

Prognostic Value of PAI1 in Invasive Breast Cancer: Evidence That Tumor-Specific Factors Are More Important Than Genetic Variation in Regulating PAI1 Expression

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Abstract

Plasminogen activator inhibitor-1 (PAI1) can promote cancer progression, and its protein expression in tumors is an independent indicator of poor prognosis in many forms of cancer. Here, we show that high PAI1 mRNA levels also predict for shorter overall survival in two independent breast cancer data sets, highlighting the importance of its transcriptional regulation. The *-675insG* (4G/5G) single-nucleotide polymorphism in the *PAI1* gene promoter has been shown to influence *PAI1* transcription, with the 4G allele eliciting higher reporter gene expression *in vitro* and higher levels of circulating PAI1 *in vivo*. Nevertheless, its genotypic distribution in 2,539 British women with invasive breast cancer was virtually identical to that seen in 1,832 matched controls ($P = 0.72$), and annual mortality rates for 4G4G, 4G5G, and 5G5G cases were 2.6%, 2.8%, and 3.1% per year, respectively

($P = 0.10$). Thus, there was no association with breast cancer incidence or outcome, and in a separate set of breast cancers, the 4G/5G single-nucleotide polymorphism showed no association with PAI1 mRNA expression ($P = 0.85$). By contrast, connective tissue growth factor (CTGF), which can regulate PAI1 expression in culture, was associated with PAI1 expression in three independent cohorts ($P \ll 0.0001$). In addition, *PAI1* gene copy number differences in the tumors were correlated with PAI1 mRNA expression ($P = 0.0005$) and seemed to affect expression independently of CTGF. Thus, local factors, such as CTGF and genomic amplification, seem to be more important than germ line genetic variation in influencing PAI1 expression and its untoward effects in breast cancer. (Cancer Epidemiol Biomarkers Prev 2006;15(11):2107–14)

Introduction

Considerable evidence indicates that PAI1, a major physiologic inhibitor of urokinase-type and tissue-type plasminogen activators, promotes cancer progression. High tumoral PAI1 protein expression is associated with poor survival in several forms of cancer (1–3) and is a strong independent prognostic factor in breast cancer, with elevated levels forecasting shorter recurrence-free and overall survival (4–8). In addition, prospective data suggest that high tumoral PAI1 levels can identify those lymph node-negative (LN⁻) breast cancer patients who are most likely to benefit from adjuvant chemotherapy (9, 10). Nevertheless, PAI1 expression is rarely used in clinical decision making, as the protein-based assays used to establish its prognostic and predictive value are poorly adapted to the limited amounts of tissue that are commonly available after routine screening and early cancer detection.

Experimental data also indicate that PAI1 promotes cancer progression. In cancer transplantation models, tumor growth,

invasion, and angiogenesis are essentially abolished in PAI1-deficient mice and are rescued by adenoviral PAI1 replacement (11, 12). In addition, data show that PAI1 promotes tumor growth in a dose-dependent and stage-dependent manner and suggest that stromal PAI1 is more important than tumor cell-derived PAI1 (13, 14). These effects seem to depend on the proteinase inhibitory activity of PAI1 (15, 16), although its ability to disrupt integrin-mediated adhesion and promote cell motility independent of its inhibitory function may also contribute (17, 18). Thus, PAI1 is not simply an indicator of progression but an active participant. Consequently, factors that affect its expression should also affect progression.

One means of regulating PAI1 is at the level of transcription. Notably, the *PAI1* (*SERPINE1*) promoter contains a common transcription-altering insertion/deletion single-nucleotide polymorphism (SNP; rs1799889) with four or five guanine nucleotides 675 bp upstream of the transcription start site (19). Gel shift, methylation interference, and DNase footprinting assays show that at least one extra protein binds the 5G allele, and reporter assays show that the 4G promoter has enhanced basal activity and is more responsive to transforming growth factor- β (TGF β) and interleukin-1 (IL-1) induction than the 5G variant (19–21). In addition, induction of *PAI1* by tumor necrosis factor- α is mediated by interaction of the transcription factor nuclear factor- κ B with a promoter element that includes the 4G/5G site, although it is not known whether the 4G/5G SNP alters its responsiveness to nuclear factor- κ B (22). Moreover, at least 37 separate studies have detected significant allelic dose-dependent correlations between carriage of the 4G allele and PAI1 protein levels *in vivo*, such that 4G4G homozygotes have the highest levels of circulating PAI1, 5G5G homozygotes have the lowest levels, and heterozygotes

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individuals have intermediate levels of circulating PAI1 (Supplementary Table S1). Indeed, multiple studies and meta-analyses have found significant associations between 4G/5G genotypes and various vascular and thrombotic diseases (23-27); yet, its role in cancer remains unclear.

Clearly, many factors influence *PAI1* promoter activity. These include TGF β , tumor necrosis factor- α , platelet-derived growth factor (PDGF), IL-1, IL-6, insulin, insulin-like growth factor-1, and glucose (21, 22, 28). TGF β , a known regulator of cancer initiation and progression, is a particularly potent inducer of *PAI1* (21), whereas PAI1 suppresses the plasmin-mediated activation of TGF β by inhibiting plasmin formation (29). Conversely, TGF β elicits the expression of connective tissue growth factor (CTGF), which enhances the TGF β -mediated induction of various TGF β -responsive genes, including *PAI1* and *CTGF* itself (30, 31). Nevertheless, it is unclear which factors affect tumoral PAI1 expression *in vivo*. Thus, we evaluated the role of germ line genetic variability and local microenvironmental and cellular factors in determining the level of PAI1 expression in breast cancer.

