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Fibrinolytics, enzyme inhibitors, and cancer survival

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The majority of cancer patients do not die from their primary tumor but from disseminated tumor cells traversing through the body to form distant metastases.[1,2] As one of the initial steps in this process, tumors secrete the vascular permeability factor VEGF that prompts the neighboring microvasculature to become permeable to fibrinogen and to other plasma proteins.[3,4] Extravasated plasma-derived fibrinogen is rapidly cleaved by the serine protease thrombin to generate cross-linked fibrin, a process which also constitutes the final step in the intravascular blood coagulation cascade.[5-8]

In cancer patients, extravascular fibrin forms a provisional extracellular matrix (tumor stroma) and, in the end, this matrix is invaded by macrophages, fibroblasts, and endothelial cells to be replaced finally by granulation tissue and connective tissue.[8,9] This array of events is not exclusive to tumor tissues but occurs in wound healing as well.[4] As fibrin is generated under physiological or pathophysiological situations, in addition to serve as a structural extracellular matrix protein and to support cell adherence and cell migration,[10-12] it binds and thereby activates tPA (tissue-type plasminogen activator), a key serine protease of the fibrinolytic/plasminogen activation system.[13,14] After that, tPA will interact with plasminogen and induce its conversion into the broad-band serine protease plasmin. In turn, plasmin will target fibrin and over time this will lead to the dissolution of the fibrin matrix.[15,16]

Interestingly, in cancer, other members of the fibrinolytic/plasminogen activation system, the urokinase-type plasminogen activator (uPA), its receptor (uPA-R, CD87), and its inhibitor PAI-1 (plasminogen activator inhibitor type-1) come into play and make use of the host plasminogen activation system to promote tumor growth and tumor cell invasiveness.[17-20] In this context it is worth mentioning that CD87 is located in the outer leaf of the plasma membrane of tumor cells, providing the anchor for binding of proteolytically active uPA. By this, focal activation of plasminogen into plasmin is achieved.[21-23] In turn, plasmin converts the zymogen pro-uPA into proteolytically active HMW-uPA. This mode of plasminogen activation is inhibited and thereby counterbalanced by PAI-1, which interacts with CD87-bound HMW-uPA to form the trimeric CD87-uPA-PAI-1 complex which is internalized by the tumor cell, stimulating tumor cell proliferation, adherence, and migration.[24]

Although the key components in these events have been identified and biochemically characterized, the ever growing and prominent role of the fibrinolytic/plasminogen system in various disease states is putting this system into the focus of clinical investigations. In this regard it is worth mentioning that plasmin-dependent pericellular proteolysis of the extracellular matrix surrounding the tumor nests takes part in tumor cell invasion and metastasis.[17,25,26] Especially uPA, its receptor uPAR, and the uPA inhibitor PAI-1 emerged as markers of poor prognosis in patients afflicted with solid malignant tumors.[27-31] In particular, elevation of uPA and/or PAI-1 protein in cancer patients indicates an elevated risk of the patient to experience early disease recurrence (metastases). Thus, shorter survival of these patients compared to cancer patients with low content of these proteolytic factors in their tumor tissue is observed.[17,26,29,32]

Most of the studies having investigated the clinical value of fibrinolytic factors uPA and PAI-1 in cancer patients have been conducted with breast cancer tumor specimens. Some of the key studies, encompassing thousands of patients with primary breast cancer, are depicted in Table 1. Elevation of uPA and PAI-1 in primary breast cancer tissue is not only associated with poor prognosis but is a marker for response/failure to certain cancer therapeutics, too.[33-36] For instance, it was shown in a multicenter clinical breast cancer therapy trial that node-negative breast cancer

Table 1. Key references demonstrating the prognostic relevance of protelytic factors of the fibrinolysis/plasminogen activation system in patients with primary breast cancer.

