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Assessment of Urokinase-Type Plasminogen Activator and Its Inhibitor PAI-1 in Breast Cancer Tissue: Historical Aspects and Future Prospects

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The Early Days in Fibrinolysis Research

The new Latin word fibrinolysis describes a process whereby a fibrin clot – the product of blood coagulation involving transformation of the liquid plasma protein fibrinogen into solidified fibrin – is lysed by proteolytic enzymes such as plasmin [1]. The serine protease plasmin is generated from the plasma protein plasminogen by the action of plasminogen activators, e.g. the tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA) [2, 3]. The proteolytic activity of plasmin is inactivated by alpha-2-antiplasmin or alpha-2-macroglobulin, that of tPA or uPA essentially by two specific inhibitors, plasminogen activator inhibitor type-1 (PAI-1) and type-2 (PAI-2) [4].
These observations are not new; Astrup described plasminogen activator activity, fibrinolysin, in 1950, which at that time was a term for any of various proteolytic enzymes, especially plasmin, capable of digesting fibrin in the bloodstream [5, 6]. It was only a few years earlier, in 1948, that MacFarlane and Biggs had reviewed previous work on the proteolytic factors of serum since different terms had been applied to identical substances, which had led to confusion [7]. The authors suggested that such names as serum trypsin, serum protease, serum tryptase, fibrinolysin, thrombolysin and others should be abandoned in favour of a more specific designation, and proposed adhering to a nomenclature suggested by Christensen and MacLeod in 1945 [8]. Here, the proteolytic enzyme of plasma is named plasmin, its precursor plasminogen, and its inhibitor antiplasmin. The term fibrinolysin was not recommended since this term was also applied to the streptococcal filtrate known to activate plasminogen in vivo. This filtrate factor became known as streptokinase.
Soon after, in 1951, Lewis and Ferguson [9] reported compounds capable of activating plasminogen in blood; in the same year, Williams [10] demonstrated the presence in urine of a substance (urokinase, uPA) able to activate plasminogen, which was later isolated from urine by Ploug et al. [11]. uPA is present in normal and malignant tissues and plasma as well, a fact which was recognised for ovarian cancer tissues in 1976 by Astedt and Holmberg [12] and a few years later (1982) for plasma by Wun et al. [13] and Tissot et al. [14]. The concentration of uPA in plasma amounts to about 3.5 ng/ml, which is low compared to the relatively high concentration of 200–300 ng/ml in urine. Regarding the second major plasminogen activator, tPA, the prefix 'tissue-type' of tPA refers to the original observation in the 1940s that tPA is present in tissues and tissue extracts [3]. The concentration of tPA in plasma amounts to 5–10 ng/ml, but varies strongly under different physiological and pathological conditions.
Although it was already known before 1970 that plasminogen activator activity may be increased in tumour tissues over non-neoplastic tissue, for some time, interest was turned away from the possible role of plasminogen activators in cancer progression [15–20], particularly because the techniques used then did not distinguish between the two types of plasminogen activators, uPA and tPA [21, 22]. In the years following Astedt and Holmberg's observation that uPA is released by human ovarian cancer cells, several other authors reported elevated uPA concentrations in tumour tissues compared to non-neoplastic tissues [23–26]. These observations prompted several investigators to restart detailed analyses of plasminogen activators, especially uPA, in tumour tissue and blood samples from cancer patients. Due to refined analytical tools and instruments, the structure of uPA, its proteolytic activation and role in the pathophysiology of tumour stroma degradation and tumour spread was investigated. This was also enhanced by the fact that, in 1985, a cell surface receptor for uPA (uPAR; CD87) was detected and it also became clear that the proteolytic activity of uPA and tPA in thrombolysis and fibrinolysis is counterbalanced by inhibitors of tPA and uPA and that uPAR is a focal adhesion point for localised uPA-mediated proteolysis in the physiological and malignant state [27, 28].
In 1966, Brakman et al. [29] described the presence of a plasminogen activator inhibitor in a group of patients with an impaired plasma fibrinolytic system, but it took another 18 years before the inhibitor PAI-1 was isolated [30]. The PAI-1 concentration in plasma is about 20 ng/ml. The other inhibitor, PAI-2, was first detected in human placental tissue [31] and was therefore named placenta-type plasminogen activator inhibitor. Later it became clear that PAI-2 is also present in various types of white blood cells and in tumour tissue [32]. The PAI-2 concentrations in plasma are usually low, but can be high (above 35 ng/ml) in pregnant women.

