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Quantitative RT-PCR assays for the determination of urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 mRNA in primary tumor tissue of breast cancer patients: Comparison to antigen quantification by ELISA

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Abstract. Urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1) play a key role in tumor-associated processes such as the degradation of extracellular matrix proteins, tissue remodeling, cell adhesion, migration, and invasion. High antigen levels of uPA and PAI-1 in tumor tissue of various solid malignant tumors, including breast cancer, are associated with poor patient prognosis. In the present study, we examined whether analysis of uPA and PAI-1 mRNA expression represents an alternative to the measurement of the respective antigen levels in breast cancer. Highly sensitive quantitative real-time PCR (QPCR) assays, based on the LightCycler technology, were established to quantify uPA and PAI-1 mRNA expression in breast cancer cell lines as well as in tumor tissue of breast cancer patients. mRNA concentrations were normalized to the expression level of the housekeeping gene h-G6PDH. The respective uPA and PAI-1 antigen concentrations were determined by established ELISA formats. QPCR mean interassay variation coefficients were 11% (uPA) and 8% (PAI-1). In breast cancer cell lines, mRNA and antigen values were highly correlated for both uPA and PAI-1 (each: r_s=0.95; $p<0.001$). In contrast, correlations between uPA/PAI-1 mRNA and protein in the breast cancer samples were found to be distinctly weaker or not significant. Thus, quantitative determination of mRNA expression for both factors does not mirror antigen levels in breast cancer tissue, possibly due to posttranscriptional regulation. Except for nodal status being inversely correlated with uPA mRNA levels, no significant interrelations were observed between uPA or PAI-1 mRNA expression and clinicopathological parameters. On the protein level, elevated uPA and PAI-1 values were associated with a negative steroid hormone receptor status. In conclusion, the implementation of mRNA quantification of uPA and PAI-1 in breast tumors is unable to serve as a one-to-one substitution for antigen determination by ELISA.

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Introduction

Today, women diagnosed with primary breast cancer have a wide range of therapeutic options and a good chance of long-term survival. Two of the novel biomarkers predicting therapy response and the course of disease, the serine protease urokinase-type plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor type 1 (PAI-1), have been extensively validated in preclinical and clinical studies and have reached level of evidence (LOE) 1, the highest level of clinical evidence for a tumor-associated biomarker (1). Moreover, by including uPA and PAI-1 in therapy decision plans, overtreatment of early-stage breast cancer patients might be prevented. In the NNBC-3-Europe trial participating centers have opted to either perform risk estimation by clinicopathological factors or by the uPA and PAI-1 concentration in tumor tissue biopsies (2).

For two decades, the involvement of the plasminogen activation system in tumor growth and metastasis has been

investigated (3). uPA and PAI-1 were found to be expressed in stromal and tumor cells, indicating their importance in tissue remodeling and desmoplasia, angiogenesis and tumor invasion (4). uPA converts the inactive zymogen plasminogen into the serine protease plasmin, a process which is accelerated by the binding of uPA to its membrane-anchored receptor uPAR (CD87) (5). Furthermore, in addition to potently increasing extracellular proteolysis, uPA - in concert with uPAR - induces signalling events affecting cell proliferation, adhesion, and migration. PAI-1 is the major physiological regulator of uPA activity. Apart from this, PAI-1 also interacts with the adhesive extracellular matrix protein vitronectin. The multifactorial effects on cell adhesion and angiogenesis ascribed to PAI-1 (6-8) could explain why, in addition to uPA, elevated tumor tissue levels of PAI-1 correlate with a poor prognosis of patients afflicted with breast cancer or other types of malignancies (1,9-12). Most of the published data regarding the clinical impact of uPA and PAI-1 in breast cancer have been collected determining the uPA and/or PAI-1 antigen content by ELISA in cytosol extracts or detergent-released membrane extracts of fresh-frozen breast cancer specimens. On the other hand, mRNA expression of uPA and/or PAI-1 in breast cancer samples in relation to patient outcome has been analyzed only in few studies (13-17). Furthermore, systematic studies with the objective to compare uPA/PAI-1 mRNA expression values with uPA/PAI-1 antigen levels in tumor tissue extracts determined by ELISA are rare, and patient numbers are small (13,17,18). Therefore, we were prompted to determine uPA and PAI-1 mRNA expression in comparison to the corresponding uPA and PAI-1 antigen levels in breast cancer tissue specimens and cell lines. Beforehand, highly sensitive quantitative real-time PCR (QPCR) assays, applying the LightCycler technology, were established.

