Breast Cancer Res Treat (2008) 111:229–240
DOI 10.1007/s10549-007-9782-6

PRECLINICAL STUDY

Urokinase receptor splice variant uPAR-del4/5-associated gene expression in breast cancer: identification of rab31 as an independent prognostic factor

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Received: 2 October 2007 / Accepted: 3 October 2007 / Published online: 20 October 2007
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Abstract
Purpose To evaluate the pure prognostic impact of the uPA-receptor splice variant uPAR-del4/5 for lymph node-negative breast cancer patients, and to identify differentially expressed genes associated with high or low uPAR-del4/5 mRNA levels.
Patients and methods mRNA transcript levels were measured by real-time PCR in tumor samples from 280 node-negative breast cancer patients who had not received adjuvant systemic therapy. Endpoints were distant metastasis-free survival (DMFS) and overall survival (OS). Gene expression analysis was performed with RNA isolated from breast cancer tissue and breast cancer cell lines using Affymetrix U133a GeneChips.
Results In multivariate analysis, uPAR-del4/5 significantly contributed to the base model of traditional prognostic factors for DMFS (HR = 3.29, $P < 0.001$) and OS (HR = 2.87, $P = 0.002$). Using microarrays, seven genes were found to be up-regulated in tumor samples and cancer cell lines with high uPAR-del4/5 mRNA expression. The gene encoding rab31, a member of the Ras oncogene family, was selected for quantitative analysis of mRNA expression in the set of 280 patients. High rab31 values were significantly associated with worse outcome of patients for DMFS (HR = 2.27, $P < 0.001$) and OS (HR = 2.01, $P = 0.008$) in multivariate analysis, independent from uPAR-del4/5. The patient subgroup with high uPAR-del4/5 and rab31 levels showed the worst DMFS and OS ($P < 0.001$, both) compared with tumors with low values of both factors.
Conclusions Our results suggest that uPAR-del4/5 and rab31 mRNA represent independent prognostic markers in breast cancer and may be components of different, but possibly associated, tumor-relevant signaling pathways.

Keywords Breast cancer · Prognosis · Quantitative PCR · rab31 · uPAR-del4/5

Electronic supplementary material The online version of this article (doi:10.1007/s10549-007-9782-6) contains supplementary material, which is available to authorized users.

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Introduction

The invasive behavior of tumor cells and their ability to form distant metastases are facilitated by cell-associated proteolytic systems which are able to degrade components of the extracellular matrix. One important part of this pericellular network of interacting proteolytic systems is the urokinase-type plasminogen activator (uPA) system [1, 2]. The main components of the uPA system are the serine protease uPA, its receptor uPAR (CD87), and its inhibitor

PAI-1. uPAR has been found to be a key molecule for pericellular proteolysis as it focuses the proteolytic activity of uPA to the cell surface. In addition, uPAR interacts with proteins such as vitronectin or integrins and by this triggers intracellular signaling events finally leading to induction of cell proliferation, adhesion, and migration [3–5]. High levels of uPA and/or PAI-1 in tumor tissues are strongly related to poor prognosis in a variety of cancer types including breast cancer [6, 7]. High levels of uPAR in breast cancer are also associated with adverse outcome. The prognostic impact of uPAR antigen determined in tumor tissue extracts, however, is less powerful than that of either uPA or PAI-1 [8–12].

uPAR is a glycosylated, glycan lipid-anchored membrane protein that consists of three structurally homologous domains (DI, DII, DIII) [13, 14]. Various molecular forms of uPAR antigen such as soluble uPAR, uPAR-DII+DIII, and liberated DI were found in tumor cell lines, experimental tumors and body fluids [15]. Furthermore, several splice variants of human uPAR have been described, including an uPAR mRNA splice variant lacking exons 4 and 5 (uPAR-del4/5) which encodes an uPAR form in which DII of uPAR is missing [16]. High uPAR-del4/5 mRNA levels in breast tumor tissues have shown to be associated with shorter disease-free survival of breast cancer patients [16, 17]. However, these studies included node-negative and node-postive patients, of whom many were treated with adjuvant systemic therapy. Therefore, the pure prognostic value could not be assessed due to possible confounding effects of treatment.

In the present study, we analyzed the mRNA expression patterns of uPAR-del4/5 as well as wild-type uPAR (uPAR-wt) in a cohort of 280 tumor tissues from lymph node-negative (LNN) breast cancer patients who did not receive adjuvant systemic therapy. Furthermore, we identified several differentially expressed genes associated with high or low uPAR-del4/5 mRNA levels, and identified rab31 as a new prognostic factor for patients with breast cancer.

Materials and methods

Study population

This study was performed on 280 available tumor tissue RNA samples of the 286 that were used previously to identify gene expression profiles which predict distant metastasis of LNN primary breast cancer [18]. The study was approved by the institutional medical ethics committee (number 02·953), and carried out in accordance with the Code of Conduct of the Federation of Medical Scientific Societies in The Netherlands (http://www.fmwv.nl/). All patients underwent primary surgery between 1980 and 1995, and none of the patients had received adjuvant systemic therapy. The median age of the patients at surgery was 52 years (range 26-83 years). A group of 214 patients (76%) had undergone breast-conserving surgery and 66 (24%) modified radical mastectomy. Assessment of estrogen receptor (ER) and progesterone receptor (PgR) status, post-operative follow-up and defining the date of diagnosis of metastasis was as described [18]. The median follow-up time of patients alive was 102 months (20–202 months) with 103 events in the analysis of distant metastasis-free survival (DMFS) and 84 events in the analysis of overall survival (OS).

