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Development of quantitative RT-PCR assays for wild-type urokinase receptor (uPAR-wt) and its splice variant uPAR-del5 *

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The receptor for the serine protease urokinase-type plasminogen activator, uPAR (CD 87), plays an important role in tumor cell invasion and metastasis of solid malignant tumors. uPAR is a highly glycosylated, glycan lipid-anchored membrane protein, consisting of three homologous domains. Each individual domain is encoded by two exons: DI by exons 2+3, DII by exons 4+5, and DIII by exons 6+7. Beside the wild-type (wt) uPAR mRNA, two splice variants either lacking exon 5 (uPAR-del5) or both exons 4 and 5 (uPAR-del4/5) have been described. Previously, we studied expression of the mRNA variant uPAR-del4/5 and uPAR mRNA encompassing exons 2, 3, and 4 (i.e. uPAR-wt plus uPAR-del5) applying real-time RT-PCR assays for quantification of the mRNA concentration.

In the present paper, we established two additional specific, robust and highly sensitive RT-PCR assays, based on the LightCycler technology, to specifically quantify either uPAR-wt or its splice variant, uPAR-del5. Expression of uPAR-wt and uPAR-del5 was analyzed in different human malignant cell lines (ovarian cancer cell lines OVMZ-6 and OVMZ-10; breast cancer cell lines MDA-MB 231, MDA-MB 231 BAG, MDA-MB 435, and aMCF-7; brain tumor cell line LN 18) as well as in a set of 174 breast cancer tissue samples. uPAR-del5 mRNA was found to be expressed very frequently at a rather low level (typically less than 1% of uPAR-wt mRNA). In tumor tissue from breast cancer patients, a statistically significant correlation between uPAR-del5 and uPAR-wt mRNA ($r = 0.779$; $P < 0.001$) was observed. There was no association between the expression level of either mRNA and clinical parameters such as nodal status, tumor size and grade. In estrogen receptor negative tumors, a significantly higher uPAR-del5 expression was found ($P = 0.023$).

The two developed quantitative RT-PCR assays described here may aid further analysis of the function and clinical relevance of uPAR-wt and one of its splice variants, uPAR-del5, in malignant tumors.

Key words: neoplasms; urinary plasminogen activator; RNA, messenger; reverse transcriptase polymerase chain reaction; RNA splicing

Introduction

Tumor cell dissemination and formation of metastases is facilitated by the interaction of diverse proteolytic systems, including serine proteases, cysteine proteases, and matrix metalloproteinases.[1] These proteases enable tumor cells to degrade the extracellular matrix and to cross natural boundaries.[2] The receptor for the serine protease urokinase-type plasminogen activator (uPAR, CD 87) is essentially involved in this process as it focuses the proteolytic activity of uPA to the cell surface. Furthermore, it interacts with a broad variety of other ligands, including vitronectin or integrins, and by this modulates proliferation, cell adhesion and migration, invasion, and angiogenesis.[3,4] High tumor levels of uPA and/or its inhibitor PAI-1 have been shown to be a predictor for poor prognosis of patients with solid tumors, including breast, gastric, esophageal, ovarian, colorectal or hepatocellular cancer.[4,5] Different therapeutic approaches have been employed to obstruct the uPA/uPAR system, using small molecules, such as antibodies or modified toxins.[6-9]

uPAR is a highly glycosylated, glycan lipid (GPI-)-anchored membrane protein, consisting of three structurally homologous domains (DI, DII, DIII).[10] In the past, a number of glycosylation variants and different molecular forms of uPAR antigen such as soluble uPAR, uPAR-DI, and uPAR-DII+III has been described and analyzed (for a summary see Luther *et al*.[11]). In some cases, certain (novel) functions or activities could be assigned to these variants: endoproteolytic processing of CD87 with removal of DI is, *e.g.*, a likely pathway for controlling cell adherence and migration [3,12,13], as the extent of glycosylation of DI strongly contributes to the affinity for its ligand uPA.[14] Furthermore, splice variants of uPAR have been identified, *i.e.* an uPAR mRNA splice variant lacking exon 5 (uPAR-del5) as well as a variant lacking exons 4 and 5 (uPAR-del4/5).[11,15] Since splice variants of genes often display a different expression pattern and biological role compared to that of the wild-type genes, especially in tumor tissue [16] , we previously studied the expression of the mRNA variant uPAR-del4/5 in a representative set of breast cancer tissues applying a real-time RT-PCR assay for quantification of the mRNA concentration.[11] The mRNA variant uPAR-del4/5 was, in fact, expressed very frequently in breast cancer tissue and, strikingly, higher uPAR-del4/5 expression was significantly associated with shorter disease-free survival of breast cancer patients. Thus, these results suggest that uPAR-del4/5 mRNA may serve as a prognostic marker in breast cancer. The aim of the present study was to establish a highly sensitive real-time RT-PCR assay based on LightCycler technology for the uPAR-del5 mRNA variant in order to be able to analyze the expression pattern of this alternatively spliced mRNA in solid malignant tumors.

