

# HER-2/neu and Urokinase-Type Plasminogen Activator and Its Inhibitor in Breast Cancer<sup>1</sup>

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## ABSTRACT

**Purpose:** Recent studies suggest that HER-2/neu specifically promotes the invasive capacity of tumor cells by up-regulating secretion of the proteolytic enzyme, urokinase-type plasminogen activator (uPA), or its inhibitor, plasminogen activator inhibitor-1 (PAI-1), in colon and gastric cancer. It was the purpose of this study to: (a) evaluate the association between HER-2/neu and uPA and PAI-1 expression in a large primary breast cancer cohort; (b) perform the first multivariate analysis, including HER-2/neu, uPA, and PAI-1 in breast cancer; and (c) define the effect of HER-2/neu overexpression on uPA and PAI-1 expression in breast cancer cells.

**Experimental Design:** HER-2/neu, uPA, and PAI-1 were measured as continuous variables by ELISA in primary breast cancer tissue extracts from 587 patients with clinical follow-up and analyzed for correlations with clinical outcome. Furthermore, a full-length human HER-2/neu cDNA was introduced into five human breast cancer cell lines to define the effects of HER-2/neu overexpression on uPA and PAI-1 expression. In addition, we tested whether HER-2/neu antibodies could reverse any given alteration of uPA and PAI-1 levels.

**Results:** Our findings indicate a weak positive association between HER-2/neu and uPA ( $r = 0.147$ ;  $P < 0.001$ ) and no association between HER-2/neu and PAI-1 ( $r = 0.07$ ;  $P = 0.085$ ). HER-2/neu overexpression ( $\geq 400$  fmol/mg) and

high levels of uPA/PAI-1 ( $\geq 5.5$  ng/mg and/or  $\geq 14$  ng/mg, respectively) were significantly associated with shorter disease-free survival (DFS;  $P < 0.001$  and  $P = 0.003$ ) and metastasis-free survival (MFS;  $P = 0.015$  and  $P < 0.001$ ). Multivariate analysis revealed prognostic independence between HER-2/neu and the uPA/PAI-1 axis for DFS and MFS. Both uPA and PAI-1 had no significant discriminatory effect among HER-2/neu-positive patients for DFS. The prognostic value of HER-2/neu overexpression for MFS, however, was significantly enhanced by elevated uPA expression ( $P = 0.053$ ). Stable transfection of the HER-2/neu gene into multiple human breast cancer cell lines resulted in consistent down-regulation of uPA or PAI-1 expression. In addition, anti-HER-2/neu antibodies did not significantly affect uPA or PAI-1 expression in human cancer cell lines naturally overexpressing HER-2/neu.

**Conclusions:** The present findings suggest that the invasive phenotype elicited by HER-2/neu overexpression in breast cancer is not a direct effect of uPA or PAI-1 expression. HER-2/neu and the uPA/PAI-1 axis have been shown to affect the invasive capacity of breast cancer independently. Determination of uPA can provide significant additional prognostic information for MFS in HER-2/neu-positive and -negative patients.

## INTRODUCTION

The human HER-2/neu (*c-erbB-2*) proto-oncogene encodes a  $M_r$  185,000 transmembrane receptor tyrosine kinase that is homologous to but distinct from the epidermal growth factor receptor, as well as other members of the type I receptor tyrosine kinase family (*i.e.*, HER-3 and HER-4; Ref. 1). Amplification of the HER-2/neu gene occurs in approximately 20–25% of human breast cancers, resulting in overexpression of the gene product (2, 3). HER-2/neu overexpression is an independent predictor of both relapse-free and overall survival in breast cancer (2, 4–6).

Recent preclinical studies suggest that HER-2/neu plays a role in tumor progression by specifically promoting the invasive capacity of tumor cells through increased cell adhesion (7) and through enhanced cell motility (8–10). In addition, recent clinical investigations (11, 12) demonstrated an association between HER-2/neu overexpression and expression of tumor-associated proteolysis in gastric and colon cancer, suggesting a direct role for HER-2/neu in invasion and metastasis through up-regulation of proteolytic enzymes. Among the proteases investigated were uPA<sup>4</sup> and its inhibitor, PAI-1, both of which have been suggested to play a central role in the degradation of the ECM (13,

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<sup>4</sup> The abbreviations used are: uPA, urokinase-type plasminogen activator; PAI, plasminogen activator inhibitor; ECM, extracellular matrix; MMP, matrix metalloproteinase; DFS, disease-free survival; MFS, metastasis-free survival.

14). Plasmin, the substrate of uPA, is a broad-spectrum proteinase that not only can directly degrade multiple ECM targets but can also cooperate with other ECM-degrading enzymes, such as members of the MMP gene family (15). Both uPA and PAI-1 have been associated with disease outcome as statistically independent prognostic markers in cancers of the breast (16–20), lung (21), colon (22), kidney (23), ovary (24, 25), and the gastrointestinal tract (26). However, thus far, the association of HER-2/neu expression with the expression of the protease, uPA, and its inhibitor, PAI-1, has not yet been thoroughly investigated in breast cancer.

To explore this association, the exact protein concentrations of HER-2/neu, uPA, and PAI-1 were assessed as continuous variables by ELISA in a consecutive series of 587 patients treated for primary breast cancer. A comprehensive comparison of the uPA and PAI-1 concentrations in HER-2/neu-overexpressing and -nonoverexpressing patients and subsequent analysis of these results provides a clearer understanding of the relationship between the uPA/PAI-1 axis and HER-2/neu. To investigate whether HER-2/neu and uPA/PAI-1 would be of independent prognostic significance, univariate and multivariate analyses for DFS and MFS were performed. To more fully elucidate the effect(s) of HER-2/neu overexpression on uPA and PAI-1 concentrations, a full-length, human HER-2/neu cDNA was introduced via a retroviral expression vector into five breast cancer cell lines (27–29). All of the parental cell lines used in this study contain a single copy of the *HER-2/neu* gene and express basal levels of the gene product, whereas the matched HER-2/neu retroviral transfectants overexpress the gene (28–30). This allows a direct comparison of uPA and PAI-1 concentrations between genetically identical parent/daughter cells, which differ only in that one member of the pair overexpresses the human *HER-2/neu* gene. Finally, the effect of the anti-HER-2/neu antibody (trastuzumab) on uPA or PAI-1 concentrations was examined in human breast and ovarian cancer cell lines that naturally overexpress the HER-2/neu receptor. The rationale for this experimental approach was to determine whether inhibition of the HER-2/neu receptor could reverse possible effects of HER-2/neu overexpression on uPA or PAI-1 concentrations in HER-2/neu-overexpressing human breast and ovarian cancer cells. The purpose of these experiments and the clinical correlation was to explore the nature and degree of the interaction between HER-2/neu and the uPA/PAI-1 axis in breast cancer and to investigate whether the regulation of expression of each of the two markers is directly linked to the other.