Cell culture

Human breast cancer cell lines BT549 and MCF-7 were purchased from ATCC (Rockville, MD) and cultured as described [16]. Cells were collected and pelleted by centrifugation at 200 $\times g$ for 10 min at room temperature. Cell pellets were stored frozen at –80°C until use in microarray analyses.

Quantification of gene expression by real-time PCR

Tissue processing, isolation of total RNA, cDNA synthesis, and evaluation of the quantity and quality of the isolated RNA were done as described [18, 19]. For quantification of uPAR-del4/5 and uPAR-wt mRNA, quantitative PCR (QPCR) assays were used as described [16].

Rab31 mRNA expression was quantified by a ready-to-go QPCR assay of TaqMan MGB (HS_00199313, Assay-on-Demand; Applied Biosystems, Darmstadt, Germany) using the protocol recommended by the manufacturer. The cDNA standards (purified PCR products) for the rab31 QPCR were generated by conventional PCR with cDNA samples from breast cancer cell lines MCF-7 and BT549. The PCR products were separated by agarose gel electrophoresis, and the specific PCR fragment was eluated and purified from the agarose gel by standard procedures (Invisorb Spin DNA Extraction kit; Invitek, Berlin, Germany). The mRNA copy number of rab31 was calculated in relation to five-log-range calibration curves of the external DNA standards (10^1–10^6 molecules).

cDNA samples were quantified at least in duplicate in independent PCR runs for the appropriate marker transcripts. The mean values of all measurements were used for further calculations. The levels of specific mRNAs were evaluated relative to the average expression levels of the medium abundance housekeeping gene hypoxanthine guanine phosphoribosyltransferase (HPRT) as described previously [19]. Relative mRNA expression ratios were used for all further calculations and statistical analyses.

Sample preparation for microarray hybridization

For the first set of tumor samples, total RNA was isolated from 50–80 mg of cryo-preserved tissue derived from eight breast cancer patients as described [16]. Total RNA from breast cancer cell lines was prepared from pellets of $20–25 \times 10^6$ cells using the RNeasy Midi Kit (Qiagen, Hilden, Germany). Biotinylated targets were prepared according to the Affymetrix Eukaryotic Hybridization protocol (Affymetrix, Santa Clara, CA) and hybridized to Affymetrix oligonucleotide microarray U133a GeneChips. Arrays were stained and scanned by standard Affymetrix protocols. Image analysis was performed by use of Affymetrix GeneChip analysis software MAS 5.0. The arrays were scaled to a target fluorescence of 500 (scaling factors 3.4–8.7). Microarray analyses of the second set of tumor samples of 280 LNN breast cancer patients were performed as described [18].

Microarray data analysis and comparison strategies

The analysis of uPAR-del4/5-dependent gene expression in tumor samples of the first set of breast cancer patients and in breast cancer cell lines was performed using GeneSpring 6.1 (Silicon Genetics, Redwood City, CA). The "Cross gene error model" was active and gene expression data were normalized to the 50th percentile of all values on that chip (per chip method) and against the median of the control samples (per gene method). To identify genes differentially expressed between groups with high or low uPAR-del4/5 mRNA levels all measurements were filtered using GeneSpring 6.1. The magnitude of difference between groups was defined using the following criterias: (i) greater than twofold difference, (ii) classification as "present" in at least half of the samples based on the Affymetrix algorithm, (iii) raw data were greater than 16 to eliminate changes within background noise. Of the second set of 280 tumors, the top 20 and the bottom 20 samples expressing uPAR-del4/5 mRNA according to the QPCR data were taken for input in a significance analysis of microarrays (SAM) test [20], which identifies differentially expressed genes. Three hundred permutations were used to calculate a false discovery rate. Genes were considered significant when the false discovery rate was less than 5% and when a minimum of twofold difference in expression levels was observed.

Statistical analysis

Differences in expression levels were assessed with the Mann-Whitney U test or Kruskal-Wallis test using patient and tumor characteristics as grouping variables [19]. The strengths of the associations between continuous variables were tested with the Spearman rank correlation (r_s). The Cox proportional hazard regression model was used to calculate the hazard ratio (HR) and its 95% confidence interval (CI) in the analysis of DMFS and OS. The associated likelihood ratio test was used to test for differences between models with variables included and excluded. For variables which showed as a continuous variable a significant correlation with DMFS, we used isotonic regression analysis [21, 22] to search for optimal cut points with criterion maximum Chi-square of a generalized logrank statistic in a Cox regression model. With isotonic regression analysis, the hazard rate for failure is estimated under the assumption of a monotone increasing failure rate with increasing, or decreasing, levels of the factor. Survival curves were generated using the method of Kaplan and Meier, and the logrank test was used to test for differences. All P values are two-sided and $P < 0.05$ was considered statistically significant. Computations were done with the use of STATA statistical package, release 9.0 (STATA Corp., College Station, TX).

Results

Identification of differentially expressed genes in samples with high versus low uPAR-del4/5 mRNA values

First, we performed genome-wide transcript expression analyses of eight primary breast cancer samples with high ($n = 4$) or low ($n = 4$) uPAR-del4/5 expression selected from a previously used cohort [16]. In addition, we generated a gene expression profile of breast cancer cell lines BT547 and MCF-7 expressing high and low mRNA levels of uPAR-del4/5, respectively [16]. According to the criteria in the Methods section, 17 genes were up- and three down-regulated more than twofold in both breast cancer samples and cell lines with high uPAR-del4/5 mRNA expression (See Supplemental Data Table 1). Next, we analyzed a second patient cohort consisting of 280 tumors of LNN breast cancer patients [18] for differentially expressed genes in the groups of patients with high versus low expression of QPCR-assessed uPAR-del4/5 mRNA values. Using SAM analysis of the top 20 and the bottom 20 cases, we found 319 genes that were up-regulated, as well as two genes that were down-regulated, by a factor of 2 (See Supplemental Data Table 2). Only seven genes were found to overlap, all of them being overexpressed in both groups: dermatopontin, cadherin-11, homeo box B6, TIMP-3, tropomyosin-1, olfactomedin-like protein, and rab31 (See Supplemental Data Table 3). Rab31 was selected first for

