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Advances in Brief

Genetic Polymorphisms in the *TGF-β1* Gene and Breast Cancer Survival: A Report from the Shanghai Breast Cancer Study

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Abstract

The effect of genetic polymorphisms in the *TGF-β1* gene at codon 10 (*T+29C*), codon 25 (*G+74C*), and the promoter region [C → T at −509 from the transcription site, (*C-509T*)] on breast cancer survival was evaluated among a cohort of 1111 patients. The median follow-up time for the cohort was 5.17 years after cancer diagnosis. No DNA sequence variation at codon 25 of the *TGF-β1* gene was found, whereas polymorphisms in *C-509T* and *T+29C* were in strong linkage disequilibrium. Patients who carried the *C* allele of *T+29C* polymorphism had a reduced 5-year disease-free survival rate (75.6% for *T/C*, and 78.2% for *C/C*) compared with the *T/T* genotype (85.1%; *P*, 0.04); the age-adjusted hazard ratio was 1.5 (95% confidence interval, 1.1–2.2). Adjustment for clinical prognostic factors slightly attenuated the association (hazard ratio, 1.4, 95% confidence interval, 1.0–1.9). Our study suggests that genetic polymorphisms in the *TGF-β1* gene may play a role in breast cancer progression.

Introduction

Transforming growth factor (TGF)-β is a pluripotent cytokine distributed widely in almost all human tissue cells (1). In normal cells, TGF-β acts as a tumor suppressor by inhibiting cellular proliferation or by promoting cellular differentiation and apoptosis (1). As cancer develops, cancer cells become resistant to the growth-inhibitive properties of TGF-β, and both the cancer cells and the stromal cells with cancer often increase their production of TGF-β (1). The paracrine TGF-β produced by cancer cells stimulates angiogenesis and cell motility, suppresses immune response, and increases the interaction of tumor cells with the extracellular matrix, leading to greater invasiveness and metastatic potential of the cancer (2).

TGF-β has three isoforms, TGF-β_1, TGF-β_2, and TGF-β_3. TGF-β_1 is the predominant isoform found in blood. Each isoform is encoded by a distinct gene and has a tissue-specific expression. The *TGF-β_1* gene is located on chromosome 19q13, and TGF-β_1 mRNA is expressed in endothelial, hematopoietic, and connective-tissue (1). Because TGF-β_1 has been implicated in both mammary development and mammary tumorigenesis (3–7), we hypothesize that functional genetic variations of the *TGF-β_1* gene may play a role in breast cancer progression. To test this hypothesis, we evaluated the association of polymorphisms of the *TGF-β_1* gene at codons 10 (*T+29C*) and 25 (*G+74C*) and the promoter region (*C-509T*) with cancer survival among a cohort of breast cancer patients who had participated in a population-based case–control study, the Shanghai Breast Cancer Study.

Materials and Methods

Participants and Study Design. Participants of the study were breast cancer patients who were recruited to the Shanghai Breast Cancer Study from 1996 to 1998 (8). Through a rapid case-ascertainment system and supplemented by the population-based Shanghai Cancer Registry, we identified 1602 women who were between the ages of 25 to 64 years and were diagnosed with a primary breast cancer between August 1996 and March 1998. Of them, 1459 (91.1%) participated in the Shanghai Breast Cancer Study. Reasons for nonparticipation included: refusal (109 cases, 6.8%), death before interview (17 cases, 1.1%), and inability to locate (17 cases, 1.1%). The median time interval between diagnosis and interview was 66 days, and 90% of the study participants were interviewed within 226 days of diagnosis. The initial institutional cancer diagnoses were confirmed by independent review of pathological slides by two senior pathologists. Blood samples (10 ml from each woman) were obtained from 1193 (82%) cases who participated in the study. These samples were typically processed within 6 h of collection, and were stored at −70°C until the relevant bioassays were conducted. Information on cancer diagnosis, disease stage (tumor-node-metastasis stage), cancer treatments, and estrogen and progesterone receptor status was abstracted from medical charts using a standard protocol.

