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Prognostic relevance of urokinase plasminogen activator and its inhibitors in patients with breast cancer

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Urokinase plasminogen activator (uPA) and its inhibitors, PAI-1 and PAI-2, play an important role in intercellular tissue degradation, thus promoting tumor cell invasion into the adjoining structures and metastasizing. Our study was aimed to assess a possible prognostic value of uPA, PAI-1 and PAI-2 in a retrospective series of 87 patients with breast cancer stage I-III, whose cytosols were stored in the archives of the Institute of Oncology in Ljubljana. The median follow-up was 35 months. The prognostic value of the established prognostic factors and uPA, PAI-1 and PAI-2 were evaluated by means of univariate statistical analysis and partial multivariate models. The obtained uPA values were very low and did not correlate with the disease-free survival, whereas PAI-1 and PAI-2 significantly influenced the time to the first recurrence. Patients with PAI-1 values above 5 ng/mg proteins had statistically significantly worse disease-free survival than the patients with lower PAI-1 values (58% vs. 85%). In the case of PAI-2, the situation was just the opposite: the patients with PAI-2 values exceeding 6.4 ng/mg proteins had statistically significantly better 3-year disease-free survival than the patients with lower values (90% vs. 60%). Both, PAI-1 and PAI-2 retained their independent prognostic value, irrespective of the addition of the established prognostic factors to partial multivariate models, and only with locally advanced disease the prognostic value of PAI-1 was greater than that of PAI-2.

Key words: breast neoplasms; urokinase; plasminogen activator inhibitor 1; plasminigen activator inhibitor 2; prognosis

Introduction

A number of extracellular proteolytic enzymes are expressed in the tumor tissue; these are involved in the invasion of tumor cells into the surrounding tissues as well as in distant dissemination process. The central role among proteolytic enzymes is attributed to the serine proteinase - urokinase plasminogen activator (uPA) and to uPA inhibitors types 1 and 2 (PAI-1 and PAI-2).[1,2,3]

A decade ago, researchers came to the idea that serine proteinases could be an indicator of the metastatic potential of tumors, and thus also a prognostic factor of cancer. In the last decade, a number of studies were carried out which were aimed to assess the prognos-

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tic relevance of uPA, PAI-1 and PAI-2 in breast cancer and other solid tumors.[4]

In his first study concerned with the prognostic value of serine proteinases, Duffy and co-workers determined uPA content in the tumor cytosols from patients with breast cancer, which were prepared for routine determination of hormone receptors.[5] The results of this study for the first time established a correlation between uPA and the prognosis in breast cancer patients. Several other authors later on also confirmed the same findings.[6-9] From the 90's on, a similar relevance was reported for PAI-1 determined in the cytosols from breast cancer patients.[10-14] The measured mean and cut-off values of uPA and PAI-1 differed from one study to another. The reason for this variability could be attributed to different tumor tissue preparation techniques and different buffers used. Because of those differences, a standard cut-off value that would delineate high values from low ones could not be determined. However, the results of investigations performed so far unequivocally speak in favor of the independent prognostic value of uPA and PAI-1.[6-14] High values of either of these two factors are associated with a higher risk of recurrence and a shorter survival. PAI-2 has not been sufficiently investigated yet. For the difference from uPA and PAI-1, high PAI-2 values were found to be associated with a favorable prognosis.[9,10] So far, the independent prognostic value of PAI-2 has been confirmed by one of the two studies performed.[9]

Our retrospective study was aimed to established whether the values of uPA, PAI-1 and PAI-2 determined in tumor cytosols prepared with phosphate buffer, which are otherwise used routinely for hormone receptor determination, correlate with the established prognostic factors, and whether they significantly influence the disease free survival of patients with breast cancer.

Materials and methods

Our retrospective study was carried out in a series of 87 patients with operable and locally advancer breast cancer, who were admitted to the Institute of Oncology for the first time in 1994 or in the first two months of 1995, and operated on for cytologically confirmed breast cancer.

Deep-frozen tumor cytosols from those patients are stored in the cytosol bank of the Institute of Oncology in Ljubljana. The prepared cytosols are kept at a temperature of minus 20°C. The cooling was never discontinued.

Patients, tumors and treatment characteristics

The data on age, menopausal status, clinical tumor size, clinical lymph node status, pathological tumor size, pathohistological tumor type, malignancy grade, axillary lymph node involvement, hormonal receptor status, and primary treatment were derived from the patient record files stored in the archives of Institute of Oncology.