Materials and Methods

Study Populations. Global mRNA expression levels were analyzed for three breast cancer data sets: Miller et al. (32), van de Vijver et al. (33), and a set⁷ from the University of California San Francisco (UCSF; Supplementary Table S2). Data sets that used a Research Genetics cDNA platform were excluded based on evidence that the PAI1 clone in this set does not match the PAI1 sequence.⁸ The UCSF data set contained expression profiles from Affymetrix HG-U133A arrays run on 118 breast cancers as well as genomic copy number information obtained by array-based comparative genomic hybridization (CGH). The Miller data set was obtained through the Gene Expression Omnibus (accession no. GSE3494). It contains comprehensive expression profiles for 251 Swedish breast cancers that were analyzed using Affymetrix U133A and B arrays covering >30,000 genes. Data from the B array were excluded because PAI1 was absent from this array and because some genes represented on both arrays were not correlated across arrays. The van de Vijver data set was obtained from Rosetta Inpharmatics. Expression profiles in this set of 295 Dutch breast cancers were obtained by mixing Cy-labeled cRNA from individual tumors with an equal amount of a pool of reverse color-labeled cRNA representing all 295 tumors equally. These were hybridized to Agilent Technologies microarrays with 24,479 biological features.

Invasive breast cancer cases for SNP analysis were drawn from SEARCH (breast), an on-going population-based study of breast cancer cases ascertained through the East Anglian Cancer Registry (34). Incident cases were diagnosed at ≤ 65 years of age after the study began on July 1, 1996, and prevalent cases were diagnosed at ≤ 55 years of age between January 1, 1991 and initiation of the study. Participants who died before initiation of the study were not included. Controls were randomly selected from the Norfolk component of the European Prospective Investigation of Cancer (EPIC) from the same geographic area as the SEARCH study (34). Vital status was available for 2,524 genotyped SEARCH participants. Controls were not matched to cases but were broadly similar in age (median = 63 years; range = 42-81) and ethnicity (>98% White). Tumor and matched normal tissue DNAs were also isolated from 129 breast cancer cases provided by the UCSF

Cancer Center Breast Oncology Program Tissue Core. Human research approval was obtained from the UCSF Committee on Human Research and the Eastern Region Multicentre Research Ethics Committee. Informed, written consent was obtained from each participant.

Genotyping. The *PAI1* -675insG SNP and SNPs in five other genes were genotyped by multiplex capillary electrophoresis-based minisequencing. Six regions of interest, including the *PAI1* region from -745 to -646, were amplified by multiplex PCR in a 25- μ L volume containing 1.4 \times PCR Buffer-II (Applied Biosystems, Foster City, CA), 4.5 mmol/L MgCl₂, 0.4 mmol/L of each deoxynucleotide, 0.3 μ mol/L of each primer, 1.25 units of AmpliTaq Gold DNA Polymerase (Applied Biosystems), and 20 ng of DNA from whole blood. To facilitate even primer annealing, each forward primer had the nonspecific sequence 5'-GCCGGTCCCAAAGGGTCACT-3' from bacteriophage M13mp18 added to its 5' end, and each reverse primer contained the T7 RNA promoter sequence 5'-TTCTAATACGACTCATTATAG-3' at its 5' end. The -675insG region was amplified using a -745/-726 forward primer (5'-TCCAACCTCAGCCAGACAAG-3') and a -646/-663 reverse primer (5'-CCGCCTCCGATGATACAC-3'). Thermal cycling conditions were 95°C for 10 minutes, 42 cycles of 95°C for 30 seconds, 66°C for 45 seconds, and 72°C for 45 seconds and a final 7 minutes elongation step at 72°C. Residual primers and deoxynucleotide triphosphates were then removed by treating 5 μ L of PCR product with 2 units of shrimp alkaline phosphatase (U.S. Biochemical, Cleveland, OH) and 10 units of exonuclease I (U.S. Biochemical) at 37°C for 1 hour followed by a 15-minute enzyme inactivation step at 80°C. Single-base extension reactions were then done in a 10- μ L volume containing ~0.15 pmol of amplified target DNA (purified multiplex PCR product), 2.5 μ L of SNaPshot Ready Reaction Premix (Applied Biosystems) with fluorescent dideoxynucleotides (R6G-ddATP, ROX-ddTTP, TAMRA-ddCTP, and R110-ddGTP), and Taq DNA polymerase and a cocktail of sense- and antisense-oriented SNP-specific primers (0.15 pmol each). The six sense-oriented and six antisense-oriented primers had free 3' ends that annealed immediately 5' to the six polymorphic sites of interest and 5' poly-T tails of various lengths. For the *PAI1* -675insG site, a 46-nucleotide sense-oriented -693/-672 oligonucleotide (5'-T24-GAGAGAGTCTGGACACGTGGGG-3') and a 30-nucleotide antisense -655/-675 oligonucleotide (5'-T9-ATGATACACGGCTGACTCCCC-3') were used. Single-base extension thermal cycling conditions were 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds. Unincorporated dideoxynucleotide triphosphates were removed by incubating single-base extension products with 0.5 unit of shrimp alkaline phosphatase at 37°C for 1 hour followed by a 15-minute 72°C inactivation step. One microliter each of shrimp alkaline phosphatase-treated single-base extension product and LIZ-120 size standard (Applied Biosystems) were added to 25 μ L of deionized water, and the 12 single-base extension products were resolved on an ABI-PRISM 3700 DNA Analyzer.

