Targeted imaging in oncologic surgery : preclinical studies utilizing near-infrared fluorescence and radioactivity
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Citation
Boonstra, M. C. (2017, April 13). *Targeted imaging in oncologic surgery : preclinical studies utilizing near-infrared fluorescence and radioactivity*. Retrieved from https://hdl.handle.net/1887/47856

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Title: Targeted imaging in oncologic surgery : preclinical studies utilizing near-infrared fluorescence and radioactivity
Issue Date: 2017-04-13

Chapter 4

Clinical applications of the Urokinase Receptor (uPAR) for cancer patients

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Current Pharmaceutical Design, volume 17, issue 19, pages 1890-1910, year 2011, issn 1381-6128/1873-4286, doi 10.2174/138161211796718233

ABSTRACT

Since decades the urokinase plasminogen activator (uPA) system has been associated with the invasion of malignant cells. The receptor of urokinase (uPAR) is one of the key players in this proteolytic cascade, because it focuses uPA's proteolytic activity to the cell surface and in addition functions as a signaling receptor. uPAR is highly expressed in virtually all human cancers, suggesting possible clinical applications as diagnostic marker, predictive tool of survival or clinical response, and as a target for therapy and imaging. This review summarizes the possibilities of uPAR in clinical applications for cancer patients.

INTRODUCTION

The urokinase-type plasminogen activator (uPA) system plays an important role in many normal physiological processes in which tissue remodeling is involved (Fig. 1A), including embryogenesis and wound healing [1]. The first association of the uPA system with cancer was found in 1961, before the function and source of uPA were even established [2]. More than 25 years later, Duffy *et al*. suggested that a high tumor tissue level of uPA could be a powerful prognostic marker for survival of breast cancer patients [3]. The less obvious association of over-expression of the uPA inhibitor PAI-1 as prognostic factor of the metastatic potential of breast tumors was made in 1992 [4]. At present, uPA and PAI-1 are actually the first biomarkers that are recommended as level-1 tumor markers by the American Society of Clinical Oncology, as predictors of recurrence and adjuvant chemotherapy response for breast cancer patients [5, 6]. The receptor of uPA (uPAR), the third essential member of this system, was identified in 1985 [7]. The binding of single chain uPA (scuPA or pro-uPA) to uPAR is a pre-requisite for efficient cell surface activation of scuPA into the two-chain proteolytic form (tcuPA), culminating into the activation of plasmin, a powerful proteolytic enzyme involved in extracellular matrix degradation

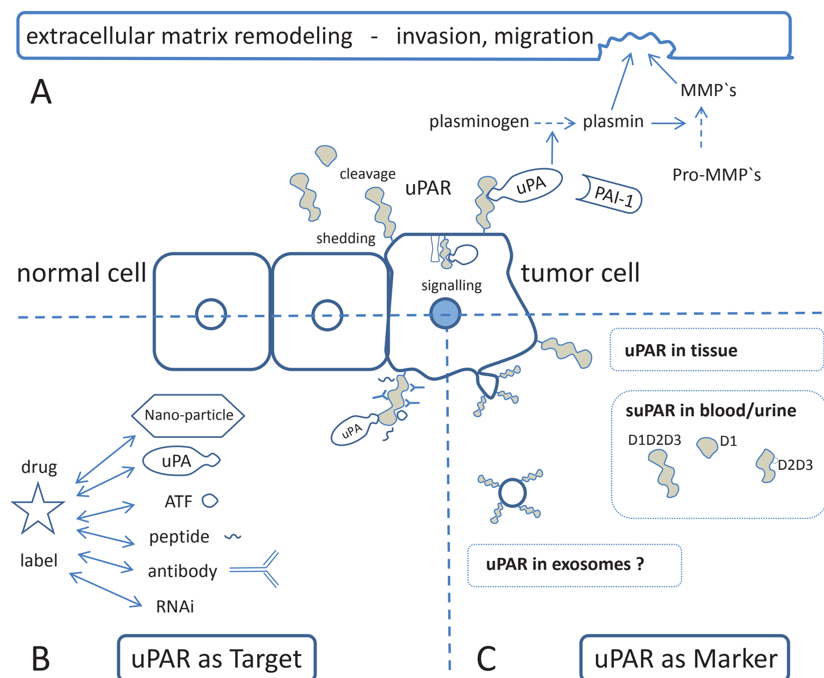

Figure 1 Schematic overview of the urokinase type plasminogen receptor (uPAR) mechanism (A), and uPARs potential clinical role in tumor targeting (B) and as tumor marker (C). ATF: aminoterminal fragment of urokinase,uPA:urokinase plasminogen activator, PAI-1: plasminogen activator inhibitor type 1, MMP: matrix metalloproteinase D: domain of uPAR

[8]. Moreover, ligand occupancy of uPAR by scuPA initiates various signaling pathways, leading to alterations in cell motility and adhesion (Fig. 1A) [1]. The association between uPAR and cancer was recognized in 1991 [9]. Since then, numerous studies have studied the expression of uPAR during carcinogenesis and metastasis, using various techniques, like immuno-histochemistry, iodinated forms of uPA, specific ELISAs and northern blots/ quantitative PCR, see Fig. 2 [10-13]. The majority of studies using tumor and adjacent normal tissues indicate that uPAR levels are enhanced in virtually all investigated cancer types (Table 1). After the discovery of the shedding of uPAR from cell membranes (Fig. 1a) by Ploug *et al*. in 1992 [14], considerable levels of soluble uPAR (suPAR) have been found in blood and urine of various inflammation- associated diseases, including rheumatoid arthritis, AIDS and most, if not all, sorts of cancer, underscoring the possibilities for uPAR as tumor marker [15-19]. In this paper we give an overview of the evidence for the clinical value of uPAR and suPAR in the diagnosis, prognosis, targeted therapy and imaging of cancer.

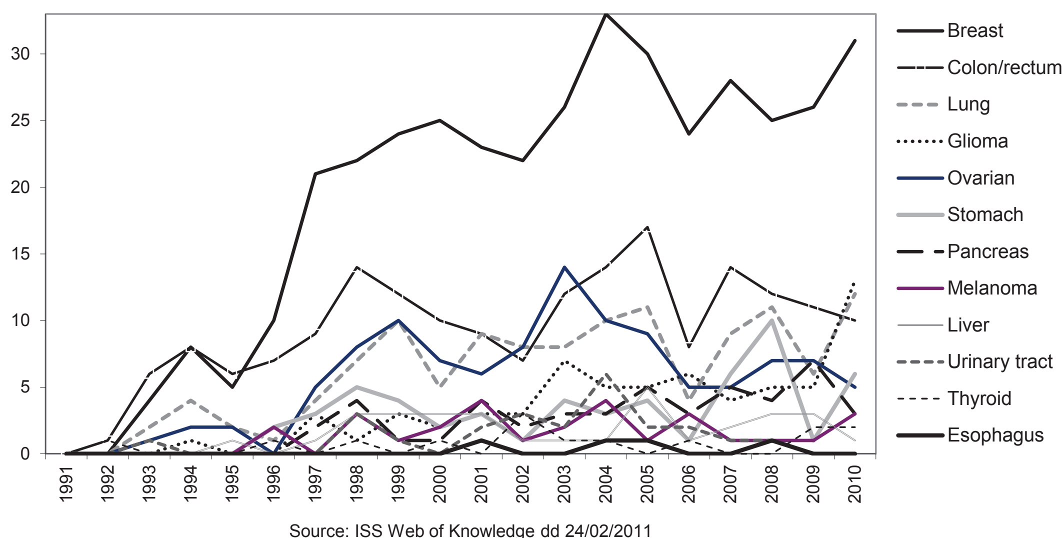

Figure 2 Number of urokinase receptor (uPAR) related publications per cancer type per year (source: ISI Web of Knowledge dd 24/02/2011).