The stage of disease was classified according to UICC-WHO criteria (UICC, 1974). While the latter criteria were used for pathohistological tumor type determination, the grade of malignancy was assessed according to Scarf-Bloom-Richardson's classification.[10] The cut-off limit for positive estrogen and progesterone receptors was set at value > 10 fmol/mg proteins.

All the patients underwent a local radical treatment. All of them also had axillary lymphadenectomy performed. In the case that only conservative surgery was feasible, the patients were additionally irradiated to the area of the operated breast. Patients with positive axillary lymph nodes received adjuvant chemotherapy. The same was also given to the patients with negative axillary lymph nodes and presence of the established unfavorable prognostic factors (e.g. large and/or poorly differentiated tumor).

The mean age of patients at diagnosis was 52 years (range 29-75 years). Other characteristics of the patients and tumors are presented in Table 1.

Tissue preparation technique and the determination of urokinase system components

Immediately upon surgery, the removed tumor tissue was stored in liquid nitrogen. In the process of cytosol preparation, the frozen tumor was first ground with a microdysmembrator. The obtained powder was suspended in phosphate buffer (5 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 1 mM monothioglycerol, 10% (v/v) glycerol, pH 7.4), and the suspension ultra-centrifuged at 100,000 x g for 45 min, at 4°C).

The total uPA concentration in the cytosol and extract was determined with IMUBIND® Tissue uPA ELISA Kit, while PAI-1 was determined with IMUBIND® Tissue PAI-1 ELISA Kit, and PAI-2 with IMUBIND® Tissue PAI-2 ELISA Kit (American Diagnostica Inc.)

Follow up

After completed primary therapy, the patients were subjected to regular follow-up examinations at the Institute of Oncology. The data on possible time and site of progression were derived from patients records.

The patients were followed up for 1-49 months (median follow up was 35 months).

Data processing

Interdependence of the urokinase system components with other primary tumor characteristics was determined on the basis of contingency tables and chi-square test. The influence of the component of urokinase system on the disease-free survival was presented by means of Kaplan-Meier's survival curves, and any differences in the survival analyzed with the log-rank test.[15,16] The multivariate regression analysis by Cox was used for the evaluation of independent prognostic value of urokinase system components.[17] Statistical analysis and graphic presentation of the results were done using „Statistica for Windows" and „BMDP" program packages.

Results

Urokinase system measurements

In 87 patients, the range of uPA values (concentrations) in the cytosol prepared with phosphate buffer was 0-1.83 ng/mg proteins, median 0.34 ng/mg proteins, the lower and the upper quarts being 0.15 and 0.53 ng/mg proteins, respectively.

In the same series of 87 patients, the range of PAI-1 levels in the cytosol prepared with phosphate buffer was 0.06-75.91 ng/mg proteins, median 6.02 ng/mg proteins, while the lower and the upper quarts were 3.77 and 8.93 ng/mg proteins, respectively.

The range of PAI-2 values in the cytosols prepared with phosphate buffer was 0.31-75.80 ng/mg proteins, median 3.35 ng/mg proteins, the lower and the upper quarts being 1.51 and 12.31 ng/mg proteins, respectively.

The influence of urokinase system components on disease-free survival

Within the median observation period of 35 months, the disease was found to recur in 28/87 patients (32%). Three patients (11%) presented with local recurrence, while 19 patients (68%) had distant metastases alone, and 6 patients (21%) had both. Three-year disease free survival of the whole group of patients was 69%. Disease free survival for the whole group of 87 patients is presented in Figure 1. We were trying to determine the cut-off values of uPA, PAI-1 and PAI-2, which

Table 1. Characteristics of 87 patients

		Number	(%)
Patients	Menopausal status		
	premenopausal	29	33
	postmenopausal	57	66
	unknow	1	1
	Tumor size		
	T1	8	9
	T2	54	62
	T3	13	15
	T4	12	14
	Nodal status		
	N0	54	62
	N1	26	30
	N2	7	8
	Stage (UICC- International Union against Cancer)		
	I	8	9
	II	60	69
	III	19	22
Tumors	Pathological tumor size		
	Tp1	1	1
	Tp2	13	15
	Tp3	57	66
	Tp4	14	16
	unknown	2	2
	Pathohistological tumor type		
	invasive ductal	76	88
	invasive lobular	8	9
	mucinous	1	1
	others	1	1
	unknown	1	1
	Differentiation grade (invasive ductal carcinoma)		
	GI	4	5
	GII	21	28
	GIII	48	63
	unknown	3	4
	Number of positive nodes		
	0	30	34
	1-3	26	30
	>3	31	36
	Estrogen receptors		
	≤10 ng/mg protein	43	49
	>10 ng/mg protein	44	51
	Progesterone receptors		
	≤10 ng/mg protein	60	69
	>10 ng/mg protein	27	31

