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# THE PROGNOSTIC SIGNIFICANCE OF UPA, UPAR AND THE CYTOKINE IL-1α IN URINARY BLADDER CANCER

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# ABSTRACT

Tumor invasion and metastasis are the major causes of cancer-related death. Proteases are important for the invasive and metastatic propensities of tumors. We have studied the mechanism underlying endogenous expression of the serine protease urokinase plasminogen activator (uPA) by human breast cancer cells. The expression of uPA mRNA was found to be affected by manipulation of the activity of the MEK-ERK signaling pathway. A minor portion of total ERK was phosphorylated (and hence active) in the MDA-MB-231 breast cancer cell line. Decreased ERK activity was associated with decreased uPA expression, whereas stimulation of ERK activity did not lead to an increase in uPA expression. In contrast, increased ERK activity was found to lead to increased expression of the cyclin kinase inhibitor p21Cip1. These data suggest that ERK activity in these cells is tuned to a level that allows rapid cell proliferation and high levels of uPA expression

Urinary bladder carcinoma is heterogeneous in nature and can follow distinct clinical courses. More than fifty percent of all patients with muscle invasive bladder tumors that develop metastasis die from their disease. It is important to identify factors that can predict the prognosis of patients with muscle invasive tumors, both to be able to offer a more individualized treatment strategy and also to be able to develop therapeutic methods that can target these factors. In a prospective study we found that the expression of uPA and its receptor (uPAR) predict the outcome of patients with bladder cancer and that elevated levels of uPAR were associated with an increased risk for development of metastasis. Moreover, we observed that high uPA expression was associated with an increased risk of death from cancer in the group of patients with muscle invasive tumors. The risk associated with elevated uPA was lower in cystectomized patients, suggesting that treatment improved the prognosis. Elevated uPAR expression did not show this pattern.

Interleukin-1α (IL-1α) is a multifunctional cytokine with many potential roles in cancer. One such role is that of inducing expression of uPA in the tumor stroma. IL-1α has been shown to be expressed by bladder cancer cell lines and has also been shown to inhibit cell proliferation and angiogenesis *in vitro*. We observed that bladder tumor cells produce IL-1α but did not observe any correlation to uPA expression. Interestingly, low IL-1α expression was associated with a relative risk of 1.76 for death in cancer in the group of patients with muscle invasive tumors. The results indicate that uPA, uPAR and IL-1α can be used as markers for refined staging of tumors. Inhibition of the uPA system and/or treatment of patients with IL-1α may be considered as future therapy of urinary bladder cancer.

**Keywords: urinary bladder cancer, urokinase, urokinase receptor, interleukin-1**

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*To My Family*

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| AP-1 | Activating protein-1 |
| ATF | Activating transcription factor |
| BCG | Bacillus calmette-guerin |
| Bcl-2 | B-cell lymphoma/leukemia-2 |
| bFGF | Basic fibroblast growth factor |
| CCDNI | The gene encoding the cyclin-D1 protein |
| CIS | Carcinoma *in situ* |
| ECM | Extracellular matrix protein |
| EGF | Epidermal growth factor |
| ER | Estrogen receptor |
| ERK | Extracellular signal-regulated kinase |
| FAK | Focal adhesion kinase |
| FAM | 6-carboxy-fluorescein |
| GAPDH | Glyceraldehyde 3-phosphatase dehydrogenase |
| GPI | Glycosyl phosphatidyl inositol |
| ICE | Interleukin converting enzyme |
| IL | Interleukin |
| JNK | c-Jun N-terminal Kinase |
| MAPK | Mitogen activated protein kinase |
| MEK | MAPK/ERK kinase |
| MMP | Matrix metalloproteases |
| CDKN2A | Cyclin dependent kinase inhibitor 2A |
| CDKN2B | Cyclin dependent kinase inhibitor 2B |
| PAI | Plasminogen activator inhibitor |
| PCNA | Proliferating cell nuclear antigen |
| PCR | Polymerase chain reaction |
| PDGF | Platelet derived growth factor |
| Rb | Retinoblastoma |
| SH2 | *Src* homology -2 |
| TAMRA | 6-carboxy-tetramethyl-rhodamine |
| TCC | Transitional cell carcinoma |
| TGF | Transforming growth factor |
| tPA | Tissue type plasminogen activator |
| uPA | Urokinase type plasminogen activator |
| VN | Vitronectin |

