Amplification of the Urokinase-Type Plasminogen Activator Receptor (*uPAR*) Gene in Ductal Pancreatic Carcinomas Identifies a Clinically High-Risk Group

Ralf Hildenbrand,^{*†} Marco Niedergethmann,[‡]
Alexander Marx,[†] Djeda Belharazem,[†]
Heike Allgayer,[§] Christiane Schleger,[†]
and Philipp Ströbel[†]

From the Institute of Pathology,^{*} Bonn; the Department of Surgery,[†] Institute of Pathology,[‡] and the Department of Experimental Surgery and Molecular Oncology of Solid Tumors,[§] Medical Faculty Mannheim, University Heidelberg and German Cancer Research Center, Heidelberg, Germany

The serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR) are known to be involved in the invasion and metastasis of many solid tumors. In this study, we analyzed the role of the uPAR/uPA system in both the development and progression of pancreatic cancer in invasive ductal adenocarcinomas of the pancreas (PDA) and their pre-malignant precursors (PanIN lesions) in 50 patients with long-term clinical follow-up. We found overexpression of the uPAR in 48 of 50 invasive carcinomas as well as in a large proportion of high-grade PanIN lesions by immunohistochemistry and *in situ* hybridization. Fluorescence *in situ* hybridization analysis showed both high- and low-level amplification of the *uPAR* gene in ~50% of cases with strictly identical patterns between invasive cancers and their accompanying precursor lesions. These results suggest that PDA may develop from PanIN lesions along an alternative rather than a sequential molecular pathway. The detection of the gene amplification of *uPAR* was a highly significant, adverse prognostic parameter ($P < 0.001$) because it likely renders the tumors more sensitive to uPA and its proliferative and anti-apoptotic signals. We conclude that the activation of the uPAR/uPA system is an early event in the development of PDA and that *uPAR* gene amplifications identify a subgroup of particularly aggressive tumors, making the uPAR/uPA system a critical and highly promising target for therapeutic in-

terventions. (Am J Pathol 2009, 174:2246–2253; DOI: 10.2353/ajpath.2009.080785)

Invasive ductal adenocarcinoma of the pancreas (PDA) represents the fourth most common cause of cancer-related death and its incidence is still increasing.¹ With an estimated number of 232,000 new cases per year, pancreatic cancer is among the most common malignancies worldwide.² Moreover, it is one of the most lethal cancers, as indicated by a mortality incidence ratio of 98%.² Once pancreatic cancer is clinically evident, it progresses at a rapid rate, and metastasis is often present already at the time of diagnosis.³ The mechanisms that regulate this aggressive growth behavior in pancreatic cancer are still poorly understood.

Several studies indicate that during cancer cell invasion and metastasis proteolytic enzymes participate in the degradation of extracellular matrix components. The serine protease urokinase-type plasminogen activator (uPA) and its receptor (uPAR, CD87) are involved in the control of extracellular matrix turnover, cell migration, invasion, and cell signaling leading to a variety of different responses under both physiological and pathological conditions.^{4–6} uPAR is a highly glycosylated 45- to 60-kDa protein that is attached to the cell membrane via glycosylphosphatidylinositol anchor. It consists of three

Supported by the Deutsche Krebshilfe (to R.H. and grant 106430 to P.S. and A.M.); the Dr. Mildred-Scheel-Stiftung für Krebsforschung (10-1507H to R.H.); the Faculty of Clinical Medicine Mannheim, University of Heidelberg (research funds to R.H.); the Alfried Krupp von Bohlen und Halbach Foundation (to H.A.); the Wilhelm Sander Stiftung (to H.A.); the Auguste-Schädel-Dantscher-Stiftung (to H.A.); the Ingrid zu Solms Foundation (to H.A.); the Dr. Hella-Buehler-Foundation (to H.A.); and the European Union [grants LSHB-CT-2003-503410 (Euro-Thymaide) and 2005105 (European Myasthenia Gravis Network) to P.S. and A.M.).

Accepted for publication February 10, 2009.

Address reprint requests to Dr. Ralf Hildenbrand, Institut für Pathologie, Heilsbachstrasse 15, 53123 Bonn, Germany. E-mail: hildenbrand@patho-bonn.de.

homologous domains of which the N-terminal one represents the uPA-binding domain.⁷ The urokinase receptor that binds to the growth factor-like domain of uPA directs membrane-associated extracellular proteolysis and signals through transmembrane proteins, thus regulating tissue regeneration, angiogenesis, cancer growth, and metastasis.^{6,8–10} Furthermore, uPA and amino-terminal fragment of uPA (ATF) induce proliferation in cancer cells by binding to uPAR. Integrins situated adjacent to uPAR carry the signals into the cell, thus stimulating proliferation that is mediated via the mitogen-activated protein kinase (MAPK) pathway.¹¹ The ability of uPA to activate the MAPK pathway by binding to uPAR was reported previously.^{6,12–14} There appears to exist a positive feedback loop between uPAR/uPA expression and MAPK: the uPAR/uPA system plays a critical role in maintaining a high level of activated MAPK and at the same time, activated MAP kinases are necessary to maintain uPA and uPAR expression.¹⁵

The present study was conducted to analyze uPAR expression and *uPAR* gene amplification in pancreatic cancer and precursor lesions and to further evaluate the role of uPAR/uPA system in pancreatic carcinogenesis.

