 4**). However, in spite of the high frequency of chromosomal gains and losses, losses of both TßR genes in this case were of the same order of magnitude as those seen in the primary tumors. Thus, these findings suggest that losses of genes that encode TGFß signaling intermediates may occur progressively as breast cancers evolve from pre-invasive to invasive to metastatic lesion.

DISCUSSION

In this study, we were able to clearly demonstrate that both of the genes that encode TGFß receptors undergo deletions in human breast cancer. Approximately one third of the primary tumors we examined exhibited allelic deletions of the TßR-I gene, while 60% of the cases revealed losses of one or both TßR-II alleles. These observations appear to be in accord with the few available previous studies in which this question has been addressed using PCR-based assays. Guo et al. (Guo, et al., 1998) were able to detect LOH of the TßR-II gene in 3 of 9 informative cases of gastric cancer, while, in our own mutational analysis of the TßR-I gene in cervical carcinomas, none of the 8 informative cases displayed allelic loss of TßR-I (Chen, et al., 1999). In addition, a number of reports have suggested that, in general, inactivation of TßR-II appears to occur more commonly than mutations of TßR-I in human neoplasms (Park, et al., 1994, Sun, et al., 1994, Kalkhoven, et al., 1995, Garrigue-Antar, et al., 1996, de Jonge, et al., 1997, Chen, et al., 1999). The greater frequency of TßR-II losses compared to TßR-I deletions in the present series may be a reflection of this same phenomenon.

Besides the TßR-I and -II genes, allelic losses of the other principal genes that mediate TGFß signaling have been reported. For example, LOH of Smad4 is frequently observed in pancreatic cancer, but much less commonly in other tumor types, including breast cancer (Barrett,

et al., 1996, Hahn, et al., 1996, Schutte, et al., 1996, Powell, et al., 1997). Although LOH of the Smad2 gene has been reported in esophageal carcinomas (Maesawa, et al., 1997), and allelic losses of Smad3 have been described in 2 of 17 colorectal cancer, no information is available for breast carcinomas(Arai, et al., 1998).

Given the almost universal resistance of breast carcinoma cell lines to TGFß *in vitro*, it is surprising that the frequency with which signaling intermediates are inactivated is relatively low. Two of the possible reasons for this apparent paradox are technical: First of all, the results of PCR-based assays can be difficult to interpret depending on the degree to which tumor specimens are contaminated with normal stromal cells. Secondly, PCR-based assays are likely to underestimate the true frequency of LOH in cases in which only a small fraction of the tumor cells are affected. Our study indicates that, at least in primary breast cancers, this can be the case. Thus, one of the advantages of FISH over PCR-based assays is its greater sensitivity for scoring LOH. Moreover, FISH has the distinct advantage of being able to identify chromosomal deletions in individual tumor cells and can, therefore, distinguish between LOH and homozygous deletions with greater confidence than PCR-based methods (Chen, et al., 1992, Fiegl, et al., 1995, Leuschner, et al., 1996). In a direct comparison with standard methods of detecting LOH based on restriction fragment length (RFLP)- or microsatellite sequence polymorphisms, the sensitivity of FISH for detecting chromosomal losses appeared to be at least equivalent and probably somewhat superior to PCR-based methods (Dalrymple, et al., 1995).

FISH hybridization efficiency using centromeric probes is high, because of the excellent signal-to-noise ratio obtained with repeat-sequence probes. Thus, FISH can be used quite accurately to quantify chromosome copy numbers. However, FISH using locus-specific DNA probes is less reliable, as the signal-to-noise ratio is decreased because the signals are smaller and less intense than centromeric signals. Thus, the ability to precisely quantify the frequency of allelic losses of individual loci is limited by a relative lack of specificity (Eastmond, et al., 1995, Xiao, et al., 1995, Wolman, 1997). The best way to determine the true hybridization efficiency of locus-specific probes is to examine non-cancerous tissue that has been procured and fixed in a manner

similar to the tumor specimens one is examining. However, our efforts to use FISH on touch preparations of normal breast tissue failed because these samples contained a preponderance of fatty tissue and a paucity of epithelial cells. Therefore, we used the alternative approach of examining touch preparations of non-cancerous axillary lymph nodes that had been removed at the time of breast cancer surgery and had been processed in a manner identical to the breast tumor itself. Under the assumption that normal nodal lymphocytes should be diploid for all genomic loci, this allowed us to estimate the hybridization efficiency of the TßR probes. Cut-off levels in determining allelic losses in the tumor specimens were then set at 2 standard deviations above the mean percentage of normal lymph node nuclei that displayed fewer than two TßR-I or TßR-II signals (29% for TßR-I, 38% for TßR-II), following criteria used in previous studies (Bentz, et al., 1993, Dalrymple, et al., 1994, Dalrymple, et al., 1995). These values are quite similar to those reported by other investigators. For example, in several different studies in which FISH was used to examine losses at the p53 locus, threshold values of between 16 and 30% appeared to best discriminate between background and true positive allelic losses (Matsumara, et al., 1992, Sauter, et al., 1992, Sauter, et al., 1994, Kobayashi, et al., 1996). In studies examining other loci, background values ranged from 8 to 24% (Cher, et al., 1995, Wiest, et al., 1997).

A practical drawback of using touch preparations for FISH studies is that appropriate cases have to be identified prospectively at the time of surgery, and touch preparations have to be made immediately prior to fixation of the tissue specimens for permanent sections. The ability to conduct this type of analysis retrospectively using archival material would greatly increase the number of tumors that could be analyzed as well as the number of different genes that could be examined. However, we were not able to obtain an acceptable hybridization efficiency using nuclei isolated from formalin-fixed, paraffin-embedded tissue samples, even when using the centromeric probes. Both the nature and duration of the initial fixation of the tissue as well as the conditions under which the paraffin block are stored are critical determinants of FISH efficiency on paraffin embedded materials (Schofield, et al., 1992, Xiao, et al., 1995). For example, formalin dramatically reduce the signal in FISH by causing DNA cross-linking such that the probes are unable to bind to their DNA targets (Simpson, et al., 1996).