Table 1 Clinicopathologic characteristics and its association with mRNA levels of biological factors ($n = 280$)

Clinicopathologic parameters	No. patients (%)	Median mRNA levels (and interquartile range)[a]		
		uPAR-wt	uPAR-del4/5	rab31
Total	280	2.58 (3.18)	0.044 (0.63)	0.74 (1.33)
Age categories[b] (years)		$P = 0.394$	$P = 0.607$	$P = 0.027$
<40	35 (13)	2.32 (2.90)	0.033 (0.050)	0.72 (1.10)
41–55	127 (45)	2.54 (3.44)	0.045 (0.064)	0.71 (1.29)
56–70	88 (31)	3.09 (3.10)	0.047 (0.065)	1.10 (1.78)
>70	30 (11)	2.44 (2.33)	0.045 (0.045)	0.56 (1.08)
Menopausal status[c]		$P = 0.713$	$P = 0.629$	$P = 0.090$
Premenopausal	137 (49)	2.47 (3.14)	0.040 (0.062)	0.70 (1.07)
Postmenopausal	143 (51)	2.72 (3.19)	0.046 (0.063)	0.90 (1.61)
Tumor size[c]		$P = 0.558$	$P = 0.695$	$P = 0.059$
≤2cm	142 (51)	2.76 (3.09)	0.045 (0.056)	0.86 (1.45)
>2cm	138 (49)	2.44 (3.25)	0.043 (0.065)	0.65 (1.17)
Tumor grade[b]		$P = 0.490$	$P = 0.501$	$P = 0.915$
Poor	145 (52)	2.73 (3.49)	0.044 (0.071)	0.72 (1.29)
Unknown	86 (31)	2.93 (3.12)	0.049 (0.062)	0.80 (1.15)
Good/moderate	49 (17)	2.32 (2.29)	0.037 (0.041)	0.69 (1.39)
ER status[c]		$P < 0.001$	$P < 0.001$	$P = 0.478$
Negative	72 (26)	4.74 (6.27)	0.087 (0.108)	0.78 (1.51)
Positive	208 (74)	2.26 (2.69)	0.037 (0.045)	0.72 (1.27)
PR status[b]		$P < 0.001$	$P < 0.001$	$P = 0.123$
Negative	106 (38)	3.63 (4.89)	0.065 (0.100)	0.58 (1.35)
Positive	162 (58)	2.29 (2.72)	0.039 (0.045)	0.86 (1.34)
Unknown	12 (4)	1.79 (1.82)	0.034 (0.021)	0.53 (0.89)

[a] Relative expression ratio mRNA/HPRT mRNA

[b] P for Kruskal-Wallis test

[c] P for Mann-Whitney U test

further analysis since another member of the Rab gene family, rab25, was recently shown to be associated with invasive growth in ovarian and breast cancer [23].

Rab31 mRNA expression in tumor samples with up-regulated uPAR-del4/5

Using QPCR, in the eight analyzed samples of the first set [16] with relatively high uPAR-del4/5 mRNA values, higher rab31 mRNA levels were detected as well ($r_s = 0.733$, $P = 0.028$). Also, the mRNA expression levels of rab31 determined by QPCR in the 280 tumor samples of the second set were significantly higher in the group with the highest uPAR-del4/5 mRNA values ($P < 0.001$, Kruskal-Wallis test; Fig. 1). The Spearman rank correlation between rab31 and uPAR-del4/5 mRNA values was moderate with $r_s = 0.514$ ($P < 0.001$).

Relation of uPAR-del4/5, uPAR-wt, and rab31 mRNA expression to patient and tumor characteristics

The normalized QPCR mRNA values of uPAR-del4/5, uPAR-wt and rab31 ranged from 0.001 to 0.77 (median 0.044), from 0.17 to 39.1 (median 2.58), and from 0.003 to 15.0 (median 0.74), respectively. The distribution pattern of mRNA values of all three factors appeared to be log-normal. The associations of patient and tumor characteristics with the expression of uPAR-del4/5, uPAR-wt and rab31 are summarized in Table 1. Significantly lower transcript levels of uPAR-del4/5 and uPAR-wt mRNA were found in ER-positive ($P < 0.001$) and PgR-positive ($P < 0.001$) tumors compared with ER- or PgR-negative tumors. Apart from that, the mRNA levels of uPAR-del4/5, uPAR-wt and rab31 did not differ significantly between tumors in relation to clinicopathologic factors, except for a significant relation of rab31 expression with age.

