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A Transforming Growth Factorβ1 Signal Peptide Variant Increases Secretion *in Vitro* and Is Associated with Increased Incidence of Invasive Breast Cancer[1]

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ABSTRACT

There is evidence that transforming growth factor (TGF)β acts as a suppressor of tumor initiation but also as a promoter of tumor progression when the antiproliferative effect of the TGFβ signaling pathway has been overridden by other oncogenic mutations. Several somatic mutations that disrupt the TGFβ-SMAD signaling pathway have been reported in human breast tumors. We have examined the association between single nucleotide polymorphisms (SNPs) in the *TGFβ1* gene and the incidence of invasive breast cancer in three case-control series, with a maximum of 3987 patients and 3867 controls, median age ~50 years, and range 22–92 years. The promoter SNP, *C-509T,* and the *T +29C* signal-peptide SNP (encoding Leu10Pro) are in strong linkage disequilibrium. They are both significantly associated with increased incidence of invasive breast cancer in a recessive manner [odds ratios: (*TT versus C*-carrier), 1.25; 95% confidence intervals 1.06–1.48; $P = 0.009$ and (ProPro *versus* Leu-carrier), 1.21; 95% confidence intervals 1.05–1.37; $P = 0.01$]. The *G-800A* SNP was not significantly associated with incidence of breast cancer. The *C-509T* SNP is not contained within a known consensus sequence for a promoter regulatory element and therefore unlikely to affect TGFβ1 expression, whereas the Leu10Pro signal peptide substitution potentially affects TGFβ1 secretion. Transfections of HeLa cells with constructs encoding either the Pro or Leu forms of TGFβ1 and driven by the cytomegalovirus promoter indicate that the signal peptide with Pro at residue 10 causes a 2.8-fold increase in secretion compared with the Leu form. These data indicate that the allele encoding Pro10 is associated with increased rates of TGFβ1 secretion and with increased incidence of invasive breast cancer for the population samples described. It is estimated that 3% of all breast cancer cases may be attributable to Pro10 homozygosity.

INTRODUCTION

First-degree relatives of breast cancer patients have an ~2-fold increased risk over the general population of developing the disease, but <25% of this excess risk is explained by inherited mutations in *BRCA1* and *BRCA2* genes (1), which are associated with substantially increased risk of breast cancer. Data from the large multiple case families suggest that other high penetrance genes will be rare. It is therefore plausible that there are multiple common low risk (low penetrance) genetic variants, which are associated with relatively small effects on risk of breast cancer in the individual but contribute substantially to the overall risk in the population (2).

In vitro and *in vivo* studies have shown that TGFβ[4] is a critical regulator of normal and transformed epithelial cell phenotypes, functioning as both a tumor suppressor and promoter of tumor progression and invasion. For most normal cell types, TGFβ acts *in vitro* as a potent inhibitor of proliferation and migration and promotes apoptosis, properties associated with tumor suppression (3, 4). However, in cells in which these suppressor functions of the TGFβ signaling pathway are overridden, TGFβ may induce cellular changes associated with malignant progression (5), invasion (6), and angiogenesis (7, 8). Both tumor suppressor and tumor-promoting activities of TGFβ have been clearly demonstrated in a variety of genetically modified mouse lines in which the TGFβ signaling pathway is ablated or modified (9). These studies support a model in which TGFβ inhibits the development of early, benign lesions but promotes invasion and metastasis when the tumor suppressor activity is overridden by oncogenic mutations in other pathways (10). In this model, the increased levels of TGFβ that are frequently detected in human tumors may contribute either to tumor suppression or progression.

Several studies have shown that somatic mutations in components of the signaling pathway of TGFβ are associated with the development of human tumors of epithelial origin (11–13), *e.g.,* it has been estimated that >80% of colorectal tumors have inhibitory mutations in the TGFβ pathway (14). There are few reports of somatic mutations in the TGFβ signaling pathway in human breast carcinomas. We have recently described four novel inhibitory mutations in the *TGFβ*R2 gene in recurrent breast tumors (15); in addition, an inactivating mutation in the *TGFβ*R1 gene has been identified in metastatic breast cancer (16), and the absence of SMAD2-P, the activated form of SMAD2, has been shown to define a small but particularly aggressive subset of breast tumors (17).