Materials and Methods

Tumor Samples and Patient Data

A prospective database of patients with pancreatic tumors has been developed in the Department of Surgery, University Hospital Mannheim, since 1987 with approval of the ethical committee of the medical faculty. One fraction of freshly removed pancreatic tissue samples was fixed in 4% buffered paraformaldehyde for 24 hours and paraffin-embedded for histological analysis, another fraction was frozen in liquid nitrogen immediately on surgical removal and maintained at -80°C until use. Some of the fresh-frozen specimens had been stored for more than 10 years. In this study, 50 R0 resected invasive ductal adenocarcinomas of the pancreas (20 female, 30 male patients) with available fresh-frozen tissue of sufficient quality operated at the Department of Surgery, Medical Faculty Mannheim, between 1994 and 2004 were enrolled. Ampullary, duodenal and biliary cancers were excluded from this study. The median age of the patients was 66.5 years (range, 41 to 79 years). Surgical procedure consisted of either a partial duodenopancreatectomy (48 patients) or a left resection of the pancreas (2 patients). Seven patients had tumor stage I, forty patients had tumor stage II, and three patients had a tumor stage III (UICC 2003). Four tumors were grade 1, thirty-three grade 2, and thirteen grade 3. Overall survival was defined as time between surgery and death. The mean overall survival (OS) was 24.3 ± 5.1 months (range, 2 to 156 months). All patients were examined every 3 to 6 months during the first 5 years of follow-up and once a year thereafter. Follow-up was performed either through direct contact with the patient or with the patients' primary physician and was terminated at patients' death. Recurrence was diagnosed either by ultrasound, computed to-

mography, magnetic resonance imaging, or through a palliative surgery with biopsy. Of the 50 patients examined 43 patients showed evidence of disease during follow-up (local relapse and/or distant metastasis) and subsequently died of their cancer. The seven remaining patients died without evidence of disease. In 28 cases the invasive adenocarcinoma was associated with low-grade pancreatic intraepithelial neoplasia (PanIN-1), in 16 cases with moderate-grade pancreatic intraepithelial neoplasia (PanIN-2), and in 26 cases with high-grade pancreatic intraepithelial neoplasia (PanIN-3). Furthermore, tissues from eight male patients (between 56 and 74 years of age, median 69 years) ($n = 8$) undergoing pancreatic resection for chronic pancreatitis were examined as controls.

Fluorescence in Situ Hybridization (FISH) of Interphase Nuclei

Three to five paraffin sections of 10 μm thickness were stained with hematoxylin and eosin. Under microscopic control tumor tissue was selectively collected with fine forceps. Extraction of interphase nuclei from microdissected tumor material was performed as described.¹⁶ Interphase FISH was performed as described previously.¹⁷ Hybridization was performed using a cosmid probe for *uPAR* (clone R28316 was kindly provided by the Lawrence Livermore National Laboratory Genome Center, Livermore, CA) and an α -satellite probe for the centromeric regions of chromosome 19 (Vysis Inc., Downers Grove, IL). The cosmid probe was labeled with digoxigenin by nick translation (Roche Diagnostics GmbH, Mannheim, Germany). Detection of cosmid probe was performed by tyramide signal amplification (TSA; Invitrogen GmbH, Karlsruhe, Germany). The α -satellite probe was purchased directly labeled (SpectrumGreen) from Vysis Inc.

Microscopy and Image Acquisition

At least 100 nonoverlapping interphase nuclei from each tumor sample were evaluated by using a fluorescence microscope (Axiophot, C; Zeiss, Göttingen, Germany) fitted with a triple band pass filter set (Vysis Inc.) allowing for the simultaneous visualization of green and red signals. Images were acquired with a cooled charge-coupled device camera (Nu200; Photometrics Inc., Tucson, AZ) and the Quips-XL FISH-imaging software (Vysis, Inc.). Each tumor cell was scored for the number of centromeric and *uPAR* signals. *uPAR* copy gain was defined as more *uPAR* signals than centromere 19 signals in more than 10% of cells. This was based on two controls of nonneoplastic pancreatic tissue showing more *uPAR* signals than centromere 19 signals in not more than 5% of cells. Hybridization efficiency was tested in lymphocytes, which were always present in interphase nuclei preparations. Only FISH experiments with high hybridization efficiency (showing at least 95% of lymphocyte nuclei with two signals for *uPAR* and two signals for centromere 19) were considered adequate for evaluation. Low-level amplification was defined as up to three more *uPAR* signals

than centromere 19 signals, high-level amplification was scored by a ratio of more than three *uPAR* signals than centromere 19 signals. FISH experiments were repeated at least twice. Nuclei without any *uPAR* or centromere 19 signals were excluded from analysis.