Materials and methods

Patients. Tumor tissue specimens originated from a population-based cohort of 105 primary breast cancer patients who had undergone breast cancer surgery between 1989 and 1996 in participating hospitals of the Comprehensive Cancer Center East in the Netherlands. The study adhered to national regulations on ethical issues and was approved by the local ethical committees. After surgical resection of the primary tumor and inspection by a pathologist, a representative part of the tumor was immediately snap-frozen and stored in liquid nitrogen. The histological grade of the tumors was determined according to Bloom-Richardson. Tumor stages were classified according to the TNM classification system. The ages of the patients at the time of surgery ranged from 30 to 88 years, with a median age of 58 years. The patients had either undergone modified radical mastectomy (n=84) or breast-preserving lumpectomy (n=21), combined with axillary lymph node dissection. Postoperative locoregional radiotherapy of the breast had been applied in 68 cases (64.8%) after incomplete resection or breast-preserving treatment, with one case of missing patient information. Axillary or supraclavicular regions were irradiated depending on the degree of nodal involvement. Fifty-four patients (51.4%) were classified as lymph node (LN)-positive, 44 patients (41.9%) were LN-negative, and the LN status of 7 patients (6.7%) was unknown.

For the analysis of the correlation between uPA/PAI-1 mRNA expression and uPA/PAI-1 antigen levels in tissue extracts, an additional set of 74 tumor tissue specimens from primary breast cancer patients, treated at the University Medical Center Dresden and the Klinikum rechts der Isar in Munich, was available.

Cell lines. The following cell lines were used: (i) the breast cancer cell lines MDA-MB-231, MDA-MB-231 BAG (subline of MDA-MB-231, stably transfected with the bacterial lacZ gene), MDA-MB-435 (provided by the Institute of Experimental Oncology and Therapy Research, Technical University of Munich, Germany), MCF-7 and aMCF-7, an adriamycine-resistant subline of MCF-7 (provided by the Max-Delbrück Center for Molecular Medicine, Berlin-Buch, Germany), (ii) the ovarian cystadenocarcinoma cell lines OV-MZ-6 and OV-MZ-10 (provided by the Städtische Kliniken Frankfurt a.M.-Höchst, Germany), and (iii) the non-malignant human keratinocyte cell line HaCaT (provided by the Department of Dermatology, Dresden University of Technology, Germany).

The cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2 in DMEM (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (Invitrogen), 1% penicillin-streptomycin (Biochrom, Berlin, Germany), 1% arginine-asparagine (Sigma, Deisenhofen, Germany), and 1% HEPES buffer (Invitrogen). After 48 h, the cells were harvested from monolayer dishes for mRNA isolation as previously described (19). For uPA and PAI-1 antigen determination by ELISA (applying the Imubind #894 and #821 ELISA kits; American Diagnostica, Stamford, CT, USA), the culture supernatant was collected, cleared by centrifugation, and stored at -20˚C until use. uPA and PAI-1 antigen levels in the supernatants were expressed as ng analyte per 10^6 cells after 48 h of cultivation.

uPA and PAI-1 antigen determination in tumor tissue extracts by ELISA. Deep-frozen tissue of 105 breast cancer specimens from Nijmegen was used to derive so-called cytosol fractions for the ELISA procedure. For this, the tumor tissues were pulverized in the frozen state and homogenized in a buffer lacking detergents (so-called EORTC buffer, but without monothioglycerol or glycerol). The protein content of the tissue extracts (cytosolic plus secreted proteins) was measured as modified by Lowry, using BSA as the standard. uPA and PAI-1 antigen contents were determined in the high-speed supernatant by published in-house ELISA formats (20-22).

In contrast, detergent extracts were prepared from homogenized tissue of the second set of 74 primary breast cancer specimens from Dresden/Munich (23-25). After solubilization of membrane-bound proteins using Tris buffer containing the non-ionic detergent Triton X-100 (1%), cell debris was separated by centrifugation, and the supernatant was stored at -20˚C until use. The protein content of these tissue extracts (cytosolic plus secreted plus membrane-released proteins) was determined with the BCA Protein kit (Pierce, Rockford, IL, USA), again using BSA as the

equences of primers and hybridization probes for uPA and PAI-1 applied in the LightCycler QPCR assay.

	Oligonucleotide sequence (5'-3')	Fragment size (bp)
uPA		
Forward primer (uPA ex10F)	AGT GTC AGC AGC CCC ACT	103
Reverse primer (uPA x10, 11A)	CCC CCT GAG TCT CCC TGG	
Hyb probe 1 (uPA FL)	AAG TCA CCA CCA AAA TGC TGT GTG CT	
Hyb probe 2 (uPA LC)	CTG ACC CAC AGT GGA AAA CAG ATT C	
PAI-1		
Forward primer (PAI-1 x5, 6F)	CTC CTG GTT CTG CCC AAG TT	132
Reverse primer (PAI-1 x6,7R)	GAG AGG CTC TTG GTC TGA AAG	
Hyb probe 1 (PAI-1 FLU)	TCG GTC ATT CCC AGG TTC TCT AGG	
Hyb probe 2 (PAI-1 LCR)	GCT TCC TGA GGT CGA CTT CAG TCT CC	

standard. uPA and PAI-1 antigen concentrations were determined applying commercially available ELISA kits (Imubind uPA ELISA #894 and Imubind PAI-1 ELISA #821). Antigen concentrations in the cytosol extracts as well as in the detergent extracts were expressed in ng analyte per mg of total protein.

