Combined Genetic Assessment of Transforming Growth Factor-β Signaling Pathway Variants May Predict Breast Cancer Risk

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Abstract

There is growing evidence that common variants of the transforming growth factor-β (TGF-β) signaling pathway may modify breast cancer risk. *In vitro* studies have shown that some variants increase TGF-β signaling, whereas others have an opposite effect. We tested the hypothesis that a combined genetic assessment of two well-characterized variants may predict breast cancer risk. Consecutive patients (n = 660) with breast cancer from the Memorial Sloan-Kettering Cancer Center (New York, NY) and healthy females (n = 880) from New York City were genotyped for the hypomorphic *TGFBR16A allele and for the *TGFB1* T29C variant that results in increased TGF-β circulating levels. Cases and controls were of similar ethnicity and geographic location. Thirty percent of cases were identified as high or low TGF-β signalers based on *TGFB1* and *TGFBR1* genotypes. There was a significantly higher proportion of high signalers (*TGFBR1/TGFBR1* and *TGFB1**CC) among controls (21.6%) than cases (15.7%; P = 0.003). The odds ratio [OR; 95% confidence interval (95% CI)] for individuals with the lowest expected TGF-β signaling level (*TGFB1**TT or *TGFB1**TC and *TGFBR1**6A) was 1.69 (1.08-2.66) when compared with individuals with the highest expected TGF-signaling levels. Breast cancer risk incurred by low signalers was most pronounced among women after age 50 years (OR, 2.05; 95% CI, 1.01-4.16). *TGFBR1**6A was associated with a significantly increased risk for breast cancer (OR, 1.46; 95% CI, 1.04-2.06), but the *TGFB1**CC genotype was not associated with any appreciable risk (OR, 0.89; 95% CI, 0.63-1.21). *TGFBR1**6A effect was most pronounced among women diagnosed after age 50 years (OR, 2.20; 95% CI, 1.25-3.87). This is the first study assessing the TGF-β signaling pathway through two common and functionally relevant *TGFBR1* and *TGFB1* variants. This approach may predict breast cancer risk in a large subset of the population.** (Cancer Res 2005; 65(8): 3454-61)

Introduction

Transforming growth factor-β1 (*TGFB1*) is a potent growth suppressor of mammary epithelial and breast carcinoma cells and acts as a negative autocrine growth inhibitor (1, 2). In normal cells, TGF-β is a potent growth inhibitor. On the other hand, it is now well appreciated that metastasis of most tumor types requires TGF-β activity and that, in advanced disease, TGF-β is clearly pro-oncogenic (3, 4). It seems therefore that for every action of TGF-β there is a counteraction that TGF-β is capable of performing (5).

Mouse models have allowed demonstration that increased TGF-β signaling is associated with decreased cancer incidence. This association has been found with both transgenic mice expressing higher *TGFB1* levels (6, 7) and mice with increased TGF-β signaling because of constitutively active *TGFBR1* receptor (8). On the other hand, the same sets of experiments have documented that the growth of tumors is fueled by increased *TGFB1* levels and by increased TGF-β signaling. Hence, these *in vivo* experiments indicate that higher *TGFB1* levels serve as a surrogate of increased TGF-β signaling and lower *TGFB1* levels a surrogate for decreased TGF-β signaling. This dichotomy of function for TGF-β serves as the basis of the hypothesis to be tested in this study.

Five polymorphisms have been identified in the *TGFB1* gene to date (9). Two are in the promoter region (C-509T and G-800A), one within the signal sequence ($Leu^{10}Pro$), one within exon 1 ($Arg^{25}Pro$), and one within exon 5 ($Thr^{263}Ile$). The C-509T single nucleotide polymorphism (SNP) is not contained within a known consensus sequence for a promoter regulatory element and does not affect breast cancer risk (10), but one report indicates that it may modify *TGFB1* expression (11). There is evidence that the $Arg^{25}Pro$ polymorphism modifies *TGFB1* circulating levels (12–14), but it has not been associated with breast cancer risk thus far. The $Leu^{10}Pro$ polymorphism has been extensively studied in relation to breast cancer risk. The CC (*TGFB1**CC) genotype ($Leu^{10}Pro$ polymorphism) was found by one group of investigators to be associated with a 64% decreased breast cancer risk in a cohort study of 3,075 White American women over age 65 years at recruitment (15). In contrast, in a pooled analysis of three European case-control studies that included 3,987 cases and 3,867 controls, the CC genotype was associated with a 21% increased risk of breast cancer. In the same study, the investigators found that the C-509T *and* the T29C SNPs but not the G-800A were in strong linkage disequilibrium and that they were both significantly associated with increased incidence of invasive breast cancer in a recessive manner. In a hospital-based study of 232 cases and 172 controls conducted in Japan, there was no significant overall association between the CC genotype and breast cancer. However, the CC genotype was

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associated with a 65% reduced risk of breast cancer in comparison with the TT genotype among premenopausal women [odds ratio (OR), 0.45; 95% confidence interval (95% CI), 0.20-0.98; ref. 16]. A German study of 500 cases and 500 controls did not find any statistically significant association between either *TGFB1**CC and *TGFB1**CT genotypes and breast cancer (17). Most recently, a large multiethnic case-control study of 1,123 breast cancer cases and 2,314 controls from Los Angeles and Hawaii also did not find any association between the *TGFB1**CC genotype and breast cancer risk (18).