In Situ Hybridization

Nonradioactive *in situ* hybridization for human *uPAR* mRNA applying digoxigenin-labeled oligodeoxynucleotides was performed as described previously.¹⁸ Briefly, paraffin sections of 10 μ m thickness were deparaffinized and incubated with Proteinase K (5 μ g/ml in 50 mmol/L Tris/HCl, pH 7.6; Roche) for 45 minutes at 37°C. After prehybridization (1 hour at 37°C; in 50 μ l 4 $\times$ standard saline citrate, 50% formamide, 1 $\times$ Denhardt's solution, 10% dextran sulfate, 150 μ g/ml salmon sperm DNA) 50 μ l of an equimolar mixture of five different 5'- and 3'-digoxigenin-labeled antisense or sense oligodeoxynucleotides to *uPAR* mRNA (total concentration: 25 ng/ml in hybridization solution) was added to the slides and incubated at 37°C overnight. The antisense nucleotides (MWG Biotech, Ebersberg, Germany) were complementary to nucleotides 121 to 150, 321 to 350, 521 to 550, 717 to 746, and 918 to 947 of *uPAR* mRNA (according to the EMBL data base accession number X51675). Control slides received the corresponding sense oligodeoxynucleotides. After hybridization, slides were washed in standard saline citrate and blocked for 30 minutes at room temperature (3% bovine serum albumin in Tris buffer). Detection was performed with an alkaline phosphatase-conjugated anti-dig antibody (30 minutes room temperature, 1:600; Roche) and subsequent immersion in Fast Red solution containing 0.1 mmol/L levamisole (Sigma, München, Germany) as chromogenic solution.

Immunohistochemistry

Immunohistochemistry was performed on 2- μ m-thick paraffin sections using a chicken polyclonal antibody (IgY) directed to affinity-purified recombinant human *uPAR*₁₋₂₇₇ expressed in Chinese hamster ovary (CHO) cells reacting with native and denaturated cell-associated *uPAR* antigen¹⁸⁻²⁰ (pAb HU277, 1:100 dilution; kindly provided by Viktor Magdolen and Manfred Schmitt; Klinische Forschergruppe der Frauenklinik, TU-München, Germany). This antibody was used because we found superior sensitivity compared with a commercially available mouse anti-*uPAR* monoclonal antibody (clone 3B10, IgG2a, no. 3936, 1:100 dilution; American Diagnostica, Stamford, CT) when staining the same series (data not shown). Immunostaining was based on an alkaline phosphatase-conjugated streptavidin-biotin detection system (Amersham Pharmacia Biotech Inc., Piscataway, NJ), using Fast Red (Roche) as a chromogen. Antigen retrieval was achieved by microwave treatment (3 $\times$ 5 minutes, 600 W). Incubation of the primary antibody was performed for 60 minutes at 37°C. Negative controls were performed by substituting nonimmune IgG and by preincubation of pAb HU277 with an excess of the CHO-

Table 1. Results of Anti-*uPAR* Immunohistochemistry and *uPAR* mRNA *in Situ* Hybridization in 50 Invasive Ductal Adenocarcinoma of the Pancreas

	Cases (n) with positive staining in			
	Cancer cells	Endothelial cells	Nonneoplastic epithelial cells	Stromal cells
pAb HU277	48	48	8	50
<i>In situ</i> hybridization	50	50	6	50

*uPAR*₁₋₂₇₇ before the staining reaction. For the detection of macrophages, endothelial cells, fibroblasts, myofibroblasts, smooth muscle cells, and lymphocytes, consecutive tissue sections were stained with mAb to CD34, CD68, CD45, smooth muscle antigen (SMA), and to fibroblast antigen (all from DAKO, Hamburg, Germany).

Proliferation and Apoptosis

For detection of proliferating cells mAb anti-Ki-67 (clone SP6; Lab Vision, Fremont, CA) was used. In each case 2000 tumor cell nuclei were evaluated. Apoptosis is achieved through activation of caspases. Recently, it has been shown that cytokeratins, in particular cytokeratin 18, are affected in early events of apoptosis. The mAb M30 (CytoDeath, Roche), which recognizes a specific caspase cleavage site within cytokeratin 18 that is not detectable in native cytokeratin 18 of normal cells, was used to detect early apoptosis in epithelial cells. In each case 2000 tumor cells were evaluated.

Tissue Extraction and Enzyme-Linked Immunosorbent Assay (ELISA)

Pancreas cancer tissue specimens were obtained at surgery and stored at -80°C until extraction. From each tissue specimen frozen sections were stained (H&E) to ensure that only cancer tissue was used. Tissue extraction and ELISA was performed as previously reported.²¹ Briefly, deep-frozen specimens of 300 to 400 mg wet weight were pulverized by a microdismembrator (Satorius, Göttingen, Germany). The resulting powder was suspended in 1.8 ml of TBS (0.002 mol/L Tris-HCl, 0.125 mol/L NaCl, pH 8.5) and 0.2 ml of the nonionic detergent Triton X-100 10% (Sigma) yielding a 1% Triton X-100 final preparation. After gentle stirring for 12 hours at 4°C, the