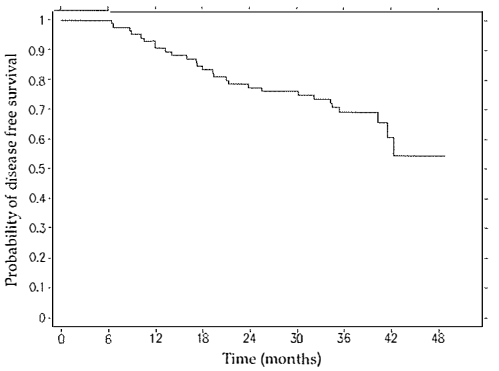

Figure 1. Relapse-free survival of 87 breast cancer patients.

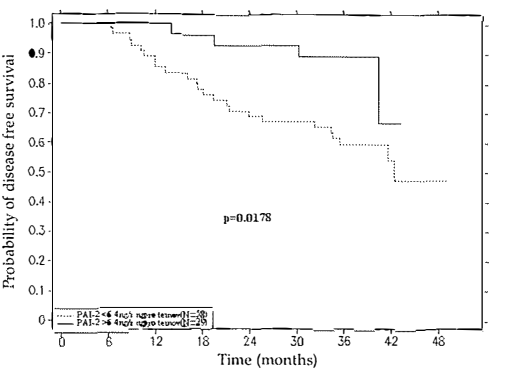

Figure 3. Relapse-frees urvival according to PAI-2.

could best differentiate patients with favorable prognosis from those with unfavorable one.

It has been found that the measured uPA values in the cytosols from our series of patients failed to correlate with the disease-free survival. The cut-off value of PAI-1 in our series of patients was 5 ng/mg proteins. The disease-free survival of patients with PAI-1 values exceeding 5 ng/mg proteins was statistically significantly worse than that of the patients with PAI-1 values under 5 ng/mg proteins (P = 0.0046) (Figure 2).

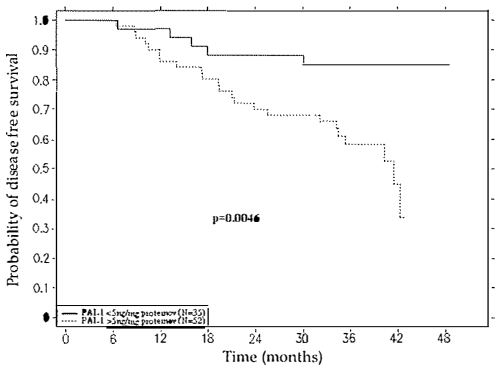

Figure 2. Relapse-free survival according to PAI-1.

The cut-off value of PAI-2 in our series of patients was 6.4 ng/mg proteins. The disease-free survival of patients with PAI-2 values exceeding 6.4 ng/mg proteins was statistically significantly better that that of the patients with PAI-2 values under 6.4 ng/mg proteins (p = 0.0178) (Figure 3).

Comparison of the influence of different prognostic factors on disease-free survival

Univariate analysis (log-rank test): The influence of menopausal status, clinical tumor size, clinical lymph node status, stage of the disease, pathohistological tumor size and grade of malignancy grade, pathohistological evidence of axillary lymph node involvement, presence of estrogen and progesterone receptors, as well as PAI-1 and PAI-2 content in the tumor on disease free survival was studied. It was found that disease-free survival was significantly influenced by 7/11 factors under study. The influence of clinical tumor size (operable cancers - T2 and T3 vs. locally advanced disease - T4) was also statistically significant. In our series, the patients with operable cancers presented with 73% 3-year survival, while those with locally advanced disease had only 20% 3-year survival rate ($p<0.00001$). Eight patients with tumors smaller than 2 cm (stage T1) were not included in the analysis.

