research article

Prognostic significance of uPA/PAI-1 level, HER2 status, and traditional histologic factors for survival in node-negative breast cancer patients

Nina Fokter Dovnik[1], Iztok Takac[1,2]

[1] Maribor University Clinical Center, Maribor, Slovenia
[2] Faculty of Medicine, University of Maribor, Maribor, Slovenia

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Correspondence to: Nina Fokter Dovnik, M.D., Department of Gynecologic Oncology and Oncology of the Breast, Maribor University Clinical Center, Ljubljanska 5, 2000 Maribor, Slovenia. Phone: +386 2 321 2178; Fax: +386 2 321 2085; E-mail: nfokter@gmail.com

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Background. The association of HER2 status with urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1 (PAI-1) levels raises the question whether uPA/PAI-1 level carries additional clinically relevant prognostic information independently from HER2 status. The aim of our study was to compare the prognostic value of uPA/PAI-1 level, HER2 status, and traditional prognostic factors for survival in node-negative breast cancer patients.

Patients and methods. A retrospective analysis of 858 node-negative breast cancer patients treated in Maribor University Clinical Center, Slovenia, in the years 2000–2009 was performed. Data were obtained from patient medical records. The median follow-up time was 100 months. Univariate and multivariate analyses of disease-free (DFS) and overall survival (OS) were performed using the Cox regression and the Cox proportional hazards model.

Results. In univariate analysis, age, tumor size, grade, lymphovascular invasion, HER2 status and UPA/PAI-1 level were associated with DFS, and age, tumor size, grade, and uPA/PAI-1 level were associated with OS. In the multivariate model, the most important determinants of DFS were age, estrogen receptor status and uPA/PAI-1 level, and the most important factors for OS were patient age and tumor grade. The HR for death from any cause in the multivariate model was 1.98 (95% CI 0.83–4.76) for patients with high uPA and/or PAI-1 compared to patients with both values low.

Conclusions. uPA/PAI-1 level clearly carries an independent prognostic value regardless of HER2 status in node-negative breast cancer and could be used in addition to HER2 and other markers to guide clinical decisions in this setting.

Key words: node-negative breast cancer; adjuvant systemic treatment; survival; uPA/PAI-1; HER2 status

Introduction

One of the greatest challenges in the treatment of node-negative breast cancer is deciding in which patients the benefit from adjuvant cytotoxic chemotherapy would outweigh its adverse effects. Traditionally, this decision has been based on clinical and histomorphologic prognostic factors, such as patient age, tumor size, tumor grade, presence of lymphovascular invasion, and steroid hormone receptor status. Human epidermal growth factor receptor 2 (HER2) status, first used as a prognostic marker, became an important factor for predicting response to anti-HER2 therapy and is now a crucial part of this decision-making.

The serine protease urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor-1 (PAI-1) are markers of tumor invasion and metastasis that have reached the highest level of evidence for clinical utility as prognostic factors in breast cancer.[1,2] Node-negative patients with high values of uPA and/or PAI-1 have been shown to benefit from adjuvant chemotherapy in a prospective randomized multicenter

therapy trial.[3,4] In addition, independent prognostic value of uPA/PAI-1 was confirmed in a pooled analysis of 8377 breast cancer patients that showed high levels of uPA and PAI-1 to be the strongest predictors of relapse-free survival and overall survival apart from lymph node status.[5] In spite of this evidence, these biomarkers are still not widely used in the clinic.[1]

Unfortunately, none of the large trials investigating the prognostic value of uPA and PAI-1 included HER2 status in the survival analysis. Only limited information is available on the relative prognostic impact of these factors when considered along with traditional prognostic markers in the same group of breast cancer patients. Therefore, it is still uncertain whether uPA and PAI-1 can give additional clinically relevant prognostic information after traditional prognostic factors and HER2 status have been taken into account.

To address this issue, we undertook the present study with the aim of comparing the prognostic impact of HER2 status, uPA, PAI-1, and traditional prognostic factors tumor size, grade, histological subtype, lymphovascular invasion, steroid hormone receptor status, and patient age on disease-free, overall, and breast cancer specific survival in node-negative breast cancer patients.

Patients and methods

Our retrospective analysis included all patients with lymph node-negative invasive breast cancer without distant metastases who underwent primary surgical treatment in Maribor University Clinical Center, Slovenia, in the ten-year period between January 1 2000 and December 31 2009. Exclusion criteria were neoadjuvant chemotherapy and presence of another active malignancy during breast cancer treatment. Considering the Helsinki Declaration principles the Slovenian National Medical Ethics Committee approved this study (Approval No. 55/11/13).