Table 2. Results of Anti-*uPAR* Immunohistochemistry and *in Situ* Hybridization in Pancreatic Intraepithelial Neoplasia Grade 1, 2, and 3 (panIN-1, panIN-2, panIN-3)

	Cases (n) with positive staining in		
	panIN-1 (n = 28)	panIN-2 (n = 16)	panIN-3 (n = 26)
pAb HU277	4	8	26
<i>In situ</i> hybridization	4	9	26

Figure 1. Immunohistochemical staining of uPAR in pancreatic carcinomas and pancreatic intraepithelial neoplasia. **A:** Anti-uPAR (pAb HU277) immunoreaction in an invasive ductal adenocarcinoma of the pancreas adjacent to nonneoplastic (normal) pancreas tissue. Cancer cells express the uPAR antigen, whereas non-neoplastic acinus cells show only a faint anti-uPAR immunoreaction. **B:** Higher magnification shows strong staining in tumor cells (arrowheads) and endothelial cells (arrows). Macrophages and fibroblasts also stain positive. **C:** Strong uPAR staining in this pancreatic intraepithelial neoplasia grade 1. **D:** Moderate uPAR staining in this example of pancreatic intraepithelial neoplasia grade III. Atypical epithelial cells as well as myofibroblasts (arrows) are stained. Alkaline phosphatase immunohistochemical stainings of paraffin sections.

suspension was subjected to centrifugation ($21,000 \times g$ for 60 minutes, 4°C) to separate cell debris. The total protein content of the extract was measured by using a conventional biuret protein reaction assay (BCA protein assay kit; Pierce, Rockford, IL). We performed an uPA and uPAR ELISA using a commercially available ELISA kit (American Diagnostica, Pfungstadt, Germany) and a conventional ELISA reader (Multiscan EX; Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. The measurements were performed in duplicates. uPA content was determined in the Triton X-100 extract and calculated per mg of tissue protein.

DNA Extraction from Paraffin Material and Quantitative Polymerase Chain Reaction (PCR)

DNA was isolated from selected tumor blocks using the Trizol method. Gene quantification was performed on the ABI Step One Plus PCR system (Applied Biosystems, Foster City, CA). Fifty ng of genomic paraffin-isolated DNA were quantified using Power Syber green master mix (Applied Biosystems) according to the manufactur-

er's specifications. The primers used were *uPAR* forward 5'-TCAGAGAAGACCAACAGGAC-3' and *uPAR* reverse, 5'-GGATGATGATGAGGTTGAGC-3'. The thermal cycling conditions were 10 minutes at 95°C denaturation, followed by 40 repeats of 15 seconds at 95°C , and 1 minute at 60°C annealing. Melting curves were achieved after a denaturation time of 15 seconds at 95°C , 1 minute at 60°C , and 15 seconds at 95°C with a temperature increase of $0.3^{\circ}\text{C}/\text{second}$. Data collection was performed during each annealing phase. During each run, a standard dilution of the DNA with a known quantity was included to permit gene quantification using the supplied software according to the instructions of the manufacturer. In each run a negative control (distilled water) was included. The measurements of gene quantification were taken three times, and the mean of these values was used for further analysis.

Statistical Analysis

Correlations between noncategorical variables were calculated using the Spearman rank sum correlation ($r = \text{Spear-}$

Figure 2. *uPAR* fluorescence *in situ* hybridization (uPAR-FISH) in pancreatic carcinomas and pancreatic intraepithelial neoplasia. **A:** High-level *uPAR* gene amplification (red signals) in this example of an invasive pancreatic carcinoma. α -Satellite DNA probes (green signals) were used to detect the centromeric regions of chromosome 19. Only a few nuclei exhibit a polysomy of chromosome 19. DAPI stain (blue) was used to highlight cell nuclei. **B:** Concomitant high-level *uPAR* gene amplification (red signals) also in the adjacent pancreatic intraepithelial neoplasia grade 3.

Table 3. Results of *uPAR* Gene Fluorescence *in Situ* Hybridization in 50 Samples of Invasive Ductal Adenocarcinoma of the Pancreas and in Tumor Cells of Pancreatic Intraepithelial Neoplasia Grade 1, 2, and 3 (panIN-1, panIN-2, panIN-3)

	PDA (n = 50)	panIN-1 (n = 28)	panIN-2 (n = 16)	panIN-3 (n = 26)
Low level	18	0	7	9
High level	8	0	0	8

man's correlation coefficient). Comparisons between different groups were performed using the Mann-Whitney *U*-test. Correlations between amplification status of the *uPAR* gene, UICC tumor stage, grading, and survival were calculated using the Kaplan-Meier method and tested using Cox-Mantel test. For all tests, a *P* value <0.05 was considered significant.

Results

uPAR Is Expressed in the Majority of Pancreatic Ductal Adenocarcinomas and Their Precursor Lesions

Fifty invasive ductal adenocarcinomas of the pancreas and associated precursor lesions were studied for expression and synthesis of *uPAR* by *in situ* hybridization, immunohistochemistry, and ELISA (Tables 1 and 2). Previous results have suggested that endothelial cells may serve as an internal staining control²¹ and were positive also in all cases analyzed here. By *in situ* hybridization, cancer cells, stromal cells (macrophages, lymphocytes, and fibroblasts), and endothelial cells showed a positive reaction with the antisense probe in all cases. In six cases also normal ductal/ductular and acinus cells showed positive *uPAR* mRNA signals.