Quantification of uPA and PAI-1 mRNA by QPCR. Total RNA from fresh-frozen breast cancer tissue samples was isolated and reverse transcribed as published previously for samples from Nijmegen (26), Dresden (27), and Munich (19). For quantification of uPA and PAI-1 mRNA, highly sensitive QPCR assays applying the LightCycler technology (Roche Diagnostics, Mannheim, Germany; software ver. 3.5) were established. Based on the cDNA sequences of uPA (GenBank accession no. NM_002658) and PAI-1 (NM_000602), gene-specific primers for amplification as well as hybridization probes for real-time monitoring of PCR reactions were designed; the resulting amplicon lengths preferably only slightly exceeding 100 bases (Table I). RT-PCR was performed with an optimized master mixture containing 0.5 μM of each amplification primer, 0.2 μM of each hybridization probe, and either 5 mM $MgCl_2$ (uPA) or 3.5 mM $MgCl_2$ (PAI-1) in a total volume of 20 μl. The amplification program started with pre-denaturation at 95˚C for 10 min, followed by 45 cycles of amplification: denaturation for 10 sec at 95˚C, annealing for 10 sec at 62˚C (uPA) or 63˚C (PAI-1), and elongation for 5 sec at 72˚C. Finally, the amplification products were cooled down to 40˚C for 30 sec. In order to verify the results of the QPCR measurements, randomly selected samples were run on 1.5% agarose gels and sequenced after purification.

Five-log-range calibration curves were generated for each PCR run using 8 glass capillaries coated with defined numbers of linearized plasmid molecules, either carrying the uPA cDNA (pUC18-uPA) or the PAI-1 cDNA (pMelBacA-PAI-1). The plasmid molecule numbers were exactly determined by HPLC calibration (Roboscreen, Leipzig, Germany). The glass capillary standards were coated with 10, 20, 50, 100, 1,000, 5,000, 10,000, and 100,000 copies, respectively.

Human glucose-6-phosphate-dehydrogenase (h-G6PDH), which has been shown to represent an appropriate house-keeping gene for breast cancer studies (19), was chosen for normalization of the data. The h-G6PDH Housekeeping Gene Set (Roche Diagnostics) was applied according to the manufacturer's protocol (amplicon length, 123 bp). All further calculations and statistical analyses were carried out using the relative mRNA expression ratios (zmol uPA or PAI-1 per amol h-G6PDH).

Statistical analyses. uPA and PAI-1 mRNA and antigen expression did not follow a Gaussian distribution. Thus, non-parametric tests (Mann-Whitney U or Kruskall-Wallis tests) were conducted. The levels of significance in correlations between continuous variables were calculated with the Spearman rank correlation (r_s). P-values ≤0.05 were considered statistically significant. All calculations were performed using the SPSS statistical package, release 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Development of QPCR assays for uPA and PAI-1 mRNA determination. The amplicon lengths of the QPCR assays were chosen as small as possible to allow analysis of uPA/PAI-1 mRNA expression not only in fresh-frozen tissue but also in formalin-fixed, paraffin-embedded tissues, considering the extensive RNA fragmentation in tissues so preserved. The lower limit is given by the technological requirement of two primers each for amplification and detection within an amplicon. In order to minimize the risk of amplifying genomic DNA, at least one primer in each assay was designed to overlap an exon boundary, i.e. to bind to two exons. In our assays, the forward primer for detection of uPA anneals within exon 10, and the reverse primer overlaps the boundary between exons 10 and 11 (Fig. 1A). These exons are separated by an intron of 989 base pairs in the genomic DNA. The forward primer for the PAI-1 assay overlaps the junction of exons 5 and 6, and the reverse primer overlaps the junction of exons 6 and 7 (Fig. 1B). Here, the intervening region between exon 5 and 6 comprises 1592 base pairs, and that between exon 6 and 7 encompasses 120 base pairs. The amplicon length for the definite uPA assay consists of 103 base pairs, and the PAI-1 amplicon is 132 base pairs long (Fig. 1; Table I).