Because the 12 sense and antisense primers had different lengths, all six SNPs were simultaneously sequenced from both directions for internal validation. We also validated the accuracy of our method using at least one alternative method for each site, including direct sequencing and a restriction fragment-based method designed for the 4G/5G SNP (35). Our minisequencing results and the results of these other approaches were 100% concordant for the *PAI1* site in 21 comparisons and >99% concordant for all six loci in >3,600 comparisons. The sense and antisense primers also yielded identical results for >99.8% of >27,000 separate genotypes (5,065 *PAI1* genotypes), and replicate samples and matched normal and tumor tissue pairs yielded identical results for 100% of >2,400 genotypes (447 *PAI1* genotypes). Only one SEARCH case and one EPIC control had discordant sense and

⁷ K. Chin, Devries S, Fridlynd J, et al. Associations of genomic and transcriptional aberrations with human breast cancer pathophysiologies. *Cancer Cell*, in press.

⁸ J.E. Korkola, personal communication.

antisense *PAI1* results and were thus excluded from our analyses. Thus, our method was >99% accurate, reproducible, and internally consistent.

Statistical Analysis. Expression-based univariate and multivariate survival analyses were done by Cox proportional hazards regression analysis using Stata-9.1. To adjust for platform differences and provide comparable meaning to the hazard ratios across all data sets, *PAI1* expression values were normalized by subtracting the mean *PAI1* expression for all cases, dividing the difference by the SD, and adding the constant five to avoid negative values. Survival curves were constructed for the highest versus lowest 50% of *PAI1*-expressing tumors and were compared using the log-rank test. Pearson correlations between *PAI1* expression and the expression of all other genes were calculated, and their corresponding two-tailed parametric *P*s were adjusted for multiple comparisons using the false discovery rate procedure of Benjamini and Hochberg (36). Because each platform contained duplicate *PAI1* probes that yielded correlated expression values ($r = 0.83-0.97$), all analyses were done using the average value from both probes. Average values were also used for all other genes with replicate probes. Genes were considered significantly correlated with *PAI1* if the false discovery rate-adjusted $P < 0.0001$.

For SNP analyses, deviations of the observed genotypic frequencies from those expected under Hardy-Weinberg equilibrium were assessed by χ^2 tests. Allelic and genotypic frequencies in cases and controls were compared by one degree and two degrees of freedom χ^2 tests, respectively. Genotype-specific risks were estimated as odds ratios by unconditional logistic regression, and 95% confidence intervals were obtained using the floating absolute risk method (34). Attributable risks were estimated as $g(r - 1) / [1 + g(r - 1)]$, where g is the genotypic frequency in the control population and r is the associated relative risk estimate. For survival analysis, time at risk was defined from the date of blood sample receipt until death due to any cause or until March 21, 2003 for surviving participants. Genotype-specific hazard ratios and 95% confidence intervals were estimated by Cox regression analysis. The proportional hazards assumption was examined and tested by adding a time \times genotype term to the model. The level of significance was determined using an overall test for heterogeneity among all three genotypes.

Results

Prognostic Value of *PAI1* mRNA Expression in Breast Cancer. As predicted from protein-based studies, high *PAI1* mRNA expression was associated with shorter overall survival in two breast cancer data sets (Table 1; Fig. 1). In the UCSF data set, *PAI1* expression was prognostic by univariate analysis but failed to provide significant additional prognostic information when tumor size and LN status (which were the only independent prognostic variables in this cohort) were accounted for in the Cox model. In the van de Vijver data set, LN status was not prognostic, whereas estrogen receptor (ER) status and a 70-gene expression signature derived from this set were (33). *PAI1* expression was also prognostic in this set when considered alone. In addition, it was an independent indicator of poor prognosis in the 151 LN⁻ cases after adjusting for ER status ($P = 0.014$) and/or the 70-gene signature ($P < 0.05$) but was not prognostic in the remaining LN⁺ cases. *PAI1* was also prognostic in the subset of 180 high-risk cases with a poor 70-gene expression signature.

Conversely, *PAI1* had no prognostic power in the Miller data set, suggesting that key differences between this cohort and the others may modify the biological effects and prognostic use of *PAI1*. Indeed, substantial differences were

seen between the patient sets (Supplementary Table S2). The proportion of ER⁻ cases in the Miller data set was significantly lower than in the van de Vijver ($P = 0.004$) and UCSF ($P < 0.0001$) data sets, and the proportion of LN⁻ cases in the Miller data set was significantly greater than in the van de Vijver ($P = 0.0009$) and UCSF ($P < 0.0001$) data sets. In addition, none of the van de Vijver cases were older than 52 years of age at diagnosis (i.e., most were premenopausal), whereas 48% of UCSF cases and 73% of Miller cases were over 52 years old. Thus, the UCSF cohort had an older age distribution than the van de Vijver cohort ($P < 0.0001$), and the Miller cohort had a far older distribution than either of the other cohorts ($P < 0.0001$). Nevertheless, *PAI1* expression continued to lack prognostic significance in the 61 Miller cases diagnosed before age 53 or in cases stratified by LN or ER status. Thus, although these differences may contribute to the presence or absence of an association between *PAI1* and survival, they are not entirely responsible and are probably indicative of other important differences that we were unable to ascertain from the data. Nevertheless, mRNA associations in the van de Vijver and UCSF cohorts are consistent with the proven prognostic value of *PAI1* and thus indicate that transcriptional controls are important in regulating tumoral *PAI1* expression.