Clinical Relevance of uPA and PAI-1: Historical Aspects

The fundamental role of the uPA/PAI-1 system in tumour invasion and metastasis has first been derived from correlations between pathophysiological phenomena and tumour tissue-associated proteolytic activity. In essence, the finding was that plasminogen activators

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are linked to degradation and remodeling of normal and cancer tissue and the surrounding extracellular matrix [2, 21, 22]. Concerning the clinical relevance of uPA, in 1985 O'Grady et al. [33] in a first comprehensive report determined total plasminogen activator proteolytic activity, uPA activity, and tPA activity in benign breast tumours and primary breast cancer. Benign tumours contained predominantly tPA activity whereas uPA activity was significantly higher in the malignant tumours compared with the benign ones. Then, in 1988, Duffy et al. [34] reported that patients with primary breast carcinomas containing high levels of uPA proteolytic activity had a significantly shorter disease-free interval than patients with low levels of activity. This finding led the authors to conclude that uPA may serve as a new prognostic marker in breast cancer. In the following years, Jänicke et al. [35, 36] presented evidence of a high correlation between elevated uPA antigen levels in the primary tumour and poor outcome of breast cancer patients, followed by a report of Jänicke et al. in 1991 [37] announcing that the same applies for the inhibitor of uPA, PAI-1, but not for tPA. Since then many independent reports applying antigen measurements (enzyme-linked immunosorbent assay (ELISA)) to quantify uPA and PAI-1 antigen content in cancer tissue extracts have been published describing such a correlation (table 1), not only for breast cancer but also for other solid malignant tumours [2, 38, 39]. These findings were important as such cancer biomarkers were urgently needed for the individualisation of oncologic therapy. Surveying the current literature (National Center for Biotechnology Information (NCBI) MedLine), it is evident that the majority of investigations has been conducted in breast cancer. In the years 1984 to July 2008, 762 articles (141 reviews) were listed in MedLine regarding the clinical relevance of uPA in cancer; for PAI-1, 536 articles (85 reviews). Regarding breast cancer, within that period of time, 336 articles (75 reviews) were listed in MedLine for uPA and 237 articles (48 reviews) for PAI-1. The peak of publications was between 2000 and 2003 (fig. 1).

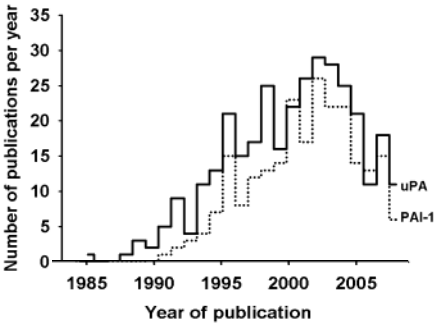

Fig. 1. Number of publications related to uPA/PAI-1 and clinical relevance in breast cancer. A survey was conducted searching the NCBI MedLine database for publications listed between 1984 and July 2008. The first publication dealing with a possible clinical relevance of uPA in breast cancer was published in 1984 by O'Grady et al. [33], followed by a publication of Jänicke et al. in 1991 for PAI-1 [37].

uPA as a Target for Therapy

Given the now known biological importance of uPA and PAI-1 in tissue remodeling, angiogenesis, cell migration, and proliferation [40–42] – not only in the physiological but also in the pathophysiological state – and the clinical relevance of these cancer biomarkers for cancer prognosis and prediction of therapy response, several approaches have been developed targeting the uPA/PAI-1 system in cancer to reduce tumour invasion and metastasis. Such preclinical strategies include the use of antisense oligonucleotides or small interfering RNAs (siRNAs) to silence the uPA gene, antibodies to uPA or uPAR as well as recombinant or synthetic uPA or uPAR analogues to prevent binding of naturally occurring uPA (and uPA:PAI-1 complexes) to uPAR,

Table 1. Publications (n = 90) are listed that focus on the prognostic relevance (disease-free and/or overall survival) of uPA and/or PAI-1 antigen determined by enzymometric assay (ELISA) in breast cancer tissue extracts. Publications encompass breast cancer collectives from a large number of different countries. The principal authors are from 14 different countries: Germany: 21; The Netherlands: 15; France: 14; Denmark: 11; Ireland: 6; Japan: 5; Sweden: 5; Slovenia: 3; Canada: 2; Italy: 2; Switzerland: 2; USA: 2; New Zealand: 1; Spain: 1