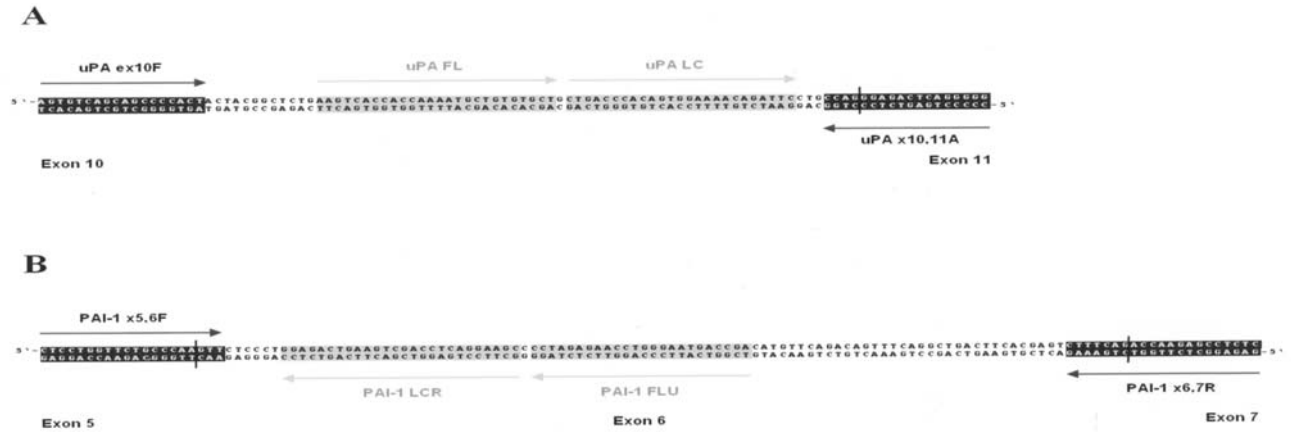

Figure 1. DNA sequence of the uPA and PAI-I amplicons generated by the LightCycler QPCR assays. The localization and orientation of the primers for uPA (A) or for PAI-1 (B) are indicated by arrows; exon/exon boundaries by vertical bars.

Table II. uPA and PAI-1 mRNA expression and antigen levels in the cell lines.[a]

	uPA	
	mRNA (zmol uPA/amol h-G6PDH)	Antigen (ng/10^6 cells per 48 h)
High expression		
OV-MZ-10	1409.77	582.76
Medium expression		
MDA-MB-231 BAG	134.82	30.11
MDA-MB-231	129.90	57.06
HaCaT	93.81	4.52
OV-MZ-6	84.87	4.24
Low expression		
aMCF-7	1.75	0.01
MCF-7	0.16	0.26
MDA-MB-435	0.09	0.01
	PAI-1	
	mRNA (zmol PAI-1/amol h-G6PDH)	Antigen (ng/10^6 cells per 48 h)
High expression		
OV-MZ-10	837.44	1883.80
MDA-MB-231	320.76	894.85
MDA-MB-231 BAG	137.11	610.42
Medium expression		
OV-MZ-6	35.74	21.70
HaCaT	20.77	33.53
aMCF-7	13.60	20.29
Low expression		
MDA-MB-435	0.41	0.06
MCF-7	0.16	1.12

[a]The mRNA transcript levels were quantified by LightCycler QPCR and normalized to h-G6PDH. Antigen levels of uPA and PAI-1 were determined in cell culture supernatants by use of the Imubind uPA #894 and PAI-1 #821 ELISA kits.

In order to calculate the absolute copy numbers of the respective amplicon, standard curves were produced by coating glass capillaries with defined numbers of linearized plasmids, determined by HPLC calibration, which harbored cDNA either encoding uPA or PAI-1. Plots of measured versus theoretical transcript numbers of uPA and PAI-1 generated from 36 and 33 independent QPCR runs, respectively, for the capillaries coated with 10-100,000 template copies in both assays are shown in Fig. 2. The correlations of mRNA values were highly significant with r_s=0.99 ($p<0.001$) in both QPCR assays. The mRNA copy numbers were obtained from repeated LightCycler measurements, the number of runs depending on interassay and intra-assay divergence. In the tumor tissue samples, the interassay variation coefficients ranged from 0 to 43% (uPA) and from 0 to 23% (PAI-1), with a mean of 11 and 8%, respectively.

Correlations of uPA and PAI-1 antigen levels determined by ELISA and mRNA expression determined by QPCR. First, the correlation between uPA/PAI-1 antigen levels and uPA/PAI-1 mRNA expression was analyzed in eight cell lines (MDA-MB-231, MDA-MB-231 BAG, MDA-MB-435, MCF-7, aMCF-7, OV-MZ-6, OV-MZ-10, and HaCaT). A high concordance was found between uPA/PAI-1 antigen levels measured by ELISA and uPA/PAI-1 mRNA expression quantified by QPCR (r_s=0.95, $p<0.001$ for both QPCR assays; Table II).

Next, we analyzed uPA and PAI-1 mRNA expression and uPA and PAI-1 antigen levels in the two sets of tumor tissue specimens encompassing 105 and 74 samples, respectively. Whereas in both sets RNA isolation was performed employing comparable technical approaches, different methods of protein extraction and uPA/PAI-1 antigen determination were used (see Materials and methods). In the first set of breast cancer samples (Nijmegen; n=105), uPA and PAI-1 antigen concentrations were determined in the cytosol fraction applying in-house ELISA formats. In the second set (Dresden/Munich; n=74), detergent extracts were used. In the first set (ELISA from cytosol extracts), a rather weak but still statistically significant correlation was found between mRNA expression and antigen levels for both uPA (r_s=0.35, $p<0.001$) and PAI-1 (r_s=0.20, p=0.045) (Fig. 3A). Regarding