By immunohistochemistry, the antibody pAb HU277 stained cancer cells in 48 of the cases (96%) (Figure 1, A–D). In two cases with positive internal controls, no *uPAR*-positive tumor cells were detected. Normal, non-neoplastic ductal/ductular and acinar cells exhibited anti-*uPAR* immunoreactions in eight cases including the same six cases that were positive also on *in situ* hybridization. Tumor cells of all 26 PanIN-3 lesions showed *uPAR* expression on immunohistochemistry and *in situ* hybridiza-

tion. Of the 16 PanIN-2 lesions, 8 cases were reactive on immunohistochemistry and 9 cases showed positive signals on *in situ* hybridization. Of 28 PanIN-1 lesions, 4 cases were positive using both methods (Tables 1 and 2).

In six of eight tissue samples with chronic pancreatitis, small ductules showed faint *uPAR* and *uPA* immunoreactivity. Only in two cases acinar cells revealed weak cytoplasmic *uPA* immunoreaction and no anti-*uPAR* immunoreaction at all. In areas of chronic inflammation and tissue remodeling, however, all cases exhibited a strong anti-*uPA* and anti-*uPAR* immunoreaction in fibroblasts, macrophages, as well as endothelial cells, in good accordance with previous reports.²²

Amplification of the uPAR Gene Is a Frequent Event in Pancreatic Adenocarcinomas and Their Precursor Lesions

Interphase FISH was performed on cancer cell nuclei isolated from 50 invasive adenocarcinomas of the pancreas. Among these cases, 8 (16%) showed high-level amplification of *uPAR* (Figure 2A), 18 cases (36%) an *uPAR* low-level amplification, and 21 cases (42%) no *uPAR* gene amplification (Table 3). Polysomy of chromosome 19 was detected in three carcinomas. *uPAR* gene amplification as detected by FISH was verified by quantitative PCR (qPCR) amplification of genomic tumor DNA in 16 randomly selected cases (*n* = 5 no amplification, *n* = 7 low level, *n* = 4 cases with high-level amplification on FISH) and by *uPAR* ELISA (Figure 3, A and B). There was a significant statistical correlation between the FISH amplification status and the qPCR results (mean copy number in tumors without *uPAR* amplification on FISH 52 ± 44.2 versus 188 ± 295.4 in tumors with low-level amplifications versus 620 ± 317.7 in tumors with high-level amplifications; Spearman test; $r = 0.57$, $P < 0.05$). Moreover, there was also a statistically significant correlation between the FISH amplification status and the *uPAR* protein level on ELISA (all 50 cases included, $r = 0.042$, $P = 0.002$). *uPAR* levels in eight chronic pancreatitis whole tissue extracts intermediate between PDA with and without *uPAR* gene amplifications [*uPAR* (ELISA) in chronic pancreatitis 3.01 ± 1.19 ng/mg versus 2.71 ± 0.5 ng/mg in PDA without gene amplification versus 4.36 ± 2.28 ng/mg in PDA with gene amplification].

Figure 3. Verification of *uPAR* FISH results by quantitative PCR and ELISA. Statistically significant difference between tumors with and without *uPAR* gene amplification as determined by FISH with respect to *uPAR* gene content as assessed by quantitative PCR from genomic tumor DNA (A) and *uPAR* protein as assessed by ELISA (B). Box-whisker plots showing mean, SE, and SD; $P < 0.05$.

Figure 4. Statistical correlation between *uPAR* gene amplification status and survival in patients with pancreatic carcinomas. Significantly shorter survival in patients with tumors with either low- or high-level amplifications of the *uPAR* gene compared with tumors without. Kaplan-Meier-analysis, Cox-Mantel-test, $P = 0.0009$. The difference between the two tumor groups with either low- or high-level gene amplifications was statistically not significant.

The 26 PanIN-3, 16 PanIN-2, and 28 PanIN-1 lesions were also evaluated for *uPAR* gene amplification (Figure 2B). The morphological grading system of PanIN lesions was partially reflected by the *uPAR* gene amplification status: 8 of 26 (31%) PanIN-3 lesions showed high-level amplification of *uPAR*, 9 cases (35%) a low-level amplification. Of the 16 PanIN-2 lesions, none showed a high-level amplification and 7 cases (44%) showed a low-level amplification. No *uPAR* gene amplification was detected in any of the 28 PanIN-1 lesions. Of note, the alterations detected in the PanIN lesions in all cases with no exceptions were identical to the alterations detected in the respective invasive tumor of the same patient. The same eight cases with high-level amplifications in the PanIN-3 lesions showed also a high-level amplification in the invasive carcinoma, and, likewise, the nine cases of PanIN-3 lesions with low-level amplification of *uPAR* showed also an *uPAR* low-level amplification in the invasive carcinoma. All of the seven cases with *uPAR* low-level ampli-

fication in PanIN-2-lesions also showed *uPAR* low-level amplification in the carcinoma (Table 3).