The hybridization efficiency we were able to obtain using nuclei isolated from frozen tumor samples was far better than on formalin-fixed cells. However, in these cases, we noted a seemingly higher frequency of TßR LOH than in the touch-preparations. As the hybridization efficiency of our centromeric probes was close to 100% in touch preparations of control lymph nodes, it is unlikely that we would have underestimated TßR gene copy numbers in the tumor touch preparations. Therefore, it is more likely that either the procedure used for isolation of nuclei from fresh frozen tissues or one of the subsequent steps in their processing had a negative impact on the hybridization efficiency of our probes, thus leading to an overestimate of the extent of TßR deletions.

It should be noted that the losses of the TßR genes that we observed may reflect, in part, allelic losses of larger chromosomal fragments that encompass the TßR-I and -II genes. The TßR-I gene has been mapped to chromosome 9q22 (Johnson, et al., 1995, Pasche, et al., 1998), and the TßR-II gene to chromosome 3p22 (Johnson, et al., 1995). Allelic losses of all or part of chromosome 3p have been observed in 30-60% of breast cancers (Ali, et al., 1989, Devilee, et al., 1989, Chen, et al., 1992, Bergthorsson, et al., 1995). The chromosomal regions with the highest frequency of losses include bands 3p13 to 3p14, and bands 3p24 to 3p26 (Bergthorsson, et al., 1995). FISH analysis demonstrated that 90% of these losses were the result of interstitial deletions, and 10% were due to loss of the entire chromosomal arm. Allelic losses in regions of chromosome 9q have also been noted in breast cancer, but the frequency of these events is significantly lower that loss on chromosome 3p (Leuschner, et al., 1996, Courjal, et al., 1997, Kerangueven, et al., 1997, Nishizaki, et al., 1997). Thus, our observation that the TßR-II gene undergoes allelic loss more frequently than TßR-I may reflect, to some extent, the general pattern of losses on these two chromosomal arms.

Just as it is possible to overestimate the degree of allelic loss, it is also possible that we actually underestimated LOH of the TBR-I and TBR-II genes because of the particular probes that we used. For both TßR genes, the probes we used were BAC clones with a total insert size of approximately 100-120 kb (Vellucci, et al., 1997). Given that the TßR-I and -II genes have a total

size of between 20 and 30 kb, each of the BAC clones include approximately 100 kb of additional flanking sequence. Thus, we would have failed to score as LOH any cases with deletions that were significantly smaller than the region corresponding to the BAC insert (for example, cases of intragenic deletion of either one of the TßR genes). Similarly, DNA breaks within the chromosomal region spanned by the BAC insert would have falsely increased the signal copy number.

Besides losses of the TßR-I and TßR-II genes in disomic nuclei, we also found that the degree of gene loss was increased in polysomic nuclei. The presence of aneuploid subpopulations of breast cancer cells has also been noted in other studies using FISH (Persons, et al., 1994, Kobayashi, et al., 1996, Simpson, et al., 1996). For example, Simpson et al. found that 40% of breast carcinoma specimens contained cells with 3 or more copies of chromosome 3 (Simpson, et al., 1996), and Leuschner et al. reported increased copies of chromosome 9 in an average 30% of breast cancer cells (Leuschner, et al., 1996). The relative increase in the extent of TßR gene loss in polysomic nuclei is also consistent with similar observations by others, and suggests that the deletion events precede polyploidization (Kobayashi, et al., 1996, Simpson, et al., 1996). This phenomenon is most clearly illustrated by the single case of recurrent breast carcinoma included in this series. This case displayed a striking degree of duplication of both chromosome 3 and chromosome 9 in the majority of nuclei. The degree of LOH of both TßR genes appeared to parallel the extent of chromosomal polyploidy. This is precisely the pattern one would expect if TßR gene loss had occurred prior to duplication of the chromosome.

Our findings raise the question at which stage of cancer development the allelic losses of the TßR genes occur, and whether they would be progressive at later stages of the disease. The current series included a single case of intraductal breast carcinoma. This intracystic papillary carcinoma was composed of an almost completely diploid cell population that did not appear to demonstrate LOH of either TßR-I or TßR-II genes. This appears to be consistent with previous reports of chromosomal losses at these loci. For example, using PCR-based assays, Radford et al

(Radford, et al., 1995) found LOH at chromosome 3p24.2-22 in fewer than 10% of cases of ductal breast carcinomas *in situ* and no evidence of LOH at chromosome 9q34.1.

According to the model put forth by Knudson (Knudson Jr., 1985), one would predict that allelic losses of the TßR genes would be associated with inactivating mutations of the second allele. Our recent finding that a particular missense mutation in the kinase domain of TßR-I is found more frequently in metastatic than in primary breast cancers supports the idea that inactivation of TGFß signaling may occur relatively late during breast cancer progression (Chen, et al., 1998). On the other hand, TßR gene losses in the single metastatic lesion in our series were not significantly higher than in primary tumors. Similarly, Simpson et al. (Simpson, et al., 1996) reported that chromosome 3 copy numbers in metastatic breast cancers were very similar to that in primary tumors (Simpson, et al., 1996). Similarly, Fiegl et al (Fiegl, et al., 1995) noted that the aneuploidy rates for chromosomes 11 and 17 in tumor-infiltrated axillary lymph nodes were also comparable to the corresponding primary tumors. Thus, our results are in accord with a model of breast cancer in which inactivation of the TGFß signaling pathway occurs late, i.e. primarily during the transition from primary invasive to metastatic lesions. However, these findings will need to be confirmed in a larger study that should not only include a greater number of *in situ* as well as metastatic cases of breast carcinoma, but in which the other components of the TGFß signaling pathway (Smad2, -3, and -4) are examined as well.

