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Prognostic relevance of uPAR-del4/5 and TIMP-3 mRNA expression levels in breast cancer ☆

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Abstract

Recently, two components of important protease systems in cancer, *i.e.*, the urokinase plasminogen activator receptor (uPAR) mRNA splice variant uPAR-del4/5 and the tissue inhibitor of matrix metalloproteinase-3 (TIMP-3), were independently reported to be of prognostic value in breast cancer. In the present study, we have evaluated the impact of both these factors on disease-free survival (DFS) in 205 breast cancer patients by assessing mRNA expression in tumour tissue by quantitative PCR. High uPAR-del4/5 mRNA expression was associated with shorter DFS in breast cancer patients ($P = 0.0363$), whereas high TIMP-3 mRNA levels were associated with a good prognosis ($P = 0.0049$). Furthermore, by combining uPAR-del4/5 with TIMP-3 values, we demonstrate that breast cancer patients with high uPAR-del4/5 and low TIMP-3 mRNA levels had a highly significantly shorter DFS in comparison to those patients with low uPAR-del4/5 and high TIMP-3 mRNA expression ($P < 0.0001$). These patients had a more than 6-fold higher risk for disease recurrence or death in multivariate analysis. Therefore, considering the prognostic impact of two proteolytic factors stemming from complementary protease systems may improve the prediction of disease recurrence in breast cancer.

Keywords: Breast cancer; Quantitative real-time PCR; Urokinase receptor transcript variants; Tissue inhibitor of metalloproteinase-3; Prognosis

1. Introduction

Extracellular proteolysis is a major contributor to tumour cell invasion and metastasis, not only because of its ability to degrade the extracellular matrix (ECM) surrounding tumour cells but also because of its impact on cell proliferation, cell adhesion and migration, and angiogenesis [1–6]. There is a pericellular network of interacting proteolytic systems, the most important being the plasminogen activator (PA) system and the matrix metalloproteinase (MMP) system, including matrix metalloproteinases (MMPs) and their inhibitors (TIMPs). The main components of the PA system are the urokinase-type PA (uPA), its receptor uPAR (CD87), and its inhibitor PAI-1 [7–9]. As its name implies, the

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serine protease uPA converts plasminogen into the enzymatically active two-chain serine protease plasmin. The latter catalyses the degradation of many ECM and basement membrane components either directly or indirectly *via* plasmin-dependent activation of other matrix degrading proteases such as the MMPs, which collectively are able to degrade all of the constituents of the ECM [10]. PAI-1 clearly displays pro-tumourigenic properties beyond its inhibitory capacity towards uPA, which may explain the finding that high tumour levels of uPA and/or PAI-1 protein are strong predictors of poor prognosis for patients afflicted with different types of solid malignant tumours [9,11,12]. Regarding uPAR, its prognostic value is not as pronounced as that of uPA and PAI-1 [13]. Still, uPAR is essential for pericellular plasminogen activation and it also functions in cell adhesion as well as in signalling pathways of migrating cells, mainly through interactions with integrins and the ECM protein vitronectin [2,14,15].

Several splice variants of human uPAR, which is encoded by seven exons, have been described and their expression was analysed in human cells and tissues [16–18]. Among these variants, there is an uPAR mRNA splice variant lacking exons 4 and 5 (uPAR-del4/5), which encodes an uPAR form, in which one of the three homologous domains (DII) of uPAR is missing. Quantification of the mRNA concentration of the uPAR-del4/5 variant in tumour tissues of breast cancer patients revealed that this mRNA transcript variant is expressed very frequently but at a rather low level. Still, elevated uPAR-del4/5 expression was significantly associated with shorter disease-free survival (DFS) of these patients [16].

The four members of the TIMP family control the activities of the MMPs. Similar to the serine protease inhibitor PAI-1, TIMP-1, TIMP-2, and maybe also TIMP-4, seem to have other, again pro-tumourigenic, functions besides their inhibitory role towards MMPs [1,19,20]. For instance, it has been shown that TIMP-2 forms a tight complex with pro-MMP-2, and TIMP-1 with pro-MMP-9, which in both cases is important for efficient cell surface-associated activation of these proforms by membrane-type (MT)-MMPs. Furthermore, both TIMP-1 and TIMP-2 display anti-apoptotic properties and, as important, growth promoting activities. This may explain the often-observed adverse prognosis for breast cancer patients whose tumours frequently express high levels of TIMP-1/2. On the other hand, TIMP-3 antagonises tumour growth, cell migration, and angiogenesis and displays pro-apoptotic properties. It is therefore considered a real suppressor of tumour growth, invasion and metastasis. TIMP-3 is also the only member of the MMP inhibitor family that binds tightly to the ECM through interaction with heparan sulphate. In addition to inhibiting MMPs, TIMP-3, but not TIMP-1, -2 or -4, is an efficient inhibitor of tumour necrosis factor-alpha converting enzyme (TACE or ADAM17), a metalloproteinase which is not a member of the MMP (matrixin) family, but belongs to the ADAM (adamalysin) family [20]. In a recent study, the clinical value of the various TIMP-1, -2, -3, and -4 mRNA expression levels for breast cancer patients was evaluated. High tumour levels of TIMP-3 mRNA, measured by quantitative PCR (QPCR), were associated with adjuvant endocrine (tamoxifen) therapy success [21].