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Material and methods

Cell lines and cell lysates, uPAR ELISA

Human ovarian cancer cell lines OVMZ-6 and OVMZ-10, human breast cancer cell lines MDA-MB 231, MDA-MB 231 BAG, MDA-MB 435, and aMCF-7 as well as the human brain tumor cell line LN 18 were cultured at 37˚C in a humidified atmosphere of 5% CO_2 and

95% air in DMEM medium (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (Invitrogen) and 1% penicilline-streptomycine (Biochrom, Berlin, Germany), 1% arginine-asparagine (Sigma, Deisenhofen, Germany) and 1% HEPES buffer (Invitrogen). Cells were harvested from monolayer dishes after two days. Total RNA from the cells was extracted using Trizol Reagent (Invitrogen). cDNA was synthesized using AMV cDNA First Strand Synthesis Kit (Roche Diagnostics, Penzberg, Germany). cDNAs from the cell lines were diluted 1:15 and aliquoted at -20 ˚C.

For uPAR antigen detection, 2 x 10^6 cells were cultured for two days on monolayer dishes, then harvested, resuspended in phosphate-buffered saline and sedimented by centrifugation (200 x *g*, 10 min, RT). Cells were disrupted by two freezing and thawing cycles, followed by a solubilization step (10 min, in 100 µl per 10^6 cells sample buffer containing 0.2% Triton X-100) and stored at -20 ˚C. uPAR antigen was determined in cell lysates by uPAR IIIF10 ELISA as described by Kotzsch and co-workers (2000). Protein content was determined using the Micro BCA protein assay kit (Pierce, Rockford, IL). uPAR antigen levels in cell lysates are expressed as ng per mg of total protein.

Patients - tissue selection

The study adhered to national regulations of The Netherlands on ethical issues and was approved by the local ethical committee. Tumor tissue was obtained from patients with unilateral breast cancer after surgical resection of the primary tumor. Patients who had received neo-adjuvant treatment, or who had a previous diagnosis of cancer or who had a carcinoma *in situ*, were excluded. Furthermore, patients with recurrent disease within one month after surgery or with distant metastases at time of diagnosis were excluded as well. After surgery, performed between November 1987 and December 1997 in participating hospitals of the Comprehensive Cancer Center East in The Netherlands, a representative part of the tumor was selected by a pathologist, frozen in liquid nitrogen, and sent to the Department of Chemical Endocrinology for routine determination of estrogen (ER) and progesterone (PgR) receptor status by ligand binding assay.[17,18] Remaining frozen tissue or tissue powder (after dismembration) prepared from this tumor was kept in liquid nitrogen. For the present study, samples were selected based on the availability of tissue stored in the tumor bank.

Patients- cDNA synthesis

Total RNA was isolated from approx. 20 mg of tissue powder using the RNeasy mini kit (Qiagen, Hilden, Germany) with on-column DNase-I treatment as previously described.[18] Reverse transcription was performed using the Reverse Transcription System (Promega Benelux BV, Leiden, The Netherlands) according to the manufacturer's protocol. After annealing of random hexamers for 10 min at 20 ˚C, cDNA synthesis was performed for 60 min at 42˚C, followed by an enzyme inactivation step for 5 min at 95 ˚C. cDNAs were diluted 1:3 and aliquoted.