On the basis of these data, we have hypothesized that polymorphisms affecting the function of genes in the TGFβ signaling pathway might also play a significant role in the development of breast cancer. We have therefore examined the incidence of breast cancer associated with SNPs in the *TGFβ1* gene that we and others (18, 19) have described. We have analyzed three polymorphisms (*C-509T, G-800A,* and *T +29C* [Leu10Pro]) in DNA samples from three series of European patients with invasive breast cancer and controls. The *T +29C* transition, which generates a Leu10Pro substitution in the signal peptide of the TGFβ1 precursor, was associated with altered risk of breast cancer. Because a change in the signal peptide sequence may affect protein secretion, *in vitro* transfection experiments have been performed to determine whether the leucine/proline substitution alters TGFβ1 secretion.

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[4] The abbreviations used are: TGF, transforming growth factor; SNP, single nucleotide polymorphism; ABC, Anglian Breast Cancer; EPIC, European Prospective Investigation of Cancer; df, degrees of freedom; LAP, latency associated peptide; v/v, volume for volume; CMV, cytomegalovirus; CI, confidence interval; OR, odds ratio.

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MATERIALS AND METHODS

Population Samples. Numerical data for the population samples from which the cases and controls were drawn are given in Table 1 (the numbers of samples analyzed are given in Table 3).

East Anglian, British (United Kingdom). Cases with invasive breast cancer were drawn from the ABC study. This is an ongoing population-based study of breast cancer cases ascertained through the East Anglian Cancer Registry (20). All study participants completed an epidemiological questionnaire and provided a blood sample for DNA extraction. Controls were randomly selected from the EPIC-Norfolk component of the EPIC (21), a prospective study of diet and cancer being carried out in the same geographical region as the ABC Study.

Heidelberg, German (HDB). Cases were drawn from a population-based study of breast cancer, conducted across the State of Baden-Württemberg in southern Germany, and matched with controls by age and area of residence (Ref. 22; Table 1). The present study was confined to the subset of cases and controls from the city of Heidelberg with at least one parent of German nationality (Table 3).

Kuopio, Finnish (FINN). Cases were drawn from the Kuopio Breast Cancer Project, a population-based study of breast cancer (23). The controls were randomly selected and individually matched to patients by age (within 5 years) and area-of-residence using the National Population Register.

Ethical Approval. Ethical approvals were obtained from the Anglia and Oxford Multicentre Research Committee in the United Kingdom, Ethics Committee of the University of Heidelberg, and Joint Ethics Committees of Kuopio University and the Kuopio University Hospital. Informed, written consent was obtained from each patient.

Genotyping. Genotyping was carried out using Taqman according to manufacturer's instructions. Primers and probes used for each polymorphism analyzed are shown in Table 2. All reactions were carried out at 62°C. All assays were carried out in 96-well arrays. Each plate contained 24 controls: 8 negatives with no DNA template and 8 positive controls for each SNP allele, in addition to 72 DNA test samples. These controls enabled semiautomated genotyping to be carried out using a 7700 Sequence Detector (Applied Biosytems, Warrington, United Kingdom). DNA samples that did not give a clear genotype result at the first attempt were not repeated because this is a high-throughput process, and, therefore, there are variations in the proportions of results obtained for each polymorphism as defined in Table 3.

Statistical Analysis of the Association Study. Deviations of the genotype frequencies in the controls from those expected under Hardy-Weinberg equilibrium were assessed by χ^2 tests. The expected proportions of common homozygotes, heterozygotes, and rare homozygotes were p^2, 2pq, and q^2, respectively, where p is the population frequency of the common allele and q is that of the rare allele. The genotype frequencies in the control groups of each series did not differ significantly from Hardy-Weinberg for any of the SNPs tested. Genotype frequencies in cases and controls were compared by 2df χ^2 tests. Genotype-specific risks were estimated as ORs by unconditional logistic regression (24). CIs for the ORs were derived using the floating absolute risk method (25). This approach yields floated standard errors and floated CIs. The method does not alter the relative risk estimates but reduces the variances attributed to the ORs that are not defined as 1 and reduces unwanted covariance between them. Results are presented for each case-control study separately and as a joint analysis. In the joint analysis, each series was treated as a separate stratum in the logistic regression model. We tested for heterogeneity between strata by comparing logistic regression models with and without a genotype-stratum interaction term using likelihood ratio tests. To test which of the two SNPs on the disease-associated haplotypes was more likely to have a functional effect, we compared three different logistic regression models (each SNP alone and both together) using likelihood ratio tests. All logistic regression analyses were performed using SPSS for Windows Version 9.0 (SPSS United Kingdom Ltd.). The population attributable risk for the Pro10 allele, under a recessive model, was estimated using the formula: $[q^2(r - 1)]/\{[q^2(r - 1)] + 1\}$, where r is the estimated relative risk for the Pro10 homozygotes as compared with other genotypes. The excess familial relative risk to siblings of breast cancer patients attributable to Pro10Leu was estimated using the formula $\lambda_s = \{0.25[q^2r\ (2)(1 - q^2)] + 0.5[q(qr + p)\ (2) + p)] - 0.75\}/\{[q^2r + (1 - q^2)]\ (2)\}$. This was expressed as a percentage of the overall familial relative risk of breast cancer, which is ~1.8 (26).