In the present study, we analysed the intratumoural expression patterns of TIMP-3 and uPAR transcript variants uPAR-del5 and uPAR-del4/5 as well as wild-type uPAR (uPAR-wt) of breast cancer patients by QPCR and their association with prognosis in a series of 205 breast cancer patients.

2. Materials and methods

2.1. Patients and tissues

Tissue specimens originated from 205 primary breast cancer patients who underwent breast cancer surgery 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 tumour, representative tumour tissue specimens were selected macroscopically by a pathologist and frozen immediately in liquid nitrogen for routine determination of oestrogen (ER) and progesterone (PgR) receptor status at the Department of Chemical Endocrinology. Remaining frozen tissue or tissue powder was kept in liquid nitrogen. Patients who had received neo-adjuvant treatment, or had a previous diagnosis of cancer or had a carcinoma *in situ* as well as patients with recurrent disease within one month after surgery or with distant metastases at time of diagnosis were excluded from the study. The patients' age at diagnosis ranged from 31 to 88 years (median 61 years). The median follow-up time of the patients was 71 months (range 2–169 months). During that time, 72 patients experienced a disease recurrence (16 locoregional, 59 distant metastases, three both) and 57 of the patients have died.

2.2. Treatment

Patients underwent modified radical mastectomy or breast conserving lumpectomy combined with axillary lymph node dissection. Postoperative locoregional radiotherapy was given to the breast after an incomplete resection or after breast conserving treatment; or to the axillary or supraclavicular regions, depending on the degree of nodal involvement. Lymph node metastases were detected in

88 patients (43%), 90 patients (44%) were lymph node-negative. Adjuvant systemic treatment was administered according to respective consensus recommendations at the time. In the absence of involved axillary lymph nodes, patients usually (94%) did not receive any adjuvant treatment. Systemic adjuvant therapy was, however, offered to all node-positive patients. Pre-menopausal patients received chemotherapy, in ER-positive and/or PgR-positive patients additional tamoxifen endocrine therapy was given. Post-menopausal node-positive patients with ER-positive and/or PgR-positive tumours received adjuvant tamoxifen endocrine therapy for two years. If the primary tumour from post-menopausal node-positive patients was hormone receptor-negative, no adjuvant chemotherapy was given. In the absence of complaints or a suspicion of relapse, patients were routinely checked once every three months during the first two years and then once every six months for the next five years, and thereafter once a year.

2.3. Preparation of RNA, cDNA and quantitative PCR analyses

Total RNA was isolated after pulverisation of deep-frozen tissue samples by a microdismembrator from approximately 20 mg of the tissue powder using the RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase-I treatment. Reverse transcription was performed using the Reverse Transcription System (Promega Benelux BV, Leiden, The Netherlands) as described previously [21]. QPCR for TIMP-3 was performed using the TaqMan assay technology and an ABI Prism 7700 device (PE Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) [21]. For quantification of uPAR-wt and the uPAR transcript variants uPAR-del4/5 and uPAR-del5, QPCR assays based on LightCycler technology were used as described in recent publications [16,18]. A 1:5 dilution of cDNA samples was used for quantification of the uPAR variants and of TIMP-3. In all QPCR assays the amount of specific PCR product was determined by detecting fluorescence signals of site-specific hybridisation probes (uPAR-wt, uPAR-del4/5, uPAR-del5) or TaqMan probes (TIMP-3, β-actin). Samples were quantified at least in duplicate (uPAR-wt, uPAR-del4/5, uPAR-del5) or in quadruplicate (TIMP-3, β-actin) in independent PCR runs for the appropriate marker transcripts. Five-log-range calibration curves (10^1–10^6 molecules for uPAR variants and TIMP-3, 10^2–10^7 molecules for β-actin) were generated for each PCR run using either ready-to-go glass capillaries coated with a defined number of molecules of uPAR variant fragments (uPAR-wt, uPAR-del4/5, uPAR-del5) or plastic tubes with DNA dilutions (TIMP-3, β-actin) as reported [16,18,21]. Additionally, positive (cDNA from the breast cancer cell line MCF-7) and negative (no template) controls were included in each of the PCR runs. In case the absolute expression data from the duplicate measurements (uPAR-wt, uPAR-del4/5, uPAR-del5) differed by more than 50% or in one of the two measurements less than 10 template molecules were detected, a third quantification was performed. Mean values were used for further calculations. All measurements were normalised to β-actin expression applying pre-developed assay reagents for the TaqMan technology (PE Applied Biosystems). Only samples with relative high β-actin transcript expression levels ($C_t < 25$) were included in the final analyses. All further calculations and statistical analyses were carried out using the relative mRNA expression ratios (amol uPAR-wt or TIMP-3/amol β-actin, and zmol uPAR-del5 or uPAR-del4/5/amol β-actin).