Factor	Reference	Year	Country	Number of patients
uPA	Jänicke; Lancet 2:1049	1989	Germany	115
uPA	Duffy; Cancer Res 50:6827	1990	Ireland	166
uPA	Jänicke; Fibrinolysis 4:69	1990	Germany	115
uPA	Reilly; Blood Coagul Fibrinol 2:47	1991	Ireland	160
uPA, PAI-1	Jänicke; Semin Thromb Hemost 17:303	1991	Germany	104
uPA, PAI-1	Foucre; Br J Cancer 64:926	1991	France	87
uPA	Ng; In Vivo 5:313	1991	Canada	63
uPA	Foekens; Cancer Res 52:6101	1992	The Netherlands	671
uPA	Spyratos; J Natl Cancer Inst 84:1266	1992	France	319
uPA, PAI-1	Grondahl-Hansen; Cancer Res 53:2513	1993	Denmark	190
uPA, PAI-1	Jänicke; BCRT 24:195	1993	Germany	247
uPA	Yamashita; Br J Cancer 67:374	1993	Japan	235
uPA	Yamashita; Br J Cancer 68:524	1993	Japan	144
uPA, PAI-1	Bouchet; Bull Cancer 81:770	1994	France	314
uPA, PAI-1	Bouchet; Br J Cancer 69:398	1994	France	314
uPA	Duffy; Cancer 74:2276	1994	Ireland	149
uPA, PAI-1	Foekens; J Clin Oncol 12:1648	1994	The Netherlands	657
uPA, PAI-1	Jänicke; Cancer Res 54:2527	1994	Germany	229
uPA	Romain; Br J Cancer 70:304	1994	France	249
uPA	Duggan; Int J Cancer 61:597	1995	Ireland	141
uPA, PAI-1	Foekens; Cancer Res 55:1423	1995	The Netherlands	1012
uPA, PAI-1	Foekens; J Natl Cancer Inst 87:751	1995	The Netherlands	235
uPA, PAI-1	Grondahl-Hansen; Clin Cancer Res 1:1079	1995	Denmark	505
uPA	Yamashita; Surgery 117:601	1995	Japan	184
uPA	Fernö; Eur J Cancer 32A:793	1996	Sweden	688
uPA, PAI-1	Fersis; GebFra 56:28	1996	Germany	155