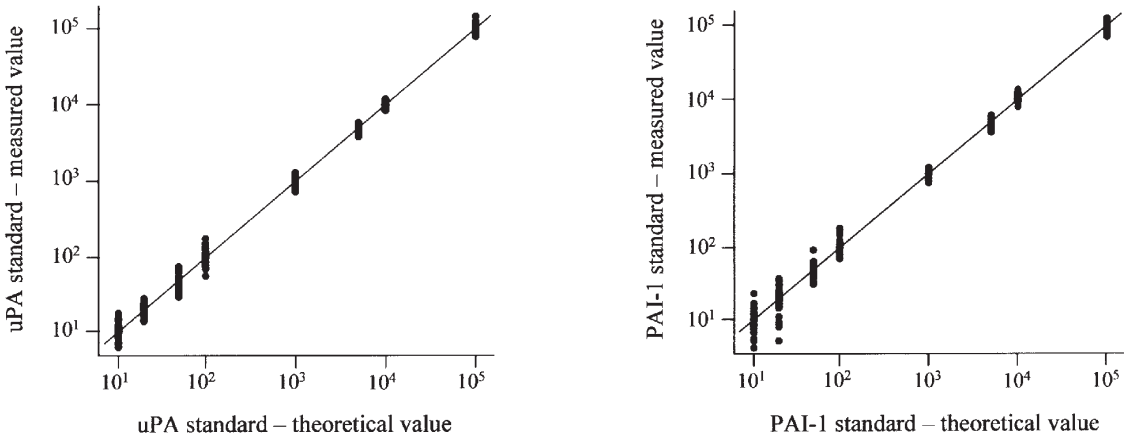

Figure 2. uPA and PAI-1 mRNA standard measurements by LightCycler QPCR. Results were obtained from 36 (uPA) and 33 (PAI-1) LightCycler PCR runs, respectively.