2.4. Statistical analyses

The association of the different marker expression values with clinicopathological parameters was determined using non-parametric Mann–Whitney or Kruskal–Wallis tests. For survival analysis DFS, defined as the time from surgery until diagnosis of recurrent disease or death was used as the follow-up end point (in accordance to the definition of DFS in the CALGB, and similar to RFS in the NSABP B-27 study protocols). The association of mRNA expression levels of uPAR variants and TIMP-3 as well as of other clinicopathological factors with DFS was analysed using the Cox univariate and multivariate proportional hazards regression models. Only patients with a complete record of all variables were included into the multivariate regression models. The multivariate models were adjusted for clinicopathological factors that may affect survival: menopausal status, tumour stage, lymph node status, ER and PgR status. Survival curves were generated by univariate Kaplan–Meier estimation using the log-rank regression model (Mantel–Cox). The statistical analyses were two-sided, and only P-values < 0.05 were considered to be statistically significant. Calculations were performed using the StatView 5.0 statistical package (SAS Institute, Cary, NC).

3. Results

3.1. Expression of TIMP-3 and uPAR variant mRNA and their association with clinicopathological parameters

TIMP-3 and uPAR variant mRNA levels were determined by specific QPCR assays in tumour samples from 205 patients with primary breast cancer. The uPAR-wt and TIMP-3 mRNA concentrations normalised to β-actin (ratio amol/amol) ranged from 0.04 to 1.18 (median 0.284) and from 0.01 to 1.43 (median 0.091), respectively. The mRNA concentrations for uPAR-del5 and

uPAR-del4/5 normalised to β-actin (ratio zmol/amol) were in the range from 0.1 to 14.4 (median 1.70) and from <0.1 to 2.65 (median 0.510), respectively. A normal distribution was found for uPAR-wt, uPAR-del5 as well as for uPAR-del4/5 mRNA values. The distribution pattern of TIMP-3 mRNA levels appeared to be log-normal (data not shown). There was no significant correlation between log-normal TIMP-3 mRNA levels and uPAR-del4/5 or uPAR-del5 mRNA values ($r = 0.125$, $P = 0.07$ and $r = 0.120$, $P = 0.09$, respectively), and only a weak correlation with uPAR-wt ($r = 0.172$, $P = 0.01$) by linear regression analysis.

The relationship between TIMP-3 and uPAR-del4/5 expression and the clinicopathological characteristics of breast cancer patients is summarised in Table 1. The levels of uPAR-del4/5, uPAR-wt (data not shown) and uPAR-del5 (data not shown) did not differ significantly between tumours in relation to clinicopathological parameters. A significant association was observed between high TIMP-3 expression and age ($P = 0.0129$), and significantly higher TIMP-3 transcript levels were found in ER-positive ($P < 0.0001$) as well as in PgR-positive ($P = 0.0025$) tumours compared to ER-negative or PgR-negative tumours. However, there was no significant association with menopausal status, tumour stage, regional lymph node metastasis or histopathological grading.

3.2. Association of TIMP-3 and uPAR variant mRNA values with DFS

The strength of association between clinicopathological and tumour biological parameters with DFS is presented in Table 2. In univariate analysis of DFS using the Cox proportional hazards model in all patients, only one of the clinicopathological parameters, the lymph node status, reached statistical significance ($P = 0.0061$, HR = 1.95, 95%CI = 1.21–3.14). TIMP-3 and uPAR variant mRNA expression levels were used as categorical variables for survival analyses. For this, optimal cut-off points for the respective mRNA expression levels were defined by optimised dichotomisation using log-rank tests, based on the ability of the respective factor to predict outcome (cut-off values: TIMP-3 at 0.105 amol/amol; uPAR-wt at 0.289 amol/amol; uPAR-del5 at 1.70 zmol/amol; uPAR-del4/5 at 0.540 zmol/amol). The expression levels of uPAR-wt and uPAR-del5 were not associated with prognosis. Breast cancer patients with high expression levels of uPAR-del4/5 mRNA