## PATIENTS AND METHODS

**Patients.** The breast cancer tissue specimens used originated from 587 patients at the Department of Obstetrics and Gynecology, University of Munich, Klinikum Grosshadern between 1992 and 1997. Clinical and pathobiological information obtained from medical records included age at diagnosis, menopausal status, lymph node metastasis, tumor size, nuclear grade, histology, and therapeutic intervention (surgical, chemotherapeutic, radiotherapeutic, and hormonal). Follow-up information for DFS and MFS was available for all of these patients. Criteria for exclusion from the study included distant metastasis at the time of diagnosis. Median follow-up time was 26 months

for DFS and 32 months for MFS. Tumors were staged according to the criteria of the International Union against Cancer. Lymph node-negative patients underwent either modified radical mastectomy or a lumpectomy, followed by radiation therapy. Lymph node-positive and high-risk node-negative patients underwent modified radical mastectomy or lumpectomy with radiation, followed by adjuvant chemotherapy and/or hormone therapy.

**Tissue Extraction and ELISA for uPA, PAI-1, and HER-2/neu.** Breast cancer tissue specimens were obtained during surgery, and tissue sections were analyzed by histological assessment in all of the cases. The remainder of each sample was stored at  $-198^{\circ}\text{C}$  in liquid nitrogen until use. Subsequently, frozen specimens of 500-mg wet weight were pulverized with a microdismembrator (Braun-Melsungen, Melsungen, Germany), suspended in 2 ml of Tris-buffered saline containing 1% Triton X-100 detergent (Sigma Chemical Co., Munich, Germany), and incubated at  $4^{\circ}\text{C}$  for 12 h, followed by ultracentrifugation at  $100,000 \times g$  for 45 min. Quantitative levels of uPA and PAI-1 were prospectively measured in the supernatants, using ELISA kits, as described (Ref. 31; Imubind uPA and Imubind PAI-1; American Diagnostica, Greenwich, CT). Antigen concentrations for uPA and PAI-1 were measured in terms of ng/mg protein. Protein concentrations were measured using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL). Assays for estrogen and progesterone receptor content were performed with enzyme immunoassays [ER-enzyme immunoassay (EIA) and PR-EIA; Abbott Laboratories, Chicago, IL], as described (32). The concentrations of HER-2/neu were measured retrospectively with a commercially available ELISA kit (Oncogene Research Products, Cambridge, MA). The assay uses a mouse monoclonal antibody for capture and a rabbit polyclonal serum for quantitation of the human HER-2/neu protein.

**Statistical Analysis.** Statistical analysis was performed using SPSS statistical software. Univariate and multivariate analyses were performed by the log rank test and Cox's regression analysis, respectively. Two group comparisons assuming equal variance were performed, using Student's *t* test (two-tail). Nonparametric methods were used (Mann-Whitney *U* test) for non-normally distributed data. *P*s of  $\leq 0.05$  were considered to be significant. Survival curves were analyzed by the method of Kaplan and Meier (33). We identified optimized cutoff values for separation of patterns with distinct prognosis using univariate analyses. After the antigen extraction method described above, a HER-2/neu cutoff value of 400 fmol/mg protein provided the maximum separation of patients with regard to DFS (log rank test;  $P = 0.0006$ ). This cutoff was chosen in accordance with cell line data in which 400 fmol/mg was also selected as a suitable cutoff value to distinguish between normal and overexpressed HER-2/neu protein levels. The selected cutoff of 400 fmol/mg was further validated using a subset of 152 patients in which the ELISA results were correlated with HER-2/neu overexpression, as detected by immunohistochemical scoring of paraffin-embedded tissue sections from the same samples. The HER-2/neu antibody, CB-11 (Novacastra, Newcastle, United Kingdom), was used for immunohistochemical staining, performed as described previously (34) in detail. The results using the CB-11 antibody for immunohistochemical scoring demonstrated a concordance of 88% with the results obtained by

ELISA, using a cutoff of 400 fmol/mg. uPA and PAI-1 cutoff values of 5.5 ng/mg and 14 ng/mg, respectively, provided maximum separation of patients with regards to DFS (log rank test;  $P = 0.046$  and  $P = 0.0076$ , respectively).

**Cell Lines and Cell Culture.** The human breast cancer cell lines, MCF-7, BT-20, MDA-MB-231, ZR-75-1, MDA-MB-435, SK-BR-3, BT-474, MDA-MB-453, MDA-MB-361, and MDA-MB-175, and the human ovarian cancer cell line, SK-OV-3, were obtained from the American Type Culture Collection (Rockville, MD). All of the cells were cultured in RPMI medium 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA). Human breast cancer cells with normal levels of HER-2/neu expression were transfected with a full-length cDNA of the human *HER-2/neu* gene, using a replication-defective retroviral expression vector, as described previously (27–29, 35). Briefly, the vector for introduction of the *HER-2/neu* gene into human cells contained the full-length human *HER-2/neu* gene-coding sequence ligated into the replication-defective retroviral expression vector, pLXSN (2, 27). This was achieved by ligating a 3.8-kb *NcoI* to *MspI* fragment containing the full *HER-2/neu*-coding sequence, without the polyadenylation signal, into an amphotrophic retroviral expression vector with a Moloney murine leukemia virus promoter, a neomycin phosphotransferase gene, and a packaging signal, but devoid of viral coding sequences. The cell lines established by these methods have been fully molecularly characterized for HER-2/neu expression at both the DNA and RNA transcript and protein levels (3, 28, 35).

uPA and PAI-1 concentrations were measured in *HER-2/neu*-transfected cells or matching parental breast cancer cells by ELISA in cytoplasmic extracts after 48 h of incubation with 1 nM of heregulin  $\beta$ 1 (Lab Vision). Similarly, cell lines naturally overexpressing HER-2/neu were incubated with either 10  $\mu$ g/ml trastuzumab or IgG for 48 h and collected for analysis. uPA and PAI-1 expression was characterized in cytoplasmic extracts by ELISA, as described above.