Of major interest in this regard is the recent report that patients with a diagnosis of breast cancer that carry the *TGFB1* C variant that results in higher circulating *TGFB1* levels have a significantly decreased survival compared with noncarriers (19). This polymorphism is represented by a SNP at position 29, which results in the substitution of leucine to proline at the 10th amino acid position ($Leu^{10}Pro$). The leucine-to-proline substitution results in significantly higher *TGFB1 in vivo* levels among *TGFB1**CC carriers (20–22). The $Leu^{10}Pro$ signal peptide substitution is well characterized for its effects on the regulation of *TGFB1* secretion. Transfection of HeLa cells with constructs encoding either proline or leucine forms of *TGFB1* and driven by the cytomegalovirus promoter show that the signal peptide with proline (C variant) at residue 10 causes a 2.8-fold increase in secretion compared with the leucine (T variant) form (10). Hence, there is both *in vitro* and *in vivo* evidence to support the conclusion that the T29C SNP is the most relevant SNP that modifies the amount of secreted and circulating *TGFB1* and affects overall TGF-β signaling. The function of the other *TGFB1* polymorphisms and their relevance to breast cancer risk remains to be further characterized.

We have previously identified *TGFBR1**6A, a common variant of *TGFBR1*. *TGFBR1**6A has a deletion of three GCG triplets coding for alanine within a nine alanine (9A) repeat sequence of *TGFBR1* (*TGFBR1**9A) exon 1, resulting in a six alanine (*TGFBR1**6A) repeat sequence (23). The 9-bp deletion that differentiates *TGFBR1**6A from *TGFBR1* is located within the predicted signal sequence cleavage region. Two separate groups of investigators have shown that *TGFBR1**6A mediates TGF-β growth inhibitory signals significantly less effectively than *TGFBR1* (24, 25). *TGFBR1**6A is a candidate tumor susceptibility allele that is associated with an increased incidence of various types of cancer (26, 27). The first report of an association between breast cancer and *TGFBR1**6A was a case-control study of 355 women with breast cancer and 248 controls by Baxter et al. (26). In that study, *TGFBR1**6A was associated with a 60% increased risk of breast cancer. These results are further supported by our recent meta-analysis of eight case-control studies that included 1,420 breast cancer cases and 1,823 controls, which showed that *TGFBR1**6A predisposes to the development of breast cancer (OR, 1.38; 95% CI, 1.14-1.67; ref. 28).

Thus, differences in TGF-β signaling, whether mediated by ligand or receptor variants, have been associated with risk for breast cancer. Various genotypic combinations may theoretically have either opposite or synergistic effects on breast cancer risk.

It is known that *TGFB1* and *TGFBR1* map to different chromosomes, 19q13.1 and 9q22, respectively. Thus, they are independently inherited. This has led us to hypothesize that the *TGFB1* T29C and the *TGFBR1**6A variants have a functional interaction with respect to breast cancer risk. Furthermore, based on genotyping of these two variants, we hypothesized that individuals with the combination of these two variants resulting in the highest predicted levels of TGF-β signaling have the lowest breast cancer risk and conversely those with the lowest levels of TGF-β signaling the highest breast cancer risk.

To test the hypothesis that a combined assessment of TGF-β pathway signaling variants may predict breast cancer risk more accurately than each variant alone, we genotyped 660 women with a diagnosis of breast cancer and 841 healthy female controls for the two most common and biologically relevant variants of the TGF-β signaling pathway, *TGFB1* T29C and *TGFBR1**6A.

Patients and Methods

Study participants. As part of institutional review board–approved protocols, we collected blood samples from 660 consecutive patients admitted to the Memorial Sloan-Kettering Cancer Center (New York, NY) with a diagnosis of breast cancer. DNA was extracted from peripheral blood following completion of diagnostic studies on these samples. Information regarding sex, age, age at breast cancer diagnosis, and ethnic status was recorded. In a subset of 99 and 97 patients, information on estrogen receptor (ER) and progesterone receptor (PR) status as assessed by immunohistochemistry was collected at the time of case collection. However, in the rest of the samples, due to destruction of personal identifiers, it was not possible to retrieve the information retrospectively. All breast cancer cases were histologically confirmed at the Memorial Sloan-Kettering Cancer Center. A population of 841 healthy female controls ages 20 to 87 years with well-defined ethnic background who had donated blood for various reasons (predominantly prenatal screening for noncancer disease) constituted the control group. Controls were of similar gender, ethnicity, and geographic location as the breast cancer cases. None of the controls had any personal history of cancer at the time of blood donation. This was ascertained by a questionnaire completed by each control. Although age categories were obtained for all participants, exact age information was not available for some controls because it was not collected prospectively. All personal identifiers were permanently removed from both cases and controls. A fraction of cases (n = 493) and controls (n = 330) included in this report have been genotyped for *TGFBR1**6A and included in a recent meta-analysis (28).