Table 1
uPAR-del4/5 and TIMP-3 mRNA levels in tumour tissue in dependence on baseline characteristics of breast cancer patients

Patients' characteristics	Number of patients[a]	UPAR-del4/5 Median value[b]	*P*	TIMP-3 Median value[c]	*P*
Total number	205	0.510 (0.53)		0.091 (0.14)	
Age category (years)[d]	205		n.s.		0.0129
<40	18	0.341 (0.49)		0.041 (0.07)	
41–55	47	0.535 (0.67)		0.077 (0.09)	
56–70	91	0.550 (0.61)		0.108 (0.14)	
>71	49	0.462 (0.51)		0.107 (0.18)	
Menopausal status[e]	205		n.s.		n.s
pre/peri	42	0.444 (0.54)		0.075 (0.12)	
post	163	0.526 (0.53)		0.099 (0.13)	
Tumor stage (pT)[d]	202		n.s.		n.s
Tl	63	0.646 (0.78)		0.108 (0.13)	
T2	114	0.515 (0.51)		0.080 (0.13)	
T3 + 4	25	0.397 (0.41)		0.071 (0.12)	
Lymph node status[e]	178		n.s.		n.s.
Negative	90	0.534 (0.67)		0.081 (0.11)	
Positive	88	0.466 (0.50)		0.085 (0.13)	
Grading (Bloom–Richardson)[e]	146		n.s.		n.s.
II	79	0.535 (0.54)		0.116 (0.16)	
III	67	0.542 (0.65)		0.096 (0.11)	
ER status (fmol/mg protein)[e]	203		(0.055)		<0.0001
Negative (<10)	65	0.582 (0.76)		0.049 (0.09)	
Positive (≥10)	138	0.496 (0.52)		0.114 (0.15)	
PgR status (fmol/mg protein)[e]	203		n.s.		0.0025
Negative (<10)	80	0.555 (0.66)		0.056 (0.01)	
Positive (≥10)	123	0.510 (0.51)		0.115 (0.15)	

[a] Total $n = 205$; because of missing values, numbers do not always add up to 205; n.s. not significant.
[b] Ratio uPAR-del4/5/β-actin, zmol/amol (inter-quartile range).
[c] Ratio TIMP-3/β-actin, amol/amol (inter-quartile range).
[d] Kruskal–Wallis test.
[e] Mann–Whitney test.

Table 2
Cox univariate analyses of disease-free survival in breast cancer patients

Factor	Number of patients[a]	Event frequency	Univariate analysis	
			HR (95%CI)[b]	*P*
Total number	205	84		
Menopausal status			1.14 (0.68–1.90)	n.s.
pre *vs.*	42	19		
post	163	65		
Tumour stage (pT)				
pT2/3/4 *vs.* pTl	139/63	61/23	1.40 (0.87–2.26)	n.s.
PT3 + pT4 *vs.* pTl + pT2	25/177	13/71	1.43 (0.79-2.59)	n.s.
Lymph node status			1.95 (1.21–3.14)	0.0061
Positive *vs.*	88	46		
Negative	90	27		
Grading (Bloom–Richardson)			0.71 (0.43–1.15)	n.s.
II *vs.*	79	31		
III	67	33		
ER status (fmol/mg protein)			0.94 (0.59–1.49)	n.s.
Positive ($\geqslant$10) *vs.*	138	56		
Negative (<10)	65	27		
PgR status (fmol/mg protein)			0.95 (0.61–1.48)	n.s.
Positive ($\geqslant$10) *vs.*	123	50		
Negative (<10)	80	33		
UPAR-wt mRNA[c]			0.89 (0.58–1.37)	n.s.
$\geqslant$0.284 *vs.*	102	42		
<0.284	103	42		
UPAR-del5 mRNA[d]			1.05 (0.68–1.61)	n.s.
$\geqslant$1.70 *vs.*	97	43		
<1.70	108	41		
UPAR-del4/5 mRNA[d]			1.59 (1.03–2.46)	0.0363
$\geqslant$0.540 *vs.*	97	49		
<0.540	108	35		
TIMP-3 mRNA[c]			1.89 (1.21–2.96)	0.0049
<0.105 *vs.*	112	52		
$\geqslant$0.105	93	32		

[a] Total $n = 205$; because of missing values, numbers do not always add up to 205.
[b] HR: hazard ratio (95% confidence interval) of univariate Cox analysis; n.s. not significant.
[c] amol mRNA/amol β-actin mRNA.
[d] zmol mRNA/amol β-actin mRNA.

had a significantly higher risk of relapse or death ($P = 0.0363$, HR = 1.59, 95%CI = 1.03–2.46). Furthermore, low TIMP-3 values were significantly associated with shorter DFS in breast cancer patients ($P = 0.0049$, HR = 1.89, 95%CI = 1.21–2.96) as determined by Cox regression analysis. These findings were confirmed by Kaplan–Meier estimation and the respective survival curves (Fig. 1, the given *P*-values were determined by log-rank test). Using uPAR variant or TIMP-3 mRNA expression levels as continuous or log-transformed, continuous factors, respectively, we found no significant association with prognosis in univariate Cox regression analysis including all patients (data not shown).