Table 1: Chronical overview of the expression of (s)uPAR in various human cancers and its prognostic value for the patient.

	Technique, (antibody)	Tumor pos/ high	Specific comment	Prognostic	Year	Ref
Bladder						
McGarvey	RT-PCR	29	T/N=2.2	nd	1998	(172)
Nakanishi	IHC(AD3936)	136/154		nd	1998	(173)
Casella	ELISA-quartiles	75/122	urine		2002	(45)
Champelovier	ICC	65/129		OS↓	2002	(174)
Seddighzadeh,	RT-PCR - median	87/175		OS↓	2002	(175)
Shariat	ELISA - median	25/51	urine	ns	2003	(50)
Shariat	ELISA - median	19/38	plasma	ns	2003	(176)
Bhuvarahmurthy	IHC	17/20		nd	2004	(177)
Bhuvarahmurthy	ISH	17/20		nd	2004	(177)
El-Kott	IHC (AD3936)	46/100		nd	2004	(178)
Vivani	IHC (R2)	23/40		nd	2004	(179)
Ecke	ELISA	31/39	serum	nd	2005	(48)
Breast						
Pyke	IHC (R2,R4)	49/51	N: 0/10	nd	1993	(11)
Bianchi	IHC (AD3936)	21/59	N: 0/14	nd	1994	(180)
Carriero	IHC (R2,3936,399)	10/10		nd	1994	(181)
Dugan	ELISA – opt cut	104/134	COP	DFS↓, OS↓	1995	(182)
Grøndahl-Hansen	ELISA –quartiles	252/505	cytosol	DFS↓, OS↓	1995	(183)
Grøndahl-Hansen	ELISA –quartiles	251/505	Triton extract	DFS ns, OS↓	1995	(183)
Costantini	IHC (AD3936)	9/10	6/9	nd	1996	(184)
Kim	IHC (AD3936)	65/104		high relapse	1997	(185)
Kennedy	IHC,	17/36		nd	1998	(186)
Hildenbrand	IHC (HU277)	22/50		nd	1998	(187)
Dublin	IHC	69/117		nd	2000	(188)
Fisher	ISH	21/23	IBC N: 4/5	nd	2000	(189)
Foekens	ELISA	2117/2780		DFS↓, OS↓	2000	(24)
Gong	ELISA - median	134/268	cytosol	OS ns	2000	(190)
Guyton	IHC (AD3937)	28/70	DCIS	DFS↓	2000	(191)
Hildenbrand	IHC (AD3932) IHC (IID7) IHC (HU277) ISH	24/50 39/50 24/50 50/50	DCIS N:46/50 DCIS N:39/50 DCIS N:9/50 N:50/50	nd nd nd nd	2000	(21)
Rha	ELISA - median	80/161		OS ns	2000	(60)
De Witte	ELISA - op cut	439/878		DFS↓, OS↓	2001	(26)
Meijer-van Gelder			♂		2001	(192)
Pacheco	NB - median	40/81		OS↓	2001	(193)
Borstnar	ELISA – opt cut	257/460	IBC	DFS ns	2002	((25)

Table 1: Chronical overview of the expression of (s)uPAR in various human cancers and its prognostic value for the patient. (continued)

	Technique, (antibody)	Tumor pos/ high	Specific comment	Prognostic	Year	Ref
Riisbro			serum/tissue		2002	(194)
Giannopoulou	IHC (AD3932)	104/173	IBC stroma 112/173	DFS↓	2007	(195)
Hurd	IHC (AD3932)	15/60	DCIS N:19/58	nd	2007	(196)
Hildenbrand	IHC (IID7)	28/30	IBC N:5/10	nd	2009	(197)
	IHC (HU277)	27/30	IBC N:4/10	nd		
	IHC (AD3936)	17/30	IBC N:3/10	nd		
	IHC (IID7)	28/30	DCIS	nd		
	IHC (HU277)	20/30	DCIS	nd		
	IHC (AD3936)	19/30	DCIS	nd		
Kotzsch	IHC (IID7)	176/270	IBC	DFS↓	2010	(198)
Brain						
Yamamoto	ISH	12/12	N:0/7	nd	1994	(199)
Yamamoto	RT-PCR	21/27		nd	1998	(200)
Garcia-Monco	ELISA - median	74/148	serum/CSF	nd	2002	(201)
Knappe	IHC	76/84		nd	2003	(202)
Salajegheh	IHC	65/65	No COP	nd	2005	(203)
Colon/rectum						
Ganesh	ELISA – opt cut	13/161		OS↓	1994	(29)
Pyke	IHC (R2,R4)	19/30		nd	1994	(20)
Suzuki	IHC (AD3937)	14/100	adenoma	nd	1998	(31)
	IHC (AD3937)	39/80	carcinoma	nd		
	ISH	30/100	adenoma	nd		
	ISH	68/80	carcinoma	nd		
Abe	ELISA - opt cut	15/90		OS↓	1999	(204)
Stephens	ELISA – median	295/591	plasma	OS↓	1999	(205)
Saito	IHC (PC)	62	stromal cells	nd	2000	(206)
Yang	IHC (AD3936)	7/59		OS↓	2000	(207)
Fernebro	ELISA-median	86/173	blood	OS↓	2001	(208)
Konno	ELISA	31/71		OS↓	2001	(209)
Baker	ELISA	?/101	T/N=4.2	nd	2003	(210)
Seetoo	IHC	29/56		OS↓	2003	(211)
Kim	ELISA	22/22		nd	2006	(212)
Kaneko	IHC	33/101		OS ns	2007	(213)
Illeman	IHC(R2)	14/14		nd	2009	(32)
	ISH	14/14				
Lomholt	TR-FIA	46/77	blood	nd	2009	(214)
		36/77	adenoma	nd		

Table 1: Chronical overview of the expression of (s)uPAR in various human cancers and its prognostic value for the patient. (continued)