DNA isolation. DNA from whole blood lymphocytes was extracted using the QIAamp DNA blood mini kit and stored at −20°C until use for genotyping.

***TGFB1* genotyping.** The first variant is a *TGFB1* T-to-C point mutation at position 10 resulting in a leucine-to-proline substitution. Part of the *TGFB1* gene was amplified by PCR according to the following conditions: initial denaturation at 95°C for 10 minutes followed by 35 cycles at 93°C for 20 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The last extension step was prolonged to 3 minutes. The reactions were carried out in a total volume of 50 μL containing 100 to 300 ng genomic DNA, 1× standard PCR buffer without $MgCl_2$, 1.5 mmol/l $MgCl_2$, 6% DMSO, 25 pmol of primers, 200 μmol/L of each deoxynucleotide triphosphate, and 1 unit Taq DNA polymerase. The primers used were 5′-TGCCGCCCTCCGGGCTGCGGCTGC*G*GC-3′ and 5′-TCTTGCAGGTGGATAGTCCCGCGG*T*CGG-3′. The PCR product is 102 bp long. The PCR product was cleaved with *Hae*III overnight. The resulting fragments from the *Hae*III digestion were separated on a 12% gel in 0.5× Tris-borate EDTA buffer and visualized with ethidium bromide. Digestion by restriction enzyme *Hae*III generates polymorphic fragments of 69, 43, and 26 bp, respectively, and a 33-bp nonpolymorphic fragment. The various genotypes were confirmed by direct sequencing.

***TGFBR1* genotyping.** The second variant is a 9-bp deletion within a stretch of nine GCG repeats coding for alanine (29). PCR amplification was done using intronic primers flanking *TGFBR1* exon 1: 5′-GAGGCGAGGTTTGCTGGGGTGAGGCA-3′ and 5′-CATGTTTGAGAAAGAGCAGGAGCGAG-3′. Genotyping was done as described previously (24). Briefly, PCR amplification was done according to the following conditions: initial denaturation at 95°C for 1 minute followed by 35 cycles at 94°C for 30 seconds and 68°C for 3 minutes. The last extension step was prolonged to 3 minutes. The reactions were carried out in a total volume of 25 μL

containing 100 to 300 ng genomic DNA using the Advantage GC-genomic kit (Clontech, Palo Alto, CA). For single-strand conformational polymorphism analysis, PCR product (5 μL) was mixed with 10 μL formamide dye. The solution was heated at 95°C for 5 minutes, placed on ice water for 1 minute, and then loaded on the single-strand conformational polymorphism gel. The single-strand DNA fragments were resolved on a 20% Tris-borate EDTA acrylamide gel. Results for the different polymorphisms were confirmed by direct sequencing.

High, intermediate, and low signalers. Previous studies have shown that *TGFBR1**6A is a hypomorphic form of *TGFBR1* (24, 25). Hence, all *TGFBR1**6A carriers were classified as low signalers, with the exception of individuals carrying both *TGFBR1**6A and the *TGFB1**CC genotype that were classified as intermediate signalers (Fig. 1). The *TGFB1* T-to-C substitution has been shown to result in higher *TGFB1* secretion (10). *TGFB1**CC carriers have significantly higher *TGFB1* circulating levels than carriers of the *TGFB1**CT and *TGFB1**TT genotypes (30). Individuals carrying the *TGFB1**CC genotype and with two copies of the wild-type *TGFBR1* (i.e., a *TGFBR1* that transduces TGF-β signals more efficiently than *TGFBR1**6A) were classified as high signalers. Individuals with other genotypic combinations were classified as intermediate signalers (Fig. 1).

Immunohistochemistry. ER and PR were stained on paraffin-embedded, formalin-fixed tissue. Slides were subjected to antigen retrieval using 0.1 mol/L citric buffer (pH 6.2) and to microwave treatment for 10 minutes. For ER, monoclonal antibody clone 1D5 (Beckman Coulter, Immunotech, Miami, FL) was diluted at 1:20. Clone 1A6 monoclonal antibody against PR (Novocastra, Newcastle, United Kingdom) was diluted at 1:150. Detection was achieved using a biotinylated secondary antibody (BA-2000, Vector Laboratories, Burlingame, CA) in conjunction with the ABC kit (Vector Laboratories). The cutoff point for ER and PR positivity was 5% per institutional standards.

