Int7G24A Variant of Transforming Growth Factor-β Receptor Type I Is Associated with Invasive Breast Cancer

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Abstract

Purpose: The transforming growth factor-β (TGF-β) signaling pathway has been frequently implicated in breast cancer. An intronic variant (*Int7G24A*) of TGF-β receptor type I (*TGFBR1*) is associated with kidney and bladder cancers in our recent study. We hypothesize that this germline variant may be involved in development and progression of breast cancer.

Experimental Design: Case-control studies were designed from archived paraffin-embedded tissue specimens from the same geographic area with a homogenous ethnic population. We analyzed 223 patients (25 with preinvasive tumors and 198 with invasive and metastatic breast cancers) and 153 noncancer controls. The *Int7G24A* was identified by PCR-RFLP. Another germline deletion (*TGFBR1***6A*) and somatic mutations in the *TGFBR1* were also analyzed by PCR and single-strand conformational polymorphism.

Results: The *Int7G24A* allele was evident in 32% of patients with preinvasive neoplasms and 48% of patients with invasive breast cancers compared with 26% controls ($P = 0.00008$). In addition, 11 (5.6%) homozygous *Int7G24A* carriers were found in patients with invasive breast cancers, whereas only 3 (2%) homozygous carriers were found in the control group. The *TGFBR1***6A* allele was not significantly associated with breast cancer patients and only one somatic mutation was found in 71 breast cancers.

Conclusion: These data suggest that the germline *Int7G24A* variant may represent a risk factor for invasive breast cancer and a marker for breast cancer progression. A separate study with a larger sample size is warranted to validate the association of the *Int7G24A* with human breast cancer.

Breast cancer is the most common form of malignancy among women in the United States with 211,240 new cases and 40,410 deaths from the disease anticipated in 2005 (1). High-penetrance germline mutations in *BRCA1* and *BRCA2* have been linked to familial breast cancer susceptibility (2, 3). Polymorphisms or genetic variations in low-penetrance genes are suspected to play a role in development and progression of sporadic breast cancers (4, 5). We have now found a genetic variant in the transforming growth factor-β (TGF-β) receptor type I (*TGFBR1*) gene that is significantly associated with patients having invasive and metastatic breast cancers.

TGF-β is a pleiotropic growth factor expressed by many cell lines and tissue types (6–9). Several important biological events are governed by this growth factor, such as cell growth, tissue differentiation, production and degradation of extracellular matrix, morphogenesis, and apoptosis (10–16). The TGF-β signal is transduced by a membrane-bound serine/threonine kinase receptor complex, including TGF-β type I and II receptors. Activated TGFBR1 phosphorylates intermediates, such as SMAD2 and SMAD3, which in turn heterotrimerize with SMAD4. These SMAD complexes translocate to the nucleus, bind to DNA in a sequence-specific manner, and regulate the transcription of target genes involved in many cellular functions (17). TGFBR1 has a rate-limiting role in the signaling pathway. Any quantitative and qualitative changes in TGFBR1 will be expected to affect TGF-β-mediated growth inhibition in normal epithelial cells. Tumor cells frequently lose responsiveness to TGF-β-mediated growth inhibition due to disruption in the TGF-β signaling pathway (18–20). Increased expression of TGF-β associated with advanced stages of human breast cancer and poor prognosis are usually the outcomes for these patients (21–24). Somatic and germline mutations in the components of the TGF-β signaling pathway have been suspected to play a role in human cancer development and progression. While failing to find somatic mutations of the *TGFBR1* gene in breast cancer, we did find a strong association between a germline variant in *TGFBR1* and the patients having advanced breast cancer. This finding suggests that this germline variant may have the potential to be an important genetic marker for susceptibility to invasive breast cancer.