# LIST OF ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

**I.** **Seddighzadeh, M.**, Zhou, J-N., Kronenwett, U., Shoshan, M C., Auer, G., Sten-Linder, M., Wiman, B. and Linder. S. ERK signalling in metastatic human MDA-MB-231 breast carcinoma cells is adapted to obtain urokinase expression and rapid cell proliferation.
*Clinical & Experimental Metastasis, 17: 649-654, 2000.*

**II.** **Seddighzadeh, M**., Steineck, G., Jansson, O., Larsson, P., Wijkström H., Norming, U., Onelöv, E. and Linder, S. Expression of uPA and uPAR is associated with the clinical course of urinary bladder neoplasms.
*Accepted for publication in the International Journal of Cancer.*

**III.** **Seddighzadeh, M.**, Steineck, G., Jansson, O., Larsson, P., Wijkström H., Adolfsson J., Portwood, N., Hansson, J. and Linder, S. Low interleukin-1α messenger RNA levels predict decreased overall survival time of patients with urinary bladder carcinoma.
*British Journal of Cancer, 84(3), 329-334, 2001.*

**IV.** **Seddighzadeh, M.**, Larsson, P., Ulfgren, A-C., Onelöv, E., Berggren, P., Tribukait, B., Thorstenson, A., Wijkström, H., Linder, S. and Steineck, G. Low IL-1α expression and death in urinary bladder cancer.
Manuscript

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# GENERAL INTRODUCTION

*Genetic instability*

Cancer results from the accumulation of mutations in genes that directly or indirectly control cell proliferation or cell death. It has been argued that an underlying genetic instability is absolutely required for the generation of the multiple mutations that are required for the tumorigenic phenotype (Loeb, 1991). Genetic alterations that affect growth-controlling genes can be divided into four major categories: 1) Deletion or insertion of few nucleotides; 2) Deletion of large chromosomal regions; 3) Alterations in chromosome numbers; 4) Chromosome translocations; 5) Gene amplification. These genetic alterations may, directly or indirectly, lead to six essential alterations in cell physiology that together dictate malignant cell growth (Hanahan and Weinberg, 2000).

*Self-sufficiency in growth signals*

Tumors have reduced dependence of exogenous growth stimulation by generating many of their own growth signals, thereby reducing their dependence on the stimulation from their normal tissue microenvironment (Deuel, 1987). Examples of tumors which show a reduced requirement for exogenous growth stimulation are human melanoma, pancreas carcinoma and ovarian carcinoma. Basic fibroblast growth factor (bFGF) is produced constitutively by melanoma cells and are considered to be important for cell growth (Rodeck et al., 1991), acidic and basic fibroblast growth factor are overexpressed in pancreas carcinoma (Kornmann et al., 1998), and TGF-$\alpha$ has an important autocrine growth stimulating role in ovarian carcinoma (Bast et al., 1993).

*Insensitivity to anti-growth signals*

Anti-growth signals can block cell proliferation either by arresting cells in the G1-phase or by inducing cells to enter into post-mitotic states. An example is the cytokine TGF−$\beta$1, which arrests human mammary epithelial cells at the G1/S boundary (Gold, 1999). Most invasive breast cancer cell lines show a reduced sensitivity to the anti-proliferative effect of TGF-$\beta$1 (Goggins et al., 1998; Reiss, 1997). Early stage cancer cells must escape the inhibitory effects of these antiproliferative signals to proliferate.

*Resistance to Apoptosis*

Programmed cell death, apoptosis, represents the major source of cell loss in the body (Wyllie et al., 1980) and is an important barrier to cancer. Mitochondria are important for the apoptosis process. Cytochrome c is released from mitochondria during apoptosis and forms a complex, called the apoptosome, with Apaf-1 and procaspase-9 (Li et al., 1997). This results in the activation of caspase-9 and, subsequently, down-stream caspases and finally the biochemical execution of cell death. Resistance to apoptosis can be acquired by various mechanisms, one of which is through over-expression of the Bcl-2 oncogene. The Bcl-2 protein is localized to the outer mitochondrial membrane and inhibits the release of cytochrome c from mitochondria

(Yang et al., 1997, Kluck et al., 1997, Cheng et al., 2001). Apoptosis resistance can also be acquired through mutations in the p53 suppressor gene, resulting in the removal of a key component of the DNA damage sensor (Harris, 1996).