Subsequently, subgroup analyses were performed for the uPAR variants and TIMP-3 by Kaplan–Meier estimation. In the patient group with low TIMP-3 mRNA levels (<0.105 amol/amol β-actin), *i.e.*, the patient group with poor prognosis, patients with high uPAR-del4/5 ($\geqslant$0.540 zmol/amol β-actin) had a significantly shorter DFS in comparison to those with low uPAR-del4/5 transcript levels ($P = 0.0085$; Fig. 2A). In contrast, no significant difference between high and low uPAR-del4/5 mRNA values was found in the subgroup with high TIMP-3 expression ($\geqslant$0.105 amol/amol β-actin, Fig. 2B). Neither uPAR-wt nor uPAR-del5 mRNA expression levels were found to be associated with DFS in any of the TIMP-3 subgroups.

Because high uPAR-del4/5 mRNA levels were correlated with poor prognosis in the patient subgroup with low TIMP-3 levels, we next assessed whether a combination of uPAR-del4/5 and TIMP-3 values might add prognostic information for patients survival. In fact, we found two significantly different subgroups of breast cancer patients. The subgroup of patients with the combination of high uPAR-del4/5 ($\geqslant$0.540 zmol/amol β-actin) and low TIMP-3 (<0.105 amol/amol β-actin) values ($n = 44$) had a relatively poor prognosis in comparison to the patient subgroup with low uPAR-del4/5 values combined with high TIMP-3 levels ($n = 40$; $P = 0.0001$, Fig. 3). No significant difference was seen when we compared the subgroup with combined low expression levels of uPAR-del4/5 and TIMP-3 ($n = 68$)

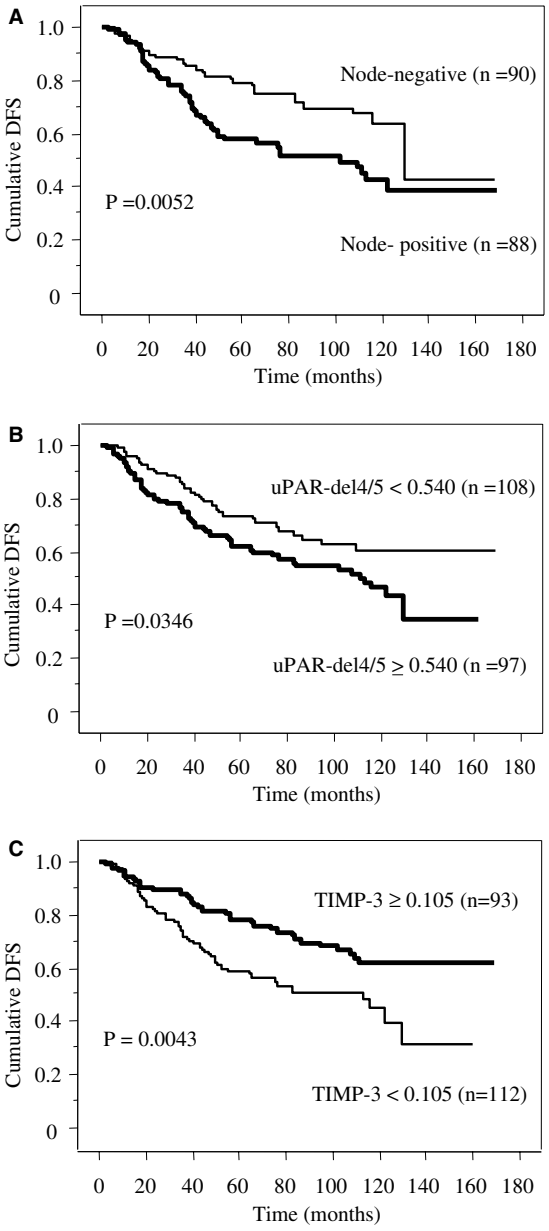

Fig. 1. Disease-free survival of breast cancer patients predicted by: (A) lymph-node status, (B) uPAR-del4/5 and (C) TIMP-3 mRNA expression. Univariate Kaplan–Meier survival analysis reveals that breast cancer patients with high uPAR-del4/5 (B) and low TIMP-3 (C) mRNA levels, respectively, have an increased risk for relapse or death (cumulative DFS; the given *P*-values were determined by log-rank test).