Quantitative real-time RT-PCR of uPAR-del5, uPAR-wt, and the housekeeping gene G6PDH

RT-PCR primers and hybridization probes were obtained from TibMolBiol (Berlin, Germany). RT-PCR was performed using the LightCycler apparatus (Roche, software version 3.5). Assays for quantification of uPAR-wt and uPAR-del5 were established, with primer sequences as follows: Ex 2F: GAC CTC TGC AGG ACC ACG AT; Ex 6,4R: CAG ATT TTC AAG CTC CAG GAC TT; Ex 5A: GGT GGC GGT CAT CCT TTG. RT-PCR was performed with a master mix with 3.0 µM $MgCl_2$, 0.6 µM of the primers, 0.2 µM of each

of the hybridization probes and 2 μl of reagent mix, in a total volume of 20 μl. The amplification program started with pre-denaturation at 95 °C, followed by 45 cycles of amplification: denaturation 10 sec at 95 °C, annealing for 15 sec at 66 °C, and elongation at 72 °C for 15 sec. A standard curve was generated for each run using eight glass capillaries (Roboscreen, Leipzig, Germany) coated with a defined number of molecules of uPAR (wild type or del5) plasmid (range from 100,000 to 10 copies). The generation of the plasmids pRcRSV-GPI-uPAR-wt (encoding uPAR-wt) and pRcRSV-GPI-uPAR-del5 have been described previously.[11] A negative control containing buffer only was included in each run. For normalization of the data h-G6PDH (human glucose-6-phosphate-dehydrogenase) Housekeeping Gene Set (Roche) was used, according to the manufacturer´s protocol. All cDNA samples displaying less than 10,000 molecules of G6PDH were considered to be of non-optimal quality and were excluded from further analyses (18 of 192 cDNA samples = 9.4%). Relative expression levels were determined calculating the ratio between absolute template molecule and G6PDH housekeeping molecule numbers. The ratios of target genes and reference gene were multiplied with factor 1,000.

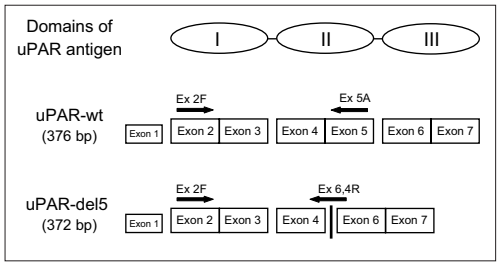

Figure 1. Detection of uPAR-wt and uPAR-del5 mRNA. uPAR consists of three homologous domains. Each individual domain is encoded by two exons: DI by exons 2+3, DII by exons 4+5, and DI-II by exons 6+7 (exon 1 encodes the signal sequence of uPAR). For specific amplification of uPAR-wt mRNA a reverse-primer directed to exon 5 was designed. In order to specifically amplify uPAR-del5 a primer overlapping the unique boundary between exons 4 and 6 was chosen. Both RT-PCR assays use the same forward primer within exon 2. The amplicon length is 376 bp for uPAR-wt and 372 bp for uPAR-del5.

Statistics

The association of uPAR mRNA levels with histomorphological and clinical parameters was analyzed using non-parametric tests (Mann-Whitney U test; Kruskal-Wallis test). Statistical analyses were performed using the SPSS 11.5 software.

Results and discussion

Development of RT-PCR assays for uPAR-del5 and uPAR-wt

For quantification of uPAR-del5 mRNA, we established a highly sensitive real-time RT-PCR assay applying the LightCycler technology. In this assay, the 5′ amplification primer (Ex 2F) is identical to the 5′ primer used in the RT-PCR assays previously described [11] for detection of uPAR-2/3/4 mRNA (encompassing exons 2, 3, and 4) and uPAR-del4/5 mRNA (encompassing exons 2, 3, and 6 and lacking exons 4 and 5, respectively). The 3′ amplification primer (Ex 6,4R) overlaps the alternative splicing site of exons 4 and 6 and, therefore, selectively binds to uPAR-del5 mRNA (and not to uPAR-wt or uPAR-del4/5). For generation of standard curves, glass capillaries coated with defined numbers of uPAR-del5 plasmid - exactly determined by HPLC calibration [19] - were used. In addition to the uPAR-del5 assay, a quantitative RT-PCR assay for uPAR-wt mRNA was established, which does neither amplify uPAR-del5 nor uPAR-del4/5 mRNA, since the 3′ amplification primer (Ex 5A) is directed to exon 5 (Figure 1). Since in both assays the 5′ and 3′ amplification primers are directed to different exons, amplification of (possible contaminating) genomic uPAR-