Stage of disease was another prognostic factor that turned out to be statistically significant for the disease free survival. In our

series, 78% of patients with stage II at the time of diagnosis survived 3 years without evidence of disease, while only 32% of those with stage III were free of recurrence after 3 years ($p<0.00001$). Patients with stage I were too few (N=8) to be included into the statistical analysis. Both clinical and pathological status of the axillary lymph nodes exerted statistically significant influence on the disease free survival. While patients with non-palpable axillary lymph nodes survived 3 years without evidence of disease in 85%, those with palpable lymph nodes had only 45% 3-year disease-free survival ($p<0.00001$). After three years, the disease recurred in 42% of patients with pathologically positive axillary lymph nodes and in only 11% of those with negative pathological lymph node findings ($p=0.0053$). The number of involved lymph nodes was also statistically significant. Thus, the patients with 1-3 positive axillary lymph nodes had 80% disease-free 3 year survival, while in those with more than three positive lymph nodes this rate was only 41% ($p<0.00001$). Further, the recurrence of disease was significantly influenced by the content (presence) of estrogen receptors in the tumor. Patients with negative estrogen receptors had lower 3-year disease-free survival than those with positive estrogen receptors, i.e. 60% vs. 80% respectively ($p=0.0409$).

Both, PAI-1 and PAI-2 contents in the tumor significantly influenced the patients' survival. It turned out that the patients with PAI-1 tumor content exceeding 5 ng/mg proteins had statistically significantly worse disease-free survival than the rest of patients under study ($p=0.0046$). Thus, the disease recurred within 3 years in 42% of patients with PAI-1 content above the cut-off value, and in only 15% of those with PAI-1 content below the cut-off value (Figure 2).

With PAI-2, however, the situation was just the opposite. Patients with PAI-2 tumor content exceeding 6.4 ng/mg proteins had statistically significantly better disease-free survival than the rest of the patients under study ($p=0.0178$). Thus, 10% of patients with PAI-2 values >6.4 ng/mg proteins presented with recurrence within 3 years, as compared to the patients with PAI-2 values below 6.4 ng/mg proteins in whom the recurrence rate was as high as 52% (Figure 3). The results of univariate analysis are presented in Table 2.

Multivariate analysis: Independent prognostic value of PAI-1 and PAI-2 was studied by the multivariate Cox's regression model. Due to insufficient number of patients, we did not include into the model all the seven factors that had shown their prognostic value in the univariate analysis; instead, we made a few partial multivariate models. Thus the remaining five factors shown as statistically relevant by univariate analysis were added one by one to the basic two factors studied (PAI-1 and PAI-2) (Table 3).

When both inhibitors of plasminogen activator alone were included into the multivariate model, PAI-1 and PAI-2 turned out to be strong prognostic factors, PAI-1 being the more relevant of the two. If only these two factors were considered, the relative risk of recurrence would increase by 5.9-times in patients with PAI-1 exceeding 5 ng/mg proteins, and by 4.4-times in those with PAI-2 values below 6.4 ng/mg proteins.

PAI-1 and PAI-2 did not loose their prognostic value by inclusion of other prognostic factors into the model. A stronger prognostic value was established only for tumor size (operable vs. locally advanced cancers) and stage (stage II vs. stage III). Both inhibitors of plasminogen activator were shown to have a stronger prognostic value that clinical and pathological status of the axillary lymph nodes. In the multivariate model with PAI-1 and PAI-2, estrogen receptors lost their prognostic value.

Table 2. Univariante analysis of disease free survival (log-rank test)

Prognostic factor	Number	Number of relapses	p
premenopausal	29	10 (34)	0.8306
postmenopausal	56	18 (32)	
Tumor size*			
T2+T3**	67	20 (30)	<0.0001
T4	12	8 (67)	
Clinical nodal status			
palpable lymph nodes	54	9 (17)	<0.0001
nonpalpable lymph node	33	19 (58)	
Stage***			
II	60	14 (23)	<0.0001
III	19	14 (74)	
Pathological tumor size			
<20 mm	14	3 (21)	0.2692
≥20 mm	71	24 (34)	
Diferentiation grade****			
II	21	4 (14)	0.0980
III	48	19 (40)	
Pathological nodal status			
negative lymph nodes	30	5 (17)	0.0053
positive lymph nodes	57	23 (40)	
Estrogene receptors			
≤10 fmol/mg protein	43	18 (42)	0.0409
<10 fmol/mg protein	44	10 (23)	
Progesterone receptors			
≤10 fmol/mg protein	60	23 (38)	0.0978
>10 fmol/mg protein	27	5 (19)	
PAI-1			
<5 ng/mg protein	35	5 (14)	0.0046
>5 ng/mg protein	52	23 (44)	
PAI-2			
<6.4 ng/mg protein	58	24 (41)	0.0178
>6.4 ng/mg protein	29	4 (14)	

* 8 patients with tumor size <2cm (T1) were excluded from the analisis because the number was to small for statistical evaluation
** Difference between T2 and T3 was not statistically significant (log rank: p=0.1254)
*** 8 patients with stage I were excluded from the analysis because the number was to small for statistical evaluation
**** invasive ductal carcinoma; 4 patients with grade I were excluded from the analysis because the number was to small for statistical evaluation

Discussion

Our retrospective study was undertaken with the aim to show whether the components of urokinase system measured in tumor cytosols prepared with phosphate buffer for biochemical hormone determination had any prognostic value.