**Collagen IV Invasion Assay.** Comparison of invasive behavior between *HER-2/neu*-transfected cells and matching parental cells was performed using a Boyden chamber assay. Human collagen IV (Becton Dickinson) gels were prepared according to the manufacturer's instructions. Approximately 75  $\mu$ l was used to coat 8- $\mu$ m pore membrane transwell 24-culture dishes (Costar). Cells ( $7.5 \times 10^5$ ) were loaded onto the top chamber in media, and 10% serum or 10% serum with 1 nM heregulin (Lab Vision) was used as a chemoattractant in the bottom chamber. Invaded cells were quantitated by direct cell count visually after 25 h for the collagen IV invasion assay. To determine whether trastuzumab was capable of reversing the invasive behavior in *HER-2/neu*-transfected cells, 5  $\mu$ g/ml trastuzumab was added to the cell culture media.

## RESULTS

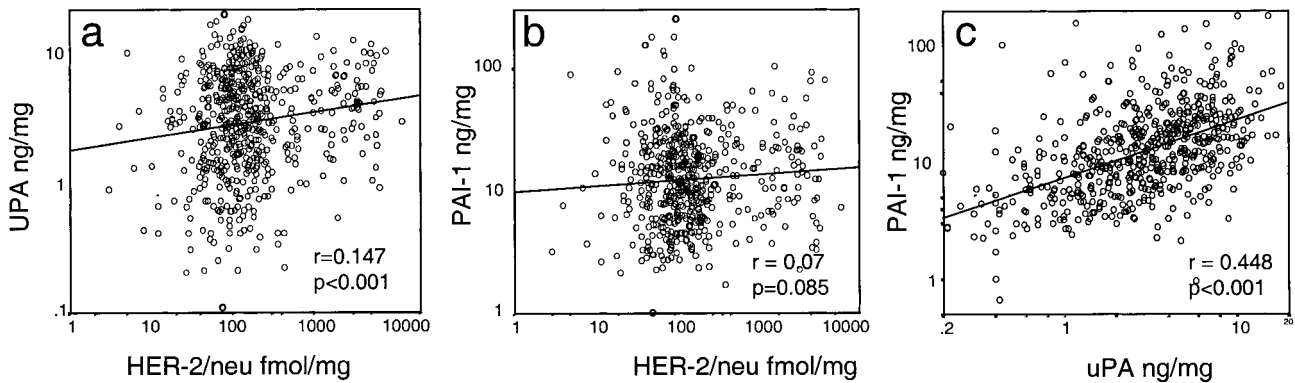
**Associations between HER-2/neu, uPA, and PAI-1 as Continuous Variables.** HER-2/neu, uPA, and PAI-1 concentrations were measured as continuous variables by ELISA in tumor samples from 587 patients with primary breast cancer. Patients as well as disease characteristics of the current study

Table 1 Patient and disease characteristics in node-negative and -positive breast cancer patients ( $n = 587$ )

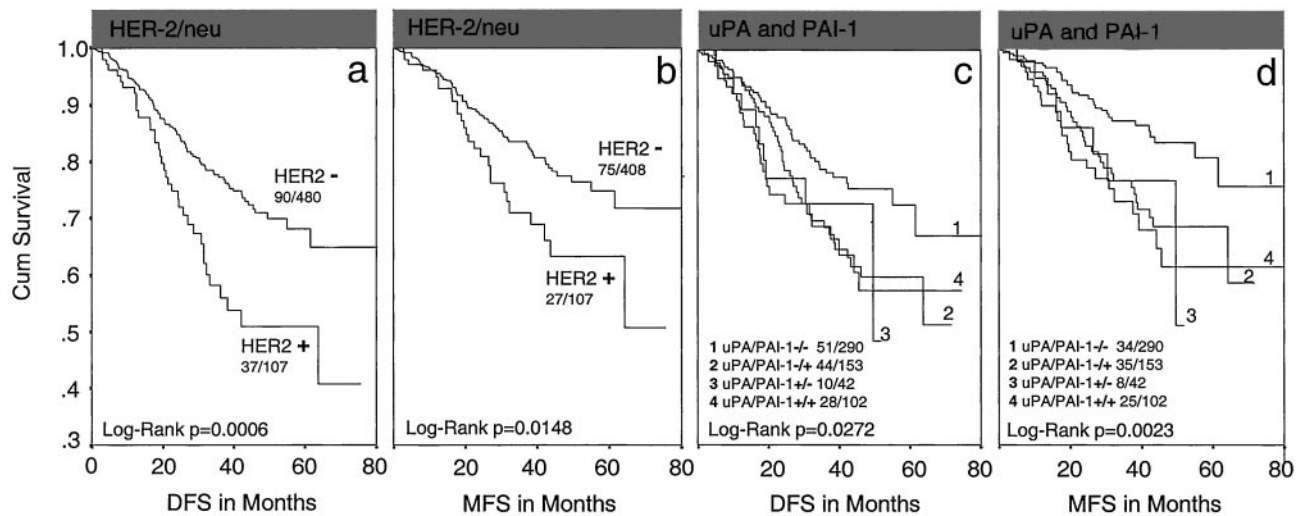
Factors	No. of patients	Percentage
Tumor size		
T1 ( $\leq 2$ cm)	216	36.8
T2 ( $> 2$ cm)	273	46.5
T3–4 ( $\geq 5$ cm)	98	17.7
No. of positive nodes		
0	283	48.2
1–3	175	29.8
4–9	57	9.7
$\geq 10$	55	9.4
Missing	17	2.9
Nuclear grade		
1–2	360	61.3
3–4	227	38.7
Menopausal status		
Pre-	142	24.2
Post-	445	75.8
Estrogen receptor status		
Negative ( $< 10$ fmol/mg)	193	32.9
Positive ( $\geq 10$ fmol/mg)	394	67.1
Progesterone receptor status		
Negative ( $< 10$ fmol/mg)	187	31.9
Positive ( $\geq 10$ fmol/mg)	400	68.1
HER-2/neu		
Negative ( $< 400$ fmol/mg)	480	81.8
Positive ( $\geq 400$ fmol/mg)	107	18.2
uPA		
Negative ( $< 5.5$ ng/mg)	443	75.5
Positive ( $\geq 5.5$ ng/mg)	144	24.5
PAI-1		
Negative ( $< 14$ ng/mg)	332	56.6
Positive ( $\geq 14$ ng/mg)	255	43.4
uPA/PAI-1		
Both negative	290	49.4
One or both positive	297	50.6