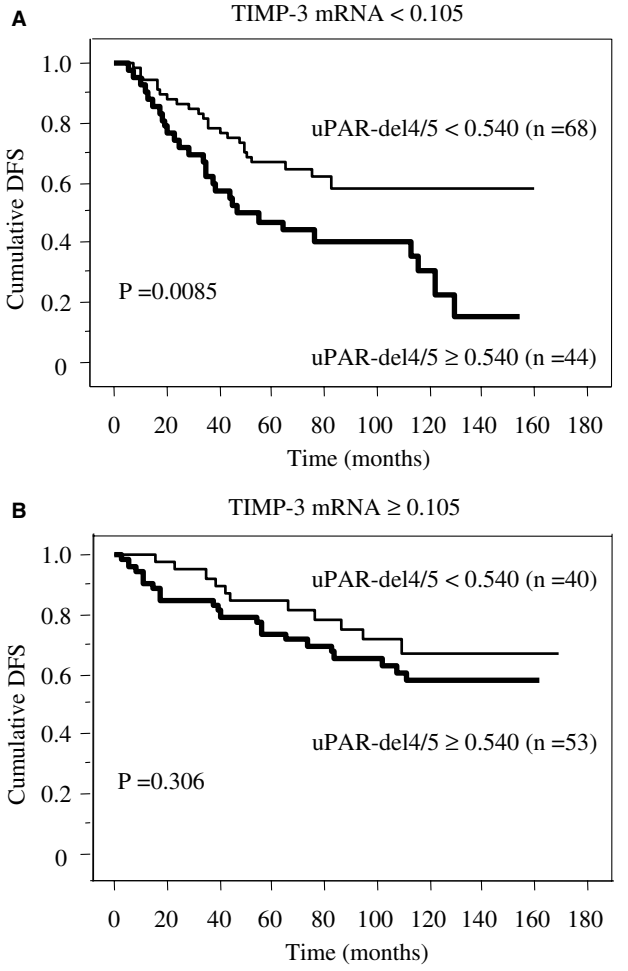

Fig. 2. Analysis of uPAR-del4/5 mRNA expression in breast cancer patients divided into subgroups with: (A) low or (B) high expression levels of TIMP-3 mRNA. In the patient group with low TIMP-3 mRNA levels (*i.e.*, the patient group with the worse prognosis), patients with high uPAR-del4/5 mRNA values have a significantly increased risk for relapse or death (cumulative DFS; the given *P*-values were determined by log-rank test).

with the patient subgroup with combined high expression levels of uPAR-del4/5 and TIMP-3 ($n = 53$; see Fig. 2A, upper line compared to Fig. 2B, lower line).

3.2.1. Multivariate analysis of TIMP-3 and uPAR variant mRNA expression in breast cancer patients

The independent relationship of TIMP-3 and the uPAR variants with DFS was studied with Cox multivariate regression analysis. In the analysed patient group including only patients with the complete dataset of clinicopathological and tumour biological parameters ($n = 176$), the mRNA values of uPAR-del4/5 and TIMP-3 remained highly significant. So, high uPAR-del4/5 levels ($P = 0.0001$, HR = 3.21, 95%CI = 1.76–5.86) and low TIMP-3 levels ($P = 0.0029$, HR = 2.29, 95%CI = 1.33–3.94) contributed independent prognostic information when adjusted for prognostically relevant clinicopathological parameters (Table 3). Surprisingly, in multivariate analysis, mRNA expression levels of uPAR-wt reached significance ($P = 0.028$). Here, low uPAR-wt levels correlated with shorter DFS. Similar results were obtained by analysis of the patient group with available histopathological grading ($n = 146$, data not shown). In the subgroup of breast cancer patients with combined uPAR-del4/5 and TIMP-3 values the combination of high uPAR-del4/5