Our retrospective study has failed to show any correlation between uPA values and disease-free survival. Contrary to our results, the findings of three published retrospective

Table 3. Multivariate Cox regression analysis

Prognostic factor	RR*	95% CI**	p
PAI-1			
(<5 ng/mg protein *vs.* >5 ng /mg protein)			
>5 ng/ mg protein	5.89	2.21 - 15.66	0.0004
PAI-2			
(>6.4 ng/mg protein *vs.* < 6.4 ng /mg protein)			
<6.4 ng /mg protein	4.26	1.47 - 7.14	0.0079
PAI-1			
(<5 ng/mg protein *vs.* >5 ng /mg protein)			
>5 ng/ mg protein	7.79	2.83 -21.46	0.0001
PAI-2			
(>6.4 ng/mg protein *vs.* < 6.4 ng/ mg protein)			
<6.4 ng /mg protein	3.57	1.20 - 10.00	0.0218
Clinical tumor size			
(T2+T3 *vs.* T4)			
T4	8.34	3.39 -20.48	<0.00001
PAI-1			
(<5 ng/mg protein *vs.* >5 ng/ mg protein)			
>5 ng /mg protein	5.65	2.83 - 21.46	0.0006
PAI-2			
(>6.4 ng/mg protein *vs.* < 6.4 ng /mg protein)			
<6.4 ng mg/protein	4	1.41 - 12.5	0.0100
Clinical nodal status			
(palpable lymph nodes *vs.* nonpalpable lymph nodes)			
nonpalpable lymph nodes	3.75	1.67 -8.43	0.0014
PAI-1			
(<5 ng/mg protein *vs.* >5 ng/ mg protein)			
>5 ng mg/protein	5.77	2.13 - 15.63	0.0006
PAI-2			
(>6.4 ng/mg protein *vs.* < 6.4 ng /mg protein)			
<6.4 ng /mg protein	5.56	1.92 - 16.67	0.0018
Stage			
(II vs. III)			
III	6.96	3.14 - 15.40	<0.00001
PAI-1			
(<5 ng/mg protein *vs.* >5 ng/ mg protein)			
>5 ng /mg protein	5.25	1.95 - 14.12	0.0010
PAI-2			
(>6.4 ng/mg protein *vs.* < 6.4 ng /mg protein)			
<6.4 ng/ mg protein	5.26	1.75 - 14.29	0.0028
Pathological nodal status			
(negative *vs.* positive)			
positive	3.81	1.39 - 10.44	0.0092
PAI-1			
(<5 ng/mg protein *vs.* >5 ng /mg protein)			
>5 ng /mg protein	5.68	2.13 - 15.13	0.0005
PAI-2			
(>6.4 ng/mg protein *vs.* < 6.4 ng/ mg protein)			
<6.4 ng /mg protein	3.85	1.33 - 11.11	0.0128
ER			
(10 fmol/mg protein *vs.* > 10 fmol/mg protein)			
> 10 fmol/mg protein	-	-	n.s.***

* Relative risk; ** confidence interval; ***not significant

studies ascribe some prognostic value to uPA measured in cytosols.[11,18,19] The authors obtained different median values (0.4 - 0.52 ng/mg proteins) and different maximum values (3.2 - 4.4 ng/mg proteins), which were invariably higher than the values measured in cytosols during our retrospective study (median 0.34 ng/mg proteins, range 0-1.83 ng/mg proteins). This discrepancy could be explained by the fact that the cytosols from the archives of the Institute of Oncology were prepared with phosphate buffer alone, whereas the cytosols used in the three retrospective studies reported were prepared with buffers and addition of EDTA. It seems that the latter substance improves the extraction of proteins, and associated with that uPA, to such an extent that the measurements become more reliable and the analytical error lesser. Apparently, the uPA values determined in the tissue that has been processed according to the technique described are just high enough to allow for the determination of cut-off value which groups breast cancer patients by prognosis.