	Technique, (antibody)	Tumor pos/ high	Specific comment	Prognostic	Year	Ref
Lomholt	TR-FIA	347/516	Serum	OS↓	2010	(215)
	TR-FIA - D1	114/484	serum	OS↓		
Thurison	ELISA - D1,D2D3	199/298	plasma	OS↓	2010	(66)
Minoo	IHC(AD3936)	`372/811	MMR proficient	OS↓	2010	(34)
Endometrium						
Foca	NB	28/34		nd	2000	(216)
Tecimer	ELISA – median	27/54		ns	2001	(217)
Memarzadeh	IHC(AF807)	35/38		nd	2002	(218)
Nordengren	ELISA – 80th perc.	37/185		DFS ns	2002	(219)
Esophagus						
Hewin	ELISA	11/18	No COP	nd	1996	(220)
Shiomi	IHC (), ISH	14/56		OS ns	2000	(221)
Kidney						
Nakanishi	IHC(AD3936)	71/154		DFS↓,OS↓	1998	(173)
Swiercz	ELISA median	25/52		nd	1998	(222)
	IHC (3936/3937)	16/16		nd		
Bhuvarahamurthy	IHC	13/18		nd	2005	(223)
	ISH	13/18		nd		
Ohba	IHC	34/106		OS↓	2005	(224)
Leukemia						
Plesner	IHC (R2,R4)	12/27		nd	1994	(225)
Lopez-Pedrera	ISH (Mo3f)	9/18		nd	1997	(226)
Lanza	IHC (3B10,VIM5)	60/74		nd	1998	(227)
Mustjoki	IHC(R3,R4)	31/38		nd	1999	(228)
	FACS	10/32	plasma	nd		
Scherrer	NB	6/33		nd	1999	(229)
Mustjoki	ELISA	30/36		nd	2000	(16)
Aref	ELISA	43	T/N=12,4	OS↓	2003	(230)
Graf	FACS	16/53	BM	DFS↓	2005	(231)
Liver						
Morita	ISH	22/31		DFS↓	1997	(232)
	IHC(AD3936)	11/20		DFS↓		
Akahane	IHC	4/32		nd	1998	(233)
De Petro	RT-PCR	23/53		OS ns	1998	(234)
Dubuisson	IHC	6/26		nd	2000	(235)
Zheng	IHC (rbPC)	19/22		nd	2000	(236)
Zhou	ELISA	13/14		nd	2000	(237)
	IHC(AD3937)	11/19		nd		
Schoedel	WB (AD)	13/21	N-, FBL-	nd	2003	(238)

Table 1: Chronical overview of the expression of (s)uPAR in various human cancers and its prognostic value for the patient. (continued)

	Technique, (antibody)	Tumor pos/ high	Specific comment	Prognostic	Year	Ref
Lung						
Pedersen	ELISA - median	42/84		OS↓	1994	(239)
Pappot	ELISA IHC	32/64 49/64	NSCLC	OSns OSns	1997	(39)
Morita	ISh	25		nd	1998	(240)
Ferrier	ELISA	5		nd	1999	(241)
Volm	IHC(3932)	76/129	NSCLC	OSns	1999	(242)
Salden	ELISA – median	44/88		ns	2000	(40)
Jumper	ELISA	22	T/N=1.5	Serum	2002	(243)
Montuori	WB	33/35	NSCLC	nd	2003	(244)
Cobos	ELISA	48	serum T/N=1.8	Nd	2003	(245)
Werle	ELISA - opt cut	19/105	T/N=1.3	OS↓	2004	(38)
Almasi	ELISA D1	32/63	SCC	OS↓	2005	(246)
Almasi	ELISA D1,D2D3	16/32	serum/plasma	OS↓	2009	(64)
Melanoma						
De Vries	IHC (AD3936)	6/11		nd	1994	(247)
Weidle	IHC	25/77		Nd	1994	(248)
De vries	IHC	15/45	all metastases	nd	1995	(249)
Ferrier	ELISA – median	23		nd	1999	(241)
Maguire	ELISA - median	23/45 26/52 13/26 8/16	benign BCC SCC melanoma	nd nd nd nd	2000	(250)
Rømer	ISH	7/14	BCC neg	nd	2001	(251)
Ferrier	IHC	33/85		nd	2002	(252)
Oral						
Nozaki	IHC	10/34		nd	1998	(253)
Lindberg	IHC(R2)	15/20		nd	2006	(254)
Baker	ELISA	38	T/N=8	nd	2007	(255)
Kumamoto	IHC	45/45		nd	2007	(256)
Bacchiocchi	IHC(R2,R4)	74/189	N:0/8	OS↓	2008	(35)
Ovarium						
Casslén	^{125}I assay	10		nd	1991	(10)
Chambers	ELISA	36	ascites	nd	1995	(257)
Chambers	IHC (AD3936)	33/103		nd	1998	(258)
Sier	ELISA - median	48/96	Serum T/N=2.0	OS↓	1998	(59)
Tecimer				DFSns, OSns	2000	(41)
Borgfeldt	ELISA - median	25/51		**OS↑**	2003	(43)

Table 1: Chronical overview of the expression of (s)uPAR in various human cancers and its prognostic value for the patient. (continued)

	Technique, (antibody)	Tumor pos/ high	Specific comment	Prognostic	Year	Ref
Sier	ELISA	12/25	urine	nd	2004	(46)
Wang	IHC(AD3936)	88/100		DFS↓	2009	(44)
Kenny	IHC(ATN615)	82/162		DFSns,OSns	2011	(42)
Pancreas						
Cantero	IHC (AD3936)	24/30		nd	1997	(259)
Harvey	IHC(AD3932)	14/27		ns	2003	(260)
Xue	RT-PCR	46	T/N=5.6	OS↓	2008	(261)
Hildenbrand	IHC(Hu277) ISH IHC(Hu277) ISH	38/70 39/70 48/50 50/50	pan IN pan IN ductal ductal	OS↓ OS↓ OS↓ OS↓	2009	(36)
Prostate						
Wood	ISH	80/117	UAR	nd	1997	(262)
Miyake					1999	(263)
Miyake	ELISA	39/72	serum	OS↓	1999	(264)
McCabe	ELISA-median	8/16	serum	nd	2000	(265)
Gavrilov	IHC ISH	25/25 19/25	all high grade all high grade	nd nd	2001	(266)
Riddick	RT-PCR	?			2005	(267)
Usher	ISH	8/16		nd	2005	(268)
Cozzi	IHC(AD3936)	94/230	N:6/40	nd	2006	(269)
Piironen	ELISA D1,D2+D3	224	Serum T/N=1.1		2006	(67)
Shariat	ELISA - median	214/429	plasma	BP-FSP↓	2007	(270)
Steuber	TR-FIAD1,D2+D3	236	T/N=1.1	nd	2007	(271)
Gupta	IHC	126/230		ns	2009	(272)
Kogianni	IHC(R4)	/169		nd	2009	(273)
Kumano	IHC(COP)	72/163		DFS↓	2009	(274)
Milanese	ELISA	30	serum T/N=1.7	DFS↓	2009	(49)
Thomas	IHC(Z0454)	33/52	BM blood	DFS↓ DFS↓	2009	(275)
Almasi	ELISA D1,D2D3	66/131	serum	OS↓	2010	(65)
Kjellman-quartiles	TR-FIA D1, D2D3	94/375	serum	OS↓	2011	(276)
Sarcoma						
Taubert	ELISA-median	40/80 39/79	Tumor serum	OS↓ OS↓	2010	(277)
Stomach						
Heiss	IHC	132/189		OS↓ DFS↓	1995	(30)
Ganesh	ELISA – opt cut	24/50		OS↓	1996	(278)
Allgayer	IHC	132/189		DFS↓,OS↓	1997	(279)