Association of uPAR-del4/5, uPAR-wt, and rab31 mRNA values with DMFS and OS: Univariate analysis

In Cox univariate regression analysis using log-transformed continuous variables, uPAR-del4/5, uPAR-wt, and rab31 were significantly related with a poor DMFS ($P = 0.049$, $P = 0.012$ and $P = 0.044$, respectively). This

Table 2 Cox univariate regression analysis for distant metastasis-free survival in breast cancer patients (n = 280)

Factor	No. patients	HR (95%CI)[a]	P
Age categories (years)			
<40	35	1	
41–55	127	0.83 (0.47–1.46)	0.512
56–70	88	0.78 (0.43–1.42)	0.419
>70	30	0.37 (0.14–0.95)	0.038
Menopausal status			
Premenopausal	137	1	
Postmenopausal	143	0.99 (0.67–1.46)	0.967
Tumor size			
≤2cm	142	1	
>2cm	138	1.09 (0.74–1.60)	0.678
Tumor grade			
Poor	145	1	
Unknown	86	0.91 (0.59–1.39)	0.665
Good/moderate	49	0.42 (0.22–0.82)	0.012
ER status			
Negative	72	1	
Positive	208	0.98 (0.63–1.54)	0.945
PgR status			
Negative	106	1	
Positive	162	0.67 (0.45–0.99)	0.046
Missing	12	0.47 (0.15–1.50)	0.202
uPAR-del4/5 mRNA[b]			
Low	242	1	
High	38	2.33 (1.44–3.77)	<0.001
uPAR-wt mRNA[b]			
Low	210	1	
High	70	1.93 (1.28–2.91)	0.002
rab31 mRNA[b]			
Low	223	1	
High	57	2.08 (1.37–3.16)	0.001
Combination-del4/5-rab31[b]			
Low/low	205	1	
Low/high	37	1.90 (1.15–3.15)	0.012
High/low	18	2.17 (1.08–4.37)	0.03
High/high	20	3.14 (1.69–5.84)	<0.001

[a] HR: hazard ratio (95% confidence interval) of univariate Cox regression analysis

[b] Relative expression ratio mRNA/HPRT mRNA dichotomized in high and low levels by cut points (0.126 for uPAR-del4/5, 4.51 for uPAR-wt and 1.96 for rab31)

justified a search for cut points to be able to analyze and to visualize these factors as categorical variables in the survival analyses. The relative expression ratios of 0.126 for uPAR-del4/5, 4.51 for uPAR-wt and 1.96 for rab31, respectively, were chosen to classify tumors as high and low. High levels of uPAR-del4/5, uPAR-wt and rab31 mRNA were strongly associated with an unfavorable DMFS (HRs were 2.33, 1.93 and 2.08, respectively; Table 2). Similarly, a high level of uPAR-del4/5 ($P < 0.001$), uPAR-wt ($P < 0.001$), and rab31 ($P = 0.003$), was associated with a poor OS with HRs of 2.74 (95%CI = 1.65–4.53), 2.38 (95%CI = 1.53–3.69), and 2.02 (95%CI = 1.28–3.20), respectively. The associations of uPAR-del4/5, uPAR-wt and rab31 mRNA levels with DMFS are visualized by Kaplan-Meier curves in Fig. 2. Except for tumor grade and PgR status, none of the traditional prognostic factors, age, menopausal status, tumor size, or ER status, was significantly associated with the length of DMFS (Table 2). This is in agreement with other studies showing that clinical parameters such as tumor size are not very strong prognostic factors in LNN breast cancer patients who had not received adjuvant systemic therapy [24, 25]. Similarly, except for PgR status, we observed no significant association of the clinicopathologic factors with OS (data not shown). In exploratory subgroup analyses, high expression levels of uPAR-del4/5, uPAR-wt, and rab31 were significantly related with a poor DMFS (and a poor OS, data not shown) in ER-negative and in premenopausal patients (Table 3). On the contrary, in ER-positive and postmenopausal patients, only for rab31 and uPAR-wt significant associations with a poor DMFS were observed. With respect to tumors ≤2 cm and >2 cm, no large differences were observed regarding the prognostic strength of any of the three factors in the analysis of DMFS, except for uPAR-wt which was not significant in patients with tumors >2 cm (Table 3). Still, we have performed an additional analysis in a subgroup of patients who were at lower risk according to consensus guidelines from the 2005 St.Gallen Consensus Conference. Since data on tumor grade were not available for all patients included in the study, tumor size (≤2 cm) and age (>35 years) of patients were combined to define a modified low risk group (n = 138). In this group high mRNA levels of uPAR-del4/5 ($P = 0.011$) and rab31 ($P = 0.005$) were significantly related with a shorter DMFS with HRs of 2.47 (95%CI = 1.23–4.96) and 2.25 (95%CI = 1.27–3.99). The association of uPAR-del4/5 and rab31 mRNA levels with DMFS visualized by Kaplan-Meier curves are shown in Supplemental Data Fig. 1. More than 50 % of patients with high mRNA expression levels of uPAR-del4/5 and rab31, respectively, in tumor tissue had a relapse, which indicates a subgroup of "low risk" patients, who may have a profit from adjuvant systemic chemotherapy.

Table 3 Cox univariate regression analysis of distant metatasis-free survival in subgroups of breast cancer patients (n = 280). (**A**) ER-negative/ER-positive patients; (**B**) pre-/postmenopausal patients; (**C**) patients with tumors ≤ 2 cm/ > 2 cm