Table 1. continued

Factor	Reference	Year	Country	Number of patients
PAI-1	Mayrhofer; GebFra 56:23	1996	Germany	197
uPA, PAI-1	Grondahl-Hansen; BCRT 43:153	1997	Denmark	250
uPA, PAI-1	Grondahl-Hansen; Clin Cancer Res 3:233	1997	Denmark	295
uPA, PAI-1	Schmitt; Br J Cancer 76:306	1997	Germany	314
uPA	Shiba; J Cancer Res Clin Oncol 123:555	1997	Japan	226
uPA	Berns; J Clin Oncol 16:121	1998	The Netherlands	401
uPA	Bouchet; Br J Cancer 77:1495	1998	France	449
uPA	Duffy; Clin Chem 44:1177	1998	Ireland	184
uPA, PAI-1	Eppenberger; J Clin Oncol 16:3129	1998	Switzerland	305
uPA, PAI-1	Harbeck; Anticancer Res 18:2187	1998	Germany	100
uPA, PAI-1	Kim; Clin Cancer Res 4:177	1998	Japan	130
uPA, PAI-1	Knoop; Br J Cancer 77:932	1998	Denmark	429
uPA, PAI-1	Kute; BCRT 47:9	1998	USA	168
uPA	Maguire; Int J Biol Markers 13:139	1998	Ireland	193
uPA, PAI-1	Meijer-van Gelder; J Clin Oncol 17:1449	1998	The Netherlands	1630
uPA	Peyrat; Clin Cancer Res 4:189	1998	France	634
uPA, PAI-1	Thomssen; Anticancer Res 18:2173	1998	Germany	103
uPA	Tetu; Hum Pathol 29:979	1998	Canada	586
uPA, PAI-1	Bouchet; J Clin Oncol 17:3048	1999	France	499
uPA	Broët; Br J Cancer 80:536	1999	France	1245
uPA, uPA:PAI-1	de Witte; Br J Cancer 79:1190	1999	The Netherlands	892
tPA, tPA:PAI-1	de Witte; Br J Cancer 80:286	1999	The Netherlands	865
uPA, PAI-1	Harbeck; BCRT 54:147	1999	Germany	316
uPA, PAI-1	Harbeck; Br J Cancer 80:419	1999	Germany	125
uPA, PAI-1	Harbeck; Int J Oncol 14:663	1999	Germany	112
uPA, PAI-1	Meijer-van Gelder; J Clin Oncol 17:1449	1999	The Netherlands	1630
PAI-1	Billgren; Eur J Cancer 36:1374	2000	Sweden	546
uPA	Ferrero-Poüs; Clin Cancer Res 6:4745	2000	France	488
uPA, PAI-1	Foekens; Cancer Res 60:636	2000	The Netherlands	2780
uPA, PAI-1	Fox; J Pathol 195:236	2000	New Zealand	136
uPA, PAI-1	Harbeck; Int J Biol Markers 15:79	2000	Germany	276
uPA, PAI-1, uPA:PAI-1	Pedersen; Cancer Res 60:6927	2000	Denmark	342
uPA, PAI-1	Harbeck; Clin Cancer Res 7:2757	2001	Germany	276
uPA, PAI-1	Jänicke; JNCI 93:913	2001	Germany	556
uPA, PAI-1	Konecny; Clin Cancer Res 7:2448	2001	USA	587
uPA, PAI-1	Malmstrom; J Clin Oncol 19:2010	2001	Sweden	237
uPA, PAI-1	Meijer-van Gelder; BCRT 68:249	2001	The Netherlands	4114
uPA, PAI-1	Romain; Int J Cancer 95:56	2001	France	237
uPA, PAI-1, uPA:PAI-1	Sten-Linder; Anticancer Res 21:2861	2001	Sweden	233
uPA, PAI-1	Borstnar; Clin Breast Cancer 3:138	2002	Slovenia	460
uPA, PAI-1	Cufer; Int J Biol Markers 17:33	2002	Slovenia	766
uPA, PAI-1	Harbeck; J Clin Oncol 20:1000	2002	Germany	761
uPA, PAI-1	Harbeck; Cancer Res 62:4617	2002	Germany	3424
uPA, PAI-1	Janz; Int J Cancer 97:278	2002	Germany	83
uPA, PAI-1	Look; JNCI 94:116	2002	The Netherlands	8377
uPA, PAI-1	Bouchet; Int J Biol Markers 18:207	2003	France	488
uPA, PAI-1	Cufer; Int J Biol Markers 18:106	2003	Slovenia	460
uPA, PAI-1	Dazzi; Cancer Invest 21:208	2003	Italy	81
uPA, PAI-1	Hansen; Br J Cancer 88:102	2003	Denmark	228
uPA, PAI-1	Schrohl; Mol Cell Prot 2:164	2003	Denmark	341
uPA, PAI-1	Zemzoum; J Clin Oncol 21:1022	2003	Germany	128
uPA, PAI-1	Dorssers; Clin Cancer Res 10:6194	2004	The Netherlands	2593
uPA, PAI-1, uPA:PAI-1	Manders; Cancer 101:486	2004	The Netherlands	576
uPA, PAI-1, uPA:PAI-1	Manders; Cancer Res 64:659	2004	The Netherlands	1119
uPA, PAI-1	Meo; Int J Biol Markers 19:282	2004	Italy	196
uPA, PAI-1	Schrohl; Clin Cancer Res 10:2289	2004	Denmark	2984
uPA	Zhou; Int J Biochem Cell Biol 37:1130	2005	Switzerland	59
uPA, PAI-1	Desruisseau; Br J Cancer 94:239	2006	France	193
uPA, PAI-1	Ryan; Ann Oncol 17:597	2006	Germany	420
uPA, PAI-1	Castelló; Thromb Res 120:753	2007	Spain	70
uPA, PAI-1	Offersen; Acta Oncol 46:782	2007	Denmark	438
uPA, PAI-1	Descotes; Clin Breast Cancer 8:168	2008	France	732
uPA, PAI-1	Linderholm; Breast May:26	2008	Sweden	219
PAI-1	Offersen; Acta Oncol 47:618	2008	Denmark	408

and naturally and synthetic serine protease inhibitors blocking uPA enzymatic activity to reduce tumour cell proliferation, invasion, and metastasis [2, 43–46].