Table 2 *Sequences of primers and probes used for genotyping*

SNP	Oligo sequences
G-800A	F: GCTATCGCCTGCACACAGC
	R: AGGACAGAAGCGGTGCCAT
	FAM: TGCCTCCAACGTCACCACCATC
	VIC: TCTGCCTCCAACATCACCACCATC
C-509T	F: TTAGCCACATGGGAGGTGCT
	R: CCAGGCGGAGAAGGCTTAA
	FAM: ACCCTTCCATCCCTCAGGTGTCCT
	VIC: CCCTTCCATCCTTCAGGTGTCCTG
Leu10Pro	F: TCTCCCTGAGGACCTCAGCTT
	R: GCAGCTTGGACAGGATCTGG
	FAM: CTGCTGCTGCTGCTGCTACCG
	VIC: CTGCTGCCGCTGCTGCTACC

Transfection Vectors for TGFβ1 Secretion Assays. TGFβ1 cDNAs were generated by PCR in two stages using DNA polymerase (Expand High Fidelity; Roche) and a cloned human TGFβ1 cDNA (generously provided by Dr. P. ten Dijke, Netherlands Cancer Institute, Amsterdam, the Netherlands) as the DNA template. cDNAs encoding the LAP portion of TGFβ1 (also provided by Dr. P. ten Dijke) were amplified using a downstream oligonucleotide (5′-GCGGTGCCGGGAGCT*CTGCAG*ATG-3′) and either Leu-UP (5′-CATGC-CGCCCTCCGGGCTGCGGCTGCTGCTGCTG-3′) or Pro-UP (5′-CATGC-CGCCCTCCGGGCTGCGGCTGCTGC*C*GCTG-3′), with a single base change shown underlined. PCR products were cloned into pGEMT-Easy (Promega, Madison, WI) to generate pGEM-LeuLAP and pGEM-ProLAP, respectively. cDNA encoding the active portion of TGFβ1 was amplified using mUP (5′-CAGCAT*CTGCAG*AGCTCCCGG-3′) and mDOWN (5′-ACCT-CAGCTGCACTTGCAGGA-3′) and cloned into pGEMT-Easy to generate pGEM-active. The underlined sequence incorporates a silent mutation (CTGCAA→CTGCAG) to generate the restriction endonuclease site, *Pst*I. TGFβ1 cDNA from pGEM-active was cloned into pGEM-LeuLAP and pGEM-ProLAP as a *Pst*I fragment to generate pGEM-L and pGEM-P, respectively. TGFβ1 cDNAs from pGEM-L and pGEM-P were cloned into pcDNA3 (Invitrogen, Paisley, United Kingdom) as *Eco*RI fragments to generate CMV-L and CMV-P, respectively. CMV-β-gal was generated by cloning a *Kpn*I-*Apa*I DNA fragment from CMVSportβ-gal (Invitrogen) into pcDNA3. All plasmid DNA was prepared using Qiagen plasmid midi-prep kits and quantified using a fluorescence-based assay (PicoGreen; Molecular Probes, Leiden, The Netherlands) according to the manufacturer's instructions. Plasmid cDNA inserts were sequenced in both orientations using an automated DNA sequencer.

Cell Culture and Transfection. HeLa cells were cultured in DMEM supplemented with 10% FCS (v/v), penicillin (100 IU/ml), and streptomycin (100

Table 1 *Data for the female population samples from which the cases and controls were drawn*

	UK (East Anglia)		HDB (Heidelberg, Germany)		FINN (Kuopio, Finland)	
	ABC cases	EPIC controls	Cases	Controls	Cases	Controls
No. eligible	5298[a]	25000	706	1381	516	514
% providing blood samples	51	64	95	82	94	96
Identification period	1991–2001	1990–1996	1990–1995		1990–1995	
Blood collection period	1996–2001	1996–1998	1990–1995		1990–1995	
Age at identification (years)	22–65[a]	45–74	<51		44–92	38–77
% ethnically Caucasian	>98		100		100	

[a] Study still ongoing. To be eligible, patients diagnosed from 1991 to 1996 had to be <56 years of age and consent to blood collection in 1996, whereas those diagnosed from 1996 onwards had to be <65 years and consent to blood collection within 1 year of diagnosis.