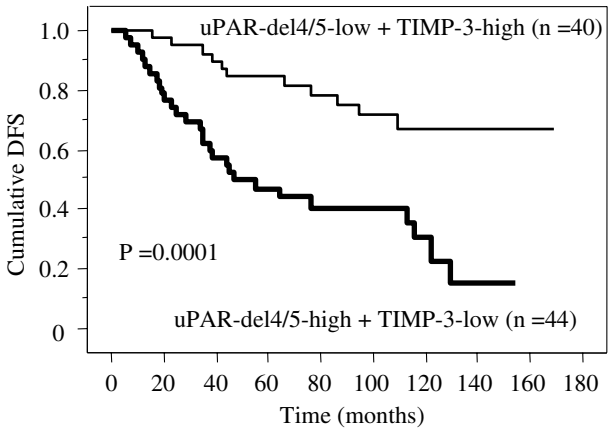

Fig. 3. Disease-free survival of breast cancer patients predicted by the combination of TIMP-3 and uPAR-del4/5 mRNA values. A subgroup with a very poor prognosis (uPAR-del4/5-high and TIMP-3-low) and a subgroup with good prognosis (uPAR-del4/5-low and TIMP-3-high) were identified (cumulative DFS; the given P-values were determined by log-rank test). Cut-off values for uPAR and TIMP-3 mRNA levels were 0.540 zmol/amol β-actin and 0.105 amol/amol β-actin, respectively.

($\geqslant$0.540 zmol/amol β-actin) and low TIMP-3 (<0.105 amol/amol β-actin) mRNA expression levels remained highly significant ($P < 0.0001$) when adjusted for clinicopathological factors with a hazard ratio (HR) of 6.16 (95%CI = 2.70–14.1), *i.e.*, patients with "uPAR-del4/5-high/TIMP-3-low" mRNA levels have a more than 6-fold higher risk for relapse or death in comparison to those with "uPAR-del4/5-low/TIMP-3-high" mRNA values (Table 3).

4. Discussion

In various types of cancer, expression of components of the PA system such as uPA and/or PAI-1 has been shown to be associated with DFS of the patients. Generally, high tumour tissue levels of uPA and/or PAI-1 protein are associated with tumour aggressiveness and poor patient outcome, not only in breast cancer but also in a variety of other malignancies such as ovarian, gastric, colorectal or lung cancer [22]. In breast cancer, both factors of the PA system were the first novel tumour biological factors to be validated at the highest level of evidence regarding their clinical utility [12]. On the other hand, the impact of uPAR protein on prognosis is more complex, and a matter of debate [13]. Different results on the prognostic relevance of uPAR protein were obtained in tumour tissue extracts using different ELISAs or by immunohistochemistry [13,23–25]. These discrepancies may be attributable to the various variants of uPAR which are present in tumour tissue, *e.g.*, cleaved uPAR forms such as soluble uPAR, uPAR-DII+III and/or uPAR-DI [26]. Moreover, alternatively spliced

Table 3
Cox multivariate analyses of disease-free survival in breast cancer patients ($n = 176$)

Factor	Multivariate analysis		Multivariate analysis[a]	
	HR (95%CI)[b]	*P*	HR (95%CI)[b]	*P*
Menopausal status	1.05 (0.59–1.88)	n.s.	1.01 (0.57–1.80)	n.s.
Pre *vs.* post				
Tumour stage (pT)				
pT2/3/4 *vs.* pTl	1.32 (0.75–2.31)	n.s.	1.25 (0.72–2.18)	n.s.
PT3 + pT4 *vs.* pTl + pT2	1.28 (0.65–2.54)	n.s.	1.29 (0.65–2.54)	n.s.
Lymph node status	1.79 (1.09–2.94)	0.0220	1.76 (1.06–2.89)	0.0274
Positive *vs.* negative				
ER status (fmol/mg protein)	1.26 (0.60–2.65)	n.s.	1.41 (0.66–2.97)	n.s.
Positive ($\geqslant$10) *vs.* negative (<10)				
PgR status (fmol/mg protein)	0.94 (0.47–1.88)	n.s.	0.93 (0.46–1.87)	n.s.
Positive ($\geqslant$10) *vs.* negative (<10)				
UPAR-wt mRNA[c]	0.49 (0.26–0.93)	0.0280	0.55 (0.31–0.97)	0.0375
$\geqslant$0.284 *vs.* <0.284				
UPAR-del5 mRNA[d]	0.87 (0.49–1.52)	n.s.	0.83 (0.47–1.47)	n.s.
$\geqslant$1.70 *vs.* <1.70				
UPAR-del4/5 mRNA[d]	3.21 (1.76–5.86)	0.0001	–	
$\geqslant$0.540 *vs.* <0.540				
TIMP-3 mRNA[c]	2.29 (1.33–3.94)	0.0029	–	
<0.105 *vs.* $\geqslant$0.105				
UPAR- del4/5/TIMP-3				
<0.540/$\geqslant$0.105 *vs.* $\geqslant$0.540/<0.105	–		6.16 (2.70–14.1)	<0.0001
<0.540/$\geqslant$0.105 *vs.* $\geqslant$0.540/$\geqslant$0.105 + <0.540/<0.105			1.78 (0.87–3.66)	n.s.