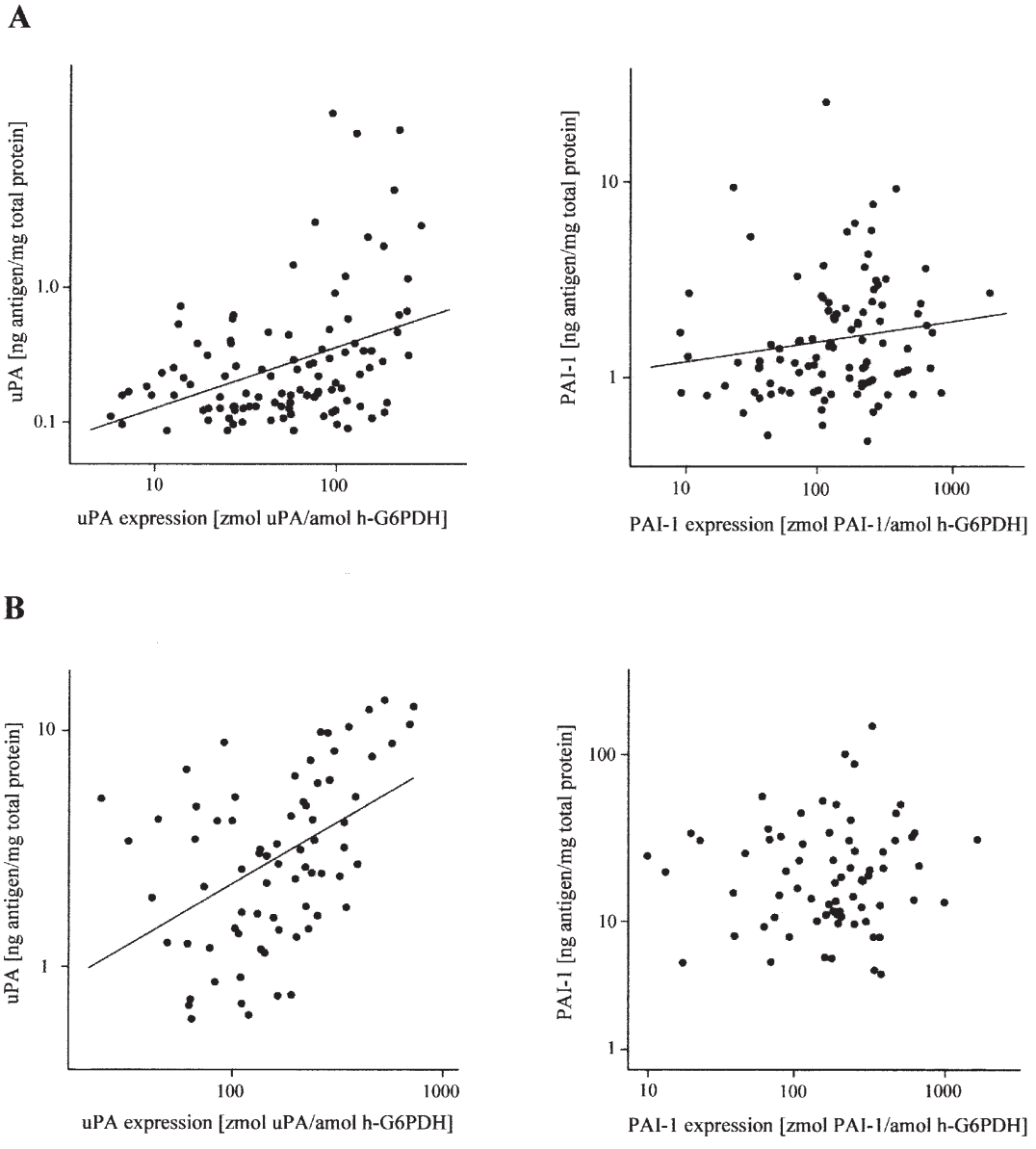

Figure 3. Correlations of uPA and PAI-1 mRNA expression with uPA and PAI-1 antigen levels determined by ELISA in tumor tissue. (A) In the first set of tumor tissue samples (Nijmegen; n=105), antigen contents were determined in the high-speed supernatant of cytosolic tumor tissue extracts applying published in-house ELISA formats (20-22). (B) In the second set of tumor tissue samples (Dresden/Munich; n=74), antigen levels were determined in detergent extracts of breast cancer tissues by use of IMUBIND uPA #894 and PAI-1 #821 ELISA kits (25). In both cases, mRNA transcript levels were quantified by LightCycler QPCR.

Table III. uPA and PAI-1 mRNA expression and antigen levels related to clinical and histomorphological parameters in breast cancer patients (n=105).[a]

Variable	uPA					PAI-1				
		QPCR		ELISA			QPCR		ELISA	
	n (%)	Median (IR[d])	p	Median (IR[d])	p	n (%)	Median (IR[d])	p	Median (IR[d])	p
Menopausal status[b]	105		0.879		0.736	104		0.837		0.126
Pre/peri	28 (26.7)	56.1 (70.4)		0.31 (0.34)		28 (26.9)	148.9 (154.7)		1.03 (1.26)	
Post	77 (73.3)	58.5 (86.0)		0.27 (0.40)		76 (73.1)	136.4 (192.1)		1.49 (1.93)	
Lymph node status[b]	98		**0.020**		0.192	97		0.056		0.540
Negative	44 (44.9)	74.0 (69.1)		0.26 (0.31)		44 (45.4)	198.2 (199.5)		1.77 (1.66)	
Positive	54 (55.1)	41.3 (72.9)		0.33 (0.46)		53 (54.6)	125.4 (185.1)		1.23 (1.97)	
Tumor size (pT)[c]	104		0.228		0.138	103		0.607		0.249
1	22 (21.2)	65.5 (73.3)		0.24 (0.22)		22 (21.3)	172.7 (175.2)		1.20 (1.90)	
2	67 (64.4)	57.2 (87.3)		0.36 (0.46)		66 (64.1)	136.4 (182.9)		1.61 (1.76)	
3/4	15 (14.4)	32.6 (78.9)		0.29 (0.45)		15 (14.6)	107.4 (224.8)		0.85 (1.65)	
Tumor grade[b]	62		0.782		0.306	61		0.642		0.380
1/2	35 (56.5)	56.8 (65.2)		0.26 (0.27)		34 (55.7)	132.4 (143.0)		1.98 (1.99)	
3	27 (43.5)	52.0 (116.8)		0.37 (0.41)		27 (44.3)	111.1 (227.3)		1.40 (2.32)	
ER status[b]	103		0.811		**0.015**	102		0.502		**0.009**
Negative	38 (36.9)	58.7 (135.2)		0.42 (0.53)		37 (36.3)	130.9 (229.5)		1.96 (2.66)	
Positive	65 (63.1)	56.8 (68.3)		0.24 (0.30)		65 (63.7)	138.8 (147.2)		1.21 (1.45)	
PgR status[b]	104		0.776		0.062	103		0.757		**0.001**
Negative	42 (40.4)	53.9 (124.2)		0.39 (0.53)		41 (39.8)	134.0 (213.9)		2.27 (2.76)	
Positive	62 (59.6)	58.0 (66.2)		0.25 (0.30)		62 (60.2)	135.3 (161.5)		1.18 (1.32)	
Age[b]	105		0.625		0.167	104		0.091		0.700
≤60 years	56 (53.3)	59.0 (82.0)		0.25 (0.38)		56 (53.8)	188.5 (150.7)		1.37 (1.93)	
>60 years	49 (46.7)	56.9 (78.5)		0.39 (0.37)		48 (46.2)	110.5 (193.8)		1.34 (1.82)	

[a]The mRNA transcript levels were quantified by LightCycler-QPCR and normalized to h-G6PDH (ratio: zmol/amol). uPA and PAI-1 antigen levels were quantified by published in-house ELISA formats from cytosolic extracts (20-22). Significant p-values are in bold print; [b]Mann-Whitney U test, [c]Kruskal-Wallis test, and [d]Interquartile range.

the second set (ELISA from detergent extracts), we also found a significant correlation between uPA mRNA expression and uPA antigen levels (r_s=0.48, p<0.001). The correlation between PAI-1 mRNA expression and PAI-1 antigen levels, however, was not significant (r_s=0.06, p=0.613) (Fig. 3B).