Clinical information on diagnosis, treatment, and follow-up was obtained from patient medical records. Survival data were completed with updated information from Slovenian Cancer Registry. Data on tumor size, histological subtype, grade, lymph node status, steroid hormone receptor status, and HER2 immunohistochemistry were obtained from original histology reports from the primary surgery. HER2 gene amplification, uPA and PAI-1 levels were obtained from our institution's Medical Genetics Laboratory. Due to economical limitations, uPA and PAI-1 could not be assessed in all patients. Some other histological data were missing in a small fraction of patients due to inconsistent hospital guidelines on histology reports in the past.

All patients underwent either modified radical mastectomy or breast conserving surgery and radiotherapy. Adjuvant systemic treatment was given according to the guidelines followed at our institution at the time and was not influenced by uPA and/or PAI-1 values. Patients who completed primary treatment were followed-up at our institution at regular intervals.

HER2 status was determined immunohistochemically and additional fluorescent in situ hybridization (FISH) with PathVysion HER-2 DNA Probe Kit (Abbott Molecular, Abbott Park, IL, USA) was performed in samples with an immunohistochemical result of 2+. Since the patients were diagnosed before the publication of new ASCO/CAP guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors[6] and human epidermal growth factor receptor 2 testing in breast cancer[7] and treated accordingly, steroid hormone receptor status and HER2 status were determined using the old guideline recommendations in order to avoid cases with diagnostic-therapeutic mismatch.

uPA and PAI-1 were analyzed prospectively from representative pieces of tumor tissue that were frozen in liquid nitrogen after histologic evaluation. The frozen samples were pulverised using a micro-dismembrator, suspended in a buffer (pH 8.5) containing 0.02 M Tris-HCl, 0.125 M NaCl and 2% Triton X-100, and shaken for three hours at 4°C. The obtained suspension was centrifuged for 30 minutes at 100,000 x g. Protein content was determined with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and uPA and PAI-1 content was determined with commercially available ELISA assay kits (American Diagnostica, Greenwich, CT, USA). uPA and PAI-1 content was expressed as nanograms of analyte per milligram of tissue protein.

The correlations between different variables were tested with Spearman's rank correlation, Kruskal-Wallis test, Mann-Whitney U test and chi-square test, depending on the type of variables. The continuous variables uPA and PAI-1 were coded as binary variables using the previously optimized cutoffs of 3 ng uPA/mg protein and 14 ng PAI-1/mg protein to distinguish between low- and high-risk patients[8], and combined into one variable (both low versus one or both high) as previously shown

to be of greatest clinical relevance.[9] Because adjuvant trastuzumab was not available for the whole cohort, the patients were divided into three groups based on HER2 status and adjuvant trastuzumab treatment for the survival analyses: HER2 negative, HER2 positive who were not treated with adjuvant trastuzumab, and HER2 positive who were treated with adjuvant trastuzumab. Disease-free survival (DFS) was calculated from the date of primary surgery until the date of disease recurrence or death from any cause, or the date of the last follow-up visit in case of no recurrence or death. Overall survival (OS) was calculated from the date of diagnosis until the date of death from any cause, or the date of the last follow-up visit. Breast cancer specific survival (BCSS) was calculated from the date of diagnosis until the date of death from breast cancer, or the date of the last follow-up visit or death from other causes for censored patients. Kaplan-Meier method was used to calculate survival curves and univariate Cox regression was used to assess the differences between the curves in univariate analysis. Multivariate analyses were performed by applying the multivariate Cox proportional hazards model. All variables regardless of univariate analysis results were initially included in the Cox model and the method used for the model was backward stepwise likelihood ratio (LR). Model if term removed was reported separately. All tests were performed at a significance level of p=0.05 and a confidence interval (CI) of 95%. All p values were two-sided. Statistical analysis was performed using the SPSS software package v. 21 (IBM, Armonk, NY, USA).