Author	Year	Country	Patients (N0)		Follow-up (months)	Factor determined	Mode of tissue extraction	Reference
Jänicke	1990	Germany	115	(54)	12.5	uPA	Detergent extract	Fibrinolysis 4:69
Schmitt	1990	Germany	115	(54)	12.5	uPA. tPA	Detergent extract	Blood Coag Fibrin 1:695
Jänicke	1991	Germany	115	(53)	26	uPA, tPA, PAI-1	Detergent extract	Sem Throm Haemost 17:303
Foekens	1992	The Netherlands	671	(273)	48	uPA	Cytosol fraction	Cancer Res 52:6101
Jänicke	1993	Germany	247	(101)	30	uPA, PAI-1	Detergent extract	BCRT 24:195
Foekens	1994	The Netherlands	657	(273)	48	uPA, PAI-1	Cytosol fraction	J Clin Oncol 12:1648
Grøhndahl-H.	1995	Denmark	505	(193)	54	uPA, PAI-1, uPAR	Cytosol fraction	Clin Cancer Res 1:1079
Foekens	1995	The Netherlands	1,012	(460)	71	uPA, PAI-1, PAI-2	Cytosol fraction	Cancer Res 55:1423
Fernö	1996	Sweden	688	(265)	42	uPA	Cytosol fraction	Eur J Cancer 32:793
Eppenberger	1998	Switzerland	305	(159)	37	uPA, PAI-1	Cytosol fraction	J Clin Oncol 16:3129
Kim	1998	Japan	130	(130)	53	uPA, PAI-1	Cytosol fraction	Clin Cancer Res 4:177
Knoop	1998	Denmark	429	(178)	61	uPA, PAI-1	Detergent extract	Br J Cancer 77:932
Kute	1998	USA	168	(168)	58	uPA, PAI-1, uPAR	Cytosol fraction	BCRT 47:9
Bouchet	1999	France	499	(233)	72	uPA, PAI-1, PAI-2, uPA-R	Cytosol fraction	J Clin Oncol 17:3048
de Witte	1999	The Netherlands	865	(434)	100	tPA, tPA:PAI-1	Cytosol fraction + Membrane fraction	Br J Cancer 80:286
Foekens	2000	The Netherlands	2,780	(1,405)	88	uPA, PAI-1, PAI-2, uPA-R	Cytosol fraction	Cancer Res 60:636
Ferrero-Pous	2000	France	488	(226)	120	uPA	Cytosol fraction	Clin Cancer Res 6:4745
de Witte	2001	The Netherlands	878	(39)	100	uPAR	Cytosol fraction + Membrane fraction	Br J Cancer 85:85
Harbeck	2001	Germany	276	(130)	109	uPA, PAI-1	Detergent extract	Clin Cancer Res 7:2757
Jänicke	2001	Germany	556	(556)	32	uPA, PAI-1	Detergent extract	JNCI 93:913
Konecny	2001	USA	587	(283)	26	uPA, PAI-1	Detergent extract	Clin Cancer Res 7:2448
Harbeck	2002	Germany	761	(269)	60	uPA, PAI-1	Detergent extract	J Clin Oncol 20:1000
Harbeck	2002	Germany	3,424	(1736)	83	uPA, PAI-1	Detergent extract	Cancer Res 62:4617
Look	2002	European study	8,377	(4,676)	79	uPA, PAI-1	Cytosol fraction Detergent extract	J Natl Cancer Inst 94:116
Luqmani	2002	Kuwait	145	(72)	48	uPA, tPA, PAI-1	Cytosol fraction	Oncol Rep 9:645
Bouchet	2003	France	488	(226)	120	uPA, PAI-1	Cytosol fraction	Int J Biol Markers 18:207
Hansen	2003	Denmark	228	(101)	144	uPA, PAI-1	Detergent extract	Br J Cancer 88:102
Look	2003	European study	8,377	(4,676)	79	uPA, PAI-1	Cytosol fraction Detergent extract	Thromb & Haemost 90:538
Pedersen	2003	Denmark	164	(164)	102	uPA, PAI-1	Detergent extract	Eur J Cancer 39:899
Schrohl	2003	Denmark	341	(164)	102	uPA, PAI-1	Detergent extract	Mol Cell Proteomics 2:164
Zemzoum	2003	Germany	128	(128)	126	uPA, PAI-1	Detergent extract	J Clin Oncol 21:1022
Desruisseau	2004	France	193	(94)	94	uPA, PAI-1	Cytosol fraction	Int J Cancer 111:733
Dorssers	2004	The Netherlands	2,593	(1311)	96	uPA, PAI-1	Cytosol fraction	Clin Cancer Res 10:6194
Manders	2004	The Netherlands	576	(576)	61	uPA, PAI-1, uPA:PAI-1	Cytosol fraction	Cancer 101:486
Manders	2004	The Netherlands	1,119	(594)	59	uPA, PAI-1, uPA:PAI-1	Cytosol fraction	Cancer Res 64:659
Meo	2004	Italy	196	(196)	65	uPA, PAI-1	Cytosol fraction	Int J Biol Markers 19:282
Zhou	2005	Switzerland	56	(56)	52	uPA	Cytosol fraction	Int J Biochem Cell Biol 37:1130