METHODS:

Tissue specimens: Breast carcinoma specimens were provided by the Program for Critical Technologies in Breast Oncology at Yale. A total of 19 cases were examined, which included 16 invasive ductal carcinomas (15 primary tumors and 1 chest wall recurrence), 2 invasive lobular carcinomas, and 1 intracystic papillary carcinoma. In the first 7 cases, nuclei were isolated from tumors that had been embedded in Tissue-Tek OCT compound (Miles, Inc., Diagnostic Division, Elkhart, IN) and snap frozen in liquid nitrogen within 20 minutes of surgical resection. Frozen sections (50 µm) were dounced and passed through a series of filters in order to isolate nuclei as described by Hedley et al. (Hedley, et al., 1983). The nuclear suspension was fixed in 3:1

methanol:acetic acid solution at -20°C. For FISH analysis, 15 µl of nuclear suspension were deposited onto pre-marked areas of silanized slides. The slides were allowed to air-dry, and were then baked in an oven at 40-50°C for 1 hour. In the remaining 12 cases, touch preparations of fresh tumor specimens were prepared directly on silanized slides immediately after surgical resection and air-dried overnight. Slides were then fixed in methanol:acetic acid (3:1) at -20°C, air-dried, and stored at -20°C in a sealed box.

Preparation of probes for FISH: As probes, we used BAC clones that contained the complete genomic sequences of the TßR-I and -II genes (approximately 20 and 30 kb, respectively) (Vellucci, et al., 1997). In addition, we used probes for the peri-centromeric regions of the corresponding chromosome 3 (for TßR-II) (Waye, et al., 1989) and -9 (for TßR-I) (Rocchi, et al., 1991). DNA probes were labeled using dNTP-digoxigenin- or dNTP-biotin. The nick translation reaction included 25 µM of either digoxigenin-11-dUTP (Boehringer Mannheim, Indiana) or biotin-16-dUTP (Boehringer Mannheim, Indiana) combined with 1 µg of DNA, 0.1 M ß-mercaptoethanol, nick translation buffer (0.5 M Tris-HCL, pH 8.0, 0.05 M $MgCl_2$, 0.05% BSA), DNAse I (Boehringer Mannheim, Indiana), and DNA Polymerase I (E.Coli) (New England Biolab, Massachusetts) in a final volume of 100 µl. The reaction was allowed to run at 15°C for 35 minutes. The length of the fragments was determined by resolving a small aliquot of the reaction product on a 1% agarose gel. When the majority of fragments were between 200 and 500 base pairs, the reaction was stopped by adding EDTA and SDS to a final concentration of 20 µM and 0.2%, respectively, followed by a 10 minute incubation at 68°C. The DNA mix [10 µl (= 500 ng) labeled TßR gene DNA, 0.5 µl (=25 ng labeled centromeric DNA, 7 µl (1µg/µl) salmon sperm DNA and 3 µl (1µg/µl) Cot1 DNA] was precipitated by adding 0.1 X volume of 3 M sodium acetate and 2.5 X volume of ethanol (100%) and incubated overnight at -70°C. After centrifugation at 13,000 rpm for 20 minutes at 4°C, the supernatant was discarded and the pellets dried in a Speed-Vac for 15 minutes. Five µl Ultrapure triple-distilled deionized formamide (American Bioanalytical, Natick, MA) was added and the tube vortexed for 10 minutes. Five µl of hybridization solution (20% dextran sulfate, 4x SSC), was then added to the tube, and vortexed

for an additional 10 minutes. The probes were denatured by incubating at 75-80°C for 10 minutes and then kept at 37°C for 20 minutes until the slides had been prepared.

***In Situ* Hybridization:** Slides were first incubated with 120 µl denaturation solution (70% deionized formamide, 2x SSC) for exactly 1.5 min at 72-75°C, then immediately taken through consecutive washes in pre-chilled (-20°C) 70%, 90%, and 100% ethanol and air-dried. Following incubation with proteinase K (0.09 µg/ml in $CaCl_2$/Tris, pH 7.5) at 37°C for 7 minutes, the slides were again taken through consecutive washes in pre-chilled (-20°C) 70%, 90%, and 100% ethanol and air-dried. Each slide was then incubated with 10 µl of hybridization solution containing labeled denatured DNA probes. A coverslip was added and sealed with rubber cement, and the slides were placed in a humid sealed box overnight at 37°C. The slides were then washed 4 times for 5 min in 50% formamide/2x SSC at 42°C, and 3 times for 5 min in 0.1X SSC at 60°C. Due to the light-sensitive nature of the fluorochromes, all subsequent steps were performed in a darkened area. Slides were incubated in the presence of blocking buffer (3% BSA, 4X SSC, 0.1% Tween-20) for 30 min at 37°C, followed by incubation with 200 µl of detector [1:400 dilution of avidin-FITC (or -Elexa) and 1:150 dilution of anti-digoxigenin-rhodamine in detection buffer (1% BSA/4X SSC/0.1% Tween-20)] for 30 min at 37°C. Following 3 washes in 4X SSC/0.1% Tween-20 for 5 min at 42°C, the slides were counter-stained with 0.1 µg/ml 4,6-Diamidino-2-phenyl-indole dihydrochloride (DAPI, Sigma, St. Louis, MO) in 2X SSC for visualization of the nuclei, and mounted using Vectashield Antifade solution (Vector Laboratories, Burlingame, MA).

Data collection and analysis: At least 150 nuclei were examined per sample. The number of centromeric signals and TßR gene signals were recorded for each nucleus. Nuclei with fewer than 2 centromeric signals were excluded from analysis. Nuclei in which the number of TßR gene signals was lower than the number of corresponding centromeric signals were scored as having undergone allelic loss. The viewer was blinded to the identity of the probes when examining the slides. Stained slides were viewed using a Zeiss Axioskop epifluorescence microscope equipped with a thermoelectrically cooled CCD camera (Photometrics T-200). A 50W mercury lamp was used for excitation. Alternatively, we used an Olympus Provis epifluorescence microscope in

conjunction with a 100W mercury lamp. Narrow band pass filters were used to obtain separate images of each fluorochrome (FITC, rhodamine, and DAPI). The three separate images of each object were then stored and overlayed on a MacIntosh Quadra 900 computer using custom designed software.