cohort are summarized in Table 1. uPA and PAI-1 concentrations ranged from 0–18.2 and 0–252.2 ng/mg protein, respectively (uPA, median, 3.0 ng/mg; PAI-1, median, 11.9 ng/mg). HER-2/neu protein concentrations ranged from 0–12,015 fmol/mg protein (median, 125 fmol/mg). Once the distribution of HER-2/neu, uPA, and PAI-1 expression for the cohort was determined, a logarithmic transformation of the data was performed, thus allowing a linear regression analysis. Among all of the 587 patients analyzed, HER-2/neu and uPA values demonstrated a positive but weak correlation ( $r = 0.147$ ;  $P < 0.001$ ; Fig. 1a). HER-2/neu and PAI-1 concentrations revealed no significant association ( $r = 0.07$ ;  $P = 0.085$ ; Fig. 1b). In contrast, uPA and PAI-1 levels correlated well ( $r = 0.488$ ;  $P < 0.001$ ; Fig. 1c), which is consistent with earlier observations by other investigators (36, 37). In accordance with these findings, HER-2/neu-positive patients ( $\geq 400$  fmol/mg protein;  $n = 107$ ) demonstrated slightly higher uPA and PAI-1 concentrations compared with HER-2/neu-negative patients (uPA: median, 3.78 versus 2.90 ng/mg;  $P = 0.052$ ; PAI-1: median, 14.6 versus 11.4 ng/mg;  $P = 0.016$ ).

**Univariate and Multivariate Analyses.** To explore the hypothesis that HER-2/neu and the uPA/PAI-1 axis are of independent prognostic significance, univariate and multivariate analyses were performed. HER-2/neu-negative patients ( $< 400$



**Fig. 1** Scatter plots of logarithmically transformed, corresponding values of HER-2/neu protein and uPA (a) or PAI-1 (b) and uPA and PAI-1 (c) protein concentrations in the primary tumors of 587 primary breast cancer patients. Pearson's correlation revealed a weak positive association between HER-2/neu and uPA concentrations ( $r = 0.147$ ;  $P < 0.001$ ), no significant association between HER-2/neu and PAI-1 protein concentrations ( $r = 0.07$ ;  $P = 0.085$ ), and a significant positive association between uPA and PAI-1 concentrations ( $r = 0.448$ ;  $P < 0.001$ ).



**Fig. 2** DFS and MFS of 587 primary breast cancer patients according to detection of HER-2/neu (a and b) and uPA and PAI-1 (c and d). The number of events and cases is shown in the corresponding figure. a, HER-2/neu low (<400 fmol/mg) versus HER-2/neu high: DFS (mean  $\pm$  SE)  $62.4 \pm 1.6$  versus  $48.5 \pm 3.3$  months; b, HER-2/neu low (<400 fmol/mg) versus HER-2/neu high: MFS (mean  $\pm$  SE)  $66.3 \pm 1.5$  versus  $55.4 \pm 3.2$  months; c, DFS (mean  $\pm$  SE): 1, uPA and PAI-1 both low (<5 ng/mg and <14.0 ng/mg, respectively),  $64.3 \pm 2.1$  months; 2, uPA low and PAI-1 high,  $41.6 \pm 2.8$  months; 3, uPA high and PAI-1 low,  $52.3 \pm 2.3$  months; and 4, uPA and PAI-1 both high,  $53.7 \pm 3.3$  months; d, MFS (mean  $\pm$  SE): 1, uPA and PAI-1 low (<5 ng/mg and <14.0 ng/mg, respectively),  $69.2 \pm 1.8$  months; 2, uPA low and PAI-1 high,  $56.1 \pm 2.2$  months; 3, uPA high and PAI-1 low,  $43.5 \pm 2.6$  months; and 4, uPA and PAI-1 both high,  $58.8 \pm 3.7$  months.

fmol/mg;  $n = 480$ ) had an improved DFS and MFS compared with HER-2/neu-positive patients ( $n = 107$ ;  $P = 0.0006$  and  $P = 0.0148$ , respectively; Fig. 2, a and b). Patients with uPA or PAI-1 concentrations below 5.5 or 14 ng/ml protein, respectively, had improved DFS and MFS (uPA: DFS,  $P = 0.046$ ; MFS,  $P = 0.0079$ ; PAI-1: DFS,  $P = 0.0076$ ; MFS,  $P = 0.0009$ ). In patients with PAI-1-negative tumors, uPA positivity did identify those with poor prognosis, whereas the discriminatory effect of uPA in the PAI-1-positive group was only marginal (Fig. 2, c and d). In accordance with previous reports (19, 38), the combination of both uPA and PAI-1 (low uPA and PAI-1 versus high uPA and/or PAI-1) allowed a better risk-group discrimination (DFS,  $P = 0.003$ ; MFS,  $P = 0.0002$ ) compared with using only one of the markers.

After univariate analysis, the variables of age, tumor size, number of positive nodes, estrogen and progesterone receptor levels, uPA/PAI-1, and HER-2/neu levels were included in the multivariate model (Table 2). uPA/PAI-1 concentrations were analyzed as one variable (both negative versus elevated uPA and/or PAI-1), because the combination of both markers allowed the best risk-group discrimination, and both markers were closely associated (Fig. 1c). In multivariate analysis for DFS, tumor size ( $P = 0.0069$ ), number of axillary nodes ( $P = 0.0002$ ), levels of uPA/PAI-1 ( $P = 0.0096$ ), and HER-2/neu ( $P = 0.024$ ) were independent prognostic markers (Table 2). In multivariate analysis for MFS, including the variables of age, tumor size, levels of uPA and/or PAI-1, levels of estrogen and progesterone receptor expression, and HER-2/neu, the variables

Table 2 Univariate and multivariate analyses (*P*s) of prognostic factors for DFS and MFS in 587 breast cancer patients

Statistical analyses were performed by the log rank test for univariate analysis and by Cox's regression analysis to evaluate the predictive power of various combinations and interactions of prognostic factors in multivariate analysis. Prognostic parameters included age, size of primary tumor (Size), number of nodes (Nodes), estrogen receptor, progesterone receptor, and HER-2/neu protein expression (HER-2/neu) as continuous variables. uPA and PAI-1 was included as a dichotomous variable (both negative or one or both positive). The multivariate analysis included 542 patients. The median follow-up was 26 months for DFS and 32 months for MFS.

Factor	DFS		MFS	
	Univariate	Multivariate	Univariate	Multivariate
Age	0.0001	0.975	<0.0001	0.26
Size	0.0004	0.0069	0.0001	0.0024 <sup>a</sup>
Nodes	0.001	0.0002	<0.0001	<0.0001
uPA/PAI-1	0.003	0.0096	0.0002	0.0016 <sup>a</sup>
Estrogen receptor	0.0014	0.15	0.0067	0.58
Progesterone receptor	<0.0001	0.14	<0.0001	0.25
HER-2/neu	0.0006	0.024	0.0148	0.064 <sup>a</sup>

<sup>a</sup> When number of nodes was not introduced into the multivariate model for MFS, HER-2/neu was of significance (HER-2/neu, *P* = 0.025; size, *P* < 0.0001; uPA/PAI-1, *P* = 0.0005).