Cytosol fraction: Mechanical disruption of tumor tissue yields the cytosol fraction in the supernatant of the subsequent centrifugation ; Detergent extract: Mechanical disruption of tumor tissue in the presence of non-ionic detergent yields the detergent fraction in the supernatant of the subsequent centrifugation.

patients stratified by elevated uPA and/or PAI-1 do benefit from adjuvant chemotherapy, indicating that cancer patients with low uPA and/or PAI-1 should be spared the burden of chemotoxic therapy.[33] Surprisingly, the finding that both uPA and PAI-1 are indicators of poor prognosis in cancer patients is in contrast to the known, classical role of the inhibitor PAI-1 to block uPA enzymatic action. This feature may be explained by the additional, multifunctional roles of uPA and PAI-1 in cell adherence, cell motility, cell signalling, and cell proliferation.[37-41]

The involvement of fibrinolytic factors in different pathophysiologies can either be direct or by (in)activation of other proteins as well as by degradation of the extracellular matrix. Understanding the pathologies associated with (anti)proteolytic action, especially the contribution of the fibrinolytic system to the disease states, and developing novel antiproteolytic therapeutic approaches is now possible owing to the availability of new biochemical and molecular biological tools and new therapeutics. Potentially, the course of the malignant disease can be altered by pharmaco-

Table 2. Diagnostic and prognostic relevance of tissue kallikreins in breast cancer assessed by measuring tissue kallikreins in tumor tissue (mRNA and/or protein).

Kallikrei n	Method	Clinical impact and clinical applications	References
KLK5	Q-RT-PCR[4]	Unfavorable prognosis · overexpressed in pre/perimenopausal, node-positive patients with ER-negative tumors · independently associated with decreased DFS and OS · independent indicator of shorter DFS and OS in node-positive patients with large tumors · associated with shorter DFS in patients with low grade tumors	Yousef, Clin Chem 48:1241; 2002
KLK7	RT-PCR	Unfavorable prognosis · gene expression significantly lower in breast cancer patients of low stage (I/II) and patients with positive progesterone receptors	Talieri, Thromb Haem 91:180; 2004
KLK9	Q-RT-PCR	Favorable prognosis · overexpressed in patients with early stage disease and small tumors · independently associated with increased DFS and OS · independent indicator of prolonged DFS and OS in patients with ER and PR-negative tumors	Yousef, BCRT 78:149, 2003
hK10	ELISA	Predictive value · higher hK10 levels independently associated with a poor response to tamoxifen therapy	Luo, Br J Cancer 86:1790; 2002
KLK13	Q-RT-PCR	Favorable prognosis · overexpressed in older, oestrogen receptor positive patients · associated with a prolonged DFS and OS · independent indicator of longer DFS and OS in node-, ER- and PR-positive patients with low grade tumors	Chang, Br J Cancer 86:1457; 2002
KLK14	Q-RT-PCR	Unfavorable prognosis · overexpressed in patients with advanced stage disease · independently associated with a shorter DFS and OS · independent indicator of DFS and OS in patients with positive nodal status, larger ER and PR-positive tumors	Yousef, Br J Cancer 87:1287, 2002
KLK15	Q-RT-PCR	Favorable prognosis · overexpressed in node-negative patients · independently associated with a longer DFS and OS · independent indicator of longer DFS and OS in	Yousef, Br J Cancer 87:1294, 2002