Table 1: Chronical overview of the expression of (s)uPAR in various human cancers and its prognostic value for the patient. (continued)

	Technique, (antibody)	Tumor pos/ high	Specific comment	Prognostic	Year	Ref
Plebani	ELISA	13/20	COP T/N=2.8	OS↓	1997	(280)
Allgayer	IHC	43/55		OS↓	1998	(281)
Kawasaki	IHC(AD3936) ISH	30/91 19/91		nd nd	1998	(282)
Ho	ELISA	32	plasma T/N=1.6	nd	1998	(283)
Taniguchi	IHC(#39)	38/102		ns	1998	(284)
Migita	IHC (R2)	16/104		ns	1999	(285)
Heiss	IHC(AD3937)	97/105		DFS↓,OS↓	2002	(286)
Kaneko	IHC(AD3936)	63/101		OS↓	2003	(287)
Lee	RT-PCR	24/35	N:23/35	OS↓	2004	(288)
Beyer	IHC(AD3937)	90/102		ns	2006	(289)
Zhang	ISH	70/105		OS↓	2006	(290)
Kita	RT-PCR RT-PCR	431/846 404/846	BM blood	DFS↓ DFS↓	2009	(291)
Alpizar-Alpizar	IHC(R2,rb-pc)	37/67	int+dif	nd	2010	(292)
Testis						
Ulisse	RT-PCR median IHC	7/14 9/10	T/N=6.25 N:3/10	nd nd	2010	(293)
Thyroid						
Kim	IHC (AD3936)	22/62		ns	2002	(294)
Ulisse	WB	13	T/N=4	nd	2006	(295)
Buergy	ELISA	69	T/N= 3.1	OS↓	2009	(296)
Nowicki	RT-PCR	21	T/N= 5.6	nd	2010	(297)
Ulisse	RT-PCR	76		DFS↓	2011	(298)

T/N=ratio tumor vs. normal
OS=overall survival, DFS=disease free survival, ▫ arrows=worse survival
BM bone marrow
IHC=immunohistochemistry, ISH=in situ hybridization, WB=western blot, NB=northern blot, PCR, TR-FIA= time-resolved fluorescence immunoassays
opt cut= optimized cut-point
rb=rabbit
pc=polyclonal antibody
NL=normal liver, FBL=fibrolamellar hepatocellular carcinoma
nd=not determined, ns=not significant,

DIAGNOSIS-PROGNOSIS

Enhanced levels of uPAR in tumor tissues have been demonstrated in numerous studies (Fig. 2). uPAR over-expression is not only associated with malignant cancer cells, but also with stromal cells, like macrophages, neutrophils, fibroblasts and endothelial cells [20, 21]. Clearly, the up-regulation of uPAR in various cell types provides biological advantage to the tumor in various pathophysiological aspects like angiogenesis, invasion, and metastasis. Therefore, uPAR levels are suggested to be associated with the progression of the tumor. Accordingly, the possible use of uPAR as tumor marker has extensively been studied in comparison with traditional diagnostic tools like tumor size, differentiation grade, invasion stage, and the presence of metastasis. By definition, tumor markers represent qualitative or quantitative alterations or deviations from normal, or a molecule, substance or process that can be detected by an assay [22]. Regarding uPAR, most studies have utilized enzyme-linked immunosorbent assays (ELISA), immunohistochemical staining, or mRNA detection to evaluate enhanced tumor uPAR expression (Table 1). Variations in the preparation of the homogenates or detergent extracts, the different procedures of tissue preparation (frozen or paraffin-embedded), and the use of different antibodies complicate the overall comparison of these data. Still, the results of most studies point in the same direction: up-regulation of the expression of uPAR in tumor cells and stromal cells, regardless of the tumor type, which is to some extent reflected in the levels of suPAR in blood and urine (Table 1). The next section summarizes the clinical value of uPAR up-regulation for specific tumor types.

Breast Carcinoma

Breast carcinoma is not only the most common cancer type in women, but also by far the most studied cancer with respect to uPAR (Fig. 2). This is mainly caused by the availability of relatively large collections of breast cancer cytosols. Clinically, lymph node involvement is considered as the most valuable prognostic factor for breast cancer. The additional value of extra markers for the assessment of patients with especially low or high risk has been extensively investigated for uPA and PAI-1 as prognostic indicators [23, 24]. There are, however, remarkably few studies directly comparing the diagnostic or prognostic value of uPA and or PAI-1 with uPAR. In a study of 460 tissue extracts from breast cancer patients, uPAR's prognostic value for 3 years disease free survival (DFS) was found to be less than for PAI-1, but slightly stronger than for uPA. Only PAI-1 turned out to be an independent marker in this cohort [25]. A comparable study, measuring uPAR in 878 patients, found high uPAR levels to be an independent predictor for overall and disease free survival [26], whereas in a previous study with basically the same group of patients uPA as well as PAI-1 were equally predictive [27]. The study design of the latter study is illustrative for the variable results obtained in studies using different materials:

The pellets and cytosols from the same tissue extract do not give comparable prognostic information, indicating that parameters like the extraction method, buffer type and pH, antibodies, etc., influence the outcome considerably. Standardized methods of tissue collection and measurement methods, like have been established for the measurement of uPA and PAI-1 as identification factors for adjuvant therapy after breast surgery, are essential for the evaluation whether the presence of uPAR in tumors could ultimately be used as a diagnostic or prognostic factor [28].

Gastrointestinal Carcinomas

Carcinomas of the gastrointestinal tract, including pancreas and hepatic cancers, cause, after lung cancer, the most cancer-related deaths world-wide. Colorectal and stomach cancer have been studied extensively for the presence of uPAR, but the groups of patients are small compared to those in breast cancer studies (Table 1). Still, the data are very similar, indicating enhanced uPAR levels in the majority of the tumors, associated with a worse survival of the patients [29, 30]. Up-regulated levels of uPAR have been found in premalignant colorectal adenomas, especially those with severe dysplasia, indicating the association with the neoplastic development of tumors [31]. Comparing the expression pattern of uPAR in a small group of primary colon tumors with their liver metastasis revealed 2 distinct uPAR profiles, correlating with specific growth patterns in especially stromal cells, which might have implications for the treatment of metastatic disease [32]. The prognostic significance of uPAR up-regulation in colon cancer has also been recognized in endothelial cells in a group of more than 400 patients [33]. These semi-quantitative immuno-histochemical studies, showing predictive value of enhanced uPAR expression not only in cancer cells but also in other cell types within the tumor, emphasize the use of homogenates/ lysates for diagnostic/prognostic purposes, because of the accumulated overall score of uPAR in this type of assay [29]. A recent RT-PCR study, establishing the prognostic value of enhanced uPAR expression in tumor cells isolated from bone marrow and peripheral blood cells in more than 800 stomach cancer patients, confirms and support these findings [34]. There are few studies published determining uPAR in cancers of the liver, pancreas and mouth and the number of included patients per study are less than 50 (Table 1). In general, uPAR levels are enhanced, but there is no or little association found with survival. More recent studies in oral and pancreatic cancer, using slightly larger groups of patients, were indeed able to identify highrisk groups based on enhanced uPAR expression, comparable with what is found in breast or colorectal cancer [35, 36]. To establish whether uPAR, or in fact any other biological marker, is a predictive tool for these cancer types, multi-center studies are necessary for these cancer types to obtain larger numbers of patients with long time follow up. Based on the experience with breast and gastrointestinal samples, in case of

uPAR the detection method should preferably be ELISAs rather than semi-quantitative immunohistochemical staining.