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Materials and Methods

Subjects. Archived paraffin-embedded surgical specimens from patients (diagnosed during 1985-1995) with breast cancer and noncancer controls were selected from the tissue bank at Wood Hudson Cancer Research Laboratory. Tissues were obtained at surgery at St. Elizabeth Medical Center (Covington/Edgewood, KY). Both cancer and control patients were from the same geographic area. Institutional review board approval for this study was received from St. Elizabeth Medical Center. Board-certified pathologists (L.D. and J.P.) reviewed a H&E-stained section from each block of breast tissue to confirm the histopathology. We analyzed 223 cases with a diagnosis of ductal carcinoma *in situ* (DCIS), infiltrating ductal carcinoma (IDC)/infiltrating lobular carcinoma (ILC), and metastatic breast cancer and 153 noncancer female controls. The ethnic background and age of each cancer patient was determined from the Kentucky Tumor Registry. Among the 223 patients with breast cancer studied, 2 (0.89%) were African American and 221 (99.1%) patients were Caucasians. The average age of cancer patients at diagnosis was 62 ± 13 years (median age, 65 years; range, 34-88 years). Nontumor control specimens were obtained from excess tissues removed during surgery from patients without a diagnosis of cancer (mean age, 62 ± 12.4 years; median age, 62 years; range, 23-92 years). Information on ethnic status was not available from the pathology reports accompanying the specimens. However, the 1990 U.S. Census figures for the geographic area served by St. Elizabeth Medical Center show that 97% are Caucasian, <2% are African American, and 1% of population are from other ethnic backgrounds. Most of the control specimens were tonsils, hernia sac, shavings, appendix, and noncancer biopsies from different organs.

Isolation of DNA from paraffin-embedded tissues. A single 8- to 10-μm-thick paraffin section from each sample block was deparaffinized (three washes with xylene for 30 minutes each) and rehydrated in decreasing concentrations of alcohol (25). For identification of the *Int7G24A* and *TGFBR1*6A* alleles of *TGFBR1* in cancer patients, a block without tumor was picked to extract constitutive DNA. For somatic mutation analysis, we selected blocks with tumor and used tissue microdissection to obtain tumor tissues for DNA extraction as described previously (25). DNA was extracted with Instagene chelex matrix solution containing 60 μg proteinase K in a shaking incubator at 37°C overnight according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Samples were boiled for 10 minutes, vortexed, and centrifuged at 7,000 × *g* for 5 minutes. The supernatant (2-8 μL) was used for PCR amplification.

Annealing control primer-PCR-RFLP. An annealing control primer system was designed to improve specificity of PCR amplification (26). The unique linker of five inosines was included in the primer design. The sequences of annealing control primer primers for *Int7G24A* allele in the *TGFBR1* gene were as follows: forward primer 5′-GTCTACCAGG-CATTCGCTTCATIIIIIGCTTAGTATTCTG-3′ and reverse primer 5′-GCTTGACTACGATACTGTGCGAIIIIICAATTCTTGAACA-3′. The PCR was done in a volume of 20 μL containing 500 nmol/L forward and reverse primers and 2 units HotStarTaq DNA polymerase (Qiagen, Valencia, CA). After an initial 15-minute denaturation at 95°C, PCR was conducted in two stages: 5 cycles of 94°C for 1 minute, 50°C for 1 minute, 72°C for 3 minutes followed by 35 cycles of 94°C for 1 minute, 65°C for 1 minute, 72°C for 1 minute with the final extension at 72°C for 5 minutes. We used *Bsr*I digestion of the annealing control primer-PCR-amplified intron 7 fragment for identification of the *Int7G24A* allele. PCR (20 μL) was added with 2 μL of 10× digestion buffer containing 2.5 units *Bsr*I. After 1.5-hour incubation at 65°C, the mixture was loaded on a 7% acrylamide gel to resolve the bands from the *Bsr*I digestion to obtain the allelic status. Negative (H_2O) and positive [known wild-type (WT) and variant carriers] controls were included throughout the experiment for quality control. The identified variant carriers had all been repeated at least twice for accuracy.

PCR and single-strand conformational polymorphism. A total of 71 breast cancers were first screened for somatic mutations in the *TGFBR1* gene by a "cold" PCR-single-strand conformational polymorphism (SSCP; ref. 27). Primer sequences for PCR amplification of the nine exons of the *TGFBR1* gene were described previously (18). The PCR was done in a volume of 20 μL containing 500 nmol/L forward and reverse primers and 2 units HotStarTaq DNA polymerase. After an initial 15-minute denaturation at 95°C, PCR was run for 40 cycles of 94°C for 30 seconds, 55°C for 40 seconds, 72°C for 1 minute followed by a 5-minute final extension at 72°C. SSCP analysis was done as described previously (28).

DNA sequencing. Suspect bands detected in cold SSCP were verified by an additional, independent PCR-SSCP analysis. DNA from adjacent normal tissue was included in this process to confirm whether the shift was somatic or germline. The confirmed shifts in the SSCP gel were excised for sequencing. After reamplification of the shifted bands, the PCR products were purified using the QIAquick PCR purification kit (Qiagen) and directly sequenced by the same forward or reverse primers used in the original PCR amplification. The sequencing was done in an automated sequencer (model 377; PE Applied Biosystems, Foster City, CA) at the DNA Core Facility of the University of Cincinnati (Cincinnati, OH).