Statistical analysis. Distributions of *TGFBR1* genotypes, *TGFB1* genotypes, age, and ethnicity were compared between cases and controls using χ^2 tests. To examine the effect of *TGFBR1* and *TGFB1* genotypes adjusting for covariates, ORs for breast cancer were estimated using unconditional logistic regression models. Both crude and adjusted ORs for breast cancer were estimated comparing carriers of *TGFBR1**6A and C allele versus noncarriers for *TGFBR1* and *TGFB1*, respectively, under dominant, additive, and recessive genetic susceptibility models. Age and ethnicity were controlled for in all analyses. Crude and adjusted ORs are similar, and we reported adjusted ORs. Additionally, adjusted ORs were estimated comparing joint states of the *TGFBR1* and *TGFB1* genotypes by *a priori* categorizing subjects as high, intermediate, and low signalers based on the known functional status of the variant alleles (refs. 10, 24, 25; Fig. 1). Trend statistics were conducted to assess whether risk of breast cancer increases according to this *a priori* categorization. A small *P* of the trend test indicates that breast cancer risk is related to the scoring based on *TGFBR1* and *TGFB1* genotypes. Whether the effects of *TGFBR1* and *TGFB1* on breast cancer differ by age was evaluated by stratified analysis and tests for multiplicative interaction. A small *P* indicates that interaction of age and gene is statistically significant on the multiplicative scale.

Sensitivity analysis was conducted to evaluate the effect of the fact that the exact age of a large number of controls is not known. In the subgroup of those with known exact age, ORs were estimated with or without adjusting age as a continuous variable. In the overall study population, ORs were estimated not only with or without adjusting age as a categorical variable but also under different scenarios by assigning controls with unknown exact age with values of lowest and highest extremes (i.e., all 20 or all 40). Analysis was also conducted for extreme scenarios where all carrier controls were assumed to be 40 years old and all noncarrier controls 20 years old and vice versa. An additional analysis was done to identify possible differences in *TGFBR1* and *TGFB1* allelic frequencies among the various age groups of controls for whom exact age was known. Because a subset of cases had data on tumor prognostic characteristics available, we conducted polytomous logistic regression to calculate ORs by comparing each of the case groups with the total controls with respect to *TGFB1* and *TGFBR1* status.

Results

There were 658 cases and 841 controls genotyped for *TGFB1* and 611 cases and 691 controls for *TGFBR1*. The mean age of cases was significantly higher than that of controls ($P < 0.01$; Table 1). The allelic frequencies of *TGFB1* and *TGFBR1* variants among controls were identical in all age groups.

Transforming growth factor-β signaling assessment. *TGFBR1* and *TGFB1* genotyping was done on 608 cases and 690 controls. There was a significantly lower proportion of high signalers (*TGFB1**CC and *TGFBR1*) among cases (15.1%) than among controls (21.4%; $P = 0.003$). The proportion of intermediate signalers (72.0% versus 68.8%) and low signalers (12.8% versus 9.7%) was similar among cases and controls, respectively. Individuals defined as low TGF-β signalers had a significantly higher risk of breast cancer than those defined as high signalers (OR, 1.69; 95% CI, 1.08-2.66). The results were similar when ethnic status and age were adjusted (Table 2). Breast cancer risk incurred by low signalers was most pronounced among women ages ≥50 years (OR, 2.05; 95% CI, 1.01-4.16; Table 3).