A						
Factor	ER-negative			ER-positive		
	No. patients	HR (95%CI)[a]	*P*	No. patients	HR (95%CI)[a]	*P*
uPAR-wt mRNA[b]						
Low	35	1		175	1	
High	37	3.52 (1.40–8.84)	0.007	33	1.65 (0.96–2.87)	0.072
uPAR- del4/5 mRNA[b]						
Low	44	1		198	1	
High	28	3.60 (1.58–8.18)	0.002	10	1.94 (0.78–4.81)	0.151
rab31 mRNA[b]						
Low	54	1		169	1	
High	18	2.66 (1.20–5.87)	0.016	39	1.83 (1.11–3.02)	0.018
B						
Factor	Premenopausal			Postmenopausal		
	No. patients	HR (95%CI)[a]	*P*	No. patients	HR (95%CI)[a]	*P*
uPAR-wt mRNA[b]						
Low	103	1		107	1	
High	34	1.94 (1.08–3.48)	0.027	36	1.92 (1.09–3.40)	0.024
uPAR- del4/5 mRNA[b]						
Low	119	1		123	1	
High	18	2.80 (1.43–5.47)	0.003	20	1.95 (0.98–3.88)	0.058
rab31 mRNA[b]						
Low	116	1		107	1	
High	21	3.50 (1.90–6.44)	<0.001	36	1.46 (0.82–2.59)	0.202
C						
Factor	Tumors ≤ 2cm			Tumors > 2cm		
	No. patients	HR (95%CI)[a]	*P*	No. patients	HR (95%CI)[a]	*P*
uPAR-wt mRNA[b]						
Low	106	1		104	1	
High	36	2.23 (1.27–3.92)	0.008	34	1.71 (0.94–3.08)	0.088
uPAR- del4/5 mRNA[b]						
Low	124	1		118	1	
High	18	2.40 (1.20–4.80)	0.024	20	2.29 (1.17–4.46)	0.026
rab31 mRNA[b]						
Low	108	1		115	1	
High	34	2.17 (1.23–3.81)	0.01	23	2.08 (1.11–3.90)	0.033

[a] HR: hazard ratio (95% confidence interval) of univariate Cox regression analysis

[b] Relative expression ratio mRNA/HPRT mRNA dichotomized in high and low levels by cut points (0.126 for uPAR-del4/5, 4.51 for uPAR-wt, and 1.96 for rab31)

Association of uPAR-del4/5, uPAR-wt, and rab31 mRNA values with DMFS and OS: Multivariate analysis

The independent relationship of uPAR-del4/5, uPAR-wt, and rab31 with DMFS and OS was studied with Cox multivariate regression analysis including age, menopausal status, tumor size, tumor grade, ER and PgR status (base model). uPAR-del4/5, uPAR-wt and rab31 mRNA values significantly contributed to the base model for DMFS when added separately, with HRs for high versus low of 3.29, 1.94 and 2.27, respectively (Table 4). When all three

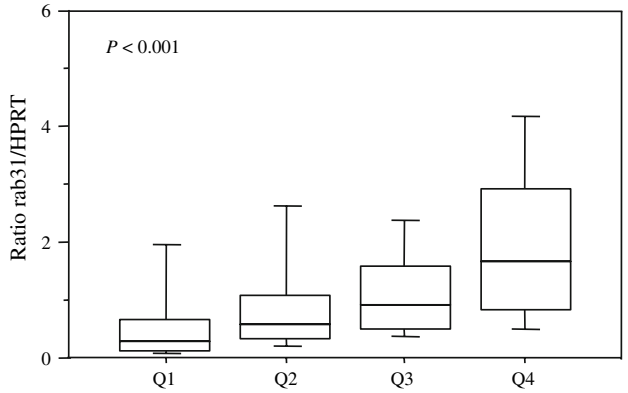

Fig. 1 Box plot of uPAR-del4/5 dependent rab31 mRNA expression. Rab31 mRNA expression is significantly higher in tumor tissue samples with high uPAR-del4/5 mRNA expression ($P < 0.001$, Kruskal-Wallis test; Q1 vs. Q2 $P = 0.357$; Q1 vs. Q3 $P = 0.127$; Q2 vs. Q3 $P = 0.526$; Q1, Q2, Q3 vs. Q4 $P < 0.001$, unpaired T-test). Q1 to Q4 – quartiles of uPAR-del4/5 mRNA values

factors were added simultaneously to the base model, uPAR-wt was no longer significant. The simultaneous addition of uPAR-del4/5 (HR = 2.43, 95%CI = 1.27–4.62, $P = 0.007$) and rab31 (HR = 1.83, 95%CI = 1.12–2.97, $P = 0.015$) resulted in an increase in Chi-square (ΔChi^2) of 18.5 (degrees of freedom, df = 2). The combination of both appeared to be superior and gave a statistically significantly better fit than the addition of either factor alone (the addition of uPAR-del4/5 resulted in a ΔChi^2 of 13.0, df = 1, and the addition of rab31 resulted in a ΔChi^2 of 11.6, df = 1). The analyses for OS showed that high expression levels of uPAR-del4/5 (HR = 2.87, 95%CI = 1.50–5.48, $P = 0.002$), uPAR-wt (HR = 2.08, 95%CI = 1.28–3.37, $P = 0.004$), and rab31 (HR = 2.01, 95%CI = 1.22–3.29, $P = 0.008$) also provided additional prognostic information over the traditional prognostic factors of the multivariate base model. After adding only uPAR-del4/5 a ΔChi^2 of 9.4 (df = 1) was observed. Combining with uPAR-wt or rab31 did not result in a significantly better fit.

Combined analysis of uPAR-del4/5 and rab31 mRNA values for DMFS and OS

We assessed whether a combination of uPAR-del4/5 and rab31 mRNA values might add information for patients' prognosis. In this analysis the patient cohort was divided into four groups based on the combination of high and low uPAR-del4/5 and rab31 mRNA values. The subgroup of patients with high uPAR-del4/5 and high rab31 values showed the worst DMFS and OS with HRs of 3.14 and 3.53, respectively, compared with tumors with low values of both (Table 2, Fig. 3). Patients with tumors having high levels of only one of the factors showed an intermediate prognosis with HRs of approximately 2.0 for DMFS (Table 2). Interestingly, concerning OS, tumors with low uPAR-del4/5 or high uPAR-del4/5 mRNA values showed good or poor prognosis, respectively, independent of rab31 mRNA values (Fig. 3b). In the analysis for DMFS, the mutual differences between the low/high, high/low and high/high groups were not of statistical significance.