*Limitless replicative potential*

Self-sufficiency in growth signals, insensitivity to antigrowth signals and resistance to apoptosis will lead to a deregulated proliferation program. In theory, this deregulated program should be sufficient to enable the generation of cell populations that constitute macroscopic tumors. But many or maybe all mammalian cells have an intrinsic program that limits their multiplication. Normal mammalian cells undergo a limited number of cell division *in vitro* (Hayflick and Moorhead, 1961, Hayflick, 1965) after which they are irreversibly arrested in a state known as replicative senescence. It has been suggested that senescence forms a barrier against tumorigenesis (Sager, 1991). The molecular mechanisms underlying senescence are becoming understood. At the ends of mammalian chromosome structures called telomeres are found that caps the chromosome and prevents end-to-end chromosomal fusions (Greider and Blackburn, 1996). The telomeric DNA is under-replicated during each round of DNA replication leading to the progressive shortening of the telomere (Harley et al., 1994) and eventually release of a senescence-inducing signal. An enzyme called telomerase catalyzes the replication of the end of the chromosomes. Tumor cells over-express this enzyme to circumvent senescence. Another mechanism related to senescence involves the ARF-gene product of the CDKN2A locus. Mouse fibroblasts defective in ARF do not undergo senescence (Kamijo et al., 1997; Krimpenfort et al., 2001).

*Sustained angiogenesis*

Cells rely on oxygen and nutrients supplied by the vasculature for survival. Incipient neoplasias must develop angiogenic ability (Bouck et al., 1996; Folkman, 1997; Hanahan and Folkman, 1996). Some examples of angiogenesis promoting factors are vascular endothelial growth factor (VEGF) and acidic and basic fibroblast factors (FGF1/2). Various inhibitors of angiogenesis have also been described, including thrombospondins, angiostatin and endostatin (Folkman, 1997). Thrombospondin-1 and –2 are secreted proteins that bind to a transmembrane receptor on endothelial cells (Bull et al., 1994). Over-expression of thrombospondin-1 in mammary carcinomas results in delayed tumor growth and a reduction of tumor microcapillaries. (Rodriguez-Manzaneque et al., 2000).

*Tissue invasion and metastasis*

The formation of tumor metastasis is a principal contributing factor to cancer morbidity and mortality (Hofmann et al., 2000; Stetler-Stevenson and Yu, 2001). Hence, tumor invasion and metastasis are central aspects of tumor biology. During development of most types of human solid tumors, cells originating from the tumor mass invade adjacent tissues and subsequently spread to distant sites where they can form new colonies. Due to the complex multistep nature of the metastatic process, only a small fraction of most primary tumor cells are capable of forming metastases (Fidler and Kripke, 1977). These few tumor cells are sufficient to render the patient prognosis highly unfavorable (Fidler, 1991). In the following section, cell signaling and tissue invasion will be described more in detail.

## Cell growth and signaling pathways

*Cell growth factors and receptors*

Independence of external growth signals can be aquired by alterations of extracellular growth signals, growth factor receptors, and signal transduction pathways. Many growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) bind and activate membrane receptors with intrinsic tyrosine kinase activity. Binding of EGF or PDGF to their respective receptors induces receptor dimerization (Heldin, 1995), leading to autophosphorylation of tyrosine residues. Phospho-tyrosines provide docking sites for downstream signal transduction molecules containing Src homology 2 (SH2) domains. Grb2 is one such SH2 containing protein that forms a complex with the nucleotide exchange factor Sos1. Interaction of Ras with Sos1 leads to GDP/GTP exchange, which in turn causes the activation of downstream molecules (Figure 1). Tyrosine kinase receptors are over-expressed in many types of cancers, which may enable cancer cells to be more responsive to the surrounding growth factors. For example, the expression of the EGF receptor is up-regulated in brain, breast, bladder and lung tumors (Gullick, 1996) and the HER2/neu receptor is over-expressed in stomach and mammary carcinomas (Slamon et al., 1987; Ross and McKenna, 2001, Kaptain et al., 2001). Overexpression is commonly due to gene amplification.