Drug candidates that emerged from synthetic uPA inhibitors shown to be effective in experimental tumour-bearing animals [46] are now in clinical phase I/II testing involving late-stage cancer patients afflicted with tumours of the breast, pancreas, ovary, or the gastrointestinal tract. Goldstein (this issue) reports the promising results of a phase Ib trial with the serine protease inhibitor WX-UK1 in patients with solid tumours, including breast cancer patients, in combination with the chemotherapeutic agent capecitabine. Also, WX-671 (MESUPRON®, an oral pro-drug of WX-UK1) was studied in a phase Ib trial with patients with head and neck cancer (Lang et al., this issue). In July 2008, Wilex AG, Munich, Germany, successfully completed recruitment of a randomised clinical phase II trial of advanced pancreatic cancer patients. Patients were treated with MESUPRON in combination with the chemotherapeutic agent gemcitabine (Gemzar®; Eli Lilly and Company, USA). MESUPRON is currently being tested clinically (phase II) in breast cancer patients in combination with the chemotherapeutic agent capecitabine.

Clinical Impact of uPA and PAI-1 in Breast Cancer

Over the years, substantial efforts have been made in breast cancer to subdivide patient populations into groups that behave differently, so that therapy can be applied more efficiently. Still, since these efforts are based on clinical outcomes related to clinical cancer size and the presence or absence of pathologically involved lymph nodes, subgroups with different biological behaviours cannot be defined correctly [47]. Nonetheless, in the last decade, basic and clinical scientists have studied a plethora of novel cancer biomarkers at the gene and protein level [48, 49]. For breast cancer, several hundreds of such markers have been reported, yet only a handful have actually gained widespread clinical use, including the steroid hormone receptors estrogen receptor (ER) and progesterone receptor (PR), the oncogene HER2, and the tumour invasion factors uPA and PAI-1 [38, 50]. This lack of acceptance, due to controversial test results, at least in part comes from the biological diversity of the breast cancer disease, poorly designed or non-validated clinical studies, non-validated tools and test systems, poor statistics, and/or low quality of the tumour material tested.

The lack of contradictory evidence on the prognostic impact of uPA and PAI-1 is quite unique for any cancer biomarker and is remarkable considering the variety of demographic conditions covered by studies in Europe and abroad [38]. Table 1 depicts key references in which studies are described showing the prognostic impact of uPA and/or PAI-1 in primary breast cancer. It is worth mentioning that uPA and PAI-1 have reached the highest level of evidence (LOE-1) according to the tumour marker utility grading system [51] by fulfilling the criteria of a prospective therapy trial (Chemo-N0) to test the clinical utility of the two cancer biomarkers [52] and by a meta-analysis (pooled analysis) encompassing 8377 patients and published databases from 18 different study centres [53]. Finally, in November 2007, determining the uPA/PAI-1 content in a breast cancer patient's primary tumour tissue was incorporated into the breast cancer treatment guidelines of the American Society of Clinical Oncology (ASCO) to provide for the appropriate adjuvant systemic treatment [50].

It is worth mentioning that no significant correlation was found between plasma and tumour tissue levels of uPA and PAI-1, indicating that determination of these factors in plasma does not reflect their concentration in tumour tissue. Therefore, measurement of uPA and PAI-1 in blood cannot be recommended for assessing prognosis in breast cancer patients [54].

Techniques for the Determination of uPA and PAI-1 in Tumour Tissues

Enzyme-Linked Immunosorbent Assay

We would like to stress that so far, clinically relevant, validated data regarding uPA and PAI-1 in breast cancer have been obtained only by measuring these two cancer biomarkers by ELISA, either in tumour tissue cytosolic fractions or in the detergent-released tumour tissue fraction [54, 55]. Such ELISAs are commercially available (e.g. FEMTELLE®; American Diagnostica Inc., Stamford, CT, USA) and robust enough for clinical routine use. The quality of the test kits is assessed and assured by the European Organization for Research and Treatment of Cancer (EORTC) PathoBiology Group (Brussels, Belgium). Use of the non-ionic detergent Triton X-100 for tumour tissue extraction is recommended since this method of extraction yields considerably more release of uPA antigen than uPA freed into the cytosol fraction. No such difference is observed for PAI-1 [55]. The test can be applied to primary tumour biopsies, core needle biopsies, and cryostat sections. Standard operating protocols for tumour tissue disintegration and uPA/PAI-1 test implementation are published and described explicitly [56, 57]. Therefore, when evaluating other ways of determining uPA and PAI-1 in breast cancer tissue, the ELISA should be considered as the gold standard.