Discussion

Compared with other components of the plasminogen activator system such as uPA and PAI-1, the prognostic relevance of uPAR protein in tumor tissue is not as clear [8–12, 26–28]. The main reasons for some of the discrepancies may be the presence of differently cleaved uPAR protein variants in tumor cytosols and detergent-extracted tumor tissue [15], or alternatively spliced forms of uPAR mRNA [16, 29, 30]. Besides that, there are reports on the prognostic value of soluble uPAR (suPAR) detected in serum samples in colorectal and breast cancer patients [31, 32]. However, recently it has been shown that the determination of suPAR (and uPA/PAI-1) in preoperative plasma samples of breast cancer patients does not reflect its (their) concentration in tumor tissue [33]. Therefore, the measurement of uPAR (and uPA/PAI-1) in blood for assessing prognosis in breast cancer was not recommended by the EORTC Receptor and Biomarker Group [33].

Recently, elevated mRNA levels of the uPAR splice variant uPAR-del4/5, but not uPAR-wt and uPAR-del5, were found to be significantly associated with disease-free survival of breast cancer patients [16, 17, 34]. In the present study, excluding possible confounding effects of post-operative therapies, high levels of uPAR-del4/5 mRNA were strongly associated with shorter DMFS and poor OS of the patients. Moreover, in multivariate regression analysis uPAR-del4/5 mRNA values significantly contributed to the base model of traditional prognostic factors for DMFS indicating that uPAR-del4/5 mRNA is an independent, pure prognostic factor in breast cancer. Even in a patients' subgroup with tumor size ≤ 2 cm and age > 35 years, high expression levels of uPAR-del4/5 and rab31 were significantly related with poor prognosis and therefore define a subgroup of "low risk" patients, who may have a profit from adjuvant systemic chemotherapy.

In contrast to previous studies using adjuvant-treated node-positive/-negative patient cohorts [16, 17], in the untreated patients analyzed here also high levels of uPAR-wt mRNA were found to be associated with an unfavorable DMFS and OS. These data are in agreement with the reported results on the association of uPAR mRNA, qualitatively measured by Northern blot analysis, with OS in

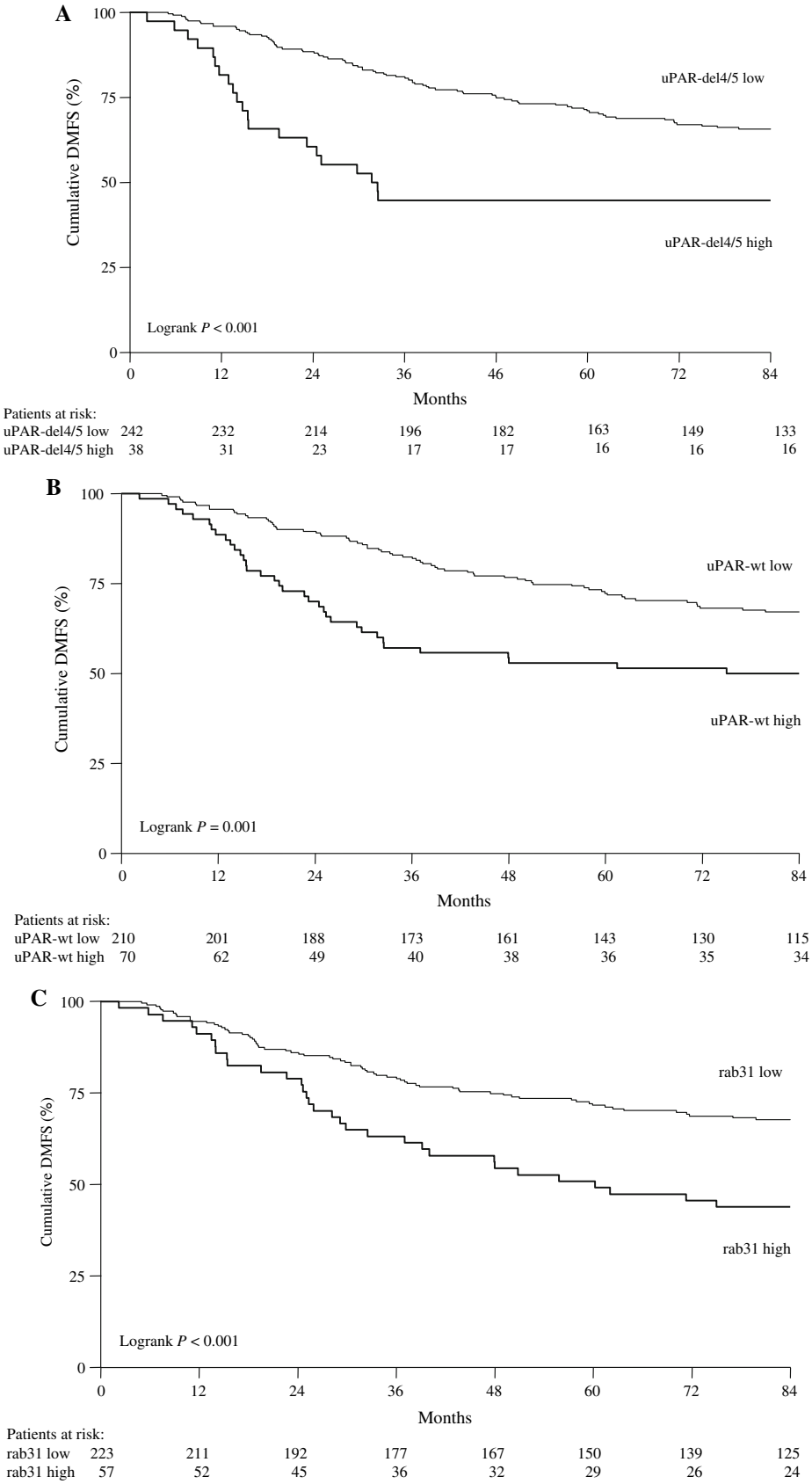

Fig. 2 Distant metastasis-free survival as a function of the levels of uPAR-del4/5 mRNA (**A**), uPAR-wt mRNA (**B**), and rab31 mRNA (**C**). The number of patients at risk at the different time points is indicated. Cut points for uPAR-del4/5: 0.126; uPAR-wt: 4.51; rab31: 1.96