[a] Analysis with combined uPAR-del4/5 and TIMP-3 values.
[b] HR: hazard ratio (95% confidence interval) of multivariate analysis; n.s. not significant.
[c] amol mRNA/amol β-actin mRNA.
[d] zmol mRNA/amol β-actin mRNA.

forms of uPAR mRNA lacking either exon 5 (uPAR-del5), exon 4 and 5 (uPAR-del4/5) or exchanging exon 7 for another final exon have been described [16,27,28]. In fact, in a cohort of 43 breast cancer patients we found that expression of uPAR-del4/5 mRNA but not wild-type uPAR mRNA was significantly associated with DFS in breast cancer [16].

In the present study, the mRNA expression profile for the splice variants uPAR-del4/5 and uPAR-del5 as well as for uPAR-wt was analysed in an independent representative patient cohort encompassing 205 breast cancer tissues. Continuous TIMP-3 and uPAR variant mRNA values were converted into categorical variables by optimal dichotomisation. Even though deriving optimal cut-off values may be associated with a considerable type I error rate [29] categorical variables were applied for all survival analyses. Using this type of analysis we could show that high levels of uPAR-del4/5 mRNA in tumour tissue are significantly associated with a poor prognosis. These results confirm our initial report obtained in a smaller cohort of patients [16]. Furthermore, in multivariate regression analysis uPAR-del4/5 contributed independent prognostic information when adjusted for clinicopathological parameters. The mRNA expression levels of uPAR-wt and its splice variant uPAR-del5 had no significant impact on prognosis. The uPAR-del4/5 transcript variant is expressed frequently but at rather low levels in breast cancer tissue. In cell culture experiments, we demonstrated that recombinant expression of this variant in hamster and human cells leads to synthesis, secretion and insertion into the cell membrane of the uPAR-del4/5 protein (16; unpublished results). uPAR-del4/5 corresponds to an uPAR form, in which one of the three homologous domains (DII) of uPAR protein is missing. It is tempting to speculate that the specific deletion of a complete domain alters one or more functions of uPAR protein (or the regulation thereof), *e.g.*, interaction with its ligands such as uPA, vitronectin, integrins, and chemotaxis (fMLP) receptors [14,26,30].

Inversely to the uPAR-del4/5 data, high transcript levels of TIMP-3 in tumour tissue were significantly related to an improved DFS in these patients. This is in accordance with the observation that overexpression of TIMP-3 in tumour cells antagonises primary tumour growth and invasion, and induces tumour cell apoptosis [20]. Since TIMP-3 mRNA levels are significantly associated with outcome of endocrine tamoxifen therapy in breast cancer, TIMP-3 may be causatively involved in treatment success through sensitisation or mediation of p53-induced apoptosis, which is important in breast cancer adjuvant and endocrine therapy [21]. In subgroup analyses, no difference between high and low uPAR-del4/5 mRNA values was found in the patient group with high TIMP-3 mRNA expression. However, in the patient group with low TIMP-3 mRNA levels, *i.e.*, the group which does not respond well to tamoxifen-based endocrine therapy, high values of uPAR-del4/5 do define a group with an even worse prognosis. This may indicate that uPAR-del4/5 mRNA expression is not directly associated with treatment success, a finding that is supported by the fact that TIMP-3 and uPAR-del4/5 are independent markers in multivariate analysis. The combination of the prognostic value of TIMP-3 and uPAR-del4/5, which represent components of complementary protease systems, may improve prediction of disease recurrence in breast cancer.

Prognostication of breast cancer patients is particularly useful for selecting patients for specific adjuvant treatments. For this purpose, it can be questioned whether a single gene has so much impact on disease progression that it can discriminate certain patient groups in a heterogeneous disease such as breast cancer. Therefore, much effort has been directed lately in defining breast cancer patients with a poor or good prognosis based on gene expression profiles [31,32]. These studies use selection methods whereby information on expression values of several tens of thousands of genes are reduced to 70–76 most informative genes. These studies, however, may be prone to false-positives [33] due to the high number of genes considered and should therefore be considered with caution before independent validation studies are performed. Alternatively, our study presented here, shows that the prognostic information of two, preferably unrelated, genes can be combined to gather information that can outperform current biomarkers [34].

In conclusion, we report here the independent but additive prognostic relevance of expression levels of uPAR-del4/5 and TIMP-3 in early breast cancer. By the approach of combining prognostic information from only two genes, patient groups with substantial differing disease outcomes were selected. Validation of the prognostic impact of the combined use of these factors in an independent group of patients is needed however, before considering our findings for routine clinical use.

Conflict of interest statement

None declared.

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