***PAI1* 4G/5G SNP Is Not Associated with Breast Cancer Incidence, Outcome, or *PAI1* Expression.** *PAI1* 4G/5G genotypes were obtained for 2,539 SEARCH cases and 1,832 EPIC controls. The cases and controls had virtually identical

Table 1. *PAI1* expression and overall survival in three breast cancer cohorts

Cohort	<i>n</i>	Hazard ratio (95% confidence interval)	<i>P</i>
UCSF			
All cases	117	1.46 (1.07-1.98)	0.016
Subgroup			
LN ⁻ cases	50	1.14 (0.52-2.47)	0.746
LN ⁺ cases	67	1.53 (1.06-2.20)	0.023
ER ⁻ cases	43	1.65 (1.06-2.58)	0.026
ER ⁺ cases	74	1.33 (0.86-2.07)	0.202
Multivariate*			
All cases	114	1.37 (0.98-1.93)	0.068
van de Vijver			
All cases	295	1.31 (1.09-1.58)	0.003
Subgroup			
LN ⁻ cases	151	1.55 (1.21-1.99)	0.001
LN ⁺ cases	144	1.13 (0.84-1.52)	0.427
ER ⁻ cases	69	1.08 (0.85-1.38)	0.508
ER ⁺ cases	226	1.34 (0.99-1.83)	0.062
High risk [†]	180	1.21 (1.01-1.45)	0.042
Multivariate [‡]			
All cases	295	1.18 (0.98-1.41)	0.083
Miller			
All cases	234	0.95 (0.72-1.24)	0.699
Subgroup			
LN ⁻ cases	149	0.83 (0.55-1.26)	0.381
LN ⁺ cases	77	0.99 (0.70-1.39)	0.957
ER ⁻ cases	30	1.93 (0.63-5.88)	0.250
ER ⁺ cases	200	0.90 (0.68-1.19)	0.454
Multivariate [§]			
All cases	223	0.95 (0.72-1.24)	0.708

NOTE: Cox proportional hazards analysis was performed for overall survival using *PAI1* expression as a normalized continuous variable. Hazard ratios and 95% confidence intervals are for 1-unit increase in the standard deviation of the log₂ expression of *PAI1* (range = 1.11-8.33).

*Multivariate analyses were adjusted for LN status, tumor size, age at diagnosis, and ER status.

[†]Cases with a poor 70-gene prognostic signature (33).

[‡]Multivariate analyses were adjusted for LN status and ER status.

[§]Multivariate analyses were adjusted for LN status, age at diagnosis, tumor size, and ER status.

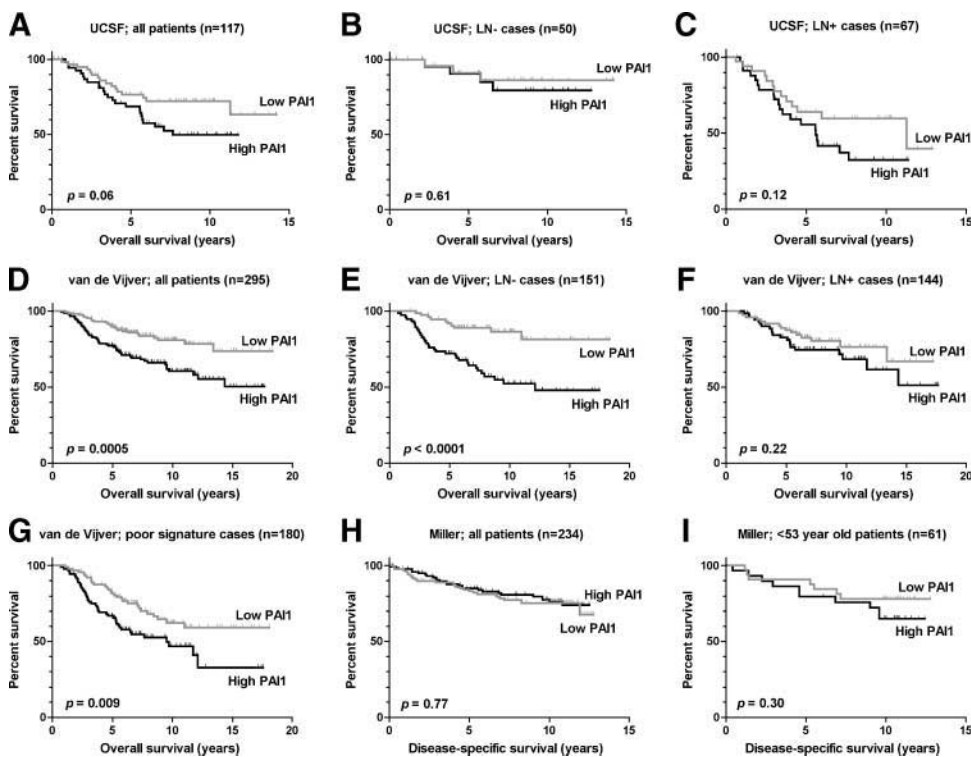


Figure 1. Kaplan-Meier plots for overall or disease-specific survival for the highest (*black*) versus lowest (*gray*) 50% of tumors in terms of their PAI1 expression. Survival curves for all outcome-informative patients (**A**, **D**, and **H**), LN⁻ cases (**B** and **E**) and LN⁺ cases (**C** and **F**) for the indicated cohorts, as well as for the subset of cases from the van de Vijver study with a poor 70-gene prognostic signature (**G**) and the subset of Miller cases younger than 53 years of age (**I**). Log-rank *P*s are provided.

allelic ($P = 0.44$) and genotypic ($P = 0.72$) distributions (Table 2). Similar 4G4G, 4G5G, and 5G5G genotypic frequencies (0.32, 0.43, and 0.25, respectively) and 4G/5G allelic frequencies (0.53/0.47) were also seen in the Caucasian subset of UCSF breast cancer cases, and no British or U.S. cohort deviated significantly from Hardy-Weinberg equilibrium ($P \geq 0.29$). Thus, the 4G/5G SNP was not associated with the incidence of invasive breast cancer.