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Table 3 *Genotype distributions and ORs in each case-control series*

A. *G-800A*

Set	GG (%)[a]	GA (%)[a]	AA (%)[a]	Attempted (% achieved)	*P*
UK					
Cases	1564 (83.2)	307 (16.3)	10 (0.5)	2302 (82)	
Controls	1090 (83.6)	204 (15.6)	10 (0.8)	1630 (80)	
OR (CIs)[b]	1.00 (0.85–1.17)[c]	1.04 (0.94–1.17)	0.70 (0.29–1.66)		0.6[d]
HDB					
Cases	386 (87.5)	53 (12.0)	2 (0.5)	447 (99)	
Controls	587 (83.7)	109 (15.6)	5 (0.7)	720 (97)	
OR (CIs)[b]	1.00 (0.74–1.34)[c]	0.74 (0.61–0.89)	0.61 (0.12–3.07)		0.2[d]
Joint					
OR (CIs)	1.00 (0.88–1.14)[c]	0.95 (0.86–1.05)	0.67 (0.31–1.44)		0.5[d]

B. *C-509T*

Set	CC (%)	CT (%)	TT (%)	Attempted (% achieved)	*P*
UK					
Cases	1181 (48.4)	1014 (41.6)	244 (10.0)	2870 (85)	
Controls	1194 (50.5)	977 (41.3)	195 (8.2)	2686 (88)	
OR (CIs)[b]	1.00 (0.97–1.03)[c]	1.04 (0.94–1.18)	1.27 (1.03–1.55)		0.08[d]
Recessive model	1.00[c]	1.00[c]	1.24 (1.02–1.50)		0.03[e]
HDB					
Cases	159 (38.1)	201 (48.2)	57 (13.7)	447 (93)	
Controls	281 (44.3)	287 (45.3)	66 (10.4)	720 (88)	
OR (CIs)[b]	1.00 (0.93–1.07)[c]	1.23 (0.96–1.60)	1.53 (1.02–2.27)		0.08[d]
Recessive model	1.00[c]	1.00[c]	1.36 (0.93–1.99)		0.1[e]
FINN					
Cases	277 (57.7)	176 (36.6)	27 (5.6)	498 (96)	
Controls	252 (55.8)	177 (39.1)	23 (5.1)	461 (98)	
OR (CIs)[b]	1.00 (0.89–1.12)[c]	0.90 (0.71–1.15)	1.07 (0.60–1.89)		0.7[d]
Recessive model	1.00[c]	1.00[c]	1.11 (0.62–1.97)		0.7[e]
Joint					
OR (CIs)[b]	1.00 (0.98–1.02)[c]	1.05 (0.95–1.16)	1.28 (1.08–1.51)		0.02[d]
Recessive model	1.00[c]	1.00[c]	1.25 (1.06–1.48)		0.009[e]

C. Leu10Pro

Set	LeuLeu (%)	LeuPro (%)	ProPro (%)	Attempted (% achieved)	*P*
UK					
Cases	99 t(37.6)	1228 (46.3)	425 (16.1)	3042 (87)	
Controls	735 (39.2)	889 (47.3)	253 (13.5)	2398 (78)	
OR (CIs)[b]	1.00 (0.96–1.04)[c]	1.02 (0.90–1.15)	1.24 (1.04–1.48)		0.06[d]
Recessive model	1.00[c]	1.00[c]	1.22 (1.03–1.45)		0.02[e]
HDB					
Cases	116 (28.0)	220 (53.0)	79 (19.0)	447 (93)	
Controls	202 (35.2)	274 (47.7)	98 (17.1)	574 (80)	
OR (CIs)[b]	1.00 (0.87–1.15)[c]	1.40 (1.08–1.80)	1.40 (0.99–1.99)		0.06[d]
Recessive model	1.00[c]	1.00[c]	1.14 (0.82–1.58)		0.4[e]
FINN					
Cases	255 (53.0)	191 (39.7)	35 (7.3)	498 (97)	
Controls	232 (51.4)	191 (42.4)	28 (6.2)	461 (98)	
OR (CIs)[b]	1.00 (0.91–1.09)[c]	0.91 (0.71–1.17)	1.14 (0.68–1.91)		0.6[d]
Recessive model	1.00[c]	1.00[c]	1.18 (0.71–1.98)		0.5[e]
Joint					
OR (CIs)[b]	1.00 (0.97–1.02)[c]	1.05 (0.94–1.16)	1.24 (1.06–1.44)		0.03[d]
Recessive model	1.00[c]	1.00[c]	1.21 (1.05–1.37)		0.01[e]