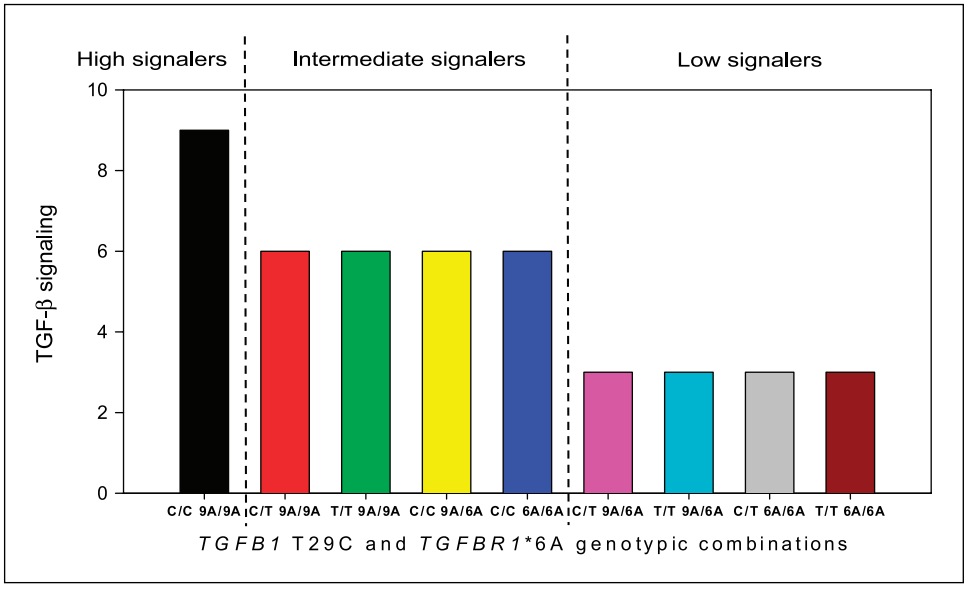

Figure 1. Genotypic combinations of *TGFB1* and *TGFBR1* functional variants and their predicted TGF-β signaling levels. *X axis,* various genotypic combinations of *TGFB1* and *TGFBR1* genotypes. *Y axis,* predicted level of TGF-β signaling based on *in vitro* functional assays and *in vivo* measurements in humans. We hypothesize that carriers of the *TGFB1**CC and *TGFBR1/TGFBR1* genotypes (high baseline TGF-β signaling) have the lowest breast cancer risk and carriers of the *TGFB1**TT or *TGFB1**CT and *TGFBR1**6A genotypes (low baseline TGF-β signaling) have the highest breast cancer risk.

Table 1. Study population

	TGFBR1 study population*			*TGFB1* study population†		
	Cases (*n* = 611), *n* (%)	Controls (*n* = 690), *n* (%)	*P*‡	Cases (*n* = 658), *n* (%)	Controls (*n* = 841), *n* (%)	*P*‡
TGFBR1 genotype						
*9A/*9A	515 (84.3)	612 (88.7)	0.03	—	—	
*9A/*6A	92 (15.1)	77 (11.2)		—	—	
*6A/*6A	4 (0.6)	1 (0.1)		—	—	
TGFB1 genotype						
TT	—	—		200 (30.4)	240 (28.5)	0.23
TC	—	—		339 (51.5)	419 (49.9)	
CC	—	—		119 (18.1)	182 (21.6)	
Age (y)§						
20-40	89 (14.6)	394 (57.1)	<0.01	97 (14.7)	534 (63.5)	<0.01
41-50	166 (27.2)	82 (11.9)		181 (27.5)	84 (10.0)	
51-60	168 (27.5)	110 (15.9)		178 (27.1)	112 (13.3)	
61-70	120 (19.6)	69 (10.0)		127 (19.3)	75 (8.9)	
71+	68 (11.1)	35 (5.1)		75 (11.4)	36 (4.3)	
Mean (SD)§	54.0 (12.7)	55.3 (11.2)		53.9 (12.9)	55.4 (11.1)	
Race						
White	512 (83.8)	541 (78.4)	<0.01	544 (82.7)	649 (77.2)	<0.01
Black	44 (7.2)	43 (6.2)		53 (8.1)	51 (6.1)	
Hispanic	25 (4.1)	80 (11.6)		27 (4.1)	110 (13.1)	
Asian	18 (3.0)	22 (3.2)		20 (3.0)	26 (3.1)	
Unknown	12 (1.9)	4 (0.6)		14 (2.1)	5 (0.5)	

*The exact age was not known for 360 controls in the lowest age category (20-40 years).
†The exact age was not known for 500 controls in the lowest age category (20-40 years).
‡*P* for χ^2 or Fisher's exact test (comparing proportions).
§Average age for controls was calculated based on those with exact age available.

***TGFBR1**6A and breast cancer risk.** *TGFBR1**6A allelic frequency was significantly higher among cases (0.082) than among controls (0.057; *P* = 0.03; Table 1). Breast cancer risk was significantly higher for *TGFBR1**6A carriers under both a dominant (OR, 1.50; 95% CI, 1.07-2.07) and a recessive (OR, 1.46; 95% CI, 1.04-2.06) model (Table 2). Breast cancer risk for *TGFBR1**6A carriers ages >50 years was almost twice higher (OR, 2.20; 95% CI, 1.25-3.87) than among women ages ≤50 years (OR, 1.18; 95% CI, 0.75-1.84; Table 3). To assess the possibility that the effect of *TGFBR1**6A on breast cancer risk is nullified by the *TGFB1* T29C variant, we restricted the analysis to *TGFB1**CC and *TGFB1**TC carriers. Breast cancer risk remained significantly higher for *TGFBR1**6A carriers (OR, 1.58; 95% CI, 1.04-2.39).

***TGFB1**CC and breast cancer risk.** About 18.1% of cases and 21.6% of controls carried the *TGFB1**CC genotype (Table 1). There was a trend toward decreased breast cancer risk for carriers of the *TGFB1**CC genotype, but it did not reach formal significance (OR, 0.89; 95% CI, 0.63-1.21). The results were similar under either an additive or a recessive model. Adjustment for age and ethnic status did not change the results (Table 2). The observed trend toward decreased breast cancer for *TGFBR1**CC carriers was similar for women above or below age 50 years (Table 3).