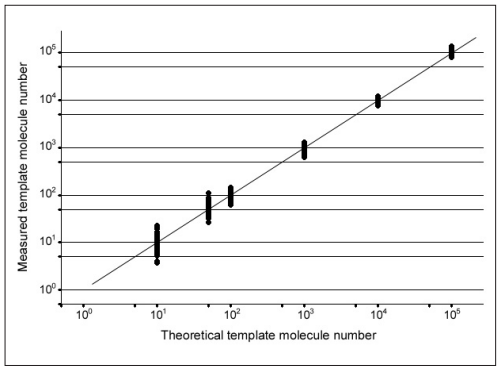

Figure 2. LightCycler PCR standard curves for quantification of uPAR-del5 mRNA. The plots of molecule numbers detected *versus* theoretical molecule numbers of uPAR-del5 were generated from 48 independent PCR runs. Correlation of the values is $r = 0.990$; $P < 0.0001$).

DNA is excluded due to the large amplicon size of > 10 kbp. With both assays, the detection limit was (at least) 10 copies of cDNA, which corresponds to the lowest glass capillary standard. Figure 2 depicts a plot of measured molecules *versus* theoretical molecule numbers of uPAR-del5 in 48 independent PCR runs for the standards ranging from 10 to 100,000 copies. In case of uPAR-wt, similar results were obtained (data not shown). To test for specificity of the uPAR-del5 and uPAR-wt assays, we analyzed samples containing large plasmid copy numbers (corresponding to about 1.5 pg DNA) of either uPAR-wt, -del5, and -del4/5 or included glass capillaries coated with 100,000 copies of the three different plasmids. In neither case, we observed an amplification signal above the buffer control for the control plasmids (data not shown). Thus, the target sequence (either uPAR-del5 or uPAR-wt) is selectively amplified with the two newly established RT-PCR assays.

Quantification of uPAR mRNA variants and uPAR antigen in cell lines

Seven different cell lines were selected and the uPAR antigen content determined applying the uPAR HU/IIIF10-ELISA.[20] As can be seen from Table 1, the uPAR antigen content ranged from undetectable (< 0.05 ng/mg, breast cancer cell line MDA-MB 435) to about 7 ng/mg total protein in the case of the ovarian cancer cell line OVMZ-10. uPAR-wt mRNA concentration (normalized to the expression of G6PDH) was determined and compared to the uPAR antigen values: the highest antigen values (OVMZ-10 and MDA-MB 231 BAG) corresponded to the highest mRNA levels, the cell line with undetectable uPAR protein levels displayed an extremely low uPAR-wt mRNA expression as well. The other cell lines displayed an intermediate expression both at the protein and mRNA level ($r = 0.786$; $P < 0.05$) (Table 1). uPAR-del5 expression was detected in all of the cell lines albeit to a low extent ranging from 0.23 to 1.53% of uPAR-wt expression.

Table 1. Detection of uPAR antigen, uPAR-wt and uPAR-del5 mRNA in seven different cancer cell lines. Cellular uPAR antigen content (expressed as ng per mg total protein) was measured using the HU/IIIF10 ELISA.[20] uPAR-wt and uPAR-del5 mRNA was quantified by the newly established RT-PCR assays and expression normalized to G6PDH mRNA .