Results

Patient characteristics

Eight hundred fifty-eight node-negative distant metastasis-free breast cancer patients who underwent primary surgery with curative intent were included in the study. The median age of the patients was 62 years (range, 24-95 years). The distribution of the traditional prognostic factors, HER2 status, uPA and PAI-1 in the study group is presented in Table 1. 787 (91.7%) patients received some kind of adjuvant systemic therapy. Of these, 132 received adjuvant chemotherapy, 522 adjuvant hormone therapy, and 133 a combination of both. Among patients who were given adjuvant chemotherapy, 79.2% received anthracycline-based therapy and the majority of the others received CMF (cyclophosphamide, methotrexate, 5-fluorouracil).

TABLE 1. Distribution of traditional prognostic factors, HER2 status, uPA and PAI-1 in the study group of node-negative breast cancer patients (N = 858)

Factors	No. of patients	%
Age	858	
≥ 40 years	821	95.7
< 40 years	37	4.3
Pathological tumor size	846	
< 2 cm	474	56.0
≥ 2 cm	372	44.0
Pathological tumor type	858	
Ductal invasive	720	83.9
Other invasive	138	16.1
Histological grade	799	
G1-2	557	69.7
G3	242	30.3
Lymphovascular invasion	795	
No	720	90.6
Yes	75	9.4
Estrogen receptor status	854	
Positive	674	78.9
Negative	180	21.1
Progesterone receptor status	803	
Positive	466	58.0
Negative	337	42.0
HER2 status	761	
Negative	610	80.2
Positive, without adjuvant trastuzumab	97	12.7
Positive, with adjuvant trastuzumab	54	7.1
uPA and PAI-1 level	332	
Both low	159	47.9
One or both high	173	52.1

Some factors could not be assessed in all tumors.

Among patients who were given hormone therapy, 28.9% received tamoxifen, 52.7% received an aromatase inhibitor, and the rest received a combination of both. Adjuvant trastuzumab was given in combination with chemotherapy in 6.4% of all patients and in 35.8% of HER2-positive patients. The median follow-up time was 100 months (range, 49–181 months).

Associations between traditional prognostic factors, HER2 status, uPA and PAI-1

Positive HER2 status was observed more frequently in younger patients ($p = 0.008$), in larger tumors ($p < 0.001$), tumor types other than ductal invasive ($p = 0.035$), tumors of a higher differentiation grade ($p < 0.001$), with lymphovascular invasion ($p = 0.018$), those with a lower expression of estrogen ($p < 0.001$) and progesterone receptors ($p = 0.002$), as well as in tumors with higher levels of uPA ($p = 0.026$) and PAI-1 ($p = 0.023$). uPA and PAI-1 levels correlated positively with each other ($r = 0.553$, $p < 0.001$). Higher uPA and PAI-1 values were seen in larger tumors ($p < 0.001$ for uPA; $p = 0.004$ for PAI-1), his-

TABLE 2. Univariate and multivariate analysis of disease–free survival in lymph node–negative breast cancer patients with a median follow–up time of 100 months. Multivariate analysis was performed in the 273 patients for whom complete data were available

	Univariate analysis		Multivariate analysis with all variables		Multivariate analysis – backward LR model		Model if term removed
	hazard ratio (95 % CI)	p	hazard ratio (95 % CI)	p	hazard ratio (95 % CI)	p	p
Age (continuous, unit = 10 years)	1.80 (1.60–2.02)	**< 0.001**	1.53 (1.20–1.96)	**0.001**	1.51 (1.19–1.93)	**0.001**	**< 0.001**
Tumor size (≥2 vs. <2 cm)	1.41 (1.08–1.83)	**0.012**	1.01 (0.56–1.80)	0.982	–	–	0.982
Tumor type (other invasive vs. ductal invasive)	1.08 (0.76–1.55)	0.658	1.28 (0.51–3.20)	0.598	–	–	0.606
Grade (G3 vs. G1–2)	1.37 (1.03–1.81)	**0.031**	1.54 (0.75–3.17)	0.240	–	–	0.240
Lymphovascular invasion (present vs. absent)	1.54 (1.03–2.29)	**0.035**	1.43 (0.65–3.15)	0.377	–	–	0.393
Estrogen receptors (negative vs. positive)	1.19 (0.87–1.61)	0.271	1.81 (0.79–4.16)	0.165	2.25 (1.24–4.09)	**0.008**	0.158
Progesterone receptors (negative vs. positive)	1.10 (0.84–1.45)	0.496	1.03 (0.48–2.23)	0.935	–	–	0.935
HER2 status		**0.005**		0.355			0.379
positive NT vs. negative	1.73 (1.21–2.47)	**0.003**	1.66 (0.83–3.31)	0.150	–	–	
positive T vs. negative	0.70 (0.35–1.43)	0.332	1.16 (0.46–2.91)	0.758	–	–	
uPA/PAI–1 (one or both high vs. both low)	2.16 (1.23–3.72)	**0.005**	1.76 (0.89–3.49)	0.106	1.99 (1.05–3.77)	**0.035**	0.098