Patients were followed up through January 2003 with a combination of active follow-up and record linkage to the death certificates kept by the Vital Statistics Unit of the Shanghai Center for Disease Control and Prevention. Of the 1459 patients included in the original study, 1290 (88.4%) were successfully contacted either in-person (n = 1241; 85.0%) or by telephone (n = 49; 3.4%) from March 2000 to December 2002. Among them, 197 patients were deceased. Through interview of patients, or next of kin for deceased patients, we obtained information on disease progress, recurrence, quality of life, and cause of death (if deceased). Survival status for the remaining 169 participants who could not be contacted in person or by telephone was established in June 2003 by linkage to the death registry. Through the linkage, 40 deaths were identified; information on the date of death and cause of death was obtained. One hundred twenty-six subjects had no match in the death registry and were assumed to be still living. Their date of last contact was assigned to be December 30, 2002, 6 months before our search of the vital statistics registry, to allow for a possible delay of entry of the death certificates into the registry. Three subjects had insufficient information for the record linkage and were excluded from the current analysis. This study was approved by the Institutional Review Board of Vanderbilt University and all of the participating institutes; consent was obtained for all study participants.

Laboratory Procedures. Genomic DNA was extracted from buffy coat fractions and used for genotyping assays with the PCR-RFLP method. The PCR primers for codon 10 and 25 were as follows: 5′-CCTCCCCACCACACCAG-3′ (forward) and 5′-CCGCAGCTTGGACAGG-3′ (reverse). The PCR primers for *C-509T* were: 5′-GAGCAATTCTTACAGGTGTCTGC-3′ (forward) and 5′-GAGGGTGTCAGTGGGAGGAG-3′ (reverse). The PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Waltham, MA). Each 30 μl of PCR mixture contained 10 ng of DNA, 1 unit of Hotstar *Taq* DNA polymerase (Qiagen, Valencia, CA), 1× Qiagen PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 6 μl of Qiagen Q-solution, and 0.5 μM each primer. The reaction mixture was initially denatured at 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 45 s. The PCR was completed by a final extension cycle at 72°C for 8 min.

The PCR products were digested with restriction endonucleases to deter-

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mine the genotype of each subject. The DNA fragments were then separated using 3% 2:1 agarose:NuSieve gel and were detected by ethidium bromide staining. For codon 10 polymorphism, the T→C substitution creates a *Msp*A1I restriction site. The PCR product (235 bp) with *T* allele was digested to four fragments (103, 67, 40, and 25 bp), and the PCR product with *C* allele was digested to five fragments (91, 67, 40, 25, and 12 bp). For codon 25 polymorphism, the G→C substitution created a *Sau*96I restriction site. The PCR product (235 bp) with *C* allele was digested to two fragments (133 and 102 bp), whereas the PCR product with *G* allele could not be cut by *Sau*96I. For *C-509T* polymorphism, the PCR products were digested with the *Eco*81I restriction endonucleases. The PCR product (81 bp) with *C* allele was digested to two fragments (42 and 39 bp), whereas the PCR product with *T* allele could not be cut by *Eco*81I.

The laboratory staff was blind to the identity of the subjects. Quality control (QC) samples were included in the genotyping assays. Each 96-well plate contained one water blank, two CEPH 1347--02 DNA, two unblinded QC DNA, and two blinded QC DNA. QC samples were distributed across separate 96-well plates. The blinded and unblinded QC samples were taken from the second tube of samples included in the study. Genotyping data were obtained from 1114 to 1118 (93–94%) cases for whom we had blood samples. The agreement rate for the QC samples was 98%. The major reasons for incomplete genotyping were insufficient DNA or unsuccessful PCR amplification.