Several types of controls were used: First, slides containing interphase nuclei from normal peripheral blood lymphocytes were included in each experiment to ensure that the hybridization efficiency (i.e. the inverse of the fraction of cells in which no signal was detected x 100%) of each probe to be >95% (Kallioniemi, et al., 1992, Matsumara, et al., 1992). The hybridization efficiency of each of the probes (the inverse of the fraction of cells in which no signal was detected) had to be >95%. Secondly, we also examined touch preparations of four normal axillary lymph nodes from different individuals that had been collected and processed in the same manner as the primary breast cancers. Because normal nodal lymphocytes should be diploid for all genomic loci, this allowed us to estimate the hybridization efficiency of the TßR probes and determine the frequency of "false-positive" TßR allelic deletions in breast tumors. We determined the mean percentage of normal lymph node nuclei that displayed fewer than two TßR-I or TßR-II signals. Cut-off levels for determining allelic losses in the tumor specimens were then set at this mean plus 2 times the standard deviation (i.e. the upper boundary of the 95% confidence interval). This corresponds to the upper boundary of the 95% confidence interval (CI), following criteria used in previous studies (Bentz, et al., 1993, Dalrymple, et al., 1994, Dalrymple, et al., 1995).

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TABLES

Table 1. Characteristics of Primary Breast Carcinomas

A. Frozen tumor specimens

Tumor sample	Patient age	Histology	Size (cm)	Hormone receptors	DNA index	% Cells S-phase	Involved lymph nodes
20	33	Invasive ductal	3.5	ER-/PR-	1.00	22%	0/11
25	64	Invasive ductal	2.2	ER+/PR-	2.00	<1%	2/7
45	31	Invasive ductal	4.0	ER-/PR-	1.75	34%	0/14
47	51	Invasive ductal	2.8	ER-/PR+	1.13	2%	1/10
49	65	Invasive ductal	1.9	ER-/PR-	2.00	25%	26/34
50	79	Invasive ductal	4.0	ER-/PR+	ND	ND	1/1
52	44	Invasive ductal	1.5	ER+/PR+	1.46	High	0/10

B. Touch preparations

Tumor sample	Patient age	Histology	Size (cm)	Hormone receptors	DNA index	% Cells S-phase	Involved lymph nodes
1T	34	Invasive ductal	2.2	ER-/PR-	1.00	6%	4/20
2T	43	Invasive ductal	1.5	ER+/PR+	1.00	1%	ND
6T	77	Intracystic papillary	2.5	ND	ND	ND	ND
7T	76	Invasive ductal	1.4	ER+/PR-	1.27	10%	ND
8T	36	Invasive ductal	4.0	ER-/PR-	1.91	48%	0/35
9T	63	Invasive ductal	2.2	ER+/PR-	1.20	12%	1/18
21T	55	Invasive lobular	1.4	ER+/PR+	1.0	3.9%	1/14
30T	46	Invasive lobular	3.0	ER+/PR+	1.0	6.5%	5/12

32T	61	Invasive ductal	1.7	ER-/PR-	1.79	13%	0/19
33T	73	Invasive ductal	3.0	ER+/PR+	2.0	<1.0	7/23
35T	54	Invasive ductal	2.5	ER+/PR+	1.0	3.4%	0/15

ND: Not done

A total of 16 primary breast cancer specimens were examined by FISH. Most of these were primary invasive ductal cancers. Frozen tumor specimens: Tumor tissue was embedded in Tissue-Tek OCT compound embedding medium (Miles, Inc., Diagnostic Division, Elkhart, IN) and snap frozen in liquid nitrogen within 20 minutes of collection. Frozen sections (50 μm) were dounced and passed through a series of filters in order to isolate nuclei. The resultant suspension was fixed in 3:1 methanol:acetic acid solution at -20°C. For FISH analysis, 15 μl of nuclear suspension were deposited onto a pre-marked area of silanized slides. The slides were allowed to air-dry, and were then baked at 40-50°C in an oven for 1 hour. Touch preparations: Touch preparations of fresh tumor specimens were prepared on silanized slides immediately after resection and air-dried overnight. Slides were then fixed in methanol:acetic acid (3:1) at -20°C, air-dried, and stored at -20°C in a sealed box.

Table 2. TßR-I & -II Allelic Losses in Primary Breast Carcinomas

A. Frozen tumor specimens

Tumor sample	TßR-I			TßR-II		
	Single copy	*Zero copy*	*Total*	*Single copy*	*Zero copy*	*Total*
20	ND	ND	ND	38%	51%	89%
25	ND	ND	ND	16%	44%	60%
45	ND	ND	ND	11%	58%	69%
47	ND	ND	ND	25%	46%	71%
49	21%	34%	55%	ND	ND	ND
50	ND	ND	ND	35%	32%	67%
52	ND	ND	ND	22%	50%	72%

B. Touch preparations

Tumor sample	TßR-I			TßR-II		
	Single copy	*Zero copy*	*Total*	*Single copy*	*Zero copy*	*Total*
1T	23%	3%	26%	20%	28%	48%
2T	34%	43%	77%	31%	31%	62%
6T	17%	1%	18%	29%	4%	33%
7T	ND	ND	ND	33%	6%	39%
8T	18%	4%	22%	ND	ND	ND
9T	28%	24%	52%	19%	35%	54%
21T	ND	ND	ND	25%	34%	59%
30T	ND	ND	ND	8%	18%	26%
32T	ND	ND	ND	34%	37%	71%
33T	16%	7%	23%	22%	4%	26%
35T	ND	ND	ND	18%	6%	25%

ND: not done

Samples were hybridized with either TßR-I or TßR-II in conjunction with a centromeric probe for the corresponding chromosome (chromosome 9 for TßR-I, chromosome 3 for TßR-II) as described in the "Materials & Methods" section. At least 150 nuclei were examined on each slide. The number of centromeric signals and TßR gene signals were recorded for each nucleus. Nuclei with fewer than 2 centromeric signals were excluded from analysis. Nuclei in which the number of

TßR gene signals was inferior to the number of corresponding centromeric signals were scored as having undergone allelic loss. The frequency of LOH of the TßR-I gene ranged from 16 to 28%, and homozygous loss from 4 to 43%. Similarly, between 8 and 38% of nuclei had undergone LOH of the TßR-II gene, and homozygous losses ranged from 4 to 58%. Results obtained by repeated hybridizations of separate slides prepared from the same tumor samples varied by less than 10%. Based on control hybridizations of touch preparation of 4 different normal lymph nodes, we defined cut-off values of 29% for TßR-I and 38% for TßR-II. Based on these findings, true positive TßR-I and TßR-II gene losses had occurred in 2 of 6 and 6 of 10 cases, respectively. There was no correlation between loss of either gene and patient age, tumor size, receptor status, % of cells in S-phase or number of involved axillary lymph nodes.