Lung Carcinoma

Although lung tumor is the most common cancer type, it is relatively infrequently studied with respect to the plasminogen activation system. This is somewhat surprising, because nicotine, the main cause of lung cancer, is shown to stimulate epithelial-mesenchymal transition (EMT) of cancer cells, mediated by the Erk/5-LOX signaling pathway via up-regulation of MMPs, urokinase and uPAR [37]. In the most recent and largest study in NSCLC patients so far, uPAR and PAI-1 were the only independent prognostic indicators amongst 10 immunohistochemically detected parameters, including uPA [38]. Earlier ELISA-based studies in tissue extracts found weak associations with survival [39, 40], suggesting that more studies with larger groups of patients are needed to determine the prognostic value of uPAR for lung cancer patients.

Bladder, Prostate and Ovarian Carcinoma

Bladder, prostate and ovarian carcinoma tissues have been studied for uPAR content (Table 1). In general, uPAR is also upregulated in these cancers, but whether high uPAR levels are prognostically relevant is still under discussion. For ovarian cancers the results are probably the most intriguing, with studies finding respectively no [41, 42], positive, [43] and negative correlation [44] with survival. Because of the different approaches of these studies, these diverse results are difficult to compare or explain. Recently, the research for especially these types of cancers has been focused on suPAR in urine and blood rather than on tumor tissue levels.

Soluble uPAR in Urine and Blood of Cancer Patients

Urine: For obvious reasons the first tumor type for which urine derived suPAR was measured and evaluated for its prognostic value was bladder cancer [15, 45]. Measuring suPAR in urine derived from bladder cancer patients could indeed provide an easy and noninvasive method to determine the state of the tumor. Interestingly, ELISA measurements specific for human uPAR showed the presence of human suPAR in urine from mice xenografted with human ovarian and breast tumors, suggesting that urine suPAR levels reflect the presence of tumor uPAR also in non-bladder cancers [46]. Enhanced urinary suPAR levels have now been detected in patients with bladder, colorectal, ovarian, prostate cancer and leukemia, see Table 1 [15, 16, 45-48]. Preliminary studies showed that the diagnostic sensitivity of suPAR for bladder carcinoma was comparable with other recently established urinary tumor markers [45, 47-50]. The mechanism how uPAR, or other proteins like MMPs, end up in the urine of cancer patients, despite the glomerular barrier, is not solved yet [46, 51]. Recent studies have shown that tumor cell

derived exosomes might be involved. Exosomes are endocytic nanovesicles that are released from cells and are present in urine of patients with urological tract cancers [52]. Tumor cell derived exosomes have been shown to contain tumor associated membrane proteins like CEA, EpCAM, PCNA, and EGFR, but also proteolytic enzymes like MMPs and urokinase [53, 54]. Recently, exosomes have been found in other body fluids like blood, ascites, and saliva and exosomes are now regarded as tools of tumor cells to communicate signals to local and remote cells or tissues [55]. Tumor cell derived exosomes have been shown to 'prepare' sentinel lymph nodes for tumor metastasis [55]. Considering the established relationship between uPAR and (micro)metastases, the discovery of uPAR in exosomes seems only a matter of time (Fig. 1C). Like for tissue determinations of uPAR, there are still some issues that need attention before the value of suPAR for diagnostic/ prognostic purposes can be confirmed. Next to full-size suPAR urine contains several suPAR-derived fragments [46, 56]. The value and detectability of these fragments need to be established before large sample sizes are measured. Comparable results require a general protocol for the sampling time, storage and dilution correction of the urine samples. Furthermore, enhanced suPAR levels could also origin from normal physiological changes like menstruation period or be induced by (temporary) inflammation, for which should be corrected [15].

Blood: Soluble uPAR was first identified in fairly high amounts in plasma and ascites of ovarian cancer patients in 1993 [57]. Soon after, enhanced levels of suPAR were reported in blood from a small group of breast cancer patients compared with healthy controls [58]. Serum suPAR levels were measured in a small group of cancer patients and a preliminary comparison was made with an established diagnostic marker [59]. In the same study, a relation between high pre-operative suPAR levels and worse survival was found, suggesting a possible role of suPAR as a prognostic marker. The measurement of suPAR from blood has obvious advantages compared with tissue derived uPAR. Detection of suPAR from blood is non-invasive and independent from surgery and could therefore be performed before therapy and during follow-up. Whether blood suPAR measurements reflect the tumor tissue levels is still under debate. A study comparing pre-operative suPAR levels in blood from 161 breast cancer patients with the level of uPAR in corresponding tumor tissue homogenates indicated a much stronger correlation for uPAR ($r2=0.61$) than for uPA or PAI-1 [60]. As for the measurement of urinary suPAR, complicating aspects for the clinical use of blood suPAR levels are the expression of uPAR on multiple cell types, the unresolved mechanism(s) of release and the existence of suPAR fragments, see Fig. 1C [61]. Different patterns in the levels of uPAR-fragments have recently been established in blood of patients with various types of cancer [62-65]. Future studies are likely to focus more on the determination of cleaved suPAR fragments rather than full-size uPAR. Because uPA is an important mediator of cleavage, liberated domain 1 of uPAR might be an indicator of enhanced overall uPA activity on the cell surface rather

than just enhanced uPAR levels [66]. The development of more specific and sensitive assays will allow the evaluation of the value of suPAR and its fragments in urine and blood, not only for prognosis of survival, but also as predictors of response to therapy, like for uPA and PAI-1 [64, 67, 68]. The few studies comparing urine and plasma levels of suPAR show that both levels are correlated, but that individual differences exist in overall levels and fragment profile, indicating the complexity of the subject [15, 18, 69]. In conclusion, the determination of uPAR seems to have diagnostic and/or prognostic value, comparable to what has been found for uPA or PAI-1. Like these parameters, uPAR will probably never be used as a single parameter test. uPAR might at best have additional value in multi-parameter assays, like recently has been shown in a cluster analysis for signaling pathways in breast cancer [70]. The value of uPAR measurements from surgically derived cancer tissue as a purely diagnostic tool is limited. However, surprisingly homogenous results have been reported for most tumor types in survival studies. The possibility that uPAR levels could divide patients into groups with a good or bad prognosis is still under investigation. The presence of soluble variant(s) in the circulation, which levels might correlate with the enhanced expression in tumors, underscores the possible use of uPAR as a diagnostic/prognostic tool. Because suPAR seems to be enhanced in a range of inflammatory responses as well, e.g. rheumatic arthritis, and HIV [71], careful interpretation of the results is required. This is similar to the use of several other genes/proteins, discovered via their role in cancer, like BRAF, MYC, RAS, RET, and SRC, as they are recently shown to play a role in inflammation as well [72].