Statistical Analysis. The primary outcomes used for this study were overall survival and disease-free survival. The end point for the analysis of overall survival was any death, and for the analysis of disease-free survival was cancer recurrence/metastasis or death related to breast cancer. Survival time was calculated as the time from cancer diagnosis to the end points of the study, censoring at the date of last contact or noncancer death (for disease-free survival only). For subjects who had died of breast cancer without information on the date of relapse or metastasis (*i.e.,* the deceased subjects identified from the death registry; $n = 40$), we used two approaches in defining disease-free survival time: (*a*) imputing the disease-free survival time by subtracting the median interval between relapse to death obtained from the subjects with known information (0.67 years) from the total survival time; (*b*). substituting the total survival time for the disease-free survival time. The two analyses produced almost identical results, and the results for the second approach were reported. The Kaplan-Meier method was used to compute 5-year survival rates and the log-rank test was applied to test the differences in survival across different genotypes. The Cox regression model was applied to evaluate the effect of the *TGF-*β_1 genotype on overall survival and disease-free survival with adjustments for age and the known prognostic factors for breast cancer, including tumor-node-metastasis status, cancer treatments and estrogen and progesterone receptor status. Graphic evaluation of Schoenfeld's residual plot indicated that the proportional hazard assumption of the Cox regression model was met. Analyses were also conducted by stratifying the data by traditional breast cancer prognostic factors to examine the potential interactive effects.

Results

Associations of Demographic and Prognostic Factors with Survival. Table 1 presents the association of demographic factors and known prognostic factors of breast cancer with overall survival among the 1111 subjects who were genotyped for *T+29C* polymorphism. As expected, stage of breast cancer (as measured by tumor-node-metastasis stage) is one of the most important predictors of overall survival. Older age at diagnosis and having received radiotherapy were related to lower survival rates, although the effect of the latter is mainly explained by disease stage. Patients who had been treated with tamoxifen had a more favorable survival rate than nonusers, whereas subjects with missing information on tamoxifen treatment had the worst survival rate. This is due mainly to the fact that information on tamoxifen use was obtained during the follow-up survey, in which a large proportion of the deceased patients could not be included. The vast majority of patients in the study had received surgery (99.3%) and adjuvant chemotherapy (93.3%). Similar results were observed when we included in the analysis subjects who did not provide a blood sample and those whose DNA sample was not successfully genotyped in the study (data not shown).

Table 1 *Overall survival by demographics and known breast cancer prognosis factors, Shanghai Breast Cancer Study*

Covariables	Levels	No. of subjects	No. of deaths	5-yr survival rate (%)	*P*
Age at diagnosis	<42	278	45	85.0	
	42–46	277	31	89.1	
	47–52	270	51	81.5	
	53–64	286	53	82.3	0.05
Education	<Middle school	138	29	80.2	
	Middle school	492	79	84.0	
	>Middle school	481	72	86.1	0.28
TNM	0–I	277	18	94.0	
	IIa	393	46	88.7	
	IIb	245	48	81.3	
	III–IV	122	48	60.9	
	Unknown	74	20	75.4	<0.01
ER	Positive	489	75	85.4	
	Negative	280	43	85.1	
	Unknown	342	62	82.6	0.52
PR	Positive	492	75	85.2	
	Negative	268	41	85.3	
	Unknown	351	64	82.7	0.49
Surgery	Yes	1104	178	84.5	
	No	0	0	—	
	Unknown	7	2	71.4	0.28
Chemotherapy	Yes	1037	167	84.6	
	No	60	9	84.9	
	Unknown	14	4	71.4	0.35
Radiotherapy	Yes	423	102	76.4	
	No	527	57	90.2	
	Unknown	161	21	86.9	<0.01
Tamoxifen use	Yes	710	84	88.8	
	No	209	29	86.7	
	Unknown	192	67	66.1	<0.01

Associations of Genotypes with Survival. The study population was not polymorphic at codon 25 of the *TGF-β1* gene. The frequencies of *T+29C* genotypes were 23.1% for *T/T*, 50.0% for *T/C*, and 26.9% for *C/C*. For the *C-509T* polymorphism, the frequencies were 23.3% for *C/C*, 50.0% for *C/T* and 26.4% for *T/T*. The genotype of both polymorphisms followed the Hardy-Weinberg equilibrium ($P = 0.90$ and 0.97), and these two polymorphisms were in strong linkage disequilibrium. Women with a variant allele of the *T+29C* or *C-509T* polymorphism had a lower disease-free survival rate than those with both wild-type alleles; 5-year disease-free survival rates were 85.2%, 77.0%, and 78.3% ($P = 0.04$) for subjects with the *T+29C T/T*, *T/C*, and *C/C* genotype, and 84.6%, 77.0% and 77.9% ($P = 0.05$) for subjects with the *C-509T C/C*, *C/T*, and *T/T* genotype (Table 2; Fig. 1). Subjects with a variant allele at these two sites also had worse overall 5-year survival rates, but the differences were not statistically significant (Table 2).