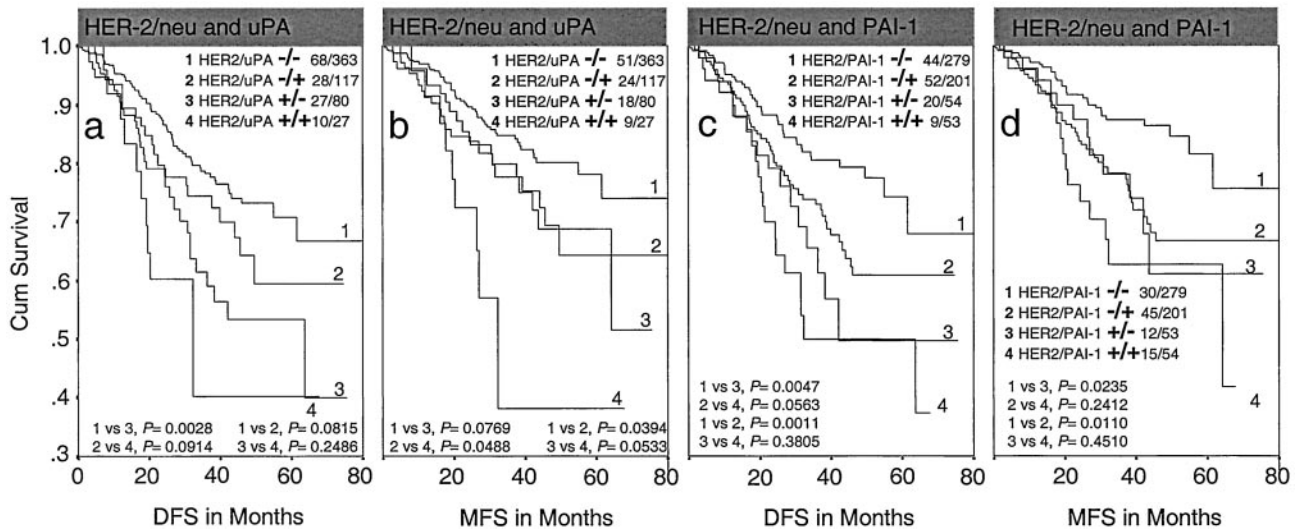
shown to be independent prognostic markers were tumor size (*P* < 0.0001), uPA/PAI-1 (*P* = 0.0005), and HER-2/neu (*P* = 0.025). However, when the number of nodes (*P* < 0.0001) was introduced into the multivariate model for MFS, HER-2/neu was of borderline significance (*P* = 0.064), and uPA/PAI-1 (*P* = 0.0016) and size (*P* < 0.0001) remained independent prognostic factors (Table 2). These findings are based upon the fact that HER-2/neu overexpression was significantly associated with nodal status (node-negative, 13.4% HER-2/neu-positive; node-positive, 23.3% HER-2/neu-positive; *P* = 0.003). However, the metastatic capacity associated with high uPA or PAI-1 concentrations is not reflected in the nodal status at diagnosis (node-negative, 24% uPA- and 42% PAI-1-positive; node-positive, 25% uPA- and 45% PAI-1-positive).

**Combined Effects of HER-2/neu and uPA or PAI-1 on DFS and MFS.** To further investigate the potential role of HER-2/neu, uPA, and PAI-1 in the development of disease recurrence or metastasis, the interactions between HER-2/neu and uPA or PAI-1 were analyzed separately for both DFS and MFS (Fig. 3, *a-d*). In patients with HER-2/neu-positive tumors, uPA positivity did not further identify patients with poor DFS (*P* = 0.2486; Fig. 3*a*), whereas, more importantly, the discriminatory effect of uPA in HER-2/neu-positive patients was significant with respect to MFS (*P* = 0.0533; Fig. 3*b*). Interestingly, a similar discriminatory effect of PAI-1 on MFS in patients with HER-2/neu-positive tumors was also observed; however, the difference was not statistically significant in this study cohort (*P* = 0.451; Fig. 3*d*). Of note, a positive uPA status did not affect the prognostic significance of HER-2/neu for DFS (Fig. 3*a*). However, a positive uPA status significantly enhanced the prognostic significance of HER-2/neu for MFS (Fig. 3*b*).

**Effect of HER-2/neu Overexpression on uPA and PAI-1 Expression in Human Breast Cancer Cells Engineered to Overexpress the HER-2/neu Gene.** A full-length *HER-2/neu* cDNA was introduced via retroviral vector into the human breast cancer cell lines, MCF-7, MDA-MB-231, MDA-MB-435, ZR-75-1, and BT-20, which are known to have a single copy of the *HER-2/neu* gene and to express normal levels of its gene product (27–29, 35). The levels of HER-2/neu overexpression in the engineered cells are comparable with but do not

exceed the levels found in actual human tumors, making it less likely that observed biological changes are artifacts of overexpression. The rationale for this experiment was to allow direct comparison of genetically identical parent/daughter cells that differ only in that one member of the pair overexpresses HER-2/neu. ELISA assays were performed for uPA and PAI-1 on these cells to determine whether increases in levels of HER-2/neu expression were accompanied by increases in levels of expression of uPA or PAI-1. Among the breast cancer cell lines investigated, MCF-7, MDA-MB-435, ZR-75-1, and BT-20 showed low basal concentrations of uPA and PAI-1, unlike MDA-MB-231 cells, which demonstrated high basal concentrations of uPA and PAI-1 (Fig. 4, *a* and *b*). HER-2/neu overexpression among the transfected cell lines did not result in any instance in up-regulation of uPA or PAI-1, but instead, resulted in significant down-regulation of uPA in MDA-MB-231/HER-2 and of PAI-1 in MDA-MB-231/HER-2, MDA-MB-435/HER-2, and MCF-7/HER-2 cells. To verify the functional significance of *HER-2/neu* gene transfection and its effect on tumor cell invasion, we also compared the cell invasiveness between three parental cell lines and their *HER-2/neu*-transfected counterparts. As a result of *HER-2/neu* transfection, MCF-7/HER-2, MDA-MB-435/HER-2, and MDA-MB-231/HER-2 cells showed a significantly higher invasive capacity compared to control cells with an up to 10-fold higher ability to invade through a collagen IV gel (Fig. 5). It is important to note that these *HER-2/neu*-transfected cell lines demonstrated increased invasiveness, despite down-regulation of both uPA and PAI-1 expression (Fig. 4, *a* and *b*).