Statistical methods. A χ^2 test or Fisher's exact test was used to determine the significance of the differences in *Int7G24A* carrier frequencies between patients with breast cancer and control groups. Tissue specimens were organized as patients with DCIS, IDC/ILC, or metastases and noncancer control groups. Logistic regression analysis was used to estimate the associations between *Int7G24A* genotypes and risk of breast cancers by calculating the odds ratio and their 95% confidence intervals. All statistical analyses were done with Statistical Analysis System software version 8 (SAS Institute, Cary, NC).

Results

Int7G24A *allele of* TGFBR1 *in breast cancer patients and noncancer controls.* A genetic G-to-A variant within intron 7 of the *TGFBR1* gene (*Int7G24A*) was identified as a germline variant previously (29). *Int7G24A* was associated with patients having kidney and bladder cancers in our recent study (30). To test whether this variant is associated with patients having breast cancer, we conducted a genetic screening of constitutive genomic DNA samples from 117 patients with invasive breast carcinomas, 81 patients with metastatic breast cancers, and 25 patients with DCIS. Additionally, 28 patients with atypical ductal hyperplasia (ADH) were also analyzed for status of the *Int7G24A* allele. A total of 153 female control specimens derived from age-matched individuals (reflecting 97% Caucasians and 2% African American according to the 1990 U.S. Census for this geographic area), who were not diagnosed with any cancer for a period of as many as 18 years, were also included in the experiment for comparison.

We applied annealing control primer-PCR techniques to improve specificity for amplifying the genomic DNA extracted from paraffin-embedded tissue samples. Annealing control primer-PCR primer requires a linker (polydeoxyinosine) between the 5′ universal sequence and 3′ specific target sequences to form a relatively long annealing control primer (see Materials and Methods). The *Int7G24A* fragment was successfully amplified following DNA extraction from sections of the paraffin-embedded tissues. The amplified intronic fragments were further digested in the same PCR tubes by *Bsr*I restriction enzyme (Fig. 1). The digested DNA samples were loaded on 6% acrylamide gel and stained with ethidium bromide. Two lower molecular weight bands represented the WT that has been cut by the *Bsr*I, whereas the high molecular weight band represents the variant allele, wherein the *Bsr*I restriction site has been eliminated.

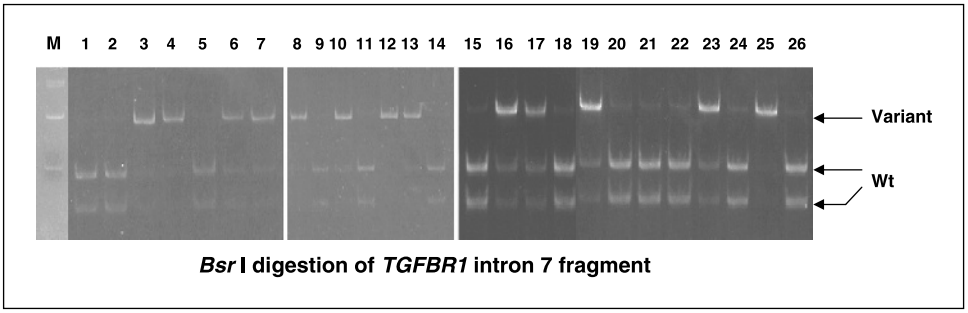

Fig. 1. Representative photographs of PCR-RFLP analysis of *Int7G24A* allele of the *TGFBR1* gene in patients with breast cancers. Twenty-six patients with breast cancers, including 4 DCIS, 13 IDC, and 9 metastases, displayed either the WT bands (two bottom bands cut by *Bsr*I restriction enzyme) or the variant band (uncut top band). Half of the cases had the heterozygous variant allele.

Forty-seven percent (55 of 117) of patients with invasive breast cancers and 49% (40 of 81) of patients with metastatic breast cancers displayed the variant allele, whereas only 26% (40 of 153) of the noncancer controls showed this variant allele (Table 1). The difference in variant carriers between controls and patients with invasive and metastatic breast cancers was highly statistically significant (χ^2 test, $P = 0.00003$). Eleven homozygous variant carriers were from patients with invasive and metastatic tumors [6 cases (5.1%) and 5 cases (6.2%), respectively]. Homozygous variant carriers were not found in 25 patients with DCIS and 28 patients with ADH. Only 3 (2.0%) homozygous variant carriers were found in a total of 153 noncancer control populations.