Notably, the 4G/5G SNP sits within one of six TGF β response elements in the *PAI1* promoter and affects *PAI1* induction by TGF β (21), whereas *PAI1* can indirectly suppress TGF β activation and the release of matrix-sequestered TGF β (29). In addition, the P (proline) allele of the nonsynonymous *TGFBI* L10P (T+29C) SNP has been associated with elevated TGF β 1 secretion in culture and an increased incidence of invasive breast cancer in three case-control sets, including the SEARCH-EPIC set (34). Therefore, we explored the possibility that the *PAI1* and *TGFBI* SNPs might interact to influence cancer risk. L10P and 4G/5G genotypes were available for 2,074 SEARCH cases and 1,766 EPIC controls. However, neither SNP was associated with breast cancer incidence when analyzed separately (odds ratio, 1.16; 95% confidence interval, 0.95-1.43 for PP versus LL homozygotes; odds ratio, 1.06; 95% confidence interval, 0.88-1.28 for 4G4G versus 5G5G homozygotes). When they were analyzed together, the distribution

Table 2. Genotypic distributions and allelic frequencies in East Anglian women with and without invasive breast cancer

Genotype	Cases (n = 2,539)	Controls (n = 1,832)	Odds ratio (95% confidence interval)
5G5G	520 (0.205)	386 (0.211)	1.00 (reference)
4G5G	1,229 (0.484)	896 (0.489)	1.02 (0.87-1.19)
4G4G	790 (0.311)	550 (0.300)	1.07 (0.90-1.26)
4G allele	0.553	0.545	
5G allele	0.447	0.455	

of the nine possible genotypic combinations in the controls was virtually identical to the expected pattern based on the observed allele frequencies and an assumption of independent inheritance ($P = 0.98$). However, a marginally significant difference between observed and expected distributions was detected among the cases ($P = 0.055$).

The relative risks for the nine possible *PAI1-TGFBI* genotypes showed an inconsistent trend in which the high-expressing *PAI1* 4G and *TGFBI* P alleles each seemed to confer an increased risk of breast cancer in an additive, allelic dose-dependent manner (Table 3). According to this semidominant model, double-homozygous 4G4G/PP individuals with four putative high-risk alleles should show a greater risk of developing cancer than individuals with three high-risk alleles, and so on, with double-homozygous 5G5G/LL individuals having the lowest relative risk. Approximately 4% of British Caucasians are 4G4G/PP double homozygotes. Thus, if the relative risk of 1.24 is correct, then ~1% of all breast cancers would be attributable to this genotype. If we instead assume that the L10P SNP is recessive for increased risk, as prior data suggest (34), and that 4G/5G is dominant, then the odds ratio for the high-risk 4G-carrier/PP group (~11% of the population) remains at 1.24 versus the 5G5G/L-carrier group; the 95% confidence interval improves to 0.97 to 1.59; and the attributable fraction increases to ~2.6%. Either way, the risks associated with a 4G/5G-L10P interaction are weak, and the population attributable risk decreases below the 3% level estimated for the *TGFBI* PP genotype alone (34), further suggesting that the 4G/5G SNP does not contribute to the risk of developing breast cancer.

Among the 2,524 genotyped SEARCH cases for which survival data were available, 291 deaths occurred over 10,517 person-years, for an overall mortality rate of 2.8% per year. Significant heterogeneity was not observed among the three genotypes; yet, annual mortality tended to decrease in an allelic dose-dependent manner from 3.1% for 5G5G cases and 2.8% for 4G5G cases to 2.6% for 4G4G cases. This was reflected in a nonsignificant increase in the unadjusted hazard ratio when using homozygous 4G4G cases as the reference group

Table 3. PAI1 4G/5G and TGFB1 L10P genotypic distributions in East Anglian women with and without invasive breast cancer

D	PAI1 genotype	TGFB1 Genotype	Cases	Controls	Genotypic odds ratio (95% confidence interval)	Additive odds ratio (95% confidence interval)
0	5G5G	LL	161	127	1.00 (reference)	1.00 (reference)
1	5G5G	LP	204	153	1.05 (0.77-1.44)	1.02 (0.78-1.33)
1	4G5G	LL	356	281	1.00 (0.76-1.32)	
2	5G5G	PP	58	42	1.09 (0.69-1.73)	1.11 (0.86-1.43)
2	4G5G	LP	487	362	1.06 (0.81-1.39)	
2	4G4G	LL	266	174	1.21 (0.89-1.63)	
3	4G5G	PP	170	104	1.29 (0.92-1.81)	1.07 (0.81-1.41)
3	4G4G	LP	271	221	0.97 (0.72-1.30)	
4	4G4G	PP	101	64	1.24 (0.84-1.84)	1.24 (0.84-1.84)

NOTE: D = dose of putative high-risk alleles. Additive odds ratios were determined for each combined allelic dosage group relative to the reference 5G5G/LL participants who had no putative high-risk alleles.

(Table 4; Fig. 2A). Thus, if anything, the high-expressing 4G allele tended to provide a slightly better, rather than worse, prognosis (proportional hazards $P = 0.10$). Likewise, there was no association with overall survival in 121 informative UCSF cases ($P = 0.74$; Fig. 2B) or in the smaller subset of 79 Caucasian patients ($P = 0.34$). Nor was the 4G/5G SNP associated with tumor stage, LN status, ER status, pathologic grade, or ductal versus lobular histology in this subset ($P \geq 0.30$).

Array-based PAI1 mRNA expression data were available for 27 UCSF cases for which 4G/5G genotypic data were also available. Among these tumors, no genotype exhibited a significantly altered level of PAI1 expression (ANOVA, $P = 0.23$; Fig. 2C), nor was there an allelic dose-dependent trend in terms of expression ($P = 0.85$), suggesting that the 4G/5G SNP does not have a substantial effect on PAI1 expression in breast cancer.