**Effect of Trastuzumab (Herceptin) on uPA and PAI-1 Concentrations in Breast and Ovarian Cancer Cells that Naturally Overexpress HER-2/neu.** Trastuzumab has specific antiproliferative activity against HER-2/neu-overexpressing human breast and ovarian cancer cells (39, 40). In addition, trastuzumab reduces the invasive capabilities of HER-2/neu-overexpressing cell lines *in vitro* (Fig. 5). Therefore, we investigated whether incubation of HER-2/neu-overexpressing human breast and ovarian cancer cells with trastuzumab was accompanied by alterations in the expression levels of uPA and/or PAI-1 (Fig. 6). Control cells were treated with human



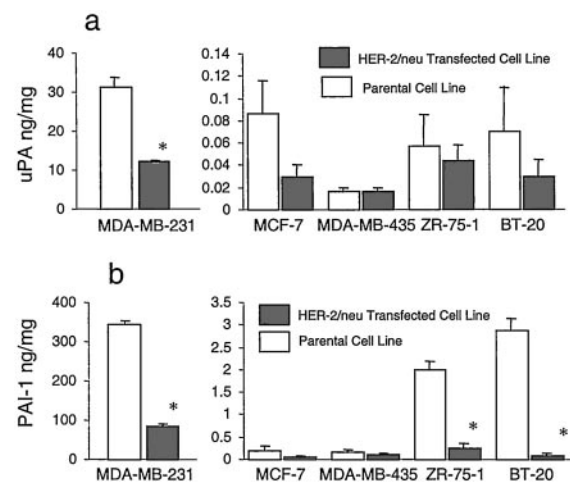
**Fig. 3** DFS and MFS of 587 primary breast cancer patients according to the detection of HER-2/neu and uPA (a and b) or PAI-1 (c and d) protein concentrations. In patients with HER-2/neu-positive tumors, uPA positivity did not further identify those with poor DFS (group 3 versus group 4;  $P = 0.2486$ ); however, it did identify those with poor MFS (group 3 versus group 4;  $P = 0.0533$ ). The number of events and cases is shown in the corresponding figure. a, DFS (mean  $\pm$  SE): 1, HER-2/neu and uPA low ( $<400$  fmol/mg and  $<5.5$  ng/mg, respectively),  $63.8 \pm 1.8$  months; 2, HER-2/neu low and uPA high,  $55.0 \pm 3.1$  months; 3, HER-2/neu high and uPA low,  $49.8 \pm 3.7$  months; and 4, HER-2/neu and uPA high,  $39.6 \pm 7.2$  months; b, MFS (mean  $\pm$  SE): 1, HER-2/neu and uPA low ( $<400$  fmol/mg and  $<5.5$  ng/mg, respectively),  $67.7 \pm 1.7$  months; 2, HER-2/neu low and uPA high,  $61.6 \pm 3.43$  months; 3, HER-2/neu high and uPA low,  $57.9 \pm 3.5$  months; and 4, HER-2/neu and uPA high,  $40.37 \pm 6.98$  months; c, DFS (mean  $\pm$  SE): 1, HER-2/neu and PAI-1 both low ( $<400$  fmol/mg and  $<14.0$  ng/mg, respectively),  $65.3 \pm 2.2$  months; 2, HER-2/neu low and PAI-1 high,  $55.4 \pm 2.2$  months; 3, HER-2/neu high and PAI-1 low,  $50.4 \pm 4.7$  months; and 4, HER-2/neu and PAI-1 both high,  $43.7 \pm 3.9$  months; d, MFS (mean  $\pm$  SE): 1, HER-2/neu and PAI-1 low ( $<400$  fmol/mg and  $<14.0$  ng/mg, respectively),  $69.6 \pm 2.0$  months; 2, HER-2/neu low and PAI-1 high,  $62.0 \pm 2.3$  months; 3, HER-2/neu high and PAI-1 low,  $57.5 \pm 4.4$  months; and 4, HER-2/neu and PAI-1 both high,  $49.4 \pm 3.8$  months.

IgG at a dose and schedule equivalent to the cell lines tested. Among the cell lines known to naturally overexpress HER-2/neu, the human breast cancer cell lines SK-BR-3, BT-474, MDA-MB-361, MDA-MB-453, and MDA-MB-175 expressed low basal levels of uPA and PAI-1, and the human ovarian cancer cell line SK-OV-3 showed elevated basal concentrations of uPA and PAI-1. However, treatment with trastuzumab did not significantly alter the levels of uPA and PAI-1 expression in any of the HER-2/neu-overexpressing cell lines tested (Fig. 6), despite the fact that trastuzumab reduced (in MCF-7/HER-2 and MDA-MB-231/HER-2) or reversed (in MDA-MB-435/HER-2) the invasive capabilities of HER-2/neu-transfected breast cancer cell lines (Fig. 5).

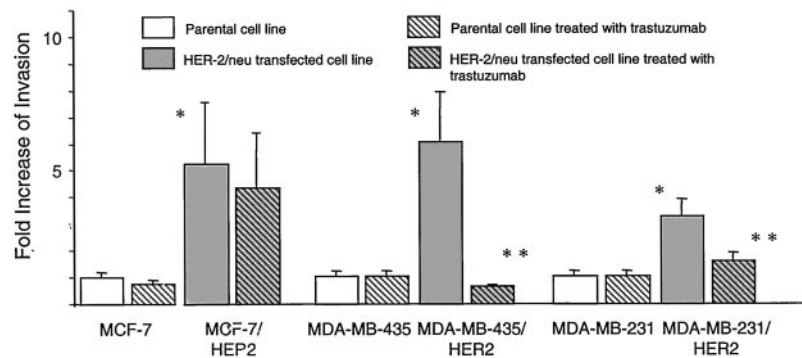
## DISCUSSION

HER-2/neu overexpression is associated with increased tumor progression and metastasis (2, 3). However, the mechanisms by which HER-2/neu regulates the metastatic phenotype are not fully understood. Overexpression of the HER-2/neu receptor results in enhanced cell proliferation (28) and increased cell motility and invasiveness (9, 10). Recent studies have described an association between HER-2/neu overexpression and the expression of proteases such as uPA (11, 12). Therefore, it was the primary purpose of this study to investigate whether the invasive phenotype elicited by HER-2/neu overexpression was directly linked to the up-regulation and expression of uPA or its inhibitor, PAI-1, in breast cancer.