logical intervention of the system resulting in the *in vivo* inhibition of plasmin and uPA by administering novel-types of synthetic serine protease inhibitors.[42-49] WX-UK1, a derivative of 3-aminophenylalanine in the L-conformation with inhibitory antiproteolytic properties, is a novel small-size synthetic inhibitor directed to serine proteases such as uPA and plasmin.[43,44] The exceptional profile of action and the safety data obtained from an *in vivo* rat breast cancer model revealed that the metastasis inhibiting effect of WX-UK1 was highly statistically significant and is not associated with side effects normally observed when applying cytotoxic cancer therapeutics, prompting the start of clinical trials with human cancer patients. Because of the different modes of action, combination of WX-UK1 with other kinds of cancer therapeutics is feasible and could be of benefit for the cancer patient. Therefore, a series of phase I/II clinical trials involving stand-alone therapy with WX-UK1 or combination therapy of WX-UK1 with the 5-fluorouracil pro-drug capecitabine (Xeloda®) have been started in several European countries and the USA (*http://www.pancreatica.org/full_articles/f2002_10_14.html; http://www.medicalnewstoday.com/index.php?newsid=8684; http://www.lifescience.de/portal/news_detail,6647,,34593,detail.html*).

To-date, about 180 human serine proteases, accounting for 32 % of the total proteases encoded by the human genome, have been identified 50. Of special interest in cancer research is the recent discovery

Table 3. Diagnostic and prognostic relevance of tissue kallikreins in ovarian cancer assessed by measuring tissue kallikreins in tumor tissue, serum, and/or ascites (mRNA and/or protein).

Kallikrein	Technique	Diagnostic value	Prognostic value	Reference
KLK4	Q-RT-PCR	yes	unfavorable	Obiezu, Clin Cancer Res 7:2380, 2001
hK4	Immunohistochemistry		favorable	Dong, Clin Cancer Res 7:2363, 2001 Davidson, Am J Clin Path 123:360; 2005
KLK5	RT-PCR	yes	unfavorable	Kim, Br J Cancer 84:643, 2001
hK5	ELISA			Dong, Clin Cancer Res 9:1710, 2003 Yousef, BBA 1628:88, 2003- Diamandis, Tumor Biol 24:299, 2003
KLK6	Q-RT-PCR	yes	unfavorable	Tanimoto, Tumor Biol 22:11, 2001
hK6	ELISA			Hoffman, Br J Cancer 87:763, 2002 Diamandis, JCO 21:1035, 2003 Diamandis, Clin Biochem 33:579, 2000
KLK7	Q-RT-PCR	yes	unfavorable	Dong Clin Cancer, Res, 9:1710, 2003 Kyriakopoulou, Clin Biochem 36:135, 2003 Tanimoto, Cancer 86:2074, 1999
KLK8	RT-PCR	yes	favorable	Magklara, Clin Cancer Res 7:806, 2001
hK8	ELISA			Shigemasa, Oncol Rep 11:1153, 2004
KLK9	Q-RT-PCR	yes	favorable	Yousef, Cancer Res 61:7811, 2001,
hK10	ELISA	yes	unfavorable	Luo, Clin Cancer Res 7:2372, 2001 Luo, Cancer Res 63:807, 2003 Shvartsman, Gyn Oncol 90:44, 2003 Luo, Clinica Chimica Acta 306:111, 2001
hK11	ELISA	yes	(un)favorable	Borgono, Int J Cancer 106:605, 2003 Diamandis, Cancer Res 62:295, 2002 Diamandis, Clin Biochem 37:823, 2004 Shigemasa, Clin Cancer Res. 10:2766, 2004
hK13	ELISA	yes	favorable	Kapadia, Clinical Chem 49:77, 2003 Scorilas, J Clin Oncol 22:678, 2004
KLK14	Q-RT-PCR	yes	favorable	Yousef, Am J Clin Pathol 119:346, 2003
hK14	ELISA			Borgono, Cancer Res 63:9032, 2003 Yousef, Cancer Res 61:3425, 2001
KLK15	Q-RT-PCR	yes	unfavorable	Yousef JCO 21:3119, 2003.

of all 15 members of the human tissue kallikrein family of genes (KLK1-15), located on chromosome 19q13.4 and belonging to the S1A subfamily of serine proteases 50-53. With the exception of hK4, all tissue kallikreins have a pro-peptide ending in Lys or Arg, suggesting that these zymogens are activated by enzymes with trypsin-like activity. Intriguingly, tissue kallikreins do activate the pro-enzyme form of uPA to generate proteolytically active HMW-uPA suggesting a role of the tissue kallikreins in a proteolytic zymogen activation cascade similar to that of the blood clotting system or the complement system 51,54-56.