It should be noted that we also observed significant correlations between uPA and PAI-1 antigen and between uPA and PAI-1 mRNA expression values in both the first set of 105 samples (uPA versus PAI-1 antigen: r_s=0.44, p<0.001; uPA versus PAI-1 mRNA: r_s=0.61, p<0.001) and the second set of 74 samples (uPA versus PAI-1 antigen: r_s=0.49, p<0.001; uPA versus PAI-1 mRNA: r_s=0.38, p=0.001). Similarly, there were high correlations between uPA and PAI-1 levels in the cell line analysis, with a correlation coefficient of r_s=0.98 (p<0.001) both between uPA and PAI-1 antigen values and uPA and PAI-1 QPCR results (data not shown).

uPA and PAI-1 mRNA expression and antigen levels in breast cancer tissue specimens and their association with clinical and histomorphological parameters. The relationship between uPA/PAI-1 mRNA expression and uPA/PAI-1 antigen levels and clinical or histomorphological variables, including menopausal status, LN status, tumor size (pT), tumor grade, estrogen receptor (ER) status, progesterone receptor (PgR) status, and the age of the patients (categorized as ≤60 years versus >60 years), was analyzed in the cohort of 105 breast cancer patients for whom complete clinical data were available. The uPA and PAI-1 mRNA concentrations (zmol/amol h-G6PDH) ranged from 27.13 to 108.68 (median 57.16) and from 72.84 to 255.28 (median 136.40), respectively. uPA and PAI-1 antigen levels (ng/mg total protein) in the cytosol extracts ranged from 0.18 to 0.56 (median 0.28) and from 0.81 to 2.64 (median 1.36), respectively. The results are summarized in Table III. The levels of uPA and PAI-1 mRNA expression did not differ significantly between tumors in relation to clinicopathological parameters, except for the LN status, which was inversely correlated with uPA mRNA expression (p=0.020; Table III). With regard to PAI-1 protein, significantly higher PAI-1 antigen levels were found in ER-negative (p=0.009) as

PgR-negative tumors (p=0.001) compared to ER-r PgR-positive tumors. For uPA protein, significantly elevated antigen levels were only found in ER-negative patients (p=0.015) compared to ER-positive patients. uPA protein was also elevated in PgR-negative tumors, but the difference only approached statistical significance (p=0.062). There was no significant association of uPA/PAI-1 antigen levels, neither with menopausal status, LN status, tumor size, tumor grade, nor with the age of the breast cancer patients.

Discussion

High protein contents of uPA and PAI-1 in primary breast tumors have been shown to indicate an unfavorable prognosis, with a strong and independent impact on disease-free and overall survival, also in the subgroup of LN-negative patients (1,9,10). With reference to the National Institutes of Health (28) and the St. Gallen consensus guidelines (29), up to 90% of LN-negative breast cancer patients are eligible to receive adjuvant systemic chemotherapy. By use of uPA and PAI-1 as additional prognostic factors, subgroups of LN-negative patients at high or low risk can be identified (30-36). Patients at high risk, as identified by elevated uPA and/or PAI-1 protein levels, benefit from adjuvant systemic chemotherapy, resulting in a substantial reduction of risk of relapse (1,31). On the other hand, a considerable number of patients at low risk of relapse could be spared the exposure to adjuvant systemic chemotherapy with its toxic side effects. In fact, determination of antigen levels of uPA and PAI-1 in primary breast cancer tissue by ELISA has already entered clinical practice for risk-adapted, individual therapy decisions, particularly in patients with LN-negative disease (1,2).

However, the measurement of uPA and PAI-1 by ELISA requires relatively large amounts of fresh-frozen tumor material, which are not always available, e.g. in small tumors (<1 cm), and requires an adequate capacity for the storage of tumor samples. Furthermore, tumor tissue material becomes more and more limited in view of the diagnosis of breast cancer at earlier stages when most tumor tissue specimens are retrieved from cryostat sections, fine needle aspirates, or core biopsies (37). Thus, alternative, less material-consuming methods for the quantitative determination of prognostic factors in tumors must be developed. In the present study, highly sensitive, robust quantitative real-time PCR assays for uPA and PAI-1, applying the LightCycler technology, were established. For the preceding mRNA isolation, only small amounts of tumor tissue were required. The amplicon lengths of the QPCR assays for uPA and PAI-1 were minimized as far as possible to potentially enable quantification of partially fragmented mRNA extracted from formalin-fixed, paraffin-embedded tissue (38). Recently, several QPCR assays for determination of uPA and/or PAI-1 mRNA expression have been introduced with rather large amplicon sizes (13-15,17,18,39,40). In contrast, our newly developed QPCR assays use amplicon lengths ranging from 103 to 132 base pairs, the shortest uPA/PAI-1 QPCR-derived cDNA fragments described so far. Furthermore, most of the other studies used SYBR-Green I for template detection, a method which requires melting-point analysis to be able to accurately identify the target amplicons. In our study, the more precise and specific FRET method was applied.