We found that neither *T+29C* single nucleotide polymorphism nor *C-509T* polymorphism was associated with demographic characteristics of breast cancer patients, neither was it related to disease stage or treatments (data not shown). *T+29C* polymorphism, however, was inversely associated with poor disease-free survival and, to a lesser extent, with overall survival. The age-adjusted hazard ratio (HR) associated with the *T+29C T/C* or the *C/C* genotype was 1.3 [95% confidence interval (CI), 0.9–1.9] for overall survival and 1.5 (95% CI, 1.1–2.2) for disease-free survival. The corresponding multivariate adjusted HR was 1.1 (95% CI, 0.8–1.7) for overall survival and 1.4 (95% CI, 1.0–1.9) for disease-free survival (Table 3). Similar results were observed for the *C-509T* polymorphism. Additional analyses stratified by the tumor-node-metastasis stage, estrogen and progesterone receptor status, tamoxifen use, chemotherapy and radiotherapy did not find a substantial variation in the associations between these

Table 2 *TGF-β_1T+29C and C-509 polymorphisms in association with 5-year overall survival and disease-free survival, Shanghai Breast Cancer Study*

			5-yr overall survival				5-yr disease-free survival			
Genotype	No. of subjects	%	No. of deaths	%	Survival rate %	*P*	No. of events[a]	%	Survival rate %	*P*
TGF-β_1 T+29C										
TT	258	23.2	35	19.4	87.5		39	17.0	85.2	
TC	553	49.8	97	53.9	83.5		127	55.2	77.0	
CC	300	27.0	48	26.7	83.6	0.30	64	27.8	78.3	0.04
TT	250	23.1	35	19.4	87.5		39	17.0	85.2	
TC/CC	832	76.9	145	80.6	83.5	0.15	191	83.0	77.5	0.01
TGF-β_1 C-509T										
CC	257	23.3	35	19.8	87.5		39	17.2	85.1	
TC	551	50.0	94	53.1	84.0		124	54.6	77.5	
TT	294	26.7	48	27.1	83.3	0.39	64	28.2	77.8	0.05
CC	257	23.3	35	19.8	87.5		39	17.2	85.1	
TC/TT	945	76.7	142	80.2	83.7	0.18	188	82.8	77.6	0.02

[a] Including relapse, metastasis, and breast cancer death.