Tissue kallikreins, often at low levels of expression, are found in several different organs.[50-53] Three of the human kallikreins (KLK1-3) are called classical kallikreins because of their earlier discovery; the 12 kallikrein genes (KLK4-15) discovered during the last few years are termed *new* kallikreins. Plasma kallikrein (KLKB1), located on chromosome 4 and expressed solely in the liver, is different from the tissue kallikreins. It is involved in blood clotting, fibrinolysis, inflammatory reactions, and regulation of blood pressure. In fact, apart from plasma kallikrein, none of the 15 tissue kallikreins except the classical hK1 have appreciable kininogenase activity.

With the identification and characterization of all

members of the tissue kallikrein gene family, accumulating reports started about 4 years ago to indicate that in addition to the classical tissue kallikreins hK1-3, the *new* tissue kallikreins hK4-15 might also be related to hormonally regulated malignancies such as that of the prostate, testis, breast, and ovary 50,51. Generally, in contrast to their upregulation in ovarian cancer, tissue kallikrein genes and proteins are down-regulated in cancer of the breast, prostate, and testis. A number of tissue kallikreins are also differently expressed in other types of cancer, e.g. in squamous-cell carcinoma, in lung adenocarinoma, acute lymphoblastic leukaemia, and in cancer of the pancreas, head, and neck.[50,51]

In prostate cancer, the *new* tissue kallikreins KLK4, 5, 10, 11, 14, 15 have been characterized at the mRNA level; hK4 and hK11 have been investigated at the protein level as well. Among the classical tissue kallikreins, hK2 and hK3 (PSA, prostate-specific antigen) are well known marker for the diagnosis, monitoring, and prognosis of prostate cancer patients. KLK5 and KLK11, determined at the mRNA level emerged as markers to predict a favorable prognosis 50,51. In testicular cancer, mRNA studies only have been conducted so far demonstrating down-regulation of KLK5, 10, 11, 13, 14 compared to normal testis tissue.[50] Regarding breast cancer (Table 2), seven out of the 12 *new* members of the human tissue kallikrein family are of prognostic/predictive value in breast cancer as assessed by (Q)-RT-PCR, except for hK10 expression, which was determined by ELISA. Among those tissue kallikreins determined by (Q)-RT-PCR, three, encompassing tissue kallikreins KLK9, 13, and 15, are markers of favorable prognosis; KLK5, KLK7, and KLK14 identify breast cancer patients with unfavorable prognosis. Tissue kallikrein hK10 is of predictive value; higher hK10 protein levels are associated with a poor response of breast cancer patients to tamoxifen therapy.

Eleven out of the 12 *new* members of the human tissue kallikrein family are of diagnostic/prognostic value in ovarian cancer (Table 3). Among these, 6 tissue kallikreins, encompassing tissue kallikreins hK4, 5, 6, 7, 10, 15, are markers of poor prognosis. Increase of tissue kallikreins hK8, 9, 11, 13, 14 identify ovarian cancer patients with a favorable prognosis. Tissue kallikreins hK4, hK6, and hK10 are highly expressed in serous epithelial ovarian tumors whereas higher expression of tissue kallikreins hK5, hK11, and hK13 is more frequently found in non-serous tumors. These data suggest that certain tissue kallikreins may be employed as determinants of prognosis in the subgroups of ovarian cancer patients, stratified by histiotype. Seven tissue kallikreins have been determined by ELISA (hK5, 6, 8, 10, 11, 13, 14), eight by RT-PCR (KLK4, 5, 6, 7, 8, 9, 14, 15), one by immunohistochemistry (hK4). These findings support the assumption that some of the tissue kallikreins are directly involved with cancer progression and metastasis. Tissue kallikreins therefore may represent not only novel tumor biomarkers but may also serve as promising therapeutic targets in cancer.

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