Eight of the 25 (32%) DCIS patients examined were heterozygous carriers of this variant (Table 1). Nine (32%) variant carriers were found in 28 patients with ADH of the breast. Although DCIS and ADH patients displayed higher frequencies of variant carrier than noncancer controls, the difference was not statistically significant. The difference between patients with preinvasive neoplasms DCIS/ADH and invasive neoplasms IDC/ILC/metastases was significant (χ^2 test, $P < 0.04$). The trend of increasing odds ratios in patients with DCIS, IDC/ILC, and metastasis was also significant ($P < 0.0001$; Table 1).

TGFBR1*6A *allele in breast cancer patients and controls.* *TGFBR1*6A*, a germline deletion variant in the coding region of the *TGFBR1* gene, was found to be a possible susceptibility allele to cancer development in our previous studies and in studies of others (31, 32). To determine a possible association of this deletion allele with breast cancer, we used the same case-control specimens to screen for the *TGFBR1*6A* carriers. We applied PCR-SSCP and successfully genotyped 115 patients with breast cancer and 129 nontumor controls (Fig. 2A). This allele was evident in 22% of the patients with invasive breast cancer and 15% of the nontumor controls (Table 2). None of 11 patients with DCIS had the *TGFBR1*6A* allele. Although the patients with invasive breast cancer had higher frequency of *TGFBR1*6A* allele, the difference between the two groups was not statistically significant.

Somatic mutation of the* TGFBR1 *gene in an invasive breast cancer. Before the case-control study, 71 tumor specimens, including DCIS, IDC, and metastatic breast cancers, were screened for somatic mutations in the nine exons of the *TGFBR1* gene. One somatic mutation was found in a 70-year-old patient with a diagnosis of IDC, who did not carry either the *TGFBR1*6A* allele or the *Int7G24A* allele. The mutation was detected by the PCR-SSCP technique (Fig. 2B). After DNA sequencing, the shifted band was found to be a G-to-A missense mutation that represented an amino acid change from valine to isoleucine at codon 153 (Fig. 2C). This mutation is located in the cytoplasmic domain of the receptor, very close to the transmembrane domain. Because of the low occurrence of somatic mutations in the *TGFBR1* gene in the breast cancers examined (1.4%), the additional 160 breast tumors were not examined for somatic mutation.

Discussion

TGF-β plays a critical role in the development and functional differentiation of the mammary gland (33, 34) and in mammary gland tumorigenesis (35, 36). Carcinomas of the human

Table 1. *Int7G24A* genotypes in breast cancer and control specimens

Specimens	Total cases	WT (%)	*Int7G24A* genotype*			Odds ratio (95% confidence interval)†	*P* (χ^2 test)
			Het V (%)	Homo V (%)	Combined (%)		
Nontumor control (female)	153	113 (73.9)	37 (24.2)	3 (2.0)	40 (26.1)		
ADH	28	19 (67.9)	9 (32.1)	0	9 (32.1)	1.34 (0.56-3.20)	0.5113
DCIS	25	17 (68.0)	8 (32.0)	0	8 (32.0)	1.33 (0.53-3.32)	0.5407
IDC/ILC	117	62 (53.0)	49 (41.9)	6 (5.1)	55 (47.0)	2.51 (1.50-4.18)	0.0004
Metastases	81	41 (50.6)	35 (43.2)	5 (6.2)	40 (49.4)	2.76 (1.57-4.85)	0.0004
IDC/ILC/metastases	198	103 (52.0)	84 (42.4)	11 (5.6)	95 (48.0)	2.61 (1.65-4.11)	<0.0001
Total cancer cases	223	120 (53.8)	92 (41.3)	11 (4.9)	103 (46.2)	2.42 (1.55-3.79)	<0.0001

*G-to-A intronic variant at 24-bp position of intron 7 of the *TGFBR1* gene; Het V, heterozygous variant carrier; Homo V, homozygous variant carrier.
†Trend for increasing odds ratios with DCIS, IDC/ILC, and metastases ($P < 0.0001$).