[a] Percentage of results in each genotype class.
[b] Floated 95% CIs.
[c] Referent group.
[d] 2df test on genotype distribution.
[e] 1df test.

μg/ml) in a humidified air/CO2 (95%/5%) incubator at 37°C. Cells were seeded (4×10^5 cells/well) in six-well plates in culture medium and transfected after 24 h with CMV-P, CMV-L, CMV-empty, and CMV-β-gal as indicated. Two separate DNA preparations were used for eight separate transfection experiments performed on triplicate cell samples. Cells were transfected, as indicated, with 2 μg of total DNA with 2 μg of lipofectin per well in 1 ml of serum-free medium according to the manufacturer's instructions. At 6 h post-transfection, the cells were aspirated and washed once with DMEM, and 2 ml of DMEM containing FCS and antibiotics were added per well. After 24 h, the cells were washed three times with DMEM and incubated in 2 ml of DMEM for periods of ≤18 h. The medium was collected, centrifuged to remove any cells, and either analyzed immediately or frozen in 0.5-ml aliquots at −80°C. The cells were washed twice with PBS and assayed for β-galactosidase activity.

TGFβ1 Assays. Before heat activation of secreted TGFβ1 in conditioned medium, the TGFβ1 was stabilized by adding 1/40 v/v of 1% fatty acid free-BSA in DMEM, and the pH was stabilized by adding 1/10 v/v of 1 M Tris-HCl (pH 7.0). The TGFβ1 samples were activated by heating at 80°C for 15 min and then cooled to room temperature. The concentration of heat-activated TGFβ1 was assayed using the R&D (Oxford, United Kingdom) assay kit specific for TGFβ1 according to instructions for cell culture supernatants. The heat-activated samples (100 μl) were diluted 1:4 in DMEM containing 100 mM TRIS-HCl (pH 7.0) and 0.02% fatty acid free-BSA to bring the TGFβ1 concentrations in the linear range of the TGFβ1 standard curve (0.03–2 ng/ml). TGFβ levels are expressed in nanograms per milliliter ± SE, and *P*s were calculated by Student's *t* test.

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RESULTS

Breast Cancer Association Studies. Initially, the United Kingdom population series was genotyped for the two promoter SNPs: *G-800A* and *C-509T*. The rarer *G-800A* polymorphism was not significantly associated with any differences in incidence of breast cancer (Table 3). The *TT* homozygotes of the *C–509T* polymorphism showed an association with breast cancer incidence that was not quite formally significant [OR (*TT versus CC*), 1.27; 95% CI, 1.03–1.55; $P = 0.08$ (2df); Table 3]. These data were extended using two additional breast cancer case-control series: HDB and FINN. The ORs for the HDB series were also close to significant [OR (*TT versus CC*), 1.53; 95% CI, 1.02–2.27; $P = 0.08$ (2df); Table 3]. The FINN series did not achieve statistical significance, although the OR (*TT versus CC*) was marginally increased (Table 3). A joint analysis of all three series was significant [OR (*TT versus CC*), 1.28; 95% CI, 1.08–1.51; $P = 0.02$ (2df)]. There was no significant evidence of heterogeneity between the results of the three series ($P = 0.9$; 2df). They are all compatible with a model that *T-509* is associated with increased incidence of breast cancer in a recessive manner [OR (*TT versus C*-carrier), 1.25; 95% CI, 1.06–1.48; $P = 0.009$ (1df); Table 3].