NT = not treated with adjuvant trastuzumab; T = treated with adjuvant trastuzumab

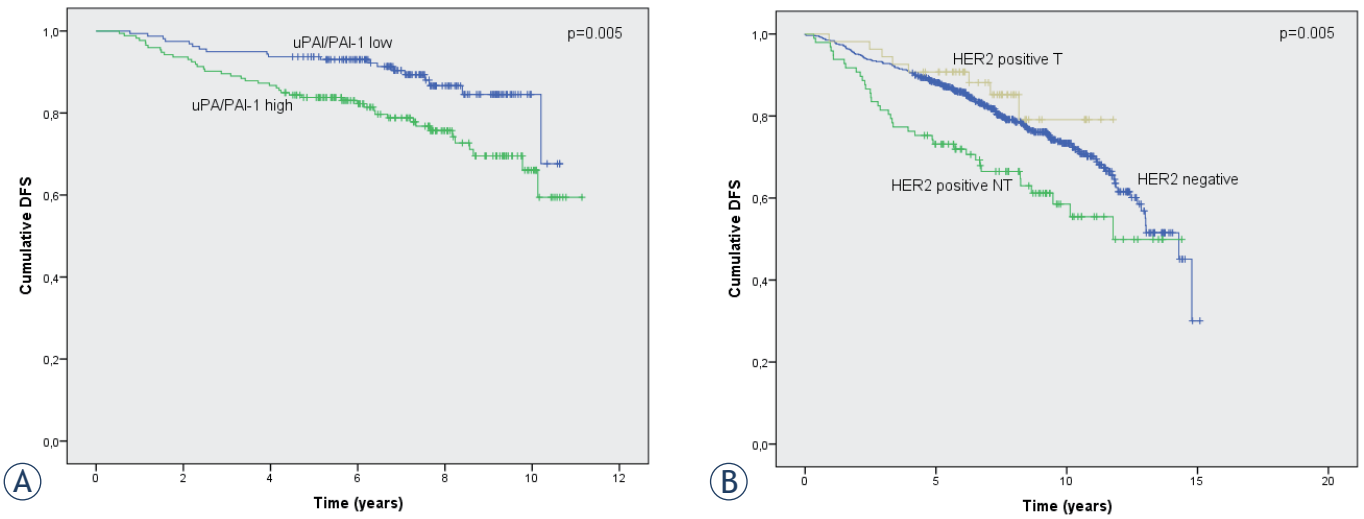

FIGURE 1. Effect of uPA/PAI-1 level and HER2 status on disease-free survival (DFS) in lymph-node negative breast cancer patients. **(A)** uPA/PAI-1 low (19 of 159 relapsed or died) versus uPA/PAI-1 high (43 of 173 relapsed or died). **(B)** HER2 negative (154 of 610 relapsed or died) versus HER2 positive not treated with adjuvant trastuzumab (NT) (37 of 97 relapsed or died) and HER2 positive treated with adjuvant trastuzumab (T) (8 of 54 relapsed or died).

NT = not treated with adjuvant trastuzumab; T = treated with adjuvant trastuzumab

tologic types other than ductal invasive ($p < 0.001$ for uPA; $p = 0.048$ for PAI-1), less differentiated tumors ($p < 0.001$ for both), and tumors with lower estrogen receptor expression ($p < 0.001$ for both). In addition, uPA but not PAI-1 was higher in tumors with lymphovascular invasion ($p = 0.047$). There were no associations between uPA or PAI-1 values and patient age or progesterone receptor status.