Cell lines		ELISA [ng/mg]	RT-PCR measurements [relative to G6PDH]		
Name	**Origin**	**HU/IIIF10**	**uPAR-wt**	**uPAR-del5**	**uPAR-del5 / uPAR-wt [%]**
MDA 435	breast	< 0.05	21	0.2	0.95
MDA 231	breast	1.99	747	2.0	0.27
LN 18	brain	2.07	137	2.1	1.53
OVMZ-6	ovary	2.18	648	5.5	0.85
aMCF-7	breast	3.19	671	3.9	0.58
MDA 231 BAG	breast	5.28	1204	2.8	0.23
OVMZ-10	ovary	6.86	3588	28.0	0.78

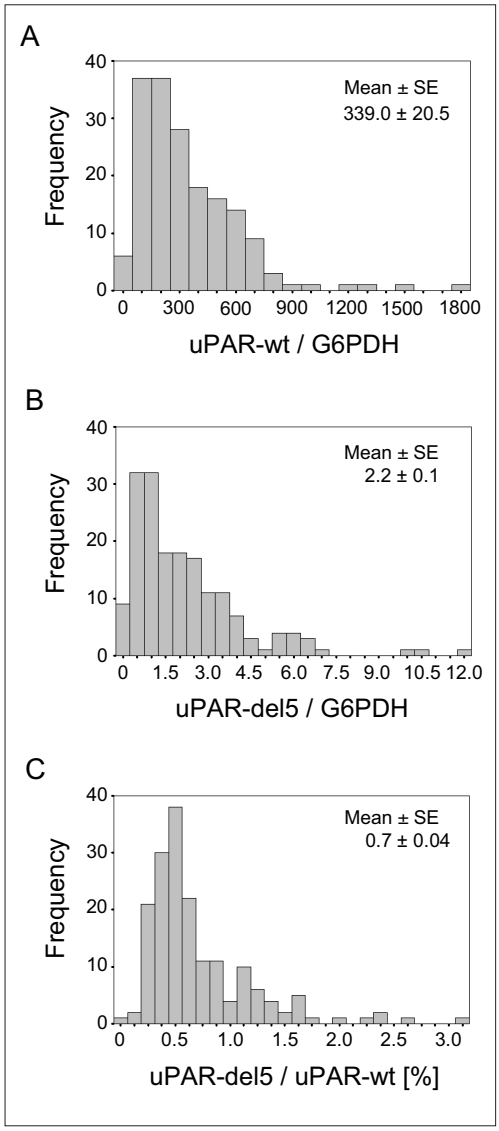

Figure 3. uPAR mRNA expression in human breast cancer tissue. 174 primary breast cancer samples were measured for uPAR-wt and uPAR-del5 mRNA with the newly established quantitative RT-PCR assays. The histograms depict the frequency distribution of **(A)** uPAR-wt expression (normalized to G6PDH), **(B)** uPAR-del5 expression (normalized to G6PDH), and **(C)** relative uPAR-del5 expression compared to uPAR-wt [%].

Quantification of uPAR mRNA variants in breast cancer tissue

In order to select an appropriate housekeeping gene for normalization of mRNA concentrations, we first analyzed expression of three different housekeeping genes (PBDG, G6PDH, and GAPDH) in a representative set (n=46) of breast cancer cDNA samples. The absolute mRNA concentrations of PBGD, G6PDH, and GAPDH strongly correlated with each other (ranging from $r = 0.880$ to 0.908, $P < 0.0001$) indicating that these genes are, in fact, expressed constitutively in breast cancer. As both genes, G6PDH and uPAR, are moderately high expressed, G6PDH was subsequently used for normalization.

We assessed the level of expression of uPAR-del5 and uPAR-wt mRNA in 174 cases of breast cancer patients (Figure 3). uPAR-wt expression was found in all cases (median relative expression level: 263). uPAR-del5 was detected in the breast cancer samples with high frequency but with a significantly lower expression level (median: 1.57). In one case, there was no uPAR-del5-amplification signal at all, in further 40 of the 174 cases (23.6%), the determined copy number was below the lowest standard included in the assay. The relative expression rate of uPAR-del5 to uPAR-wt was between 0 and 3.1% (median $uPAR\text{-}del5_{rel}/uPAR\text{-}wt_{rel}$: 0.53%). The mRNA concentrations of uPAR-wt and uPAR-del5 significantly correlated with each other ($r = 0.779$; $P < 0.001$). We observed no statistically significant association of uPAR-wt or uPAR-del5 expression with clinical parameters such as nodal status, tumor size or grade (Table 2). A significantly higher uPAR-del5 expression was found in estrogen receptor negative tumors ($P = 0.023$).