Table 3. Tumor Cell Ploidy and Individual Chromosome Copy Numbers

A. Frozen tumor specimens

	Flow Cytometry		Chromosome 3		Chromosome 9	
Tumor sample	Diploid (%)	Aneuploid (%)	2 copies (%)	>2 copies (%)	2 copies (%)	>2 copies (%)
20	100	0	90	10	ND	ND
25	40	60	50	50	ND	ND
45	40	60	86	14	ND	ND
47	4	96	97	3	ND	ND
49	44	56	ND	ND	96	4
50	ND	ND	58	42	ND	ND
52	82	18	85	15	ND	ND

B. Touch preparations

	Flow Cytometry		Chromosome 3		Chromosome 9	
Tumor sample	Diploid (%)	Aneuploid (%)	2 copies (%)	>2 copies (%)	2 copies (%)	>2 copies (%)
1T	100	0	100	0	100	0
2T	100	0	99	1	99	1
6T	ND	ND	ND	ND	100	0
7T	64	36	100	0	ND	ND
8T	83	17	ND	ND	98	2
9T	40	60	100	0	68	32
21T	100	0	97	3	ND	ND
30T	100	0	93	7	ND	ND
32T	89	11	95	5	ND	ND
33T	50	50	56	43	97	3
35T	100	0	ND	ND	100	0

ND: Not done

Comparison between ploidy and chromosome 3 and -9 copy numbers. In general, there was concordance between the DNA index obtained by routine flow cytometry of isolated tumor nuclei and the copy numbers of chromosomes 3 and -9 determined by FISH.

FIGURE LEGENDS

Figure 1. Detection of TßR genes in breast carcinoma touch preparations using FISH. A. Example of a touch preparation of primary breast carcinoma hybridized with TßR-I and centromeric probes. Stained slides were viewed using a Zeiss Axioskop epifluorescence microscope equipped with a thermoelectrically cooled CCD camera (Photometrics T-200). A xenon lamp was used for excitation. Narrow band pass filters were used to obtain separate images of each fluorochrome (FITC, rhodamine, and DAPI). The three separate images of one object were stored and then overlayed on a MacIntosh Quadra 900 computer using custom designed software. **B.** Frequency distributions of centromeric and TßR gene copy numbers in diploid breast carcinoma specimen, 2T. The number of centromeric signals and TßR gene signals were recorded for each nucleus. Nuclei with fewer than 2 centromeric signals were excluded from analysis. In each case, a total of 150 nuclei were counted in a blinded fashion. Nuclei in which the number of TßR gene signals was inferior to the number of corresponding centromeric signals were scored as having undergone allelic loss.

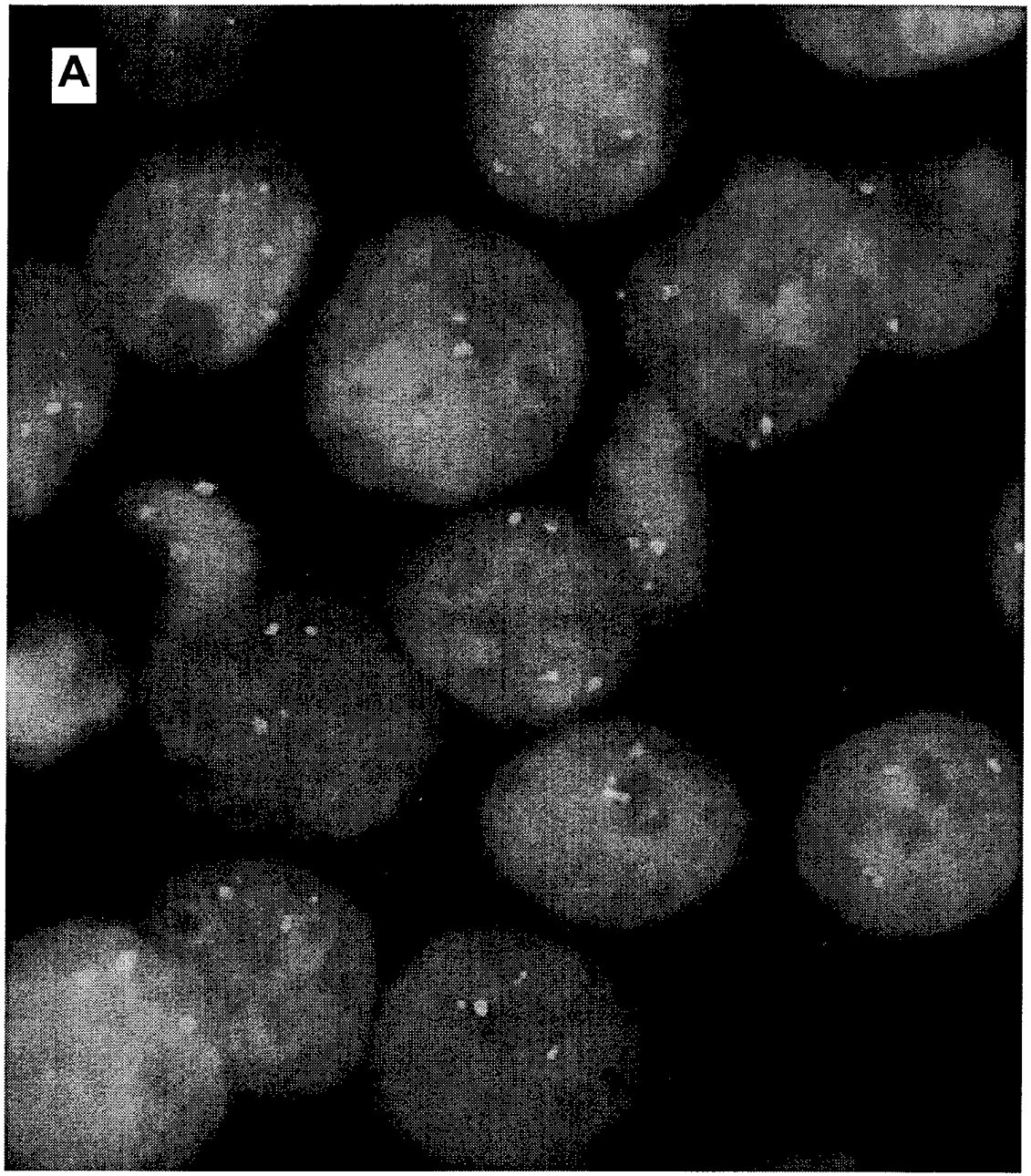
Figure 2. Fraction of breast tumor cells with TßR-I and -II gene in breast cancer specimens. Each bar represents an individual case. As controls, TßR-I and -II signal copy numbers were also determined in tumor-free axillary lymph nodes compared with breast carcinomas (n=4). The average fraction of nodal lymphocytes with <2 TßR signals was 19% (95% CI: 9-29) for TßR-I and 21% (95% CI: 3-38) for TßR-II. Using the upper boundaries of the 95% confidence intervals as threshold values (29% for TßR-I and 38% for TßR-II), tumor cell subpopulations with TßR-I deletions were present in 2 of 6 (33%), and TßR-II deletions in 6 of 10 (60%) touch preparations examined. It is likely that the results obtained using nuclei isolated from frozen tumor material represent an overestimate of the extent of TßR LOH.