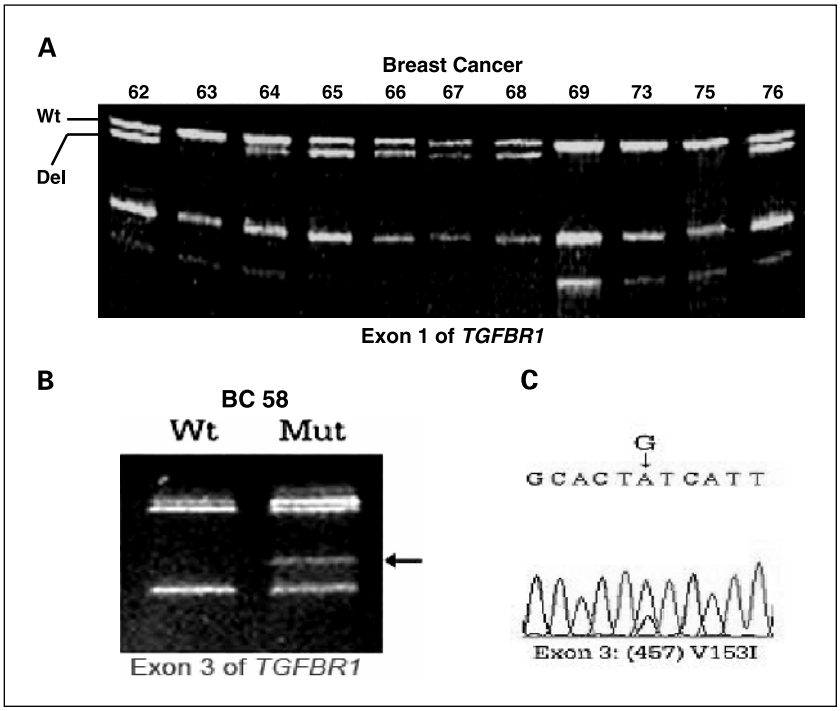

Fig. 2. PCR-SSCP analysis of the *TGFBR1* gene in patients with breast cancers. *A,* WT and del(GGC)$_3$ (*Del*; *TGFBR1*6A*) alleles in exon 1 of *TGFBR1*. Six patients with breast cancer (62, 64-68, and 76) are heterozygous for Del allele. The rest of the patients (63, 69, 73, and 75) are homozygous for WT allele. *B,* WT and mutant (*Mut*) bands were revealed by PCR-SSCP analysis of breast cancers. *Arrow,* shifted band representing somatic change. *C,* a G-to-A missense mutation was found by automated sequencing, a exchange from valine to isoleucine.

breast are considered to be sensitive to TGF-β at early stages and become refractory to TGF-β at late stages (37, 38). In fact, TGF-β switches from a suppressor to prometastatic factor in a model of breast cancer progression (38). However, the genetic and epigenetic changes that underlie this transition during cancer development and progression remain unclear. Reduced expression of TGFBR2 and increased secretion of TGF-β have been reported in advanced breast cancer and implicated in loss of sensitivity to TGF-β-mediated growth inhibition during breast cancer progression (22–24, 39). We now find that an intronic variant of *TGFBR1* is significantly associated with patients having invasive and metastatic breast cancers.

The *Int7G24A* variant was analyzed in this case-control study that included patients with DCIS, IDC/ILC, and metastatic breast cancers and age-matched female controls. As summarized in Table 1, patients with either IDC/ILC or metastases were significantly associated with the variant allele (47% patients with IDC/ILC and 49% metastases versus 26% controls; $P < 0.0004$). Patients with DCIS had less frequent carriers of the *Int7G24A* allele than patients with IDC/ILC. The incidence of the variant allele in patients with DCIS was only slightly increased over the variant frequency in the control population (32% of patients with DCIS versus 26% of controls; $P = 0.54$). The incidences of the variant in DCIS and ADH patients were identical. However, the odds ratios showed a significant trend for the association of the *Int7G24A* with progression from DCIS to metastatic breast cancer ($P < 0.0001$).

The strong association of this variant with patients having advanced breast cancer implies that the variant allele imparts modifications in the signaling pathway contributing to deregulation of the TGFBR1 signaling axis during breast cancer development and progression. In support of this notion, 11 (5.6%) homozygous carriers of the variant were found exclusively in patients with invasive and metastatic breast cancers but not in patients with DCIS and ADH, and only 3 (2%) homozygous carriers of the variant were present in a total of 153 noncancer control individuals. This is consistent with our hypothesis that the variant allele could predispose the carrier to breast tumor progression. A similar conclusion was made from studies of TGFBR2 (39).