TABLE 3. Univariate and multivariate analysis of overall survival in lymph node-negative breast cancer patients with a median follow-up time of 100 months. Multivariate analysis was performed in the 273 patients for whom complete data were available

	Univariate analysis		Multivariate analysis with all variables		Multivariate analysis – backward LR model		Model if term removed
	hazard ratio (95 % CI)	p	hazard ratio (95 % CI)	p	hazard ratio (95 % CI)	p	p
Age (continuous, unit = 10 years)	2.18 (1.88–2.52)	**< 0.001**	1.61 (1.19–2.17)	**0.002**	1.68 (1.25–2.27)	**0.001**	**0.001**
Tumor size (≥2 vs. <2 cm)	1.38 (1.02–1.88)	**0.036**	0.94 (0.47–1.88)	0.865	–	–	0.865
Tumor type (other invasive vs. ductal invasive)	1.24 (0.83–1.85)	0.298	2.24 (0.76–6.59)	0.142	–	–	0.165
Grade (G3 vs. G1-2)	1.40 (1.01–1.94)	**0.042**	2.39 (0.95–6.02)	0.066	2.69 (1.34–5.40)	**0.005**	0.064
Lymphovascular invasion (present vs. absent)	1.34 (0.83–2.16)	0.236	1.36 (0.46–4.01)	0.582	–	–	0.594
Estrogen receptors (negative vs. positive)	1.24 (0.88–1.75)	0.224	1.80 (0.67–4.81)	0.242	–	–	0.233
Progesterone receptors (negative vs. positive)	1.01 (0.74–1.38)	0.955	0.62 (0.25–1.54)	0.299	–	–	0.279
HER2 status		0.190		0.843			0.837
positive NT vs. negative	1.37 (0.89–2.09)	0.152	0.76 (0.31–1.90)	0.562	–	–	
positive T vs. negative	0.63 (0.26–1.55)	0.318	0.90 (0.26–3.18)	0.871	–	–	
uPA/PAI-1 (one or both high vs. both low)	2.73 (1.33–5.58)	**0.006**	1.98 (0.83–4.76)	0.126	–	–	0.114

NT = not treated with adjuvant trastuzumab; T = treated with adjuvant trastuzumab

Disease-free survival

A total of 228 events occurred in the DFS analysis. Univariate and multivariate analyses of DFS are presented in Table 2. In univariate analysis, older age, tumors larger than 2 cm, grade 3, with evident lymphovascular invasion, and high uPA and/or PAI-1 (Figure 1A) were associated with worse DFS. HER2 positive patients who were not treated with adjuvant trastuzumab had significantly worse DFS than HER2 negative patients. DFS of HER2 positive patients who received adjuvant trastuzumab was not significantly different than DFS of HER2 negative patients (Figure 1B). In backward stepwise LR multivariate model, the three variables that remained significant for DFS after the final step were patient age at diagnosis, estrogen receptor status and uPA/PAI-1 level. uPA/PAI-1 level was the second most important variable after patient age in the model if term removed (Table 2).

In multivariate analysis, the HR for disease recurrence or death from any cause in estrogen receptor positive patients with high uPA and/or PAI-1 was 2.78 (95% CI, 1.28–6.03; p = 0.010) compared to those with both values low. The corresponding HR values for estrogen receptor negative, HER2 negative, HER2 positive patients who did not receive adjuvant trastuzumab, HER2 positive patients who received adjuvant trastuzumab, and triple negative patients, were 1.11 (95% CI, 0.33–3.76; p = 0.863), 1.60 (95% CI, 0.70–3.68; p = 0.268), 9.25 (95% CI, 1.06–80.82; p = 0.044), 3.31 (95% CI, 0.10–109.67; p = 0.503), and 1.08 (95% CI, 0.30–3.86; p = 0.902), respectively.

Overall survival

A total of 172 events occurred in the OS analysis. Univariate and multivariate OS analyses are shown in Table 3. Patient age at diagnosis, tumor size, grade, and uPA/PAI-1 levels (Figure 2a) were found to have a significant impact on OS in univariate analyses. HER2 (Figure 2B) and the other traditional prognostic factors were not found to influence OS in our series of node-negative breast cancer patients. In backward stepwise LR multivariate model, the remaining variables significantly associated with OS after the last step were patient age and tumor grade (Table 3). uPA/PAI-1 level was the third most important factor in the model if term removed and was removed from the model at the last step.