To determine whether this observed association was attributable to the *C–509T* polymorphism or to another *TGFβ1* polymorphism in linkage disequilibrium with it, we considered the other known *TGFβ1* SNPs. The five common, potentially functional SNPs generate six haplotypes (18), and the simplest sequential pathway for their derivation from the ancestral haplotype is shown in Fig. 1. The frequencies shown in Fig. 1 are those estimated from the United Kingdom controls or data in Syrris *et al.* (18). There is little evidence for intermarker recombination; these common haplotypes together constitute 96% of all of the observed haplotypes. Only the Pro10-encoding allele is both sufficiently frequent (13.5% homozygosity; Table 3) and carried on haplotypes with the *T-509* allele to be a potential cause of the observed association between T-509 homozygosity (8.2%; Table 3) and the incidence of breast cancer. The Pro10-encoding allele occurs on haplotypes with both *T-509* and *C-509*, but the converse (*T-509* with Leu10) is very rarely observed. The Ile263- and Pro25-encoding alleles define very rare subsets of the *T–509*- and *C-509*-carrying haplotypes, respectively (Fig. 1), and these are too rare to explain the observed association. We therefore investigated the association of the Pro10Leu polymorphism with breast cancer risk and found that the Pro10 allele was also associated with increased risk of breast cancer in the United Kingdom [OR: (ProPro *versus* LeuLeu), 1.24; 95% CI 1.04–1.48; $P = 0.06$ (2df); Table 3]. Again, similar results were obtained in HDB [OR: (ProPro *versus* LeuLeu), 1.4; 95% CI 0.99–1.99; $P = 0.06$ (2df); Table 3]. As with the *C-509T* polymorphism, no strong association was seen in the FINN sample, but the joint analysis of all three series was significant: [OR (ProPro *versus* LeuLeu), 1.24; 95% CI, 1.06–1.44; $P = 0.03$; Table 3]. No significant differences in the magnitude of the ORs were observed when the cases were stratified by patient age, tumor stage, or grade at diagnosis, where recorded (data not shown). Although the HDB series provide some evidence for a codominant mode of inheritance, overall, these data fit a recessive model: [OR (ProPro *versus* Leu-carrier), 1.21; 95% CI, 1.05–1.37; $P = 0.01$ (1df); Table 3]. We conclude that rare allele homozygosities at both the *C-509T* and Leu10Pro loci, which are in very strong linkage disequilibrium, are associated with a moderately increased incidence of invasive breast cancer.

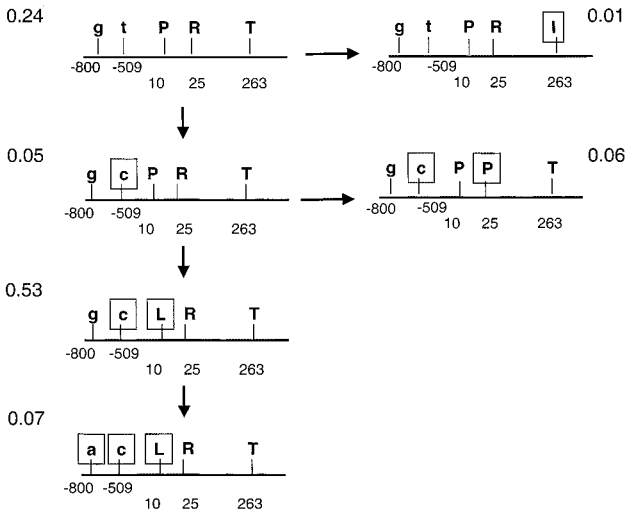

Fig. 1. Relationship between the TGFβ1 haplotypes examined. A diagram showing the most parsimonious order of common mutational events in the human *TGFβ1* gene. The ancestral haplotype (*top left*) was determined from comparisons between species (18). The frequencies shown are those estimated from the United Kingdom controls. A majority (96%) of all haplotypes observed does not require a recombination event to explain their existence. The haplotypes directly examined in the present study are each shown (*left*). Other known haplotypes, forming subgroups of the ones studies here (18), are also shown (*right*). Promoter base changes and amino acid positions are *numbered*. Single letter amino acid codes are used.

As a consequence of the strong linkage disequilibrium between the *C-509T* and Leu10Pro SNPs, it is not easy to determine which is more likely to be the cause of the observed association. In an attempt to resolve this, we fitted logistic regression models in which we allowed for the effects of homozygosity at both *C-509T* and Leu10Pro, both individually and jointly. The model incorporating both SNPs did not fit significantly better than the model with Pro10Leu alone ($P = 0.3$), but there was weak evidence that the model with *C-509T* alone fitted less well than the joint model ($P = 0.07$).