Figure 3. Chromosome polyploidy and TßR allelic losses. Nuclei that had undergone chromosome duplication were analyzed separately. Approximately 14% of cells carried greater than 2 copies of chromosome 3, and approximately 7% of cells had undergone duplication of chromosome 9. In the nuclei which had undergone duplication of chromosome 3, allelic losses of

the TßR-II gene were noted in 26% of cells, whereas the nuclei in which there was duplication of chromosome 9, TßR-I gene copies were lost in 8%. Even though these represented relatively small subpopulations of tumor cells, the frequency of TßR-I or –II losses was proportionately greater in cells that carried an increased number of the corresponding chromosome.

Nuclei that had undergone chromosome duplication were analyzed separately. Approximately 14% of cells carried more than 2 copies of chromosome 3, and approximately 7% of cells had undergone duplication of chromosome 9. The frequency of TßR-I or –II losses was proportionately greater in cells that carried an increased number of the corresponding chromosome

Figure 4. TßR-I and -II gene copy numbers in aneuploid recurrent breast carcinoma. The higher frequency of TßR gene losses among aneuploid populations is best illustrated in a case of recurrent breast carcinoma on the chest wall. Whereas the frequency of TßR-II gene loss in the diploid cell population was relatively low, the nuclei that had undergone extensive duplication of chromosome 3 simultaneously displayed decreases of TßR-II gene copy numbers.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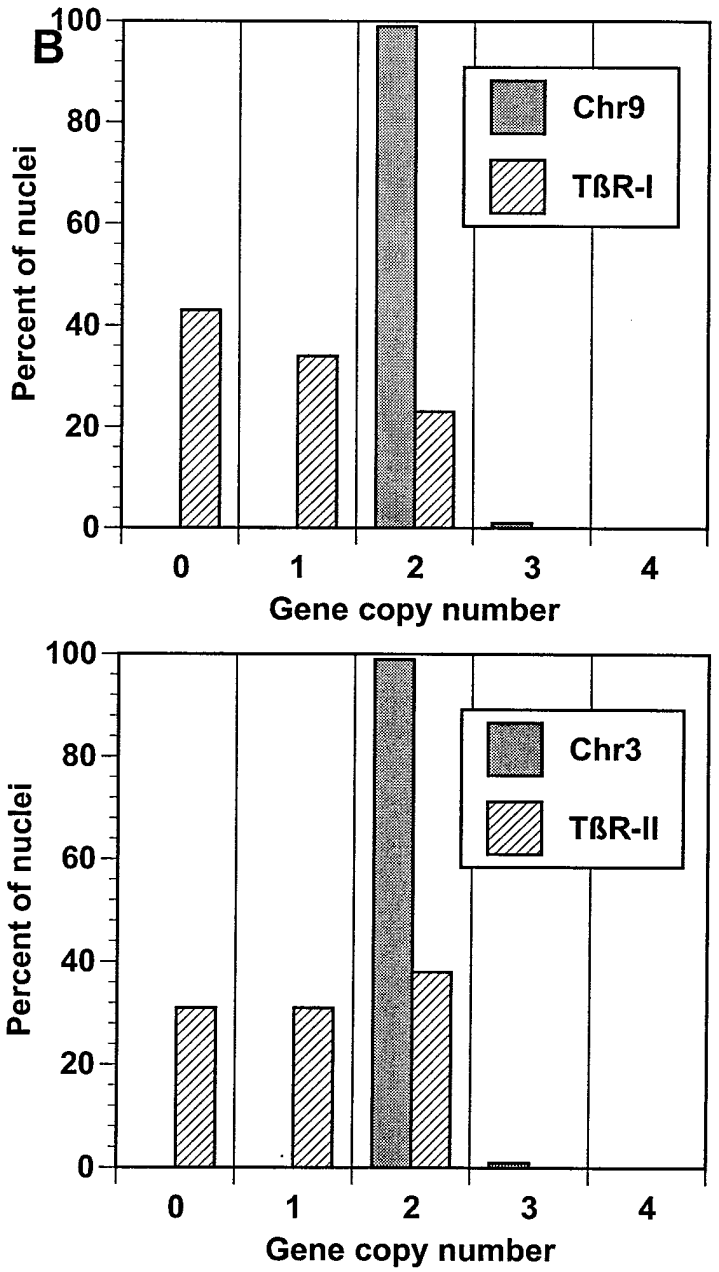
B
Percent of nuclei
100
80
60
40
20
0
Chr9
TßR-I
0
1
2
3
4
Gene copy number
Percent of nuclei
100
80
60
40
20
0
Chr3
TßR-II
0
1
2
3
4
Gene copy number