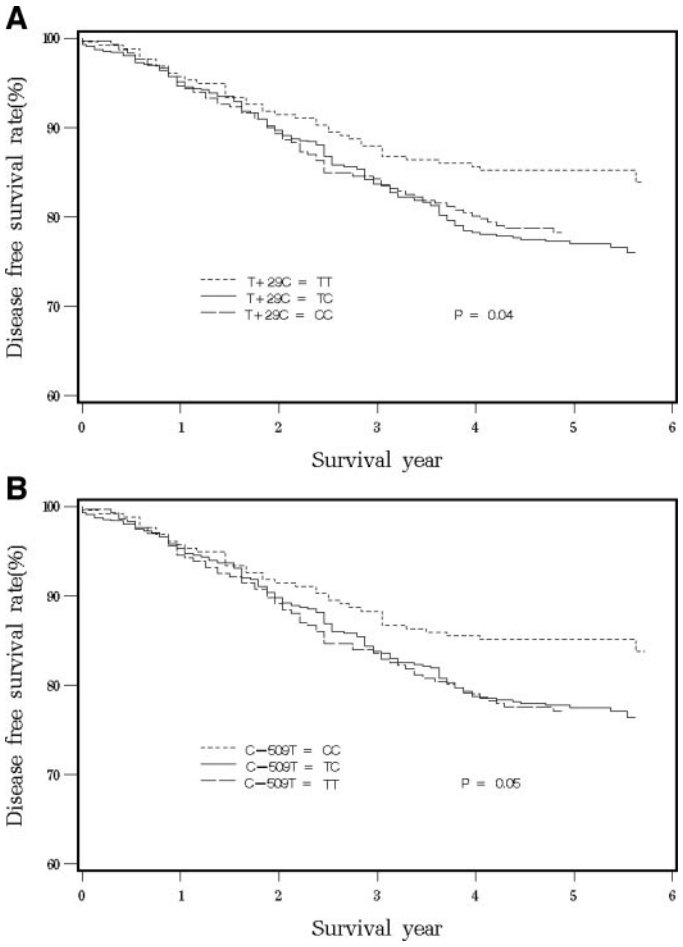

Fig. 1. Disease-free survival among breast cancer patients by *T+29C* and *C-509T* polymorphisms. *A*, patients carrying the variant *C* allele(s) at the *T+29C* polymorphism had a lower disease-free survival rate than those who have two copies of the wild-type alleles. *B*, patients carrying the variant *T* allele(s) at the *C-509T* polymorphism had a lower disease-free survival rate than those who had two copies of the wild-type alleles.

two polymorphisms and overall or disease-free survival (data not shown). The statistical power of the study, however, is limited for a formal test of a moderate interaction between *TGF-β_1* polymorphism and clinical prognostic factors on breast cancer survival.

Discussion

Grainger *et al.* (9), in a study of 170 pairs of female twins, found that plasma concentration of active plus acid-activatable latent TGF-β_1 is predominantly under genetic control (heritability estimate 54%). They also showed that *C-509T* polymorphism was significantly associated with plasma TGF-β_1 level. The *T+29C* polymorphism, resulting in a leucine to proline substitution, has also been previously linked to an elevated serum level of TGF-β_1 (10), as well as increased secretion of TGF-β_1 in an *in vitro* study (11). Our study and an earlier study (11) have both found that *C-509T* and *T+29C* polymorphisms are in strong linkage disequilibrium. Therefore, it remains unknown which of these two polymorphisms is functionally significant.

In a cohort study involving 3075 women aged 65 years or older, Ziv *et al.* (12) reported that subjects with the *C/C TGF-β_1* genotype had a 64% reduced risk of developing breast cancer as compared with women with the *C/T* or *T/T* genotype. Results from a recent report of three case-control series of 3987 cases and 3867 controls confirmed that the *T/T* genotype was related to an increased risk of invasive breast cancer, but that the magnitude of the risk was much smaller than in the earlier study (odds ratio, 1.21 for *C/C versus T* carrier; Ref. 11). *C-509T* polymorphism, in strong linkage disequilibrium with *T+29C* polymorphism, was also related to a similar risk. These findings are biologically plausible, because TGF-β_1 is a potent growth inhibitor and can induce apoptosis (1, 13).

Table 3 *TGF-β_1 T+29C and C-509T polymorphisms in association with overall survival and disease-free survival, Shanghai Breast Cancer Study*

	HR[a] (95%)	HR[b] (95%)
T+29C		
Overall survival		
TT	1.0	1.0
TC	1.4 (0.9–2.0)	1.2 (0.8–1.8)
CC	1.2 (0.8–1.9)	1.1 (0.7–1.6)
TC/CC	1.3 (0.9–1.9)	1.1 (0.8–1.7)
Disease-free survival		
TT	1.0	1.0
TC	1.6 (1.1–2.2)	1.4 (1.0–2.1)
CC	1.5 (1.0–2.2)	1.3 (0.9–1.9)
TC/CC	1.5 (1.1–2.2)	1.4 (1.0–1.9)
C-509T		
Overall survival		
CC	1.0	1.0
TC	1.3 (0.9–1.9)	1.1 (0.8–1.7)
TT	1.3 (0.8–1.9)	1.0 (0.7–1.6)
TC/TT	1.3 (0.9–1.9)	1.1 (0.7–1.6)
Disease-free survival		
CC	1.0	1.0
TC	1.5 (1.1–2.2)	1.4 (0.9–2.0)
TT	1.5 (1.0–2.2)	1.3 (0.8–1.9)
TC/TT	1.5 (1.1–2.1)	1.3 (0.9–1.9)

[a] HR, hazard ratio adjusted for age (<42, 42–46, 47–52, 53–64).