TGFβ1 Secretion. The *C-509T* promoter polymorphism does not lie within a known regulatory sequence, and its role in transcription is therefore unclear. Furthermore, the above analysis suggested that that this polymorphism alone was unlikely to account for the observed associations. However, the Pro10Leu substitution in the signal peptide potentially affects secretion of the TGFβ1 protein, and this was assayed in HeLa cells. The amounts of TGFβ1 secreted in response to transfection with the CMV-P and CMV-L vectors as a function of time after transfection are shown in Fig. 2*a*. The amount of TGFβ1 secreted from CMV-P-transfected cells was consistently greater than from CMV-L-transfected cells, and for both forms, secretion was maximal by 12 h and constant to 18 h. The mean ratio of TGFβ1 secreted when driven by the two constructs (CMV-P:CMV-L) was 2.4 ± 0.1 (range 2.1–2.7), and this ratio did not vary significantly over the 18-h time course. The decrease in rate of TGFβ1 accumulation with time probably reflects the quiescent state of the cells induced by incubation for prolonged periods in DMEM after transfection.

A series of experiments similar to those in Fig. 2*a* was performed to compare amounts of TGFβ1 secreted at 18 h after transfection with the CMV-P and CMV-L vectors and to determine whether cotransfections with CMV-E or CMV-β-gal affected TGFβ1 secretion. The data in Fig. 2*b* show that in three separate experiments, neither CMV-E nor CMV-β-gal significantly affected the amount of TGFβ1 secreted in response to CMV-P or CMV-L. Furthermore, no significant differences in amounts of TGFβ1 secreted in response to CMV-P or CMV-L (or in the ratios of secreted TGFβ1) were observed using two separate preparations of the CMV-P and CMV-L vectors (data not shown). The addition of BSA (0.02%) to the incubation medium during transfection or to the incubation medium after transfection made no significant difference to the amounts of TGFβ1 secreted. The absolute amounts of TGFβ1 secreted varied significantly between independent transfection experiments, reflecting the efficiency of the transfections, but transfection efficiencies within experiments deter-

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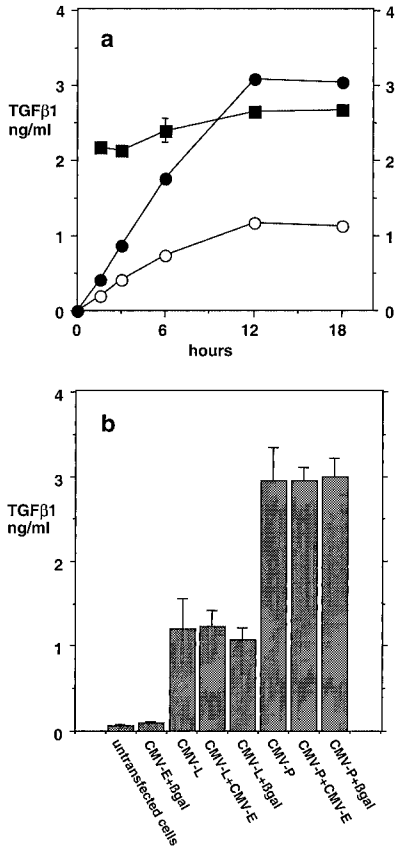

Fig. 2. Secretion of TGFβ1 from HeLa cells transfected with CMV-P and CMV-L. In *a*, serum-free DMEM medium containing secreted TGFβ1 was collected at the times indicated from HeLa cells that had been transfected with either the CMV-P (●) or CMV-L (○) construct (no β-gal) and was assayed for TGFβ1 after activation, as described in "Materials and Methods." Data from a representative time course experiment ($n = 3$) are shown as the means ± SE for triplicate determinations of TGFβ1, and the ratio for the amounts of TGFβ1 secreted for the transfected constructs (■) is also shown. In *b*, serum-free medium was collected after 18 h and assayed for TGFβ1 as in *a* from HeLa cells that had been transfected with CMV-P ± CMV-β-gal, CMV-L ± CMV-β-gal, CMV-P ± CMV-E, CMV-L ± CMV-E, or CMV-β-gal + CMV-E. Data are means ± SE from triplicate determinations of TGFβ1 in three separate experiments.

mined by β-galacosidase assays did not vary significantly. The ratio of secreted Leu and Pro forms of TGFβ1 for all experiments ($n = 8$) was 2.8 ± 0.2.