Estrogen receptor, progesterone receptor, and stage at diagnosis. Information on ER and PR status and stage at diagnosis was available for 152 patients. There was no association between *TGFBR1**6A and ER and PR status and stage at diagnosis (Table 4). On the contrary, both *TGFB1**CT and *TGFB1**CC were more likely to have advanced stage as assessed by the presence of lymph node metastasis and stage III or IV at diagnosis (Table 4). Patients carrying the *TGFB1**CC genotype were more likely to have ER-negative and PR-negative tumors (Table 4).

Discussion

TGFBR1 and *TGFB1* map to different chromosomes but are essential components of the same signaling pathway. We have hypothesized previously that a combined assesment of the functionally relevant common variants may help better characterize cancer risk and may predict disease aggressiveness (31, 32). In a previous study, *TGFRB1**6A was associated with increased breast cancer risk among women with a mean age at diagnosis of 38 years. These women had been selected based on age at onset under 40 years, a family member with breast cancer irrespective of age at diagnosis, or bilated breast cancer irrespective of family history or age at disease onset (26). The mean age at diagnosis of breast cancer cases presented in this study is similar to the mean age at diagnosis of breast cancer in the general population. Our findings of a significant association of *TGFBR1**6A with breast cancer suggest that the effect of *TGFBR1**6A can be extended beyond familial, early-onset, and bilateral breast cancer. The data add to the growing body of evidence that *TGFBR1**6A may act as breast tumor susceptibility gene (26–28). Interestingly, a subset analysis of our results indicate that *TGFBR1**6A effects are most prominent among women ages >50 years. The hypomorphic

*TGFBR1**6A allele was not associated with more aggressive tumor behavior, which is in agreement with a recent report indicating that transgenic mice with decreased TGF-β signaling have an increased incidence of breast cancer but a reduced incidence of breast cancer metastases (8).

TGFB1 exerts pleiotropic effects in the oncogenesis of breast cancer in a contextual manner (i.e., it suppresses tumorigenesis at an early stage by direct inhibition of angiogenesis and tumor cell growth). However, overproduction of *TGFB1* by advanced tumors may accelerate disease progression through indirect stimulation of angiogenesis and immune suppression (33). Evidence also exists that TGF-β signaling contributes to the metastasis in breast cancer (34, 35) and that TGF-β signaling blockade inhibits mammary tumor cell viability, migration, and metastasis (36). The contribution of TGF-β to the malignant phenotype of breast cancer cells is particularly prominent in cell lines that retain the TGF-β signal transduction system but have lost TGF-β-induced growth inhibition. Such is the case in breast cancer cells with a hyperactive Ras pathway (35, 37, 38).

Both stromal and epithelial cells from *TGFB1**CC are likely surrounded by more *TGFB1* than cells from *TGFB1**TT individuals given the fact that the *TGFB1* Leu^{10}Pro (T29C) results in higher *in vitro TGFB1* secretion and the *TGFB1**CC genotype is associated with higher *TGFB1* serum levels (10, 39). In our report, the higher frequency of lymph node metastases and the more advanced stage at diagnosis in carriers of the *TGFB1**CC and *TGFB1**CT genotypes are in agreement with these laboratory findings and with the recent report of a significantly reduced 5-year survival among patients with breast cancer that carry the *TGFB1**CC and *TGFB1**CT genotypes (40).

Taken together, these results are additional evidence that increased TGF-β signaling due to a naturally occurring variant is associated with a more aggressive tumor behavior. If confirmed in larger studies, *TGFB1* genotype may become a new prognostic marker for women diagnosed with breast cancer and the TGF-β signaling pathway may become a molecular target for therapeutic interventions. The additional finding of an increased proportion of ER-negative and PR-negative tumors among

Table 2. Adjusted ORs of breast cancer by *TGFBR1*, *TGFB1* genotypes, and TGF-β predicted signaling status