GENETIC BACKGROUND, UPAR SINGLE NUCLEOTIDE POLYMORPHISMS(SNPS)

Single nucleotide polymorphisms (SNP) are DNA sequence variations in a single nucleotide, which are inherited in a Mendelian way and therefore vary between populations. SNPs in coding sequences could affect the protein, leading to truncated or even inactive forms. For uPAR, more than 25 SNPs are identified, including 4 in the promoter region of the gene. These promoter-located SNPs may result in changed transcription efficiencies. Compared with other cancer-associated proteins like MMPs, relatively little research has been performed on the association of the uPAR genotype with cancer [73]. This is probably because the uPAR gene (PLAUR) is located in a relatively stable region, in contrast to uPA/PLAU, with exceptionally high differentiation of allele frequencies [74]. The first cancer-related uPAR-genotyping study determined L317P (rs4760) in a Caucasian cohort of patients with lung cancer [75]. A relation of this coding region-located SNP with overall survival prognosis was not established, but because of the low power of the study, a certain association could not be excluded. In the same study, two SNPs in respectively the PAI-1 and PAI-2 gene were indeed associated with the outcome of

the patients, whereas a SNP for uPA was not [75]. Variations in rs4760 are frequently found in Caucasian populations. The promoter-located uPAR SNP rs344781 variations are rather common in Asia and have been investigated in patients with various cancer types [76- 78]. In a study comprising 102 patients with hepato-cellular cancer (HCC) in a Taiwanese population, heterozygote individuals (T/C) and carriers of the C/C variant had a significant risk (3 fold and 2 fold AOR respectively) for HCC compared with T/T wild-type homozygotes [76]. However, this relation was absent in a study consisting of 253 patients with oral carcinoma by the same authors [77]. Genotype frequencies of variant homozygotes of uPAR were significantly different between 375 non-small cell lung cancer patients and 380 control subjects [78]. Individuals with homozygous uPAR variant CC had lower ORs for NSCLC (adenocarcinoma and squamous cell carcinoma) compared with those carrying wild-type allele (TT or TC genotype). Subjects carrying a T allele genotype had a tendency to develop advanced disease [78]. SNP rs344781 has recently be studied in a large Caucasian population with systemic sclerosis and was found to be associated with vascular complications [79]. The uPAR rs344781 GG gene variant is associated with vasculopathy and impaired angiogenesis, which might influence microvessel densities and could therefore be protective against cancer progression [80]. Next to SNPS, also other genetic variations of the uPAR gene have been described [81]. Determination of a mRNA splice variant of uPAR, (exons 4 and 5) in a group of 43 breast cancer patients revealed a significant association with shorter DFS [82]. In conclusion, the evaluation of the association of uPAR gene variations with diseases like cancer has just begun. Less than a handful of SNPs have been investigated in small groups of cancer patients. The present data are not consistent enough to draw firm conclusions. The determination of uPAR SNPs might turn out to be a valuable tool as predictor, especially in case of uPA(R)-targeted therapy, like has been proposed for other monoclonal antibody -based treatments of cancer [83].

TARGETING OF UPAR - THERAPY

Over-expression of the urokinase receptor on cancer cells and tumor-surrounding stromal cells in a broad range of tumor types makes uPAR a potential and attractive target for therapeutical applications [84-87]. Several strategies are being investigated: Like several other tumor-associated receptors like HER2/neu and EGF, uPAR is used as a plain recognizing tool for tumor cells to deliver anti-cancer agents to evoke cell death. Alternatively, interference with uPA-uPAR interactions blocks the activity of the proteolytic enzyme, hereby down-regulating the ability of the tumor cells to invade and metastasize. For the latter, also the options of gene therapy have been explored. In this section we give an evaluation of the different approaches of using uPAR in a therapeutical setting.

Peptide Antagonists and Fusion Proteins

The first uPAR targeting peptide was developed by isolating the N-terminal fragment of uPA (ATF), containing the first 135 amino acids including the growth factor domain in 1987 (Fig. 3B) [88]. ATF has high affinity for uPAR and competes with uPA, reducing the enzymatic activity *in vitro* and in animal model systems [89- 91]. The possibility to conjugate ATF to a functional moiety has led to several hybrid proteins with different functionalities. ATF linked to a radio-isotope was successfully used for alpha-emitter therapy of advanced ovarian cancers in a nude mouse model [92]. Examples of ATF-based fusion proteins are, HSA-ATF with human serum albumin [93], PAI-2-ATF with human uPA inhibitor PAI-2 [94], UTI-ATF with human urinary trypsin inhibitor [95], BPTI-ATF with bovine pancreas trypsin inhibitor [96], TIMP1-ATF with human inhibitor of MMPs [97, 98], ATF-methioninase [99], and ATFVAS with vasostatin, an inhibitor of angiogenesis [100]. These constructs inhibit specific protein activity localized at the cell surface. ATF has also been conjugated to bacterial or plant cytotoxins like gelonin [101], saporin [102], anthrax [103], diphteria toxin [104], and pseudomonas toxin [105]. Synthetic peptides, based on the growth factor domain (GFD) of uPA or binding specifically to domain D3 of uPAR offer several advantages compared to ATF with respect to size or affinity [106-111] (Fig. 3B). ATF or GFD-based constructs are meant to induce (tumor)cell death after delivery via internalization. The majority of these ATF constructs were tested in a proof-