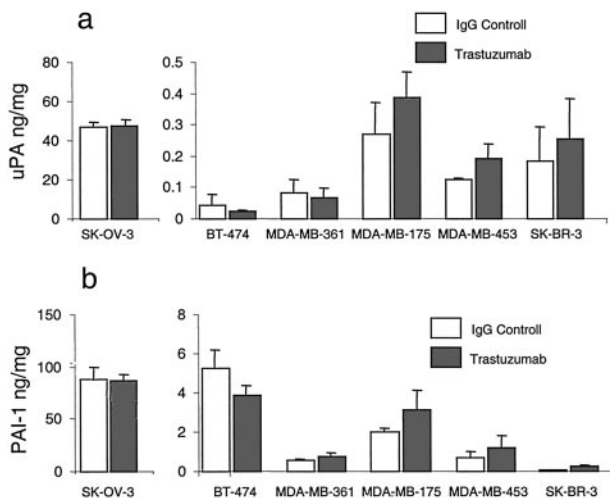
Both studies (11, 12), which documented an association



**Fig. 4** uPA (a) and PAI-1 (b) concentrations in HER-2/neu-transfected cells or matching parental breast cancer cell lines. A full-length HER-2/neu cDNA was introduced via retroviral vector into human breast cancer cells that are known to have a single copy of the HER-2/neu gene and express normal levels of the gene product. The transfected cell lines have been fully characterized at the DNA, RNA, and protein levels (27, 28, 52). The functional activity of the HER-2/neu protein in transfected cell lines was verified earlier by immunoblotting techniques (28). MDA-MB-231 human breast cancer cells express high basal concentrations of uPA and PAI-1 compared with MDA-MB-435, BT-20, ZR-75-1, and MCF-7 breast cancer cells. HER-2/neu-transfected cells exhibited significantly lower uPA concentrations in MDA-MB-231/HER-2 breast cancer cells and significantly lower PAI-1 concentrations in MDA-MB-231/HER-2, BT-20/HER-2, and MDA-MB-435/HER-2 breast cancer cells. \*, significant difference.



**Fig. 5** Invasive behavior of HER-2/neu-transfected and matched parental MDA-MB-231, MDA-MB-435, and MCF-7 cells. HER-2/neu transfection strongly increased the invasive capabilities of the human breast cancer cell lines MCF-7/HER-2, MDA-MB-231/HER-2, and MDA-MB-435/HER-2. Treatment of HER-2/neu-transfected cells with trastuzumab (1  $\mu$ g/ml) was capable of reducing (MCF-7/HER-2) or reversing (MDA-MB-231/HER-2 and MDA-MB-435/HER-2) the invasive characteristics associated with HER-2/neu overexpression. \*, significant difference between transfected and control cell lines; \*\*, significant difference between cell lines incubated with or without trastuzumab.



**Fig. 6** Comparison of uPA (a) and PAI-1 (b) concentrations after treatment with trastuzumab. Human breast and ovarian cancer cell lines that demonstrate natural HER-2/neu overexpression (BT-474, MDA-MB-361, MDA-MB-175, SK-BR-3, and SK-OV-3) were incubated with 10  $\mu$ g/ml of either trastuzumab or IgG. The HER-2/neu antibody treatment did not significantly alter uPA or PAI-1 expression. However, there was a general trend for uPA and PAI-1 to be up-regulated by treatment with trastuzumab.

between HER-2/neu and uPA expression, characterized the relationship using dichotomous cutoff values based on immunohistochemical staining of paraffin-embedded tissue. Because such a classification would possibly oversimplify the interaction, we decided to characterize the relationship between HER-2/neu and uPA or PAI-1 by analyzing expression as continuous variables. Using ELISA, the actual antigen levels for both markers can be measured in units of fmol/mg and ng/mg protein, respectively. Furthermore, the use of frozen breast cancer samples for HER-2/neu, uPA, and PAI-1 analyses minimizes the well-recognized impact of reduced immunogenicity attributable to fixation, which has been observed when using paraffin-embedded tissue (30). However, this approach can suffer from

errors that could result in a reduction of reported levels as opposed to the actual levels, because of dilutional artifacts known to exist in tissue homogenates of mixed cell type populations (3). However, the same effect occurs with the measurement of uPA, PAI-1, and HER-2/neu, because all of the three are affected to the same degree by the stromal cell artifacts. Because it was the primary focus of the present study to precisely characterize the relationship between HER-2/neu and uPA and PAI-1, quantitation of the HER-2/neu, uPA, and PAI-1 proteins as continuous variables by ELISA therefore appeared to be the most appropriate method.

In the present study, the strength of the association between HER-2/neu and uPA is very low ( $r = 0.147$ ), suggesting that the association between uPA and HER-2/neu is more likely to be based on indirect interactions rather than on direct regulation. A previous study (11) demonstrated a stronger association ( $r = 0.4$ ) between HER-2/neu and uPA expression among 58 patients with colon cancer by using computerized image analysis of cytoplasmic areas on immunohistochemically stained paraffin-embedded tissue. However, considering the reported rate of HER-2/neu gene amplification (6.8%; Ref. 41) and protein expression (20–43%; Refs. 41, 42) described for colon cancer, this positive association was detected in a very small group of patients. In addition, the diagnostic value of positive cytoplasmic staining for membrane-bound HER-2/neu receptors was questioned earlier (30).

Similarly, a study of 203 gastric cancer patients demonstrated a significant association between HER-2/neu positivity and the expression of uPA and other proteolytic factors interacting with the uPA system (12). The investigators of this study used a semiquantitative scoring system of membranous and cytoplasmic staining and reported a high rate of HER-2/neu positivity (2+/3+) in 53% of gastric cancer patients, as opposed to a lower incidence of HER-2/neu overexpression (9–34%; Refs. 43, 44) or gene amplification (11.7%; Ref. 45) reported elsewhere. A high proportion of patients also demonstrated elevated expression (2+/3+) of uPA (75%) and PAI-1 (71%). Furthermore, the clinical significance of the observed correlation remains unclear, because the association was tested by

using a semiquantitative scoring system for both variables, rather than a prognostically valid cutoff for each variable.

Mechanisms that could help explain the observed weak association between HER-2/neu and uPA in the present clinical study can be related to known indirect interactions between tumor cells and adjacent host cells such as fibroblasts or endothelial cells also expressing uPA and PAI-1 (46). A recent study (47) demonstrated that expression of uPA or PAI-1 in fibroblasts was closely associated with the clinical and pathological features (nuclear grade, tumor size, and invasion) of the primary breast cancer cells. Interestingly, a further recent study (48) in transgenic chimeric mice expressing a MMP (MMP-9) only in cells of hematopoietic origin demonstrated that MMP-9 originating from hematopoietic cells can strongly contribute to the invasive behavior of tumor cells lacking MMP-9.