Breast cancer specific survival

A total of 65 events occurred in the BCSS analysis. Univariate and multivariate BCSS analyses

TABLE 4. Univariate and multivariate analysis of breast cancer specific survival in lymph node-negative breast cancer patients with a median follow-up time of 100 months. Multivariate analysis was performed in the 273 patients for whom complete data were available

	Univariate analysis		Multivariate analysis with all variables		Multivariate analysis – backward LR model		Model if term removed
	hazard ratio (95 % CI)	p	hazard ratio (95 % CI)	p	hazard ratio (95 % CI)	p	p
Age (continuous, unit = 10 years)	1.16 (0.95–1.41)	0.148	0.76 (0.51–1.12)	0.162	–	–	0.160
Tumor size (≥2 vs. <2 cm)	2.44 (1.45–4.13)	**0.001**	1.72 (0.55–5.38)	0.350	–	–	0.340
Tumor type (other invasive vs. ductal invasive)	1.20 (0.63–2.29)	0.587	3.33 (0.64–17.23)	0.152	–	–	0.196
Grade (G3 vs. G1-2)	3.58 (2.12–6.05)	**< 0.001**	7.10 (1.23–40.86)	**0.028**	10.34 (2.33–45.97)	**0.002**	**0.014**
Lymphovascular invasion (present vs. absent)	1.48 (0.70–3.12)	0.305	1.43 (0.36–5.65)	0.608	–	–	0.618
Estrogen receptors (negative vs. positive)	2.21 (1.33–3.66)	**0.002**	1.24 (0.28–5.36)	0.778	–	–	0.776
Progesterone receptors (negative vs. positive)	1.74 (1.06–2.86)	**0.028**	0.81 (0.17–3.77)	0.789	–	–	0.788
HER2 status		**0.031**		0.663			0.614
positive NT vs. negative	2.18 (1.21–3.93)	**0.009**	1.29 (0.39–4.31)	0.683	–	–	
positive T vs. negative	0.96 (0.30–3.10)	0.944	0.46 (0.06–3.68)	0.460	–	–	
uPA/PAI-1 (one or both high vs. both low)	6.46 (1.47–28.43)	**0.014**	2.79 (0.57–13.67)	0.205	–	–	0.166

NT = not treated with adjuvant trastuzumab; T = treated with adjuvant trastuzumab

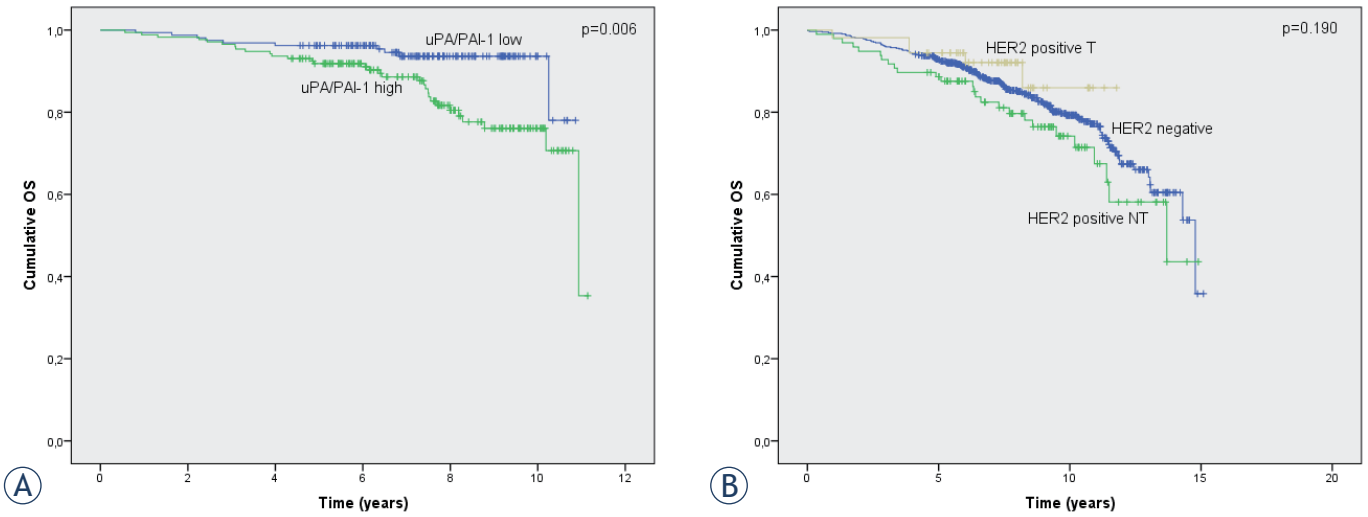

FIGURE 2. Effect of uPA/PAI-1 level and HER2 status on overall survival (OS) in lymph-node negative breast cancer patients. **(A)** uPA/PAI-1 low (10 of 159 died) versus uPA/PAI-1 high (31 of 173 died). **(B)** HER2 negative (118 of 610 died) versus HER2 positive not treated with adjuvant trastuzumab (26 of 97 died) and HER2 positive treated with adjuvant trastuzumab (5 of 54 died).