*Ras signaling pathways*

Ras signaling pathways control cell growth, differentiation and apoptosis (Satoh and Kaziro, 1992, Khosravi-Far and Der, 1994). Ras proteins are positioned at the inner surface of the plasma membrane where they serve as binary molecular switches that transduce extracellular ligand-mediated stimuli to cytoplasmic kinases. The Raf-1 serine/threonine kinase is an important effector of Ras function (Moodie and Wolfman, 1994) that, upon activation, phosphorylates and activates two mitogen activated protein kinase (MAPK) kinases, MEK-1 and MEK-2. Activated MEKs phosphorylate threonine and tyrosine residues in two MAPKs, designated p42 $^{MAPK}$/ERK2 and p44 $^{MAPK}$/ERK1, and thereby activate them (Crews et al., 1992). Activated MAPKs translocate to the nucleus where they phosphorylate and activate a variety of substrates, including the Elk-1 nuclear transcription factor (Marais et al., 1993). Ras can also activate MEKK-1, a serine /threonine kinase (Lange-Carter et al., 1993; Sanchez et al., 1994). MEKK-1 activates SEK1, a dual -specificity kinase that phosphorylates and activates the c-JUN N- terminal kinases (JNKs) 1 and 2 (Yan et al., 1994; Minden et al., 1994; Sanchez et al., 1994; Derijard et al., 1995). JNK in turn activates the ATF-2 and JUN nuclear transcription factors (Figure 1). ATF-2 and the JUN can dimerize with other transcription factors to stimulate transcription from AP-1 binding sites (Karin, 1995). However, whereas ERK-activation is generally associated with growth stimulatory responses, JNK-activation is associated with stress responses that result in apoptosis (Xia et al., 1995, Chen et al., 1996; Yang et al., 1997; Tournier et al., 2000).