Gene/genotype	*n* (cases/controls)	OR (95% CI) for breast cancer risk*	OR (95% CI) for breast cancer risk†
TGFBR1			
Dominant model			
9A/9A	515/612	1.00	1.00
9A/6A or 6A/6A	96/78	1.46 (1.06-2.02)‡	1.50 (1.07-2.11)‡
Additive model			
9A/9A	515/612	1.00	1.00
9A/6A	92/77	1.42 (1.03-1.96)‡	1.46 (1.04-2.06)‡
6A/6A	4/1	4.75 (0.53-42.66)	4.40 (0.48-40.52)
Recessive model			
9A/9A or 9A/6A	607/689	1.00	1.00
6A/6A	4/1	4.54 (0.51-40.73)	4.19 (0.46-38.48)
TGFB1			
Dominant model			
TT	200/240	1.00	1.00
TC/CC	458/601	0.91 (0.73-1.14)	0.98 (0.77-1.25)
Additive model			
TT	200/240	1.00	1.00
TC	339/419	0.97 (0.78-1.23)	1.02 (0.79-1.32)
CC	119/182	0.79 (0.58-1.06)	0.89 (0.63-1.21)
Recessive model			
TC or TT	539/659	1.00	1.00
CC	119/182	0.80 (0.62-1.03)	0.86 (0.65-1.14)
TGF-β predicted signaling status			
High signalers			
CC/9A9A	92/148	1.00	1.00
Intermediate signalers			
TT/9A9A, CC/9A6A, CC/6A6A, or TC/9A9A	438/475	1.48 (1.11-1.98)‡	1.27 (0.93-1.74)
Low signalers			
TT/6A6A, TT/9A6A, TC/9A6A, or TC/6A6A	78/67	1.87 (1.23-2.84)‡	1.69 (1.08-2.66)‡
P for trend		0.02‡	0.02‡

*Crude ORs.
†ORs were adjusted for ethnic groups and age as categorical variables.
‡$P < 0.05$.

carriers of the *TGFB1**CC genotype points to the fact that increased TGF-β signaling results in more aggressive tumor behavior in the absence of ER and PR overexpression.

This may explain the conflicting results of the Ziv et al. (10), Dunning et al. (15), and Le Marchand et al. (18) studies. Among premenopausal women, the proportion of tumors overexpressing ER and PR is lower than among postmenopausal women. Hence, the growth of ER-negative and PR-negative tumors from premenopausal women may benefit from increased *TGFB1* levels, whereas growth of the predominantly ER-positive and PR-positive tumors from postmenopausal women may not be similarly affected by higher *TGFB1* levels. Subjects enrolled in the Ziv et al. study were ages ≥65 years with a mean age of 70 years. Subjects in the Le Marchand et al. study were predominantly postmenopausal with a mean age of 63 years. Conversely, the Dunning et al. study reported a combined analysis of three case-control studies, including breast cancer cases with a mean age of 50 years, and showed that *TGFB1**CC and *TGFB1**CT carriers had a slight but significant increased risk of invasive breast cancer. Although not significant, our results show a trend similar to that of the former study. The mean age of our cases was 5 years higher than the Dunning study, 15 and 10 years lower than the Ziv et al. and Le Marchand et al. studies, respectively. Given that the mean age at menopause in the general population is 50 years, the proportion of postmenopausal women in our population is likely higher than in the Dunning et al. study and may explain the nonsignificant trend toward a *TGFB1**CC protective effect. The findings that differences in TGF-β signaling effects are more pronounced among postmenopausal women further support this explanation. However, another plausible explanation for these conflicting results is the functional interaction between *TGFBR1**6A and the *TGFB1* T29C polymorphism shown in this report.

Our study has several limitations. Due to destruction of personal identifiers, we only had exact age information in a subset of our controls. For the remainder of controls, only the age range was available. Furthermore, cases and controls were not matched for variables known to be associated with breast cancer. However, we did analyses controlling for age. We also conducted sensitivity analyses using hypothetical models to show that the effect of the lack of detailed age information in a portion of our samples was negligible. It is possible that age differences in cases and controls affected the allele frequencies observed. Nonetheless, this would be expected to create a bias toward the null hypothesis because it would overestimate the deleterious allele frequency in controls given that a fraction of younger women who would have developed breast cancer were not removed from the control group. Thus, all the younger mean age of controls could have resulted in a bias toward the null hypothesis, resulting in a weaker association. In addition, an additional limitation of our study is the lack of analysis of other *TGFB1* polymorphisms, some of which may modify *TGFB1* circulating levels (41).

Another potential drawback is the lack of complete pathologic information in our cases. Due to the destruction of the personal identifiers, we could not retrospectively collect data on ER/PR and the lymph node status in a subset of patients. However, taking into account the limited number of cases with complete histopathologic information, our significant results in the advanced stage and ER-negative population merit further investigation in a large prospective study.