Amplification of the uPAR Gene in Pancreatic Adenocarcinomas Is a Prognostically Adverse Parameter

On Kaplan-Meier analysis (Cox-Mantel test), presence of either a low ($P = 0.017$) or a high level ($P = 0.0009$) *uPAR* gene amplification was an adverse prognostic parameter compared with cases without detectable amplifications (median survival in patients without detectable *uPAR* gene amplifications 24.5 months, patients with low-level amplifications 12.0 months, patients with high-level amplifications 7.5 months) (Figure 4). The difference in mortality between cases with low- and with high-level amplifications was statistically not significant ($P = 0.25$). All other parameters tested (tumor stage, lymph node metastasis, tumor grading) were statistically not significant.

uPA Expression Correlates with Increased Proliferation and Reduced Apoptosis in Pancreatic Adenocarcinomas

Protein extracts from the 50 invasive adenocarcinomas were analyzed for uPA content by ELISA and correlated with proliferation and apoptosis rates of the same tumors as assessed by quantitative immunohistochemistry. The mean uPA expression level in these tumors was 10.46 ± 2.41 ng/mg (mean $\pm$ SEM; range, 0.7 to 25.6 ng/mg). The mean Ki-67-associated proliferation rate was $20.1 \pm 4.3\%$ (mean $\pm$ SEM; range, 3 to 57%). The mean M30-associated apoptosis rate was $14.1 \pm 2.7\%$ (mean $\pm$ SEM; range: 2 to 33%). There was a positive correlation between uPA expression and proliferation ($r = 0.77$, 95% confidence interval (95% CI), 0.61 to 0.87; $P < 0.001$) (Figure 5A) and a negative correlation between uPA expression and apoptosis ($r = -0.41$; 95% confidence interval (95% CI), -0.69 to -0.11 ($P < 0.001$) (Figure 5B). The proliferation level was significantly higher in tumors with high-level amplifications of the *uPAR* gene

Figure 5. Correlation between uPA content measured by ELISA and proliferation and apoptosis rates determined by quantitative immunohistochemistry. **A:** Strong positive correlation between uPA level in tumor tissue extracts and Ki-67-associated proliferation rate [$r = 0.77$; 95% confidence interval (95% CI): 0.61 to 0.87; $P < 0.001$]. Significantly higher proliferation rate in tumors with high-level amplifications of the *uPAR* gene compared with tumors without amplification. **B:** Moderate negative correlation between uPA level and M30-associated apoptosis rate ($r = -0.41$; 95% confidence interval (95% CI): -0.69 to -0.11 ; $P < 0.001$). The difference in apoptosis was not different between tumors with and without *uPAR* gene amplifications.

than in tumors without amplification (Ki-67: 14 ± 9.6 versus 30 ± 14 ; $P < 0.01$), while the rate of apoptosis was not statistically different.

Discussion

The reasons for the aggressiveness and dismal prognosis of pancreatic cancer are still poorly understood. Several studies indicated that the urokinase system plays a central role in the control of extracellular matrix turnover, cell migration, invasion, cell signaling, angiogenesis, and metastasis.²³ A moderate to strong expression of uPA and uPAR in pancreatic cancer has been previously described.²⁴ Our studies using immunohistochemistry and *in situ* hybridization confirm a strong expression of uPAR in virtually all pancreatic carcinomas and their precursors. Previous work from our and other groups demonstrated a high frequency of high-level amplifications of the chromosomal region 19q12-13.2 in pancreatic cancer by using comparative genomic hybridization.^{25,26} Using several complementary approaches, we here show that the *uPAR* gene (located on chromosome 19q13.2) is a likely biological target of these amplification events. In this study, 58% of cases showed either a low or a high level *uPAR* gene amplification. Importantly, *uPAR* gene amplifications were already detectable in precursor lesions and the *uPAR* gene amplification status in invasive carcinomas and the precursor lesions in the same specimens was remarkably homogeneous. Within a given sample, the pattern of high- or low-level *uPAR* gene amplifications was identical in the invasive carcinoma and the precursor lesion in all cases. Moreover, the morphological grading system of PanIN lesions was reflected by the *uPAR* gene amplification status: *uPAR* gene amplifications were observed only in PanIN-2 and -3 lesions and high-level amplifications only in PanIN-3 lesions. This was somewhat paralleled by immunohistochemistry because all of the high-grade precursor lesions (PanIN-3) and a substantial proportion of PanIN-2 lesions showed overexpression of uPAR, whereas PanIN-1 lesions and nonneoplastic tissues were usually negative. Similar results have been described in precancerous lesions of the breast (DCIS).¹⁸ Our interpretation of these observations is that *uPAR* gene amplification is an early event that is obviously not indispensable for carcinogenesis (given the 40% of carcinomas without detectable gene amplifications). Moreover, our findings are difficult to reconcile with a linear or sequential model of carcinogenesis, in which low-grade PanIN lesions acquire several hits and thereby progress to high-grade lesions and finally invasive carcinoma.²⁷ By contrast, our findings fit better to an alternative model in which pancreatic carcinomas develop along different molecular pathways: preneoplastic lesions without *uPAR* gene amplifications appear to progress into carcinomas without *uPAR* gene amplifications rather than acquiring this alteration later in the process of carcinogenesis. In this respect, PDA would resemble the situation in breast carcinomas, in which many clinicomorphological and molecular data suggest that low-grade ductal carcinoma *in situ* (DCIS) tends to

progress to low-grade invasive breast cancer, and high-grade DCIS tends to progress to high-grade invasive cancers.^{28–31}