NT = not treated with adjuvant trastuzumab; T = treated with adjuvant trastuzumab

are shown in Table 4. Tumor size, grade, estrogen and progesterone receptor status, HER2 status and uPA/PAI-1 levels were associated with BCSS in univariate analyses. The only variable retained in the final multivariate model using the backward stepwise LR method was tumor grade. uPA/PAI-1 level was the third most important factor in the model if term removed.

Discussion

The results of our study indicate an important prognostic value of uPA/PAI-1 level in node-negative breast cancer patients. Even though uPA and PAI-1 values were associated with most of the prognostic factors currently in use for clinical decision-making in the adjuvant setting, multivariate analysis showed that uPA/PAI-1 level carries additional, independent prognostic information. This was particularly true for DFS analysis where uPA/PAI-1 level was the second most important variable in the survival model after age and was retained in the backward LR model after the final step along with age and estrogen receptor status. In OS analysis, the final model included only age and tumor grade, which is probably a reflection of the very strong association between higher uPA and PAI-1 values and less differentiated tumors. However, in the multivariate model that included all variables, the HR for death from any cause was 1.98 (95% CI 0.83–4.76) for patients with high uPA and/or PAI-1 compared to patients with both values low, which shows a substantial possibility of an important effect on OS that would have probably remained statistically significant in a larger sample. The same three variables were the most important in the multivariate model of BCSS as well. Here, the singular prognostic importance of tumor grade was even more evident. As opposed to DFS and OS which both included deaths from other causes as events in the analysis, younger age seemed associated with worse BCSS. It is important to emphasize that BCSS analysis must be interpreted with caution due to the small number of events. Considering the HRs and confidence intervals, it is probable that both age and uPA/PAI-1 level importantly influence BCSS and would have retained statistical significance in a larger sample. On the other hand, HER2 status only showed prognostic significance in univariate DFS and BCSS analyses when comparing HER2 negative to HER2 positive patients who were not treated with adjuvant trastuzumab. It did not remain an important determinant of DFS and BCSS in multivariate analyses and lost all prognostic value in OS analysis.

A subgroup analysis of DFS according to estrogen receptor and HER2 status showed that apart from the no longer relevant group of HER2 positive patients who did not receive adjuvant trastuzumab treatment, uPA/PAI-1 level was prognostically by far the most important in estrogen receptor positive tumors. In contrast, its prognostic value in multivariate analysis was practically null in HER2 positive patients treated with adjuvant trastuzumab and in triple negative patients. Conveniently, estrogen receptor positive patients are the ones with the highest uncertainty regarding the benefit of adjuvant chemotherapy, while the other two subgroups are generally recommended adjuvant chemotherapy ± anti-HER2 therapy regardless of other prognostic factors.

Our findings confirm those of numerous other studies that have reported uPA and PAI-1 to be statistically independent prognostic factors in lymph node-negative breast cancer patients.[10-18] However, these studies did not include HER2 status which is now an important part of the decision about adjuvant systemic therapy. Because of the observed associations between overexpression of the HER2/neu protein and tumor proteolytic factors in breast and in other cancers[19-21], HER2/neu has been suggested to up-regulate the proteolytic enzymes, including uPA and PAI-1, and thus play a direct role in tumor invasion and metastasis.[19] This possible association might be one of the additional reasons besides methodological difficulties why uPA and PAI-1 testing is still not that frequently used in the clinic in spite of the excellent evidence of its clinical utility.[1] Even so, the main reason for this inconsistency are practical issues. uPA and PAI-1 are determined using validated ELISA assay kits as described above. This measurement requires relatively large amounts of fresh or freshly frozen tumor tissue, which is not practical for clinical use, especially in needle or surgical biopsies and in small tumors. Besides, the results may not be available at the time of the histology report due to sample pooling. However, attempts to develop immunohistochemistry assays on formalin-fixed and paraffin-embedded tissues are ongoing.[1]