Table 4 Cox multivariate regression analysis of distant metastasis-free survival in breast cancer patients (n = 280)

Factor	No. patients	HR (95%CI)[a,b]	P
Age categories (years)			
<40	35	1	
41–55	127	0.72 (0.40–1.29)	0.27
56–70	88	0.42 (0.18–0.98)	0.044
>70	30	0.21 (0.07–0.64)	0.006
Menopausal status			
Premenopausal	137	1	
Postmenopausal	143	1.68 (0.90–3.15)	0.11
Tumor size			
≤2cm	142	1	
>2cm	138	1.01 (0.67–1.51)	0.98
Tumor grade			
Poor	145	1	
Unknown	86	1.09 (0.70–1.71)	0.7
Good/moderate	49	0.47 (0.24–0.93)	0.03
ER status			
Negative	72	1	
Positive	208	1.40 (0.84–2.35)	0.2
PgR status			
Negative	106	1	
Positive	162	0.60 (0.38–0.95)	0.029
Missing	12	0.46 (0.14–1.50)	0.2
		Additions to the base model	
uPAR-del4/5 mRNA[c]			
Low	242	1	
High	38	3.29 (1.78–6.10)	<0.001
uPAR-wt mRNA[c]			
Low	210	1	
High	70	1.94 (1.24–3.04)	0.005
rab31 mRNA[c]			
Low	223	1	
High	57	2.27 (1.45–3.57)	<0.001

[a] HR: hazard ratio (95% confidence interval) of multivariate Cox regression analysis

[b] uPAR variants and rab31 were separately added to the base model including age, menopausal status, tumor size, tumor grade, ER status and PgR status

[c] Relative expression ratio mRNA/HPRT mRNA dichotomized in high and low levels by cut points (0.126 for uPAR-del4/5, 4.51 for uPAR-wt and 1.96 for rab31)

breast cancer patients [35]. Still, when combining uPAR-wt and uPAR-del4/5 mRNA values in multivariate analysis for DMFS and OS, uPAR-wt did not further contribute to the base model in which uPAR-del4/5 was included. Therefore, the prognostic impact of the uPAR splice variant uPAR-del4/5 seems to be more powerful compared with that of uPAR-wt.

The potential tumor biological role of uPAR-del4/5 beyond its prognostic relevance is still unclear. Previously, we could demonstrate that recombinant expression of the uPAR-del4/5 variant, lacking complete domain DII of uPAR, in hamster (CHO-) cells and human cancer cells leads to synthesis, secretion and insertion into the cell membrane of uPAR-del4/5 protein [16; unpublished data]. It is tempting to speculate that the specific deletion of a complete domain alters one or more functions of the uPAR protein (or the regulation thereof), e.g. interaction with its ligands such as uPA or vitronectin, and the lateral interaction with integrins, fMLP receptors or cross-talking receptors like EGFR which are necessary for uPAR signal transduction [3, 5, 15]. In fact, overexpression of uPAR-del4/5 in three different breast cancer cell lines (MDA-MB231, MCF-7, and CAMA-1) leads to siginificant changes in cell adhesion to vitronectin as well as to other ECM proteins such as collagen IV and fibronectin, whereas adhesion to laminin remained unchanged [unpublished data].

To further explore the potential biological role of uPAR-del4/5, e.g. with respect to possible interactions with components of signaling pathways, we aimed at identifying differentially expressed genes associated with high uPAR-del4/5 mRNA levels by microarray analyses. Seven genes were found to be strongly up-regulated in tumors with high uPAR-del4/5 mRNA expression. For the gene encoding rab31, selected as the first factor for further analysis, we found a strong, significant relation between high rab31 mRNA values and worse outcome of the patients. Similar to uPAR-del4/5 and uPAR-wt, rab31 mRNA significantly contributed to the base multivariate model, and thus may serve as a novel prognostic marker in breast cancer. Simultaneous addition of all three factors to the base model caused a loss of the prognostic relevance of uPAR-wt, but did not affect the prognostic power of rab31 and uPAR-del4/5 mRNA.

Rab31 belongs to the Ras superfamily of low-molecular-weight GPT-binding proteins that regulate and coordinate consecutive stages of intracellular transport between organelles [36, 37]. However, there is accumulating evidence that Rab proteins are also involved in intracellular signal transduction, receptor internalization and recycling [38]. Recently, different Rab proteins have been shown to regulate internalization and recycling pathways of integrins or growth factor receptors like EGFR, thereby controling/driving processes such as cell adhesion, proliferation and migration [39, 40].