Figure 1

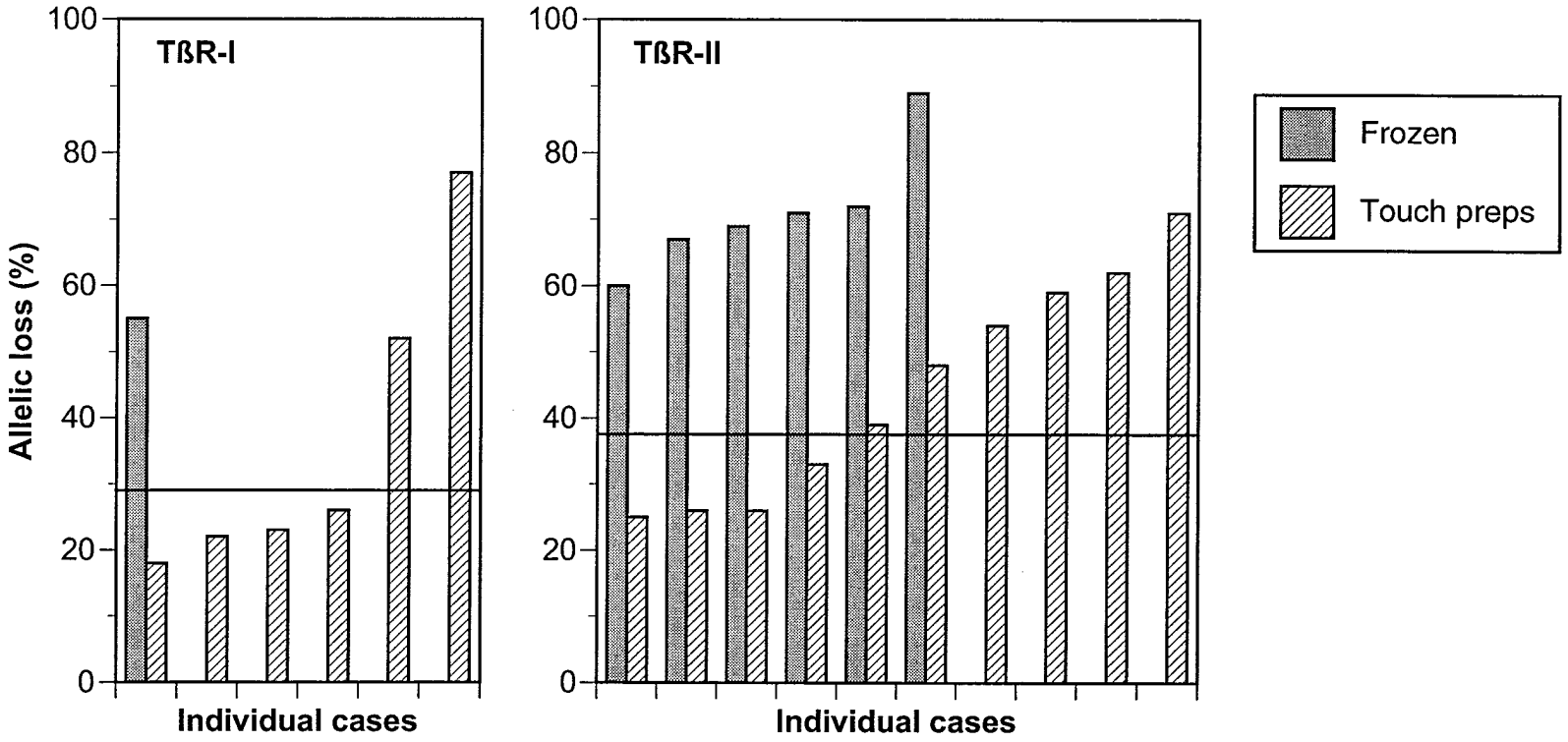
TßR-I
TßR-II
Allelic loss (%)
100
80
60
40
20
0
Individual cases
Frozen
Touch preps