Immunohistochemistry

The use of fixed, archived paraffin-embedded tissue specimens, enabling more widely available determination of uPA and PAI-1, e.g. by applying specific antibodies in immunohistochemistry, is hampered by the fact that both uPA and PAI-1 antigens are presented by tumour cells and surrounding stroma cells and that these biomarkers are released into the tissue as well, making reliable scoring rather difficult. Still, a first comparison of uPA values obtained by ELISA and by immunohistochemical score was already published in 1990 by Jänicke et al. [36]. A statistically significant increase in the uPA values determined by ELISA was noted with increasing staining intensity in immunohistochemistry. Such a correlation was also published for PAI-1 by Reilly et al. [58]. Various antibodies to uPA and PAI-1 generated in animals have been established and tested by different groups; an overview of published work describing distribution of the uPA/PAI-1 antigens in breast cancer tumour tissue specimens is presented in table 2. Work is in progress utilising novel approaches to scan immunohistochemically stained breast cancer tumour tissue specimens by use of a high-resolution virtual microscope (figs. 2 and 3) combined with automatic image analysis systems. The aim of such studies is to provide an alternative to determination of uPA/PAI-1 in tumour tissue extracts by ELISA to allow worldwide quantification of these cancer biomarkers in routinely available breast cancer specimens, including preoperative core needle biopsies.

mRNA and DNA Methylation

Since assessment of uPA and PAI-1 expression status in breast cancer tumour tissues by ELISA requires fresh or fresh-frozen tissue, alternative methods of cancer biomarker analysis using formalin-fixed biopsy material have been investigated. One option is assessment of uPA and PAI-1 marker expression at the transcriptional level. Thus, recently, highly sensitive quantitative reverse transcription-polymerase chain reaction (RT-PCR) assays requiring only small amounts of mRNA and using formalin-fixed tissue specimens as the test material were established by Biermann et al. [59]. Interestingly, for uPA

Table 2. A list of publications is presented dealing primarily with application of antibodies to uPA and/or PAI-1 to localise and/or quantify these cancer biomarkers in breast cancer tumour tissue specimens

Factor	Reference	Year	Country	Breast cancer cases	Comments
uPA	Jänicke; Fibrinolysis 4:69	1990	Germany	115	strong staining; independent prognostic factor for early relapse; ELISA/IHC comparison
PAI-1	Reilly; Int J Cancer 50:208	1992	Ireland	43	ELISA/IHC comparison for PAI-1
uPA	Hildenbrand; Pathol Res Pract 191:403	1995	Germany	42	correlation between microvessel density, angioinvasion and uPA/PAI-1 levels; ELISA/IHC comparison
uPA	Hildenbrand; Br J Cancer 72:818	1995	Germany	42	correlation between microvessel density, vascular invasion, uPA level, macrophage content and proliferation rate; ELISA/IHC comparison
uPA	Hubbard; Eur J Cancer 31A:103	1995	UK	134	overexpression of uPA is not correlated with presence or absence of ERs
uPA	Göhring; Anticancer Res 16:1011	1996	Germany	281	58% positivity for uPA
uPA	Solomayer; Br J Cancer 76:812	1997	Germany	280	uPA-positive tumour: shorter metastasis-free interval; poor prognosis for patients with both TAG12 and uPA positivity, followed by TAG12 positivity; patients with disseminated tumour cells: uPA-positivity correlated with poor prognosis
uPA, PAI-1	Umeda; J Pathol 183:388	1997	Japan	73	uPA associated with poor prognosis
uPA	Kennedy; Br J Cancer 77:1638	1998	Ireland	36	significant correlation between uPA and uPAR staining
uPA, PAI-1	Ferrier; Br J Cancer 79:1534	1999	The Netherlands	28	uPA, tPA, PAI-1 and uPAR expression levels assessed by ELISA or IHC
uPA, PAI-1	Jahkola; Br J Cancer 80:167	1999	Finland	158	fibroblastic PAI-1 expression associated with local recurrence and distant metastasis; diffuse stromal uPA associated with local recurrence
uPA, PAI-1	Dublin; Am J Pathol 157:1219	2000	UK	142	significant association between strong expression of uPA and uPAR and tumour size; in intraductal carcinomas strong expression of uPA, uPAR, and PAI-1
uPA, PAI-1	Schneider; In Vivo 14:507	2000	Spain	189	uPA:PAI-1 positivity correlated with ER expression, PR expression, favourable nuclear grade
uPA	Nielsen; Lab Invest 81:1485	2001	Denmark	31	close correlation between intensity of uPA immuno-staining and uPA protein content measured by ELISA
uPA	Tetu; Cancer 92:2957	2001	Canada	557	uPA positivity associated with poor outcome; high uPA expression was associated with poor nuclear grade and lack of hormone receptor content
uPA, PAI-1	Alvarez-Millan; Oncology 62:286	2002	Spain	28	PAI-1 reflects poor prognosis; uPA:PAI-1 complex accumulation in tumour cells reflects good prognosis
uPA, PAI-1	Zhao; Breast Cancer 9:118	2002	China/Japan	20	PAI-1 in tumour cells associated with histological grade; PAI-1 in stromal cells associated with histological grade and comedo-type necrosis
uPA, PAI-1	Schneider; Br J Cancer 88:96	2003	Spain	212	statistically significant interaction between the presence of uPA:PAI-1 complexes and PR positivity for axillary metastasis
uPA, PAI-1	Castello; Thromb Res 120:753	2007	Spain	70	uPA/PAI-1 mRNA and antigen levels increased in N+ patients
uPA, PAI-1	Haas; Virchow's Archive 452:277	2007	Germany	55	fibroblastic inflammatory reaction around the biopsy channel affects stromal uPA and PAI-1 expression, which may lead to increased levels of uPA/PAI-1 in ELISA
uPA, PAI-1	Minisini; Am J Clin Pathol 128:112	2007	Italy	199	correlation between uPA and ER expression; negative uPA expression associated with negative steroid hormone receptor expression, high tumour grade, and high proliferation index
uPA	Surowiak; Anticancer Res 27:2917	2007	Germany	45	expression of uPA associated with overall and relapse-free survival