For the difference from uPA, PAI-1 and PAI-2 in our study correlated with disease-free survival. The univariate analysis has shown a statistically significant influence of PAI-1 and PAI-2 content in tumor cytosols on disease-free survival. Patients with higher PAI-1 values presented with recurrence more frequently than those with lower PAI-1 values. With PAI-2 the situation was just the opposite: the recurrence rates within three years in patients with higher PAI-2 values were lower. A similar influence of PAI-1 on disease-free survival was also established by univariate analysis in some other studies investigating the prognostic value of PAI-1. [6,7,9,11,14,20] Also the results of both studies on the prognostic value of PAI-2 are more or less consistent with our study.[9,10] While the French study -likewise ours - has confirmed the association of high PAI-2 levels with a favorable prognosis, Foeckens *et al.* in their study on 1012 patients failed to confirm a correlation between PAI-2 values and disease-free survival or overall survival. However, when their patients were grouped according to uPA tumor content, the patients with higher uPA values also had the cut-off value of PAI-2 determined, which distinguished the patients by prognosis. It has been found that the patients with higher uPA content had a better prognosis if they also had high PAI-2 values.[10]

Apart from PAI-1 and PAI-2, in our group of patients a statistically significant influence on the disease-free survival was also exerted by the established prognostic factors: clinical tumor size, clinical lymph node status, stage of the disease, pathological evidence of axillary lymph node involvement, and the presence of estrogen receptors in the tumor. In the evaluation of stage, stages II and III were compared, which meant a comparison between operable and locally advanced tumors, since a majority of stage III patients had locally advanced tumors. Thus the patients with locally advanced cancers and stage III had by all expectations worse disease-free survival. A statistically significant influence on the disease-free survival was also exerted by clinical and pathological lymph node status. Our univariate analysis has also shown that a worse prognosis was associated with negative estrogen receptors, while the presence of progesterone receptors in the tumor failed to provide prognostically relevant information. In our analysis menopausal status, pathological tumor size and grade of malignancy of invasive ductal cancers did not show prognostic value for disease-free survival. The reason for the absence of prognostic value of pathological size and grade of tumors could be attributed to a relatively small number of patients included, as well as to a small number of events in the groups of tumors smaller than 2 cm and in moderately differentiated tumors.

In multivariate models, both PAI-1 and PAI-2 showed an independent prognostic

value, the value of the former being somewhat higher. In our study, only clinical tumor size and stage were stronger prognostic factors than both inhibitors. In these two prognostic factors we actually used similarly formed groups, which are defined prevailingly by locally advanced disease, and thus providing a similar information. In our study, clinical and pathological lymph node status have shown a lower prognostic power than both inhibitors, while estrogen receptors have lost their independent prognostic value to PAI-1 and PAI-2. Thus, our study has revealed that PAI-1 and PAI-2 are strong independent prognostic factors, and that only locally advanced disease provides a more relevant information on the outcome of disease.

Independent prognostic value of PAI-1 for the disease-free survival of all breast cancer patients was also established by German[7,20], Dutch[6], and French[9] researchers. In all those studies, apart from pathological lymph node status, PAI-1 was found to be the strongest prognostic factor. Only the French study, which investigated not only PAI-1 but also the independent prognostic value of PAI-2, has confirmed that only pathological lymph node status has a stronger independent prognostic value.[9]

Our study has therefore established the value of PAI-1 and PAI-2 contents in tumor cytosols for the prognosis of disease in breast cancer patients. Based on the results obtained, we believe that in the cases when only cytosol which does not enable a reliable uPA determination is available, PAI-1 and PAI-2 can provide a sufficient information for foretelling the outcome of disease.

It is presumed that a combination of both inhibitors, and perhaps also their combination with other components of the urokinase system or other proteinases might provide even better information for foretelling the outcome of disease, however, such an analysis would require a larger number of patients. It should be also necessary to establish the prognostic value of urokinase system components in individual subgroups of patients, distributed according to menopausal status, lymph node involvement, hormonal status, etc. Such an approach would enable us to detect the patients at an increased risk of recurrence within the prognostically more favorable groups. Nevertheless, such an analysis as well would require a considerably larger number of patients. Apart from that, it would be interesting to find out whether immunohistochemically determined components of the urokinase system would provide a similar information, or would such determination - likewise in the case of cathepsin D - undermine the beliefs about their prognostic value.[21] We plan to carry out a prospective study, which would touch upon at least some of the hypotheses presented here.

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