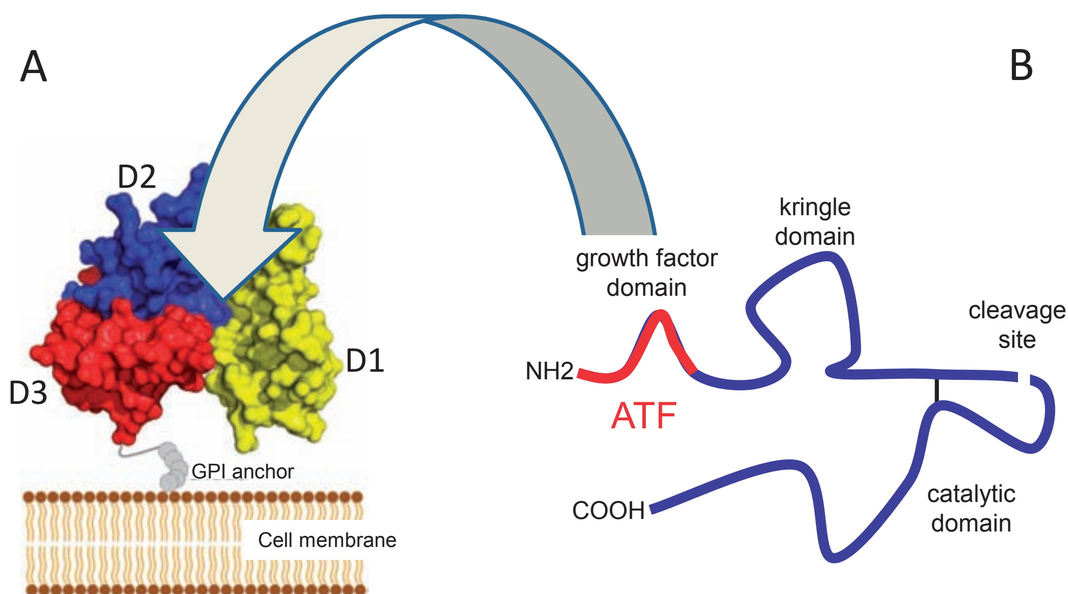

Figure 3 Diagrams of uPAR, urokinase and ATF. A) uPAR is attached to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. The three domains are depicted by numbers D1-D3. The arrow indicates the interaction site with the amino-terminal fragment or the growth factor domain of urokinase. (courtesy of Paola Llinas [171]). B) Schematic overview of urokinase. The amino-terminal fragment (ATF) and the growth factor domain (GFD), relevant for targeting and imaging purposes, are indicated.

of-principle 0like study, using human tumors in animal models. Despite the positive results in most studies, showing tumor regression and dormancy, ATF-constructs have not been clinically evaluated yet. A general difficulty with the testing of ATF and ATF-hybrids in xenograft models and the translation of the results to the clinic is the strong species specificity of the binding between uPA and uPAR [112, 113]. Either the human tumor cells or the stromal cells of animal origin will be targeted, depending on the species of ATF [97]. Obviously, also data from syngeneic animal models cannot be directly translated to the clinic, because findings found in animal cancers do not always reflect the situation in humans [85, 114, 115]. Problems might be expected with the use of ATF-targeted cytotoxins in humans, because of immune responses against the specific toxins as shown with other toxin-fusion proteins [116]. A more specific disadvantage is that AT, like uPA, will only bind unoccupied uPAR, which might be a disadvantage compared to certain antibodies.

Antibodies

More than 200 monoclonal antibodies are being tested in clinical trials, around 20 are FDA approved, and some of these antibodies are commonly accepted as therapeutical intervention, e.g. bevacizumab, cetuximab, gemtuzumab, ibritumomab, panitumumab, trastuzumab. Besides the blockage of the receptor from their ligand, therapeutic antibodies can have natural cell-destructive capabilities via complement activation. Moreover these antibodies can also be used as targeting component in combination with a functional moiety, like a radio-ligand, drug or toxin (Fig. 1B). Large numbers of anti-uPAR antibodies against various epitopes have been developed and tested *in vitro* and in animal models [117, 118]. Antibodies directed against rat uPAR decreased tumor volume by apoptosis in a syngeneic breast cancer model [119]. Recently, positive results were achieved using ATN-658 antibodies against human uPAR in xenografted mice with ovarian, colon and prostate cancer [120-122]. Interestingly, this antibody is not selected for its uPA blocking capacity. This antibody is able to bind to D2D3 of uPAR, even when uPA is bound. A humanized version of the antibody is expected to enter a phase 1 clinical cancer study [122]. Overall, despite encouraging results in animal tumor models, therapeutical applications based on uPAR antibodies are still not available. Complicating factors are uPARs multi-ligand nature [123, 124], the different configurations of uPAR (D2D3 after release of D1), and the possibility of release from the cell (suPAR) [1]. These issues could probably be solved by the choice of uPAR epitope to target. Other difficulties to be solved are the association of uPAR up-regulation with the invasive front of the tumor, which would impede penetration and the enhanced expression of uPAR by a range of stromal cells.

Nanoparticles

An alternative way of delivering therapeutical active moieties to tumors is through targeted particles, which offer great transport capacity, but with rapid uptake and clearance by the liver and spleen and limited penetration in poorly vascularized or necrotic tumor regions [125]. The use of uPAR as a target was initially tested *in vitro* with micro-silica particles coated with uPA and antibodies against uPAR [126]. Recently, smaller nanoparticles directed to uPAR were described. A GFD-derived peptide in a self-assembled liposome was used to deliver DNA to uPAR positive cells [127]. ATF-conjugated multifunctional nanoparticles, detectable with MRI and near infrared imaging and containing a toxin have been developed for the treatment of prostate cancer [128]. Nanorods, rod-shaped gold nanocrystals conjugated with ATF showed similar results as EGFR and integrin $\alpha_v\beta_3$ targeting ligands *in vitro* [125]. These studies indicate the potential of nano particles in cancer treatment, but the data are still preliminary and the possibilities have to be verified further in animal models.

Interventional Gene Therapy

A promising approach to regulate uPAR expression is to block or interfere in the protein synthesis using antisense nucleotide inhibition, RNA interference (RNAi) or other gene-based approaches. An anti-sense uPAR transcript was used for the first time in 1994 to demonstrate reduced invasive potential of a highly invasive cell line *in vitro*, and in the chorioallantoic membrane model system [129].The *in vivo* use of antigene approaches for down-regulating uPAR as a potential therapy for cancer has been extensively reviewed by Pillay *et al*. [130]. Various tumor types have been treated with different approaches and model systems. The reported results were in general positive, ranging from partial reduction of colon, prostate tumors and gliomas to complete inhibition of primary breast tumors or metastases [131-140]. Especially RNA interference has proven to be an efficient method to block uPAR expression [141, 142]. Recent studies have been focusing on combinations with other genes and the effect on of uPAR expression blockage on angiogenesis [143-147]. All together, uPAR interference therapy seems to be a potential approach for cancer treatment. Although inhibition of enhanced uPAR expression by cancer cells, endothelial cells, and tumor-associated fibroblasts will down-regulate tumor development, the effect on uPAR expressing infiltrating cells is largely unknown, but could be opposite. Nevertheless, various preclinical studies with different tumor types show extensive anti-cancer effects suggesting a prompt translation into a clinical setting [130, 148].