Among the few studies that considered the clinical relevance of both HER2 and proteolytic enzymes for survival in breast cancer patients, the results are somewhat conflicting. Harbeck *et al.*[22] showed that PAI-1 was the only independent prognostic factor for DFS and OS when considered along with tumor size, tumor grade, steroid hormone receptor status, uPA, HER2 status, MIB1, SPF, p53, and cathepsin D in 100 node-negative breast cancer patients. Similarly, Bouchet *et al.*[23] found no additional prognostic information of HER2/neu protein levels when evaluated in multivariate analysis together with uPA, PAI-1 and traditional histologic factors in the subgroup of 226 node-negative patients. They reported DFS to be independently influenced by PAI-1 and tumor size and OS by PAI-1 and uPA levels. Recently, Buta *et al.*[24] also reported

superior DFS in 73 node-negative, postmenopausal, steroid hormone receptor positive breast cancer patients with smaller tumors and low PAI-1, independent of HER2 status. On the other hand, Konecny *et al*.[19] reported that both uPA/PAI-1 level and HER2 status independently influenced DFS in addition to tumor size and nodal status in a group of 542 patients with a short follow-up not selected by nodal status. The same factors were found to be important in multivariate OS analysis as well, but this time HER2 status did not quite reach statistical significance. Interestingly, among 118 node-negative patients with long-term follow-up, Zemzoum *et al*.[25] found uPA/PAI-1 to be the only variable independently influencing DFS, and HER2 status to be the most important factor in the multivariate analysis of OS. uPA/PAI-1 and tumor grade were of borderline significance for OS. Complicating the matter even further, without reference to uPA and/or PAI-1, Schmidt *et al*.[26] reported HER2 status to be prognostically significant in node-negative breast cancer patients only when determined by FISH and not when determined immunohistochemically. In our group of 273 node-negative patients with long-term follow-up who were available for multivariate analysis, the combination of uPA and PAI-1 levels clearly carried independent prognostic value for DFS and was important although not formally statistically significant in multivariate analysis of OS as well, while HER2 status determined with the usual combination of immunohistochemistry and FISH did not prove to be an important determinant of DFS or OS in multivariate analyses regardless of adjuvant anti-HER2 treatment.

Most of the studies confirming the prognostic value of uPA and PAI-1 in breast cancer patients have focused on patients who received no adjuvant systemic treatment. In fact, the prognostic information from these biomarkers seems diminished in patients who receive adjuvant treatment, particularly adjuvant hormone therapy[27,28], indicating a possible predictive role of uPA and PAI-1 for response to therapy. However, the prognostic importance of uPA/PAI-1 level was evident in our group of patients although the vast majority received adjuvant hormone therapy and a significant fraction were given adjuvant chemotherapy. Contrastingly, based on our results, HER2 status is clearly more important in its predictive than in its prognostic role.

Our study has several limitations, the principal one being its retrospective character and the associated possibility of bias. A major problem is missing data, particularly on uPA and PAI-1 values which could only be determined in about 40% of the patients because of our institution's economical limitations and the fact that these markers still largely serve only academic purposes. Because of inconsistent histology reports in the past, some other information is missing in a fraction of patients. The total number of patients with available information was used for each univariate analysis and multivariate analyses were performed in the 273 patients in whom complete data were available. Even so, we are aware of only one study comparing the prognostic values of uPA, PAI-1, HER2, and traditional prognostic factors that included a slightly larger subgroup of 283 lymph node-negative patients.[19] Another possible limitation is the comparatively high proportion of deaths from causes other than breast cancer, which may have somewhat obscured the results of both DFS and OS analysis and is probably also the cause of such a marked association of older age and worse prognosis, an assumption confirmed by the fact that older age was associated with improved breast cancer specific survival. Furthermore, our results must be considered in the light of the unavoidable multiple analyses and the possibility of a type I statistical error. To facilitate interpretation, 95% confidence intervals and not just the p values have been stated wherever possible. On the other hand, one of the main strengths of our study in addition to the combination of analyzed prognostic factors is the long follow-up which was in the range of 49-181 months, the median of 100 months being more than three times the median follow-up of the larger study by Konecny *et al*.[19] Moreover, we believe that the scientifically sound and straightforward statistical analysis is a strength of our study as well.

Based on the results from our retrospective analysis of node-negative breast cancer patients with long-term follow-up, we conclude that the combined uPA/PAI-1 level carries important additional prognostic information, particularly for DFS, even after all traditional prognostic factors as well as HER2 status have been taken into account. We believe that routine use of uPA/PAI-1 level would further improve risk stratification and adjuvant therapy decisions in this setting, especially in estrogen receptor positive patients.

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