Correlations between PAI1 mRNA Expression and Other Factors. Three global gene expression data sets were used to identify genes that might regulate PAI1 expression, or that might be influenced by PAI1 or regulated in common with it. Of ~24,000 arrayed genes, 532 were significantly correlated with PAI1 at a false discovery rate-adjusted $P < 0.0001$ (Supplementary Table S3). Of these, 68 were correlated with PAI1 in two data sets, and 32 were correlated in all three data sets. Thus, not only were these 32 genes significantly correlated within each data set, but the probability that any one would be significantly correlated in all three data sets by chance was $\sim 1 \times 10^{-6}$. Furthermore, only five genes were among the 40 most highly correlated genes in all three data sets: CTGF, the facilitated glucose transporter SLC2A3, the cysteine-rich angiogenic inducer CYR61, the bone morphogenetic protein antagonist gremlin (GREM1), and the extracellular matrix protein fibronectin (FN1). CTGF was by far the most highly correlated gene in the UCSF ($r = 0.68$) and Miller ($r = 0.66$) data sets and ranked ninth in the van de Vijver data set ($r = 0.45$). Likewise, SLC2A3 ranked first, second, and ninth among PAI1-correlated genes in the van de Vijver ($r = 0.57$), Miller ($r = 0.59$), and UCSF ($r = 0.54$) data sets, respectively. The probability of CTGF or SLC2A3 being so highly ranked in all three sets by chance was $< 4 \times 10^{-12}$. Thus, PAI1 was more highly and consistently correlated with CTGF and SLC2A3 than with any other gene.

Other consistently correlated genes included angiotensin-like 2 (ANGPTL2), mitogen-inducible 2 (MIG2), growth-arrest and DNA-damage-inducible β (GADD45B), tristetraprolin (ZFP36), urokinase-type plasminogen activator and its receptor (PLAU and PLAUR), inhibin- β A (INHBA), IL6, laminin- β 1 (LAMB1), nidogen (NID), versican (CSPG2), PDGF receptor- β (PDGFRB), and TGF β -inducible early growth response (TIEG/KLF10). Notably, PAI1 and TGF β were not correlated with one another in any data set ($r = 0.10$ -0.29, $P = 0.02$ -0.38). However,

the mRNA expression of TGF β does not necessarily reflect its activity, as TGF β is extensively regulated post-transcriptionally (37), whereas PAI1 is a robust indicator of TGF β activity (38). Moreover, at least 15 (47%) of the 32 genes that were correlated with PAI1 in all data sets respond to TGF β , as do many of the less consistently correlated genes, such as TGFB1 (TGF β -induced, 68 kDa) and TGFB111 (TGF β 1-induced transcript 1). Thus, although TGF β and PAI1 were not correlated at the mRNA level, local TGF β activity probably affects tumoral PAI1 expression.

Genomic and epigenetic changes in PAI1 may also affect its overall expression in tumors. Notably, a P1-derived artificial chromosome (RP4-747G18) that contains the entire PAI1 gene plus 24.4 kb of upstream DNA was among the 4,325 array comparative genomic hybridization probes in the UCSF data set. Comparison of DNA copy number results for this probe and expression estimates for PAI1 revealed that this region was amplified in 21 (30%) of the 70 cases for which genomic and expression data were available, and that the copy number ratios for all 70 cases were positively correlated with average PAI1 expression ($r = 0.40$, $P = 0.0005$). Thus, PAI1 copy number changes also seem to influence overall PAI1 mRNA levels in breast cancer. Moreover, gene copy number differences (which act *in cis*) and CTGF (which acts *in trans*) should affect PAI1 expression independently. That is, tumors with high CTGF expression and PAI1 amplification should have the highest levels of PAI1 expression. Indeed, PAI1 expression was more highly correlated with the product of CTGF expression \times the PAI1 copy number ratio ($r = 0.73$, $P < 0.0001$) than with either variable alone. Furthermore, partial correlations for PAI1 expression versus CTGF ($r = 0.74$, $P < 0.001$) and copy number ($r = 0.33$, $P = 0.005$) indicate that the interaction between CTGF and PAI1 is even stronger when gene amplification is taken into account.

Discussion

Gene ablation and reconstitution data show that PAI1 can promote cancer progression (11-14), and correlative data consistently indicate that its elevated expression in breast

Table 4. Genotypic mortality rates and relative hazards

Genotype	n	Time at risk (y)	Annual % mortality	Hazard ratio (95% confidence interval)
4G4G	784	3,269.6	2.57	1.00 (reference)
4G5G	1,222	5,106.8	2.76	1.07 (0.82-1.40)
5G5G	518	2,141.1	3.08	1.20 (0.87-1.66)

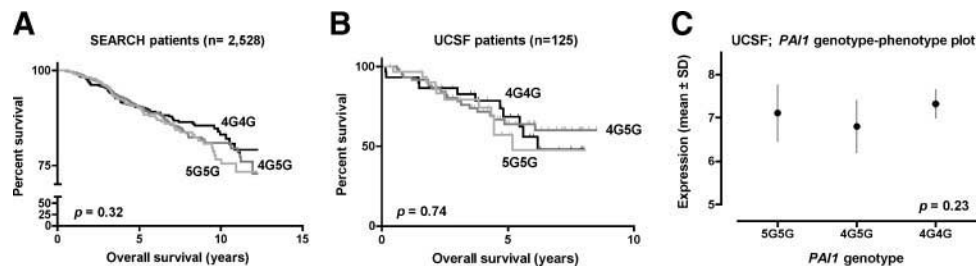


Figure 2. The *PAI1* 4G/5G SNP is not associated with overall survival or tumoral *PAI1* mRNA expression in breast cancer. Kaplan-Meier survival curves for the putative high expression 4G4G (black), intermediate expression 4G5G (dark gray), and low expression 5G5G (light gray) genotypes for all outcome-informative SEARCH (A) and UCSF (B) patients. Mean tumoral *PAI1* mRNA expression levels obtained by microarray expression profiling in a subset of UCSF cases for the corresponding *PAI1* genotypes (C). Two-tailed log-rank test for trend (A and B) and ANOVA (C) *P*s are provided.