TARGETING OF UPAR - IMAGING

Apart from tumor characteristics like stage and differentiation grade, complete surgical removal is pivotal for the prognosis of the cancer patient. Although there are numerous imaging technologies in pre-operative settings available to assess the extent of the tumor, during surgery only ultrasonography can be used. Therefore, surgeons rely on visual inspection and palpation to detect residual disease. As a consequence, the resection margins are not always tumor-free. Despite curative-intended surgery, up to 30% of gastrointestinal cancer patients develop local recurrences as the only site of relapse [149]. The same holds for the surgical treatment of liver metastases from colorectal cancer, for which local recurrences are mainly determined by resection margin status. In breast-conserving surgery for non-palpable lesions, irradical resection rates up to 40 percent are reported. Often secondary surgery is required with associated morbidity. Image-guided surgery (IGS) is the technology where the surgeon intra-operatively is guided by images of the tumor. IGS is based on a fluorescent label which could be conjugated to a tumor targeting determinant [150-153]. Near-infrared fluorescent probes (NIRF) are mostly used because this region of the spectrum offers advantages such as high photon penetration, low autofluorescence and even more important, the NIR spectrum is invisible to the human eye and therefore NIR light will not interfere with the surgical field. The targeting component consists generally of a cell-surface recognizing protein or peptide [154, 155]. In this section we will give an overview of the studies which used uPAR for tumor imaging purposes and evaluate the clinical potential for imaging purposes. Because uPAR is up-regulated in most tumor types and only moderately expressed in normal tissues [1, 156], uPAR is considered a possible candidate for tumor imaging. The principle use of uPAR for primary tumor imaging was shown *in vitro* in human breast carcinomas with 125-Iodine-labeled scuPA in 1994 [157]. One year later, uPAR expression was found on disseminated tumor cells in bone marrow biopsies, suggesting a role for uPAR also as target for micro-metastasis imaging [158]. uPAR plays an important role in lymphatic dissemination of tumor cells and micro-metastases formation, as has been shown for stomach cancers: 67% of metastatic lymph nodes stained strongly for uPAR, with a higher intensity than in the corresponding tumor [159, 160]. Recently, an integrated bioinformatics analysis, using publicly available genomic profiles has elucidated uPAR as one of the most potential imaging targets, next to well known imaging targets like somatostatin receptor, HER2/neu, integrin $\alpha_v\beta_3$, and epidermal growth factor receptor [161]. Like for therapeutic purposes, there are several options to target uPAR: labeled (pro)uPA or uPA-derived fragments (ATF, GFD, peptides, nano particles), or anti-uPAR antibodies or smaller versions. The uPA activatable compounds, not directly targeting the uPA-receptor, will not be discussed.

Labeled uPA, Derived Fragments, and Peptides

The use of labeled full-size or fragments of uPA for imaging implies that mainly unoccupied uPAR will be detected (Fig. 1B). Free and occupied uPAR differ in mobility and localization on the cell membrane [162]. Although clear data about uPAR occupancy in tumors are not available, in most cancers uPAR up-regulation coincides with a rise in uPA and PAI-1. Therefore, in tumors the majority of uPAR is expected to be occupied or even internalized and will not be available for imaging by uPA-based targeting peptides or proteins [163]. Radioactive labeled linear uPA-based peptide binding to human uPAR, labeled were used to image human glioblastoma, breast tumors and intraperitoneal disseminated ovarian tumors in immunodeficient mice using microPET-scan and other detection systems [92, 164, 165]. Analysis of the biodistribution showed high tumor uptake with tumor/background ratios from 4 to almost 10, with rapid elimination from the blood via the renal/urinary route. ATF or uPA-derived peptides are shown to be particularly suited to coat to iron oxide (IO), or other nanocrystals. Imaging studies using these nanoparticles have been performed in orthopically xenografted nude mice with human breast, prostate and pancreas tumors [125, 128, 166, 167]. Because of their high loading capacity, nanoparticles could even be used to carry simultaneously therapeutical as well as imaging components [168]. Another possibility is an uPAR-targeted nano particle consisting of iron oxide, for detection by MRI, carrying a NIR fluorescence probe [166]. The data from these animal models indicate that labeled uPAR peptide antagonists may find application in imaging and therapy of uPAR-expressing cancers in patients. The point discussed in the previous therapeutic targeting section that animal models might not be representative enough for human tumors might be less relevant for imaging [85], because for imaging the issue which cells are exactly targeted is less important, as long as they are present within or directly around the tumor. The use of relatively small uPA-derived peptides offers a number of advantages. First, because of their origin these peptides are minimally immunogenic. Furthermore, the size and weight of a peptide or protein are major determinants of the route of excretion, i.e. via the liver or kidney. Generally, a protein size below 60 kDa, the renal threshold for glomerular filtration, results in clearance via the renal system, accompanied by high accumulation in the kidney. The use of ATF (15kDA) or even smaller peptides would be favorable for the imaging of liver neoplasms, but would be a specific problem for kidney tumors in terms of background. Recent strategies to reduce renal absorption of peptides and antibodies include co-injection of cationic antibodies or gelofusine and will also reduce the accumulation of the imaging ligand in the liver [92, 169].

Antibodies

Because of their large size (150.000 kDa), injected antibodies possess longer half-life and prolonged elimination times than ATF or smaller peptides. Antibodies have the

advantage that they can be targeted to specific epitopes on the uPAR receptor and could for instance be designed to recognize all forms of uPAR (grand total) or particular forms, like complexed to uPA, or fragmented. Dullin *et al*. published recently the use of an uPAR antibody labeled with Cy5.5, to visualize mammary carcinomas in an orthotopic mouse model *in vivo*, showing tumor specificity versus the control antibody [170]. The disadvantage of using relative large antibodies could be reduced or eliminated by using antibody fragments like F(ab)2, Fab or scFv with kDa's of respectively 110, 50 and 45, or camel-based nanobodies (kDa 15). Use of these antibody fragments will decrease liver uptake, reducing background signaling. In summary, despite a long historical interest in the role of the plasminogen activation system and cancer, relatively few studies have been performed using uPAR targeted ligands in animal cancer models for *in vivo* imaging. The preliminary results with ATF and specific uPAR-targeting peptides and antibodies have been encouraging and considering the growing arsenal of peptides and antibodies available we will probably see more of this application in the near future.

CONCLUSION AND PERSPECTIVES

Despite more than 25 years of research, the clinical applications of uPAR for cancer therapy seem still less pronounced than previously expected. This is partly due to the complicated role(s) of uPAR in various biological systems, which are only recently being elucidated. Also the characteristic that enhanced uPAR expression is found on cancer cells as well as tumor-associated stromal cells does not contribute to a fast translation from laboratory findings to the clinic. Still, there are several promising developments that encourage further evaluation of uPAR's role in cancer care. There could indeed be a role for uPAR and/or suPAR as predictive tumor marker(s), probably in a panel with others. Especially for the identification of patients with poor prognosis for neo-adjuvant treatment and, perhaps even more interesting, as predictor of therapy response. The new antibodies and the more specific and sensitive detection techniques which are developed, used in larger groups of patients, will confirm previous research and extend our vision on the possible usefulness of (s)uPAR as biomarker. Also the development of drugs which target tumors via uPAR-recognition has proven its potential in animal models. Especially, because these drugs will not only challenge the malignant cells, but also supporting stromal cells like fibroblasts, macrophages and angiogenic endothelial cells. The presence of uPAR on these cells could be an important advantage for the third application, image guided surgery. The proteins used presently for tumor targeting are either present on cancer cells (CEA, EGFR, EpCAM), angiogenic endothelial cells ($\alpha_v\beta_3$) or tumor-specific stromal cells, like macrophages in necrotic areas; uPAR is highly expressed on exactly those cells.

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