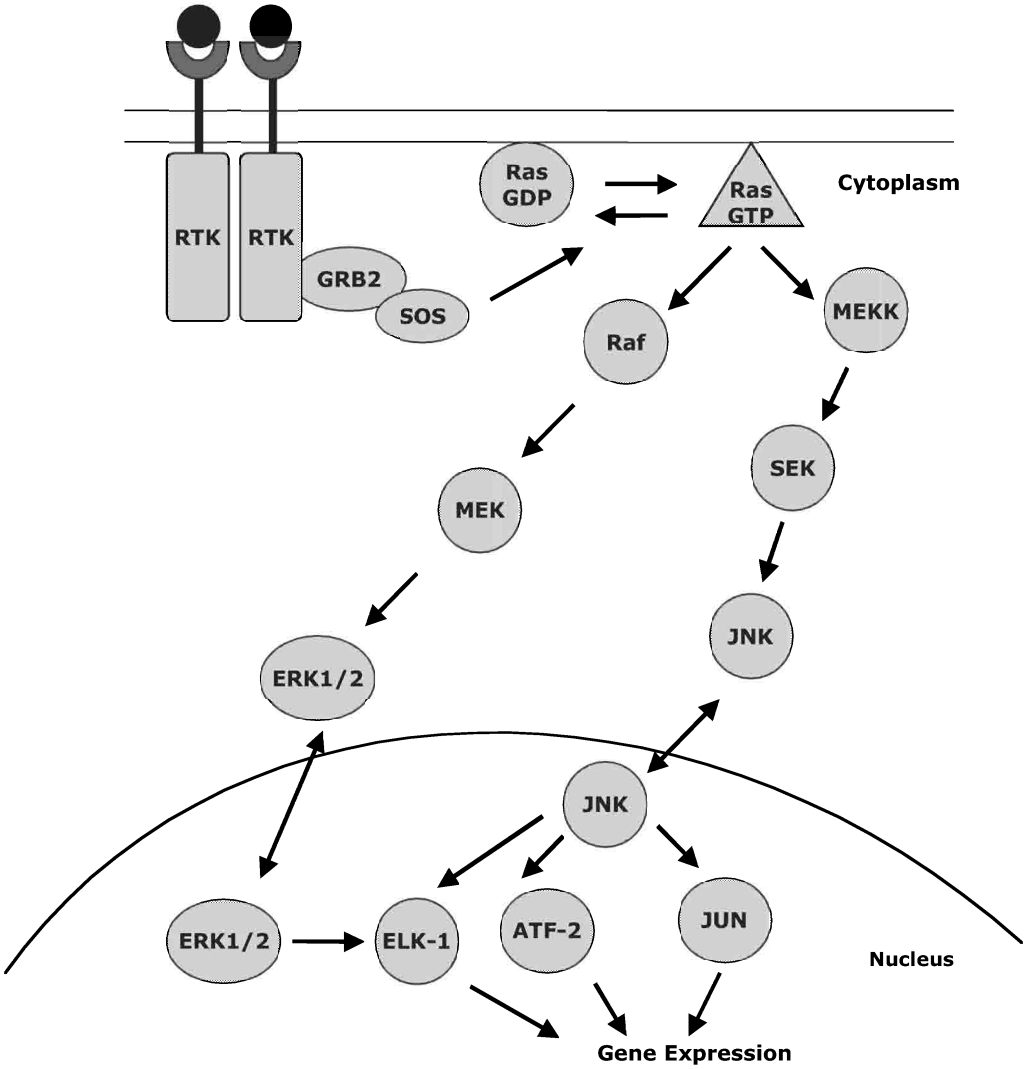


**Figure 1. ERK and JNK signaling pathways.** Receptor tyrosine kinases become phosphorylated upon binding to a ligand. This results in the activation of Ras and subsequently the phosphorylation of ERK and JNK. The phosphorylated forms of these kinases translocate to the nucleus where they in turn activate different types of transcription factors.

## Cancer cell invasion and metastasis

*The anatomy of the metastatic process*

When the proliferation of the epithelial cells is not confined to the basal lamina, the disorder is called dysplasia. Superficial cells of dysplasias still show some signs of differentiation. Dysplasia can progress to carcinoma *in situ* where the pattern of cell division and differentiation becomes more disrupted. This type of lesion can give rise to malignant carcinoma where cells break the basal lamina and begin to invade the underlying connective tissue (Figure 2) (Farber and Cameron, 1980).

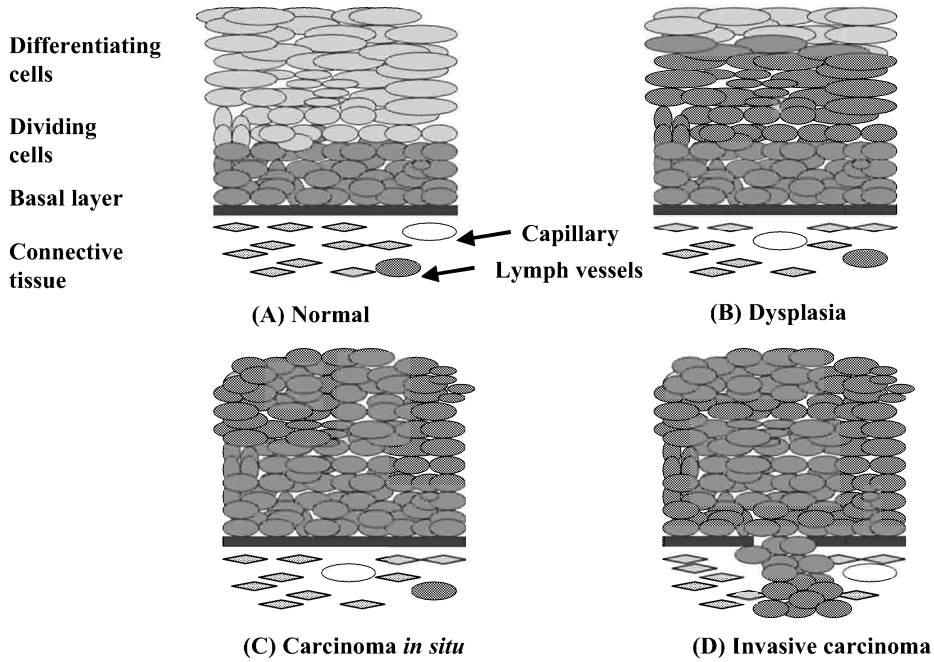


**Figure 2. The stage of progression from normal tissue to carcinoma (A-D).** In dysplasias, the cells show some sign of differentiation. In carcinoma *in situ,* the cells of all layers are proliferating and are undifferentiated. In invasive carcinoma, the proliferating cells