In the present study, a high correlation between secreted uPA/PAI-1 protein and mRNA measurements was found in cell lines of r_s=0.95, each. When applying the QPCR assays to tumor samples, correlations between uPA/PAI-1 antigen levels in tissue extracts and mRNA tissue expression were considerably weaker, although in most cases still significant. Cell lines often lack representativeness as breast cancer *in vivo* displays greater heterogeneity and complexity than breast cancer cell lines (43). Our results strongly indicate that uPA and PAI-1 antigen concentrations, independent of the extraction method used (either so-called cytosol or detergent extracts), do not completely reflect the respective mRNA expression levels. This may be due to posttranscriptional regulation processes which have been previously described for components of the plasminogen activation system, including uPA and PAI-1 (44). Our observations of rather weak correlations between mRNA and protein expression in tumor tissue are in line with results obtained by Spyratos *et al* (13), who found, in a small cohort of 21 patients, only borderline significance between uPA protein and mRNA levels and a significant correlation of PAI-1 protein and mRNA. Furthermore, no significant correlations at all were observed in a cohort of 54 breast cancer patients between uPA and PAI-1 protein and mRNA measurements (18). Only recently, the same research group reported significant, albeit weak, correlations between mRNA and antigen values for uPA and PAI-1 in tumor tissue of another cohort of 70 breast cancer patients (17).

Corresponding to our results, in studies by Spyratos *et al* (13), Urban *et al* (15), and Castelló *et al* (17), positive correlations between uPA and PAI-1 mRNA values were also observed. Several other groups have previously found significant correlations between uPA and PAI-1 antigen levels in tumor tissue (17,30,34,35,41,42,45,46). These findings may not be surprising considering the various interactions of the members of the plasminogen activation system and the synergic effects ascribed to uPA and PAI-1 in tumor growth and metastasis.

The levels of uPA and PAI-1 mRNA did not differ significantly between tumors in relation to clinicopathological parameters, with the exception of a significant relationship between high uPA mRNA concentrations and a negative LN status, which has not been described in the literature so far. Conversely, our findings that uPA and PAI-1 antigen levels were significantly higher in ER-negative and, in the case of PAI-1, also in PgR-negative patients, as compared to the receptor-positive patient samples, have been supported by a number of previous studies (35,41,42).

So far, there are few studies in which uPA and PAI-1 mRNA expression has been linked to patient prognosis (13-17). In the study by Spyratos *et al* (13), high uPA and PAI-1 mRNA levels were significantly associated with shorter disease-free survival in a population of 130 primary breast cancers, independent of hormone receptor or LN status. Leissner *et al* (14), who included 87 patients, all LN-positive/hormone receptor-positive, observed that high PAI-1 mRNA expression was significantly associated with a shorter metastasis-free survival, whereas uPA mRNA levels were not

of prognostic relevance. In a subset of ErbB2-positive breast cancer patients, Urban *et al* (15) identified uPA mRNA, among 60 other genes measured by QPCR, as the most significant marker associated with metastasis-free survival (MFS). This result was validated in two microarray-derived data sets. Furthermore, in the QPCR data set, PAI-1 mRNA was found to be significantly associated with MFS by univariate Cox regression, independent of the ErbB2 status. In another study, analyzing PAI-1 mRNA expression in three microarray breast cancer data sets, Sternlicht *et al* (16) reported an unfavorable prognostic strength in the univariate Cox analysis of only two of the three independent patient cohorts. On the other hand, Castelló *et al* (17) found no indication for higher uPA or PAI-1 mRNA levels (in contrast to high PAI-1 antigen levels) in tumor tissue of patients who suffered a relapse as compared to relapse-free patients. However, it should be noted that the analyzed cohort comprised only 70 patients, of whom 6 had suffered a relapse. Finally, in a recent study (47), we evaluated expression of *KLK7* mRNA, encoding the serine protease human tissue kallikrein-related peptidase 7, in tumor specimens of 155 breast cancer patients. High *KLK7* mRNA expression was found to be significantly associated with a better outcome for the patients according to both univariate and multivariate Cox survival analysis. The same patient cohort was also used to quantify the expression of uPA and PAI-1 mRNA, respectively (uPA/PAI-1 antigen data were not available for the entire cohort). Here, the expression levels for both uPA and PAI-1 mRNA were not associated with patient prognosis (data not shown).

Still, as shown in several but not all of the above described studies, quantitative determination of uPA and/or PAI-1 mRNA expression levels may exhibit prognostic information for patients afflicted with breast cancer. Taking into account the weak correlations between antigen and mRNA values, uPA and/or PAI-1 mRNA quantification may potentially add independent information concerning patient outcome in addition to uPA and/or PAI-1 antigen levels. Up to now, unfortunately, there are no data available directly comparing the prognostic strength of uPA/PAI-1 protein versus mRNA determination. Such clinical studies may be the prerequisite for the introduction of the highly sensitive, reproducible, and rapid QPCR assays for uPA and PAI-1 into clinical routine.

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