Figure 2

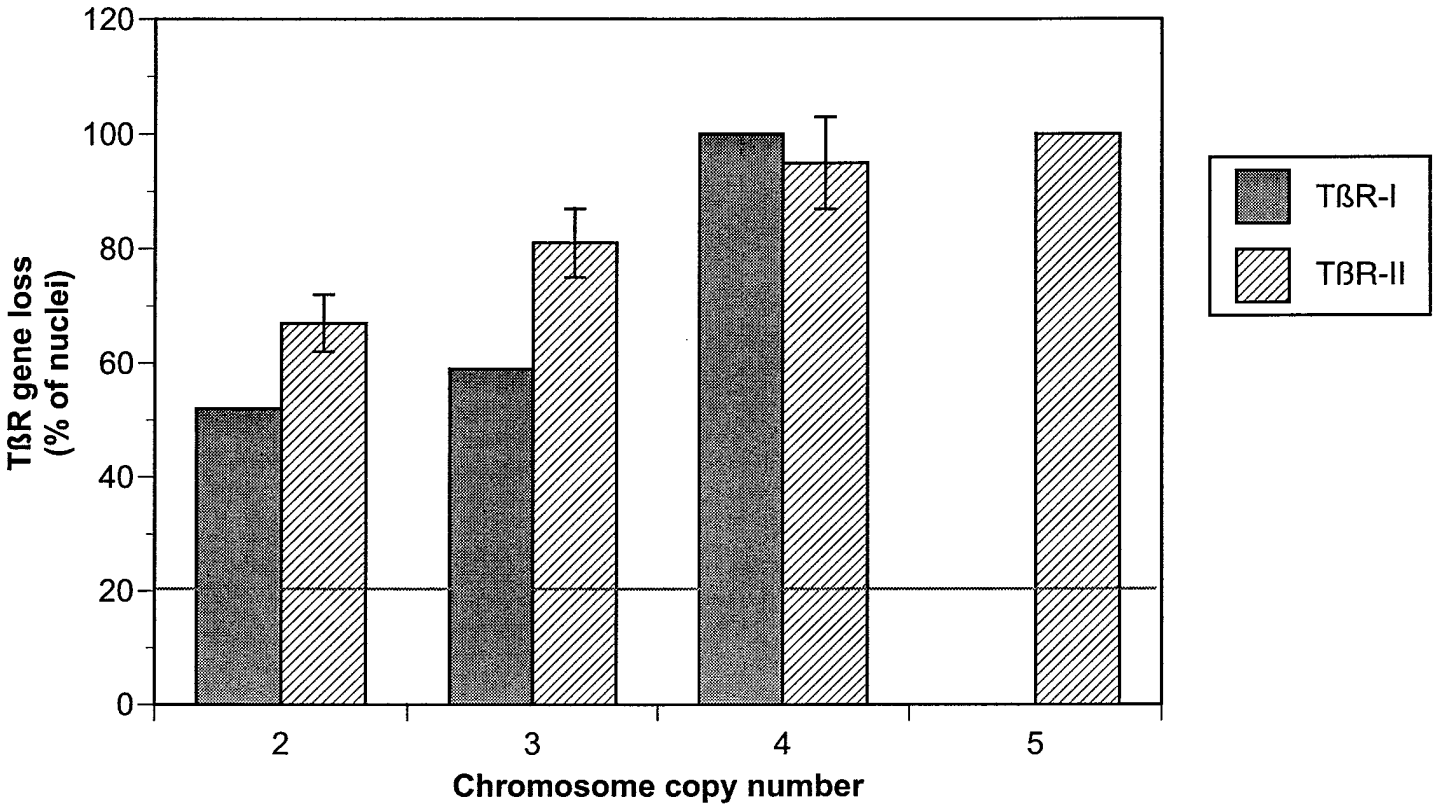
120
100
80
60
40
20
0
TßR gene loss
(% of nuclei)
2
3
4
5
Chromosome copy number
TßR-I
TßR-II

Figure 3

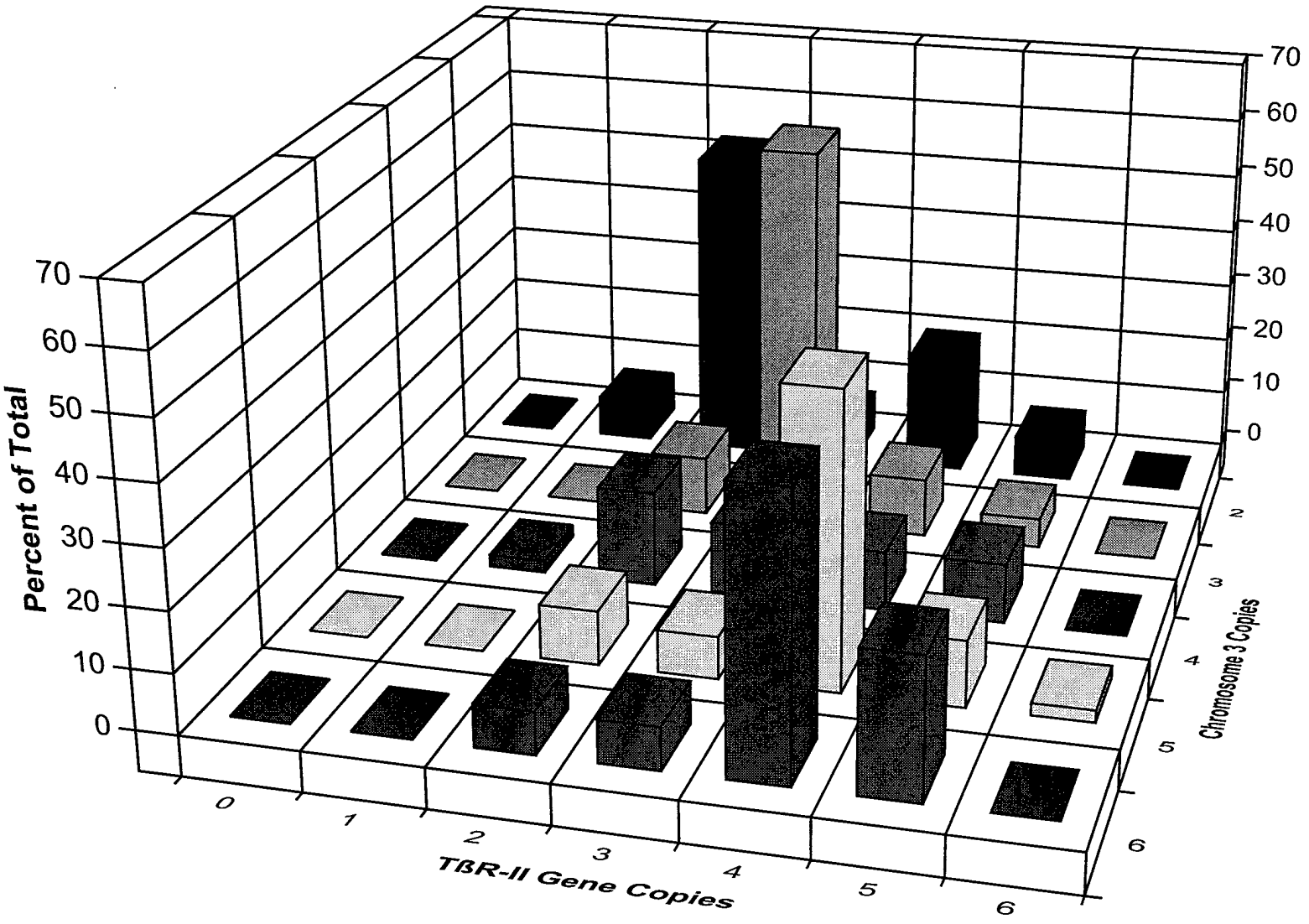
Percent of Total
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
TßR-II Gene Copies
70
60
50
40
30
20
10
0
2
3
4
5
6
Chromosome 3 Copies

Figure 4