tumors is an independent indicator of poor prognosis (4-8). Here, we found that high *PAI1* mRNA expression is also associated with shorter overall survival in breast cancer. Nevertheless, the transcription-altering *PAI1* 4G/5G SNP was not associated with breast cancer incidence, outcome, or tumoral *PAI1* mRNA expression. Thus, local factors seem to be more important than germ line genetic variability in determining the level of *PAI1* expression in breast cancer. Furthermore, our data show that *PAI1* mRNA levels correlate with CTGF and the level of *PAI1* gene amplification, suggesting that they play an important role in regulating the expression and adverse effects of *PAI1* in breast cancer.

Because *PAI1* mRNA expression correlates with its protein expression in breast cancer (39), we reasoned that its mRNA expression should also be prognostic. Indeed, elevated *PAI1* mRNA expression has been associated with shorter disease-free survival in 130 breast cancers (39). Here, we show that tumoral *PAI1* mRNA expression is also an independent or borderline independent prognostic indicator for overall survival in two breast cancer cohorts. Furthermore, the absence of an association in a third cohort suggests that there are demographic and molecular factors that modify the effects of *PAI1* and might aid in identifying cancers for which *PAI1* has the greatest prognostic value. Indeed, the cohorts had significantly different age, ER status, and LN status distributions; however, none of these seemed to explain the between-cohort differences in the prognostic power of *PAI1*. In one cohort, *PAI1* was prognostic in LN⁻ cases, whereas in another, it was prognostic in the LN⁺ subset. Thus, it seems critical to determine what dictates the differential activity and prognostic use of *PAI1*. Nonetheless, our mRNA data were consistent with protein-based observations and support the hypothesis that transcriptional regulation is an important factor in controlling the tumoral levels of *PAI1*.

One factor that can affect *PAI1* transcription is the 4G/5G SNP; yet, our data indicate that it is not associated with breast cancer incidence or survival. Given the common nature of this SNP and the size of our cohort, we had >95% power to detect an odds ratio of 1.5 at *P* < 0.001. By comparison, the five other studies that have examined this SNP in cancer had an average of only 92 cases and 122 controls per study and were thus far less apt to detect a true association (40-44). Despite or due to their limited size, three of these studies detected borderline associations between the high-expressing 4G allele and breast (40, 41) or general cancer incidence (42), whereas no associations were seen in ovarian (43) or colorectal cancer (44). In both breast cancer studies, the 4G allele was more common among cases than controls, and 4G carriers were more apt to have cancer than 5G homozygotes, particularly when the studies were combined. However, these associations

disappear in a meta-analysis that includes SEARCH-EPIC data (Supplementary Table S4). To our knowledge, no functional *PAI1* polymorphism other than the 4G/5G SNP has been described, although a nonsynonymous *PAI1* signal sequence SNP of unknown functional significance (rs6092, Ala¹⁵Thr) has been associated with colorectal cancer risk (45). However, the genotypic distribution of the cases in this study was disconcertingly far from Hardy-Weinberg equilibrium, and this same SNP was not associated with risk in a small ovarian cancer study (43).

The high-expressing 4G4G genotype has also been associated with more advanced colorectal cancers (44) as well as larger diameter and higher-grade breast cancers (40), whereas no such associations were seen in another breast cancer cohort (41). Indeed, these few significant associations are outnumbered by multiple null comparisons and were seen in subsets with an average of only 38 cases (range = 17-55), thus increasing the likelihood that they represent false-positive associations. By comparison, our far larger study revealed no association with outcome and, if anything, showed an unexpected tendency for patients with higher expressing genotypes to have prolonged rather than diminished survival. Thus, our data support the conclusion that cancer-associated signals are more important than germ line genetic variability in determining the expression and ill effects of *PAI1* in breast cancer.

PAI1 protein levels tend to be 7- to 10-fold higher in tumors than normal tissues and can vary >1,000-fold between tumors (2-4). Notably, this is far wider than the 2- to 6-fold differences in reporter gene expression seen for 4G/5G alleles in culture (19, 20). Furthermore, although the 4G/5G SNP contributes to the regulation of humoral *PAI1* *in vivo*, it only accounts for 2% to 3% of the variability in circulating levels (46-49), and mean plasma levels are only 1.2- to 2.5-fold higher in 4G4G homozygotes than in 5G5G homozygotes (Supplementary Table S1). Likewise, it is unclear whether the 4G/5G SNP has any appreciable effect on tumoral *PAI1* levels or whether it is overpowered by other factors. One study found no association between the 4G/5G SNP and *PAI1* protein levels in 40 colorectal cancers (50), whereas another found that the high-expressing 4G allele was associated with increased tumoral *PAI1* expression in 104 breast cancers (40). Our own analyses revealed no such association in a subset of UCSF breast cancers, which is consistent with the lack of association between the 4G/5G SNP and outcome in the SEARCH and UCSF cohorts. Thus, although subtle genotype-driven differences in *PAI1* expression could exert profound effects over an entire lifetime or during the prolonged evolution of a cancer, most data suggest that the 4G/5G SNP has little effect on tumoral *PAI1* expression, which raises the question of what factors are important.