Also, uPAR acts as a modulatory molecule in cell adhesion and migration upon uPA binding and interaction with integrins, fMLP receptors or EGFR which are necessary for uPAR signal transduction [5, 41, 42]. The type of adaptor molecule (and the signaling pathways activated

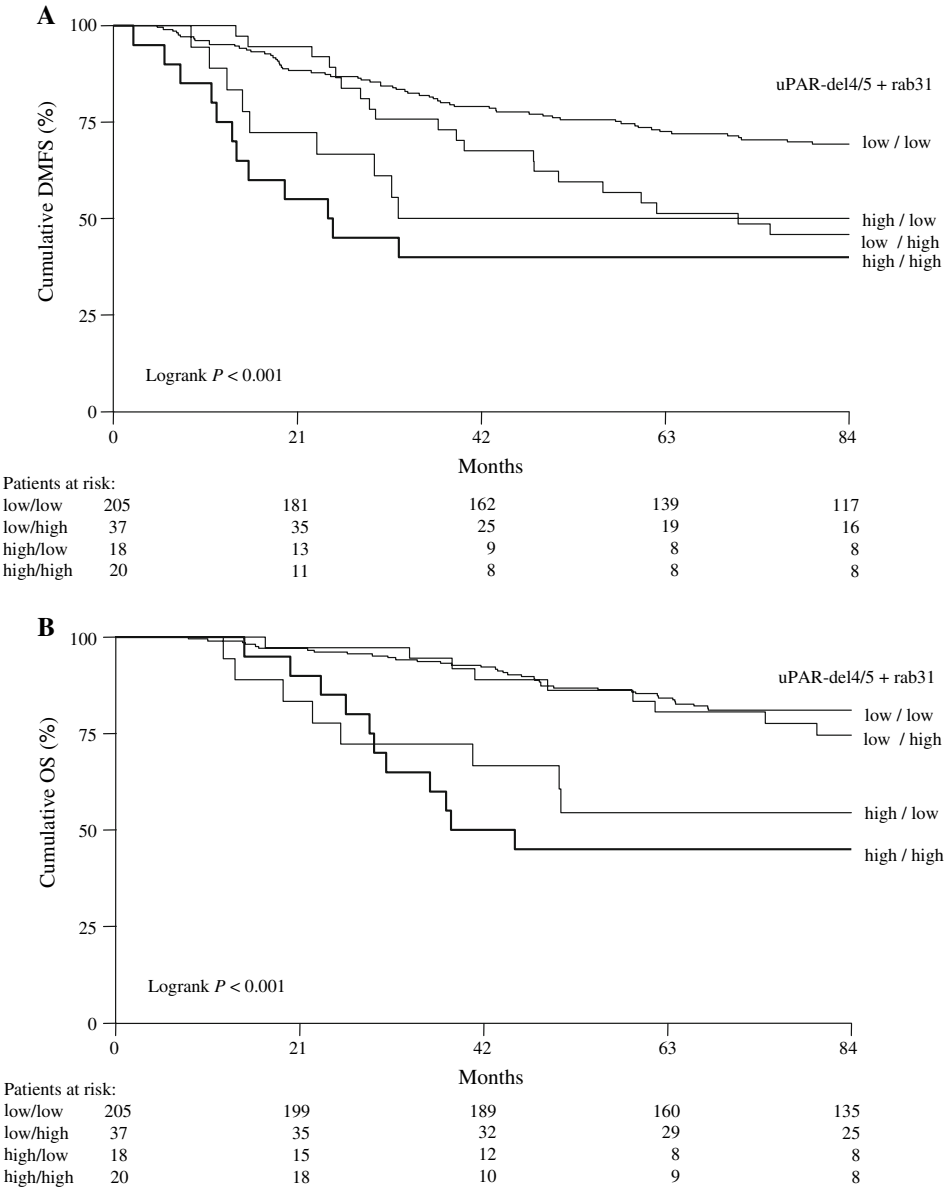

Fig. 3 Distant metastasis-free survival (**A**) and overall survival (**B**) as a function of the combined uPAR-del4/5 and rab31 mRNA expression status. The number of patients at risk at the different time points is indicated. Cut points for uPAR-del4/5 and rab31 mRNA levels were 0.126 and 1.96, respectively

thereof) seems to be regulated by cleavage of uPAR by uPA or other proteinases at the linker region between domains DI and DII [43]. Thus, the biological activity of the uPAR-del4/5 protein variant, lacking this protease-sensitive sequence, may be altered due to its resistance against limited proteolysis.

Both, QPCR and microarray data show that expression of rab31 is correlated with uPAR-del4/5 (and uPAR-wt) expression and, therefore, endocytosis and recycling of uPAR (and uPAR-/uPAR-del4/5-mediated cell migration *via* integrins/EGFR) may also be regulated by a Rab protein-driven pathway. However, the interrelationship between rab31 and uPAR-del4/5 (and uPAR-wt) was not strong ($r_s = 0.514$). Furthermore, uPAR-del4/5 and rab31 independently contributed to patients' prognosis in multivariate analysis. Thus it seems more likely that rab31 mRNA is—independent of uPAR-del4/5—differentially expressed in tumors with high versus low aggressiveness. In fact, there is growing evidence that dysregulation of Rab gene expression may be a more generalized component of carcinogenesis, tumor growth and invasion [44, 45]. Members of the Rab family have been found up-regulated in a variety of tumors and preneoplastic alterations [46–49], and in ovarian and breast cancer patients high rab25 mRNA tumor levels were significantly associated with poor prognosis [23]. Recently, rab31 was identified among 11 genes to be overexpressed only in ER-positive breast cancer patients by microarray analyses [50]. In contrast, in the present study we did not detect significantly different expression levels of rab31 in ER-positive or -negative tumors.

In conclusion, we report here for the first time the independent but additive pure prognostic relevance of mRNA expression levels of uPAR-del4/5 and rab31 in lymph node-negative breast cancer patients. The combination of the prognostic value of rab31 and uPAR-del4/5, which represent components of different but possibly associated signaling pathways involved in cell migration and proliferation, may improve prediction of disease recurrence in breast cancer.

Acknowledgements This study was supported by grants from the Deutsche Krebshilfe e.V., Germany (Grant No. 106 185). We thank Antje Zobjack, Annelie Zürich, and Andrea Lohse for their excellent technical assistance.

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