Table 2. *TGFBR1*6A* allele in control and breast cancer specimens

Specimens	Total cases	WT	*TGFBR1*6A**			Odds ratio (95% confidence interval)	P (χ^2 test)
			Het	Homo	Combined (%)†		
Nontumor control (female)	129	111	18	1	19 (14.7)		
DCIS	11	11	0	0	0 (0)		
IDC/ILC/metastases	104	81	23	0	23 (22.1)	1.67 (0.85-3.25)	0.137

*Germline deletion of three alanine residues resulting in *TGFBR1*6A* allele.
†Combined heterozygous and homozygous *TGFBR1*6A* allele.

The functional role of this *TGFBR1* variant has yet to be discovered. Published studies linking mutations close to the intron-exon boundaries in *BRCA1* (40), *BRCA2* (41), *TP53* (42, 43), and the androgen receptor gene (44) indicate that these changes either affected transcription or altered RNA splicing. For example, a G-to-T transversion in position +5 of the donor splice site in intron 6 of the androgen receptor gene was found to affect RNA splicing and contributed to partial androgen insensitivity syndrome (44). In a study of a series of 40 patients and families with germline *TP53* mutations, 18% of the mutations in *TP53* affected RNA splicing (43). Most of the intronic mutations affecting RNA splicing are located at the 5′ donor site of the intron. The variant reported in the present study resides in the +24 position of the donor splice site. Whether this variant will affect splicing awaits further study. Although we will not rule out the possibility that *Int7G24A* is a marker representing a *TGFBR1* haplotype, no statistically significant difference was reported in a haplotype analysis of *TGFBR1* in a recent report by Jin et al. (45).

Zhang et al. reported that homozygous but not heterozygous carriers of this same variant are at increased risk of developing non–small cell lung cancer (46). In our recent study of renal cell carcinoma and transitional cell carcinoma of the urinary bladder, a strong association of both the heterozygous and the homozygous *Int7G24A* allele with susceptibility to renal cell carcinoma and transitional cell carcinoma was identified when compared with age-matched nontumor controls (30). In the study of patients with kidney and bladder cancers, we did not find any significant differences between two age groups (≤61 and >61 years), high and low tumor grades, and male and female carriers with and without this genetic variant. Therefore, the association of the *Int7G24A* with renal cell carcinoma and transitional cell carcinoma may suggest that it contributes to cancer susceptibility rather than to tumor progression. However, in the present study of breast cancer, the variant allele is associated only with invasive and metastatic breast cancer. Further studies of additional DCIS patients will address the question of whether the variant allele carriers in DCIS patients have an increased risk of progression to invasive breast cancer.

We and others reported previously an in-frame germline deletion variant (*TGFBR1*6A*) in the *TGFBR1* gene, resulting in loss of three alanine residues in the receptor (31, 32). *TGFBR1*6A* has been implicated as a tumor susceptibility allele both in our studies and in those of others (29, 32, 47–49). Kaklamani et al. recently reported that *TGFBR1*6A* [del$(GGC)_3$] was associated with a significantly increased risk for breast cancer (47). In their study, 15.7% of the cases harbored the *TGFBR1*6A* allele compared with 11.3% of the controls (P = 0.03). We now find that 22.1% of the cases of invasive breast cancer versus 14.7% of controls are carriers of this same deletion allele (Table 2). Although the 7.4% difference between cases and controls in our study is higher than the 4.4% difference of cases over controls in their study, our results were not statistically significant due to a small sample size. However, both studies suggest that the deletion allele of *TGFBR1* may play a role in susceptibility to breast cancer. Recently, Jin et al. reported that *TGFBR1*6A* allele is not significantly associated with breast cancer in Finnish and Polish populations (45). An independent study is needed to confirm the role of the *TGFBR1*6A* allele in breast cancer development and progression.

Among 71 breast cancer cases analyzed for somatic mutations in nine exons of the *TGFBR1* gene, we found 1 (1.4%) patient that had a mutation in exon 3 of the *TGFBR1* gene. Interestingly, this patient did not carry either the TGFBR1*6A allele or the *Int7G24A* variant of the *TGFBR1* gene. This is similar to the previously reported incidence of 6% somatic mutations of *TGFBR1* in breast cancer (18).

Whereas somatic mutations in the *TGFBR1* gene are not frequent events in breast cancers, we now show that an intronic variant (*Int7G24A*) of the *TGFBR1* gene is associated with invasive and metastatic breast cancer. Homozygous carriers for this variant were also frequent in patients with invasive and metastatic breast cancer. Together, these data suggest that this intronic variant may contribute to breast cancer progression. Additional experiments with larger samples of DCIS patients will determine if the variant allele is associated with enhanced risk of tumor progression in DCIS patients.

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