Table 3. Adjusted ORs of breast cancer by age groups (>50 or ≤50 years)

Gene/age groups	Genotypes	*n* (cases/controls)	OR (95% CI)*	*P* for testing multiplicative interaction
TGFBR1				
Age ≤50 y	9A/9A	217/417	1.00	0.09
	9A/6A or 6A/6A	38/59	1.18 (0.75-1.84)	
Age >50 y	9A/9A	298/195	1.00	
	9A/6A or 6A/6A	58/19	2.20 (1.25-3.87)†	
TGFB1				
Age ≤50 y	TT or TC	223/477	1.00	0.99
	CC	55/141	0.85 (0.57-1.29)	
Age >50 y	TT or TC	316/182	1.00	
	CC	64/41	0.87 (0.56-1.35)	
Joint status of *TGFBR1* and *TGFB1*‡				
Age ≤50 y	High signalers	44/112	1.00	0.65
	Intermediate signalers	177/314	1.33 (0.84-2.10)	
	Low signalers	32/50	1.49 (0.77-2.87)	
	P for trend		0.19	
Age >50 y	High signalers	48/36	1.00	
	Intermediate signalers	261/161	1.23 (0.76-1.98)	
	Low signalers	46/17	2.05 (1.01-4.16)†	
	P for trend		0.06	

*ORs were adjusted for ethnic groups and age within age strata.
†$P < 0.05$.
‡Low signalers were those with TT/6A6A, TT/9A6A, TC/9A6A, or TC/6A6A; intermediate signalers were those with TT/9A9A, CC/9A6A, CC/6A6A, or TC/9A9A; and high signalers were those with CC/9A9A.

Table 4. Association of *TGFB1* and *TGFBR1* genotypes with ER, PR, and stage at diagnosis

	TGFB1 genotype, adjusted OR (95% CI)				*TGFBR1* genotype, adjusted OR (95% CI)		
	n (cases/controls)	TT	TC	CC	*n* (cases/controls)	9A/9A	9A/6A or 6A/6A
ER status							
ER positive vs controls	72/841	1.00	1.27 (0.70-2.29)	0.94 (0.44-2.03)	56/690	1.00	1.51 (0.67-3.42)
ER negative vs controls	27/841	1.00	0.26 (0.10-0.64)*	0.36 (0.12-1.10)	21/690	1.00	2.07 (0.67-6.44)
PR status							
PR positive vs controls	53/841	1.00	1.10 (0.59-2.13)	0.90 (0.39-2.11)	41/690	1.00	1.54 (0.61-3.85)
PR negative vs controls	44/841	1.00	0.55 (0.28-1.09)	0.51 (0.21-1.28)	35/690	1.00	1.86 (0.73-4.76)
Joint status of ER and PR							
Both are positive vs controls	51/841	1.00	1.37 (0.62-3.05)	0.87 (0.36-2.11)	39/690	1.00	1.65 (0.65-4.17)
Either is negative vs controls	21/841	1.00	0.89 (0.30-2.65)	1.44 (0.40-5.16)	18/690	1.00	1.13 (0.25-5.09)
Both are negative vs controls	25/841	1.00	0.97 (0.25-3.84)	0.28 (0.08-1.00)*	19/690	1.00	2.36 (0.75-7.44)
Stage							
Stage I/II vs controls	117/841	1.00	0.83 (0.53-1.30)	0.62 (0.34-1.14)	90/690	1.00	1.61 (0.85-3.06)
Stage III/IV vs controls	30/841	1.00	0.46 (0.21-1.00)*	0.26 (0.07-0.93)*	24/690	1.00	0.81 (0.18-3.57)
Lymph nodes							
0	48/841	1.00	1.34 (0.64-2.80)	1.47 (0.62-3.45)	32/690	1.00	1.28 (0.43-3.82)
≥1	44/841	1.00	0.44 (0.22-0.86)*	0.35 (0.14-0.90)*	40/690	1.00	1.57 (0.63-3.93)

*$P < 0.05$.

The results presented here reflect a nonselected population of patients with breast cancer. *TGFBR1**6A carriers (15.7% of cases and 11.3% of controls) and high TGF-β signalers (15.1% of cases and 21.4% of controls) make up >30% of the population of both cases and controls. This is evidence that variants of the TGF-β signaling pathway are likely to modify breast cancer risk in a large subset of the population. Studies are in progress to determine the contribution of the TGF-β signaling pathway variants to familial and sporadic breast cancer.

The combined analysis of high versus low signalers is the first indication in humans that altered TGF-β signaling modifies breast cancer risk. It identifies high signalers as a subgroup of individuals with increased TGF-β signaling and decreased breast cancer risk, although, taken separately, the proportion of *TGFB1**CC and *TGFBR1/TGFBR1* carriers was similar among cases and controls. It also shows that combination of naturally occurring TGF-β signaling pathway variants probably result in functional differences large enough *in vivo* to modify breast cancer risk, similar to what has been shown recently in transgenic mouse models (8). These results warrant validation in well-designed case-control studies to explore further the role of TGF-β signaling pathway variants with respect to breast cancer risk and outcome.

Acknowledgments

Received 8/17/2004; revised 12/15/2004; accepted 2/3/2005.

Grant support: National Cancer Institute grants CA90386 and CA89018, Avon Breast Cancer Career Development Award (B. Pasche), Mander Foundation, Lomangino and Weissenbach-Southworth Family Research Funds, Lymphoma Foundation, and New York Cancer Project administered and funded by AMDeC Foundation, Inc.

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