DISCUSSION

Breast Cancer Risks. The moderately increased risk of breast cancer (1.2–1.3-fold) associated with *TGFβ1 T-509* or Pro10 homozygosity is comparable in magnitude to other recognized breast cancer risk factors, *e.g.*, current use of oral contraceptives (1.24; 95% CI 1.15–1.33; Ref. 27) or hormone replacement therapy for >5 years (1.35; 95% CI 1.21–1.49; Ref. 28). In this study, the effect of the polymorphisms appears to be recessive, although the HDB series gives heterozygote ORs of similar magnitude to the rare homozygote risk (Table 3). As a recessive allele, Pro10 would contribute only 0.2% to the excess sibling risk of breast cancer, which is 1.8-fold in the population (see "Materials and Methods"). However, we estimate that 3% of all breast cancer cases may be attributable to Pro10 homozygosity, calculated as defined in "Materials and Methods."

Because Pro10 homozygotes only comprise 13% of the population, very large sample sizes are required to detect the modest risk associated with this genotype. Thus, the combined data from three series (a maximum of 3987 patients and 3867 controls) had >95% power to detect a relative risk of 1.5 at $P < 0.001$ but only ~50% power to detect a relative risk of 1.2 at $P < 0.01$. These estimates clearly illustrate the need for large sample sizes to identify moderate risk genotypes.

Ziv *et al.* (29) have recently evaluated the *T +29C* (Leu10Pro) polymorphism in a nested case-control study, based on 146 breast cancer cases from a cohort of 3075 women aged >65. In marked contrast to our study, Pro10 homozygosity was associated with a reduced risk of developing breast cancer (risk ratio 0.36; 95% CI 0.17–0.75). Our much larger study clearly excludes a reduced risk of this magnitude for the cases and controls studied (median age ~50 years; range 22–92 years). Comparing the two studies, we note that the cases in the cohort of Ziv *et al.* had, on average, a later age at diagnosis (median age: 70 years) and included 13% noninvasive cancers, which were specifically excluded from our study.

The data for association of the Leu10Pro polymorphism in TGFβ1 with invasive breast cancer can be compared with the associations reported for several other diseases, *e.g.*, Pro10 homozygosity is associated with a decrease in the rate of loss of bone density and a therapeutic response to vitamin D in postmenopausal Japanese women with osteoporosis, but is a risk factor for spinal osteophytosis (30). Pro10 alleles are also associated with reduced incidence of rheumatoid arthritis (31) but with increased risk of systemic sclerosis (32), asthma (33), pulmonary dysfunction with cystic fibrosis (34), and progression of liver fibrosis with chronic hepatitis virus C (35). Pro10 homozygosity is associated with myocardial infarction in Japanese men (36) and Western patients with end stage dilated cardiomyopathy (37) but not with coronary artery disease (18). Graft vascular disease after heart transplant is associated with Pro10 homozygosity (38), whereas renal insufficiency after transplantation is reduced (39). Reported associations of Pro10 alleles with higher systolic blood pressure depended on ethnicity and sex (40, 41).

Effect of the Variants on TGFβ1 Secretion. The Pro/Leu substitution in the signal peptide of TGFβ1 has a substantial effect on protein secretion driven by the CMV promoter in the absence of any component of the TGFβ1 promoter. These data therefore demonstrate that Pro10Leu alone has a functional effect *in vitro* independent of any effect of the *C-509T* promoter polymorphism. A significant effect of the Leu10Pro polymorphism on the amount of TGFβ1 secreted *in vivo* may provide an explanation for its reported associations with a variety of diseases. We have reported previously that the amount of TGFβ in serum is higher for Pro10 homozygotes than Leu10 homozygotes (42), and other studies are consistent with this observation (36). It is therefore plausible that local secretion of TGFβ by breast tumors and/or local stromal cells is also higher for Pro10 homozygotes. As noted earlier, current theories for the effects of TGFβ on tumor development are that increased amounts of TGFβ1 activity may suppress the early stages of tumor formation but promote the invasiveness, metastasis, and angiogenesis of tumors. In the present study, cases with noninvasive breast cancer have been excluded, and therefore, we can conclude only that the Pro10 homozygotes have an increased incidence of invasive disease. Any effects of Pro10 homozygosity on the initiation of breast cancer remain to be determined.

It is of note that the antiestrogen tamoxifen is reported to cause increased TGFβ accumulation in breast tumors (43, 44). The data presented therefore raise the clinically significant question of what effect the TGFβ produced in response to tamoxifen may have on the invasive stages of breast tumor development.

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