[b] Adjusted for age (<42, 42–46, 47–52, 53–64), education (<middle school, middle school, >middle school), tumor-node-metastasis (0–I, II, III, IV, unknown), radiotherapy (yes, no, unknown), chemotherapy (yes, no, unknown), estrogen receptor status (positive, negative, unknown), progesterone receptor status (positive, negative, unknown), and tamoxifen use (yes, no, unknown).

During multistage tumorigenesis, the growth-inhibitory and apoptotic effects of TGF-β are lost, and other TGF-β responses in favor of malignant tumor progression prevail (13–15). It has been well documented that TGF-β can enhance epithelial-to-mesenchymal transition, down-regulate cellular adhesion molecules, augment expression of metalloproteases, increase tumor cell motility and tumor angiogenesis, and cause local and systemic immunosuppression, leading tumors to an invasive metastatic status (4–7, 13, 14). Therefore, it is conceivable that patients with the variant allele of *T+29C* or *C-509T* polymorphisms may have reduced cancer survival, because the variant allele is associated with an elevated *TGF-β_1* level. To our knowledge, only one study has evaluated the association of *TGF-β_1* polymorphisms with overall survival of breast cancer (16). *T+29C* polymorphism was found to be related to a slightly increased, although not statistically significant, risk of death (HR, 1.3; 95% CI, 0.8–2.1 for *C/C versus T/T T+29C* genotype; Ref. 16). This finding was similar to what we have found in our study, conducted among Chinese women, in which the *C/C* genotype was related to an age-adjusted risk of 1.2 (95% CI, 0.8–1.9). Noticeably, when disease-free survival was assessed, women carrying the variant *C* allele were found to have a significantly increased risk of the disease progressing or dying of breast cancer (HR, 1.5; 95% CI, 1.1–2.2), and the risk remained marginally significant, even after adjustment for all of the traditional prognostic factors (HR, 1.4; 95% CI, 1.0–1.9). This suggests that the effect of *T+29C* polymorphism on breast cancer survival is most likely mediated through its effect on tumor progression, such as metastases and relapse, and such an effect is likely to be diluted when overall survival is assessed. Similar results were found for the *C-509T* polymorphism, in which the polymorphism was significantly related to reduced disease-free survival, but nonsignificantly associated with overall survival.

Our finding is supported by a recent study of 44 advanced breast cancer patients. This study found that plasma TGF-β_1 levels were significantly elevated among patients with stage IIIB/IV breast cancer compared with healthy controls. Furthermore, the association appeared to be independent of tumor size, site of distant metastases, steroid hormone receptor content, and age at cancer onset (17). Follow-up with six of those patients suggested that higher levels of TGF-β_1 might be related to poor response to disease treatment. Several other studies have also shown that plasma TGF-β_1 levels were higher among breast cancer patients than controls (18), and plasma TGF-β_1 levels were inversely related to breast cancer progression (19). However, other studies have failed to find any difference in plasma TGF-β_1 levels between breast cancer patients and healthy controls (20–22). The variability in plasma sample preparation and assay methods, as well as the small samples involved, may have contributed to the controversy (23).

Our study has a number of strengths. The population-based patient setting, high response rate, and high follow-up rate have minimized any potential selection bias. The large sample size and long follow-up period have provided the study with relatively high statistical power. Information on cancer characteristics and treatment was obtained for the vast majority of patients, allowing an evaluation of possible effect modifications. The genotype information was available for 76% of study participants, raising a concern of potential selection bias. However, we did not find any appreciable difference in disease-free survival between patients with and without genotype data. Furthermore, it is unlikely that *TGF-β_1* polymorphism would have affected patients' decision to donate a blood sample to the study. There may be some errors in estimating disease-free survival in the study, because this estimation was based on self-reported or death certificate information. Again, these errors are likely to be random and unlikely to be related to *TGF-β_1* genotype and therefore, should not affect the association observed in this study.

In summary, we found that *T+29C* and *C-509* polymorphisms in the *TGF-β_1* gene were inversely associated with disease-free survival among breast cancer patients. This finding is biologically plausible and may have significant implications for identification of high-risk breast cancer patients for further treatment and close follow-up.

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