The detection of *uPAR* gene amplification (low and high level) was the only statistically significant predictor in this analysis and had an adverse prognostic impact, suggesting that *uPAR* gene amplifications identify a particularly aggressive subgroup of PDA. As shown here, tumors with high-level amplifications of the *uPAR* gene showed also a concomitant increase of uPAR protein and increased proliferation. Remarkably, the mean uPAR levels in pancreatic cancers appear much higher than in many other tumors, such as breast carcinomas.³² Increased uPAR protein expression (ELISA) were also observed in cases of chronic pancreatitis comparable with PDA without *uPAR* gene amplification.

Expression of the uPAR ligand uPA was correlated with increased proliferation and reduced apoptosis. These findings suggest that *uPAR* gene amplifications render the tumor more sensitive to the pro-survival and anti-apoptotic signals of uPA. Recent reports showed that uPAR/uPA-induced proliferation involves activation of ERKs and p38 MAP kinases^{11,12} either directly or via release of different growth factors and through dynamic control of cell-matrix interactions. In agreement with previous reports,²⁴ we found strong uPAR expression in tumor-associated macrophages and fibroblasts of the tumor stroma. This increased expression in the stroma as well as in tumor cells implies a specific stromal response to tumor infiltration that may contribute to tumor invasiveness. Another possible mechanism involves tumor neovascularization. We have previously described a good correlation between microvessel density and uPA expression in breast carcinomas.²¹

In summary, our findings show that the urokinase system plays an important role in the biology and clinical behavior of PDA. Amplifications of the *uPAR* gene appear to be an early event in carcinogenesis and may be helpful to identify a distinct molecular pathway of PDA development, associated with a particularly poor prognosis. Interference with the urokinase system may be a promising therapeutic strategy to prolong survival of patients with PDA.

References

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ: Cancer statistics, 2008. *CA Cancer J Clin* 2008, 58:71–96
2. Parkin DM, Bray F, Ferlay J, Pisani P: Global cancer statistics, 2002. *CA Cancer J Clin* 2005, 55:74–108
3. Niedergethmann M, Alves F, Neff JK, Heidrich B, Aramin N, Li L, Pilarsky C, Grutzmann R, Allgayer H, Post S, Gretz N: Gene expression profiling of liver metastases and tumour invasion in pancreatic cancer using an orthotopic SCID mouse model. *Br J Cancer* 2007, 97:1432–1440
4. Dass K, Ahmad A, Azmi AS, Sarkar SH, Sarkar FH: Evolving role of uPA/uPAR system in human cancers. *Cancer Treat Rev* 2008, 34:122–136
5. Duffy MJ, McGowan PM, Gallagher WM: Cancer invasion and metastasis: changing views. *J Pathol* 2008, 214:283–293
6. Hildenbrand R, Gandhari M, Stroebel P, Marx A, Allgayer H, Arens N: The urokinase-system—role of cell proliferation and apoptosis. *Histol Histopathol* 2008, 23:227–236