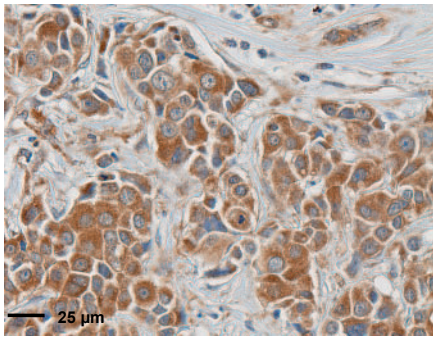

Fig. 2. uPA protein expression in ductal invasive breast cancer. Brown: uPA antigen, blue: haematoxylin (nuclei). Scanned with a Hamamatsu NanoZoomer virtual microscope (Hamamatsu Instruments, Herrsching, Germany) at 400 × magnification. Mouse monoclonal antibody #3689 (American Diagnostica Inc., Stamford, CT, USA). No antigen retrieval applied. Peroxidase-based labeled streptavidin biotin (LSAB) method with chromogen 3,3'-diaminobenzidine (DAB).

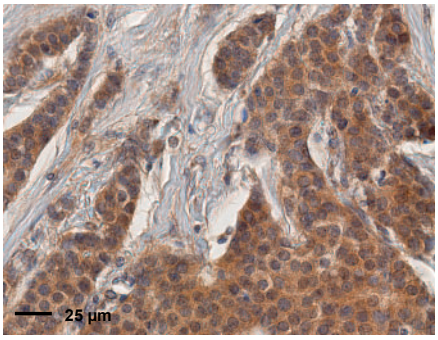

Fig. 3. PAI-1 protein expression in ductal invasive breast cancer. Brown: PAI-1 antigen, blue: haematoxylin (nuclei). Scanned with a Hamamatsu NanoZoomer virtual microscope (Hamamatsu Instruments, Herrsching, Germany) at 400 × magnification. Mouse monoclonal antibody #3785 (American Diagnostica Inc., Stamford, CT, USA). No antigen retrieval applied. Peroxidase-based LSAB method with chromogen DAB.