With regards to markers of tumor proteolysis, we have confirmed the prognostic impact of both uPA and PAI-1 in primary breast cancer patients using optimized cutoff values of 5.5 ng/mg for uPA and 14.0 ng/mg for PAI-1 (16–20). A direct comparison of the present cutoff values with previously reported ones, however, is difficult, because the values reported elsewhere vary, based on differences in extraction methods (18, 19), antibodies used (49), and in study populations examined (38, 50). Despite these differences, however, the proportion of cases with high uPA (24.5%) or high PAI-1 (43.4%) expression in the present study is comparable with previous studies for uPA (24–32%; Refs. 18, 49) and PAI-1 (23–44%; Refs. 19, 49). Importantly, the observation that patients with low levels of both uPA and PAI-1 have a better prognosis than patients with elevated concentrations of either uPA or PAI-1 concurs with studies reported previously (38, 50).

We were also able to confirm the prognostic significance of HER-2/neu in the present breast cancer cohort by using a cutoff value of 400 fmol/mg protein, which allowed the best discrimination between high- and low-risk groups. This cutoff for HER-2/neu overexpression was initially optimized by log rank statistics, chosen in accordance with data from fully characterized human breast cancer cell lines, and finally validated among a cohort of 152 primary breast cancer patients previously characterized for HER-2/neu overexpression by immunohistochemical staining. After univariate analysis, we were further able to demonstrate prognostic independence between HER-2/neu overexpression and elevated levels of uPA and/or PAI-1 in multivariate analysis, which supports our hypothesis that the clinically invasive phenotype associated with HER-2/neu overexpression is independent of uPA and/or PAI-1 expression.

Finally, to determine whether a similar phenomenon was present in experimental biological systems, additional human breast cancer cell line experiments were performed. In an attempt to define the effect of HER-2/neu overexpression on the regulation of uPA and PAI-1, we directly compared the expression of uPA and PAI-1 between genetically identical parent/daughter cells, which differ only in that one member of the pair overexpresses the human *HER-2/neu* gene. Using these approaches, we found that HER-2/neu overexpression was not sufficient to induce uPA or PAI-1 expression among the human breast cancer cell lines examined. In fact, HER-2/neu-transfected cell lines consistently exhibited lower uPA and PAI-1 concentrations compared with the parental cell lines. Interest-

ingly, these findings are in strong contrast to the increased invasiveness observed among the *HER-2/neu*-transfected human breast cancer cell lines *in vitro*, further supporting the hypothesis that factors other than uPA or PAI-1 must be responsible for the invasive phenotype. The rationale to investigate the effect of HER-2/neu antibodies on the expression of uPA and PAI-1 in naturally overexpressing human breast and ovarian cancer cell lines was that any observed effect elicited by HER-2/neu overexpression should be reversed by treatment with inhibitory HER-2/neu antibodies. Our results clearly show that HER-2/neu antibodies did not substantially affect the expression levels of uPA and PAI-1 among the HER-2/neu-overexpressing cell lines but did reverse the invasive capabilities of HER-2/neu-transfected cell lines.

Thus far, the effect of HER-2/neu gene expression on uPA expression has only been studied *in vitro* in one human lung cancer cell line, in which stable transfection of the *HER-2/neu* gene, with subsequent overexpression of HER-2/neu receptors, led to elevated secretion of uPA (51). The investigators of this study also compared the urokinase promoter activity between two different cell lines, one with HER-2/neu overexpression (B104-1) and one with low HER-2/neu expression (NIH 3T3), and found that the former showed increased uPA promoter activity. However, the effects could be unique to either of the cell lines examined. Comparable with the results of the present study, in which HER-2/neu antibodies did not affect expression levels of uPA or PAI-1 in naturally HER-2/neu-overexpressing cell lines, Wiechen *et al.* (52) previously demonstrated that inhibition of HER-2/neu in naturally overexpressing SKOV-3 human ovarian cancer cells by HER-2/neu-specific single-chain antibodies decreased transformation abilities; however, it did not affect the secretion of uPA. Preclinical data (53) also suggest that the tissue-invasive phenotype elicited by growth factor (epidermal growth factor receptor) stimulation can be preserved in the absence of a functional plasminogen-activator system.

Regarding the clinical significance of HER-2/neu, uPA, and PAI-1, the combined effects of HER-2/neu and the uPA/PAI-1 axis have thus far only been evaluated among 112 node-negative breast cancer patients (54). In this study, HER-2/neu amplification indicated a poor prognosis among uPA- and PAI-1-negative patients; however, the effect of *HER-2/neu* amplification on uPA- and/or PAI-1-positive patients was not further investigated. Therefore, the present study is the first investigation to fully analyze the effects HER-2/neu and the uPA/PAI-1 axis on prognosis in primary breast cancer, and it confirms the prognostic significance of HER-2/neu expression in uPA- and PAI-1-negative patients. In addition, it also demonstrates that HER-2/neu overexpression indicates a poor prognosis among uPA- and PAI-1-positive patients. Most interestingly, HER-2/neu overexpression had a strong impact on the overall relapse rate, which includes locoregional recurrences, regardless of the uPA or PAI-1 status. However, the risk for tumor cell dissemination with subsequent development of metastasis was significantly affected and enhanced by coexpression of elevated uPA levels among HER-2/neu-positive patients. This report clearly suggests that the expression of uPA, which facilitates degradation of the ECM, can significantly increase the risk to develop a distant disease recurrence that is primarily associated with HER-2/neu overexpression. A possible explanation for the ob-



servation that the discriminatory effect of uPA expression among HER-2/neu-positive patients was greater for MFS as compared with DFS might be related to the fact that DFS, as opposed to MFS, also includes locoregional recurrences as relapse events. HER-2/neu overexpression, as a marker of tumor cell proliferation, is possibly a greater risk factor for the rapid development of locoregional recurrences as compared with elevated levels of uPA expression. The risk to develop distant disease recurrence, however, was strongly enhanced by coexpression of uPA and HER-2/neu. These observations support the hypothesis that HER-2/neu can be considered as a marker of increased tumor cell proliferation and invasive capabilities and that uPA and its inhibitor, PAI-1, may be sole markers of invasive capabilities.

In summary, data of the present study clearly demonstrate that the invasive phenotype elicited by HER-2/neu overexpression in breast cancer is not primarily related to up-regulation of uPA or its inhibitor, PAI-1. The prognostic independence of HER-2/neu and the uPA/PAI-1 axis suggest that determination of both tumor biological factors can significantly improve risk group assessment in breast cancer patients.

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