Conclusions

In the present study, we developed a quantitative real-time RT-PCR method to specifically quantify uPAR-wt mRNA (excluding both

Table 2. uPAR-wt and uPAR-del5 mRNA expression and clinical parameters. Associations of uPAR-wt and uPAR-del5 expression with clinical parameters were analyzed by using non-parametric tests. All tests were performed at significance level of a < 0.05.

174 breast cancer patients			uPAR-wt / G6PDH		uPAR-del5 / G6PDH	
Variable	**Total**	**No. of patients (%)**	**Mean ± SE**	***P* value**	**Mean ± SE**	***P* value**
Menopausal status [a]	174					
pre/peri		39 (22.4)	378 ± 53	**0.397** (ns)	2.2 ± 0.3	**0.785** (ns)
post		135 (77.6)	327 ± 22		2.1 ± 0.2	
Nodal status [a]	149					
negative		79 (53.0)	360 ± 31	**0.058** (ns)	2.5 ± 0.3	**0.070** (ns)
positive		70 (47.0)	291 ± 30		1.9 ± 0.2	
x		25				
Size (pT) [b]	171					
1		55 (32.2)	398 ± 38	**0.101** (ns)	2.6 ± 0.3	**0.240** (ns)
2		97 (56.7)	286 ± 20		1.9 ± 0.2	
3		15 (8.8)	405 ± 118		2.1 ± 0.4	
4		4 (2.3)	369 ± 133		1.5 ± 0.6	
x		3				
Grade [a]	123					
I/II		65 (52.8)	275 ± 23	**0.116** (ns)	1.7 ± 0.2	**0.112** (ns)
III		58 (47.2)	397 ± 47		2.4 ± 0.3	
x		51				
ER status [a]	174					
negative		56 (32.2)	427 ± 48	**0.066** (ns)	2.8 ± 0.3	**0.023**
positive		118 (67.8)	297 ± 19		1.9 ± 0.1	
PgR status [a]	174					
negative		67 (38.5)	390 ± 39	**0.274** (ns)	2.6 ± 0.3	**0.153** (ns)
positive		107 (61.5)	307 ± 22		1.9 ± 0.2	

a Mann-Whitney-U Test
b Kruskal-Wallis Test
x Unknown status

splice variants uPAR-del5 and uPAR-del4/5) and uPAR-del5 mRNA in breast cancer. The assays are rapid, robust, and sensitive. As these assays require only minute amounts of cDNA, they are well suited for studies when the amount of sample is limited. For validation of the uPAR-wt assay, we initially measured uPAR mRNA expression in cell lines of different origin and observed that the measured mRNA expression levels corresponded well to the respective antigen levels determined by a uPAR ELISA (HU/IIIF10). In the tumor cell lines, we also detected low expression of uPAR-del5 mRNA. *In vivo* expression of this uPAR splice variant was subsequently proven by analyzing breast tumor samples. Similar to uPAR-wt, we found no significant association of uPAR-del5 expression in rela-

tion to the nodal status, tumor size or grade in the analyzed patients cohort.

The cDNA sequence of the uPAR-del5 variant has been originally published by Casey *et al*. [15] Recently, we verified expression of uPAR-del5 in tumor cells by amplification of uPAR mRNA with primers directed to exon 1 and 6, respectively, followed by direct sequencing of the resulting PCR products.[11] Further evidence for the *in vivo* occurrence of uPAR-del5 comes from searching available nucleotide databases, in which a series of independent submissions of the cDNA sequence encoding uPAR-del5 obtained from various sources (*e.g.* accessions AX281707, AA481366, BM543893 or BM767461) is found. Thus, the uPAR-del5 splice variant seems to be often expressed in human cells. Previously, we have generated stably transfected Chinese hamster ovary cells, which harbor an expression plasmid encoding uPAR-del5. By ELISA, flow cytofluorometry, and Western blot analysis, we confirmed synthesis, secretion and cell surface-association of uPAR-del5. These experiments indicate that uPAR-del5 mRNA is translated and processed in a similar manner as wild-type uPAR. Therefore, experiments are on the way to search for (new) functions of uPAR-del5 (and the other expressed splice variant uPAR-del4/5) which may play a role for tumor invasion and metastasis.

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