7. Danø K, Behrendt N, Hoyer-Hansen G, Johnsen M, Lund LR, Ploug M, Romer J: Plasminogen activation and cancer. *Thromb Haemost* 2005, 93:676–681
8. Schmitt M, Harbeck N, Thomssen C, Wilhelm O, Magdolen V, Reuning U, Ulm K, Hofler H, Janicke F, Graeff H: Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. *Thromb Haemost* 1997, 78:285–296
9. Hildenbrand R, Wolf G, Bohme B, Bleyl U, Steinborn A: Urokinase plasminogen activator receptor (CD87) expression of tumor-associated macrophages in ductal carcinoma in situ, breast cancer, and resident macrophages of normal breast tissue. *J Leukoc Biol* 1999, 66:40–49
10. Hildenbrand R, Arens N: Protein and mRNA expression of uPAR and PAI-1 in myoepithelial cells of early breast cancer lesions and normal breast tissue. *Br J Cancer* 2004, 91:564–571
11. Gandhari M, Arens N, Majety M, Dorn-Beineke A, Hildenbrand R: Urokinase-type plasminogen activator induces proliferation in breast cancer cells. *Int J Oncol* 2006, 28:1463–1470
12. Aguirre-Ghiso JA, Liu D, Mignatti A, Kovalski K, Ossowski L: Urokinase receptor and fibronectin regulate the ERK(MAPK) to p38(MAPK) activity ratios that determine carcinoma cell proliferation or dormancy in vivo. *Mol Biol Cell* 2001, 12:863–879
13. Aguirre Ghiso JA, Kovalski K, Ossowski L: Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. *J Cell Biol* 1999, 147:89–104
14. Tang H, Kerins DM, Hao Q, Inagami T, Vaughan DE: The urokinase-type plasminogen activator receptor mediates tyrosine phosphorylation of focal adhesion proteins and activation of mitogen-activated protein kinase in cultured endothelial cells. *J Biol Chem* 1998, 273:18268–18272
15. Ma Z, Webb DJ, Jo M, Gonias SL: Endogenously produced urokinase-type plasminogen activator is a major determinant of the basal level of activated ERK/MAP kinase and prevents apoptosis in MDA-MB-231 breast cancer cells. *J Cell Sci* 2001, 114:3387–3396
16. Liehr T, Grehl H, Rautenstrauss B: FISH analysis of interphase nuclei extracted from paraffin-embedded tissue. *Trends Genet* 1995, 11:377–378
17. Schleger C, Verbeke C, Hildenbrand R, Zentgraf H, Bleyl U: c-MYC activation in primary and metastatic ductal adenocarcinoma of the pancreas: incidence, mechanisms, and clinical significance. *Mod Pathol* 2002, 15:462–469
18. Hildenbrand R, Leitz M, Magdolen V, Luther T, Albrecht S, Graeff H, Stutte HJ, Bleyl U, Schmitt M: Validation of immunolocalization of the urokinase receptor expression in ductal carcinoma in situ of the breast: comparison with detection by non-isotopic in-situ hybridization. *Histopathology* 2000, 36:499–504
19. Magdolen V, Rettenberger P, Lopens A, Oi H, Lottspeich F, Kellermann J, Creutzburg S, Goretzki L, Weidle UH, Wilhelm O, Schmitt M, Graeff H: Expression of the human urokinase-type plasminogen activator receptor in *E. coli* and Chinese hamster ovary cells: purification of the recombinant proteins and generation of polyclonal antibodies in chicken. *Electrophoresis* 1995, 16:813–816
20. Hildenbrand R, Glienke W, Magdolen V, Graeff H, Stutte HJ, Schmitt M: Urokinase receptor localization in breast cancer and benign lesions assessed by in situ hybridization and immunohistochemistry. *Histochem Cell Biol* 1998, 110:27–32
21. Hildenbrand R, Dilger I, Horlin A, Stutte HJ: Urokinase and macrophages in tumour angiogenesis. *Br J Cancer* 1995, 72:818–823
22. Friess H, Cantero D, Graber H, Tang WH, Guo X, Kashiwagi M, Zimmermann A, Gold L, Korc M, Buchler MW: Enhanced urokinase plasminogen activation in chronic pancreatitis suggests a role in its pathogenesis. *Gastroenterology* 1997, 113:904–913
23. Mazar AP: Urokinase plasminogen activator receptor choreographs multiple ligand interactions: implications for tumor progression and therapy. *Clin Cancer Res* 2008, 14:5649–5655
24. Cantero D, Friess H, Defflorin J, Zimmermann A, Brundler MA, Riesle E, Korc M, Buchler MW: Enhanced expression of urokinase plasminogen activator and its receptor in pancreatic carcinoma. *Br J Cancer* 1997, 75:388–395
25. Schleger C, Arens N, Zentgraf H, Bleyl U, Verbeke C: Identification of frequent chromosomal aberrations in ductal adenocarcinoma of the pancreas by comparative genomic hybridization (CGH). *J Pathol* 2000, 191:27–32
26. Harada T, Baril P, Gangeswaran R, Kelly G, Chelala C, Bhakta V, Caulee K, Mahon PC, Lemoine NR: Identification of genetic alterations in pancreatic cancer by the combined use of tissue microdissection and array-based comparative genomic hybridisation. *Br J Cancer* 2007, 96:373–382
27. Furukawa T, Sunamura M, Horii A: Molecular mechanisms of pancreatic carcinogenesis. *Cancer Sci* 2006, 97:1–7
28. Wiechmann L, Kuerer HM: The molecular journey from ductal carcinoma in situ to invasive breast cancer. *Cancer* 2008, 112:2130–2142
29. O'Connell P, Pekkel V, Fuqua SA, Osborne CK, Clark GM, Allred DC: Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. *J Natl Cancer Inst* 1998, 90:697–703
30. Hwang ES, DeVries S, Chew KL, Moore II DH, Kerlikowske K, Thor A, Ljung BM, Waldman FM: Patterns of chromosomal alterations in breast ductal carcinoma in situ. *Clin Cancer Res* 2004, 10:5160–5167
31. Stratton MR, Collins N, Lakhani SR, Sloane JP: Loss of heterozygosity in ductal carcinoma in situ of the breast. *J Pathol* 1995, 175:195–201
32. Duggan C, Maguire T, McDermott E, O'Higgins N, Fennelly JJ, Duffy MJ: Urokinase plasminogen activator and urokinase plasminogen activator receptor in breast cancer. *Int J Cancer* 1995, 61:597–600