and PAI-1, when assessing breast cancer cell lines, a significant correlation of transcript and antigen (protein) levels was noted; this, however, did not apply to breast cancer tissue samples, confirming earlier studies in which also no significant correlation of mRNA level with antigen expression was found. Spyratos et al. [60], by assessing breast cancer tissue specimens, found only borderline correlation of uPA antigen with mRNA expression but a significant correlation for PAI-1. Although these results point to the fact that for clinical decision-making the uPA/PAI-1 ELISAs cannot be replaced at present by uPA/PAI-1 mRNA determination, transcription level determination of these cancer biomarkers may have prognostic impact in certain patient subpopulations, for instance by predicting nodal status, malignant transformation, distant metastasis, disease recurrence, or disease-free survival. Significant differences in the topographical distribution of transcription and protein expression levels of uPA and PAI-1 were noted by Castello et al. [61] when comparing uPA/PAI-1 expression levels assessed by in situ hybridisation to those obtained by immunohistochemical analyses. Lamy et al. [62] used a very novel approach, nucleic acid sequence-based amplification (NASBA), and showed high concordance between NASBA and uPA/PAI-1 antigen expression determined by ELISA.

Although still a matter of debate, especially for PAI-1, transcription can also be influenced by genetic factors leading to nucleotide polymorphisms (e.g. 4G/5G) of the PAI-1 gene [63, 64]. In contrast to these studies, Sternlicht et al. [65], screening more than 2500 tumour tissue samples of breast cancer patients, did not find such an association of PAI-1 polymorphism with mRNA levels and frequency differences between tumour and control collectives, and also no association with annual mortality rates between the different allele subsets. Thus, until now, allele assessment of the 4G/5G PAI-1 polymorphism shows no consistent association with clinical factors.

Epigenetics represents an additional level of gene transcription control [66]. Methylation of cytosine residues in so-called CpG dinucleotide repeats in specific gene promoter regions can influence transcriptional activity, associating methylated CpGs with transcriptional silencing of the respective gene. This mechanism is facilitated by a complex machinery of enzymes, including DNA methyl transferases (DNMTs), demethylases, methylated DNA binding proteins (MBDs) and histone-modifying enzymes, linking DNA methylation with transcriptional repressive chromatin status [65]. Since epigenetic markers are DNA based, assessment of DNA methylation markers can easily be carried out in formalin-fixed, paraffin-embedded biopsies using DNA array technology, sequencing, or PCR-based assays. Xing et al. [67] and Gao et al. [68] showed an association of uPA and PAI-1 promoter methylation status with respective mRNA expression in breast cancer cell lines and an association with invasive capacity, which could be modulated by methylating (S-adenosylmethionine, SAM), demethylating (5-azacytidine, decitabine), or histone deacetylase-inhibiting drugs (Trichostatin, TSA). In breast cancer tissues, Paknejshan et al. [69] found a strong correlation of uPA DNA methylation status with its respective mRNA levels and increased demethylation of the promoter region with increasing tumour grading. New approaches for silencing of uPA or PAI-1 transcription involve RNA interference (short hairpin RNA (shRNA), antisense RNA) as demonstrated by Meyret-Figuieres et al. [70], Arens et al. [71], and Ishii et al. [72]. Future breast cancer studies may thus, in addition to uPA/PAI-1 antigen and mRNA measurements, consider assessment of nucleotide modifications and epigenetic variations of the uPA/PAI-1 genes to provide additional clinical information eventually leading to improved management of the breast cancer disease.

Conclusions

The cancer biomarkers uPA and PAI-1 are linked to tumour invasion and metastasis in patients afflicted with solid malignant tumours, such as breast cancer. The prognostic and predictive value of these proteolytic factors was shown in numerous validated retrospective and prospective studies, including a multicentre clinical trial (Chemo-N0). Thus, uPA and PAI-1 were awarded the highest level of evidence, LOE-1, based on the ASCO tumour marker utility grading system. So far, most of the clinically relevant data have been collected by quantitatively determining the uPA and PAI-1 antigens contents in primary breast cancer tumour tissue extracts by certified ELISA tests. Although these tests are highly validated and quality-assured, alternative techniques not requiring fresh-frozen tissue are currently being explored. So far, none of the alternative ways of assessment at the gene or protein level has yielded satisfactory results, but research in this direction is encouraged. In particular, improved immunohistochemistry formats and quantitative assessment of epigenetic modifications of uPA and PAI-1 may provide new tools and vistas to determine these important cancer biomarkers even in small tissue samples such as core needle biopsies or single cells. Comparison of the clinical impact of uPA and PAI-1 in breast cancer to breast cancer mRNA signatures, such as the Amsterdam 70-gene signature (Mammaprint®), the Rotterdam 76-gene signature, the Oncotype DX®, or the H/I signature, have not yet been published [73, 74].

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