To identify potential upstream regulators of *PAI1* in breast cancer, we examined three comprehensive mRNA expression data sets for genes that were consistently associated with *PAI1*. Notably, *CTGF* was far more strongly correlated with *PAI1* than any of several thousand other genes in two data sets and was the ninth most highly correlated gene in another. Thus, not only were these associations highly significant within each set, but the likelihood that *CTGF* would be so highly ranked in all three sets by chance was minuscule. Moreover, *CTGF* has been shown to enhance $TGF\beta$ -mediated induction of *PAI1* by stimulating expression of the transcription factor *TIEG*, which in turn suppresses transcription of the $TGF\beta$ antagonist *SMAD7* (Fig. 3; refs. 30, 31). However, *SMAD7* was positively rather than inversely correlated with *PAI1* in two data sets, suggesting that an alternative mechanism may be involved. By contrast, *TIEG*, which has been shown to enhance *PAI1* induction (38), was directly correlated with *PAI1* in all data sets, as was *FN1*, which is also induced by $TGF\beta$ and *CTGF* (31). Thus, *TIEG* probably coregulates *PAI1* and *FN1* in response to local $TGF\beta$ /*CTGF* activity. Moreover, not only does *CTGF* enhance the effects of $TGF\beta$, but its own expression is induced by $TGF\beta$ (30, 31). Thus, the involvement of *CTGF* suggests that $TGF\beta$ is also involved. Indeed, 47% of the genes associated with *PAI1* in all three data sets are induced by $TGF\beta$. Furthermore, although $TGF\beta$ and *PAI1* were not correlated in any data set, the expression of $TGF\beta$ does not necessarily reflect its actual activity (37), whereas *PAI1* is an established indicator of $TGF\beta$ activity (38). Alternatively, other $TGF\beta$ super-family members may be involved. Indeed, activins have been shown to induce *PAI1* expression through the same $TGF\beta$ response elements (51). Moreover, the inhibin- β A subunit that homodimerizes to form activin-A was significantly correlated with *PAI1* in all three data sets, and the type I activin receptor (*ACVR1*) and type II $TGF\beta$ receptor (*TGFBR2*) were significantly associated with *PAI1* in one data set. Thus, our data support the notion that *PAI1* levels in breast cancer are strongly influenced by $TGF\beta$ and/or activin-A acting in concert with *CTGF* and *TIEG*.

Another highly correlated gene was the glucose transporter *SLC2A3*. Notably, *SLC2A3* was one of three *PAI1*-correlated genes (including *COL4A2* and *AKAP2*) in the prognostic 70-gene set identified by van de Vijver et al. (33). Thus, its presence in this set may reflect the functional and prognostic importance of *PAI1*, and *PAI1* may add value to the 70-gene signature, as its prognostic power was independent of the 70-gene profile. Moreover, as glucose has been shown to regulate

PAI1 expression (22), *SLC2A3* may be intimately involved in the glucose-mediated regulation of *PAI1* in breast tumors. Likewise, *IL-6* was consistently correlated with *PAI1* and can enhance *PAI1* expression in culture (28), suggesting that it too may influence *PAI1* expression in breast cancer. In addition, *PDGF* may contribute, as its receptor was associated with *PAI1* in all data sets and because it can also influence *PAI1* expression (22). Thus, although *PAI1* expression will depend on the combined effects of many inputs, *CTGF* and *SLC2A3* may be particularly important in breast cancer, given their consistency and level of correlation.

A better understanding of the regulation and role of *PAI1* in breast cancer will undoubtedly require integration of many complementary approaches and will almost certainly enhance its use as a prognostic and predictive tool or therapeutic target. Indeed, some breast cancers may be more sensitive to the effects of *PAI1* than others and thus more informative when *PAI1* is used to predict their prognosis or therapeutic responsiveness. Thus, determining the basis for these differences could prove particularly beneficial. In addition, our data suggest that transcriptional controls play an important role in the overall regulation of *PAI1*; yet, the transcription-altering 4G/5G SNP is apparently unimportant. Thus, inter-individual genetic differences in *PAI1* expression are probably overcome by more profound cancer-associated signals that are responsible for the up-regulation and ill effects of *PAI1* in cancer. Indeed, our data suggest that amplification of the *PAI1* gene may contribute to its elevated expression in some cancers, and that microenvironmental and cellular factors, such as *CTGF* and *SLC2A3*, are key regulators of *PAI1* expression in breast cancer and thus ripe for further study.

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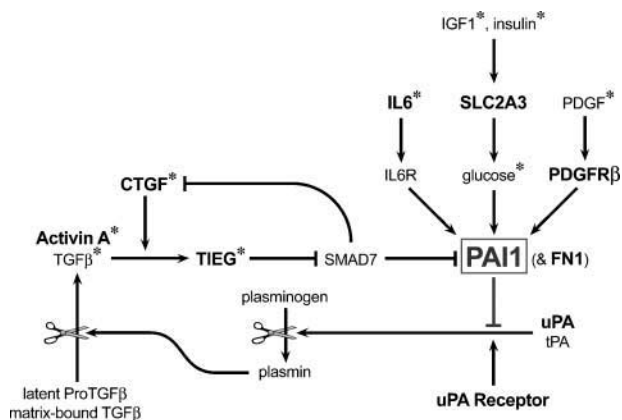


Figure 3. Model depicting the signaling pathways and feedback mechanisms that regulate *PAI1* expression. Factors that were correlated with *PAI1* expression in all three breast cancer data sets are highlighted in bold. *FN1* is included because it is coregulated in parallel with *PAI1* by $TGF\beta$ and *CTGF* (31). *, factors that have been shown to promote *PAI1* expression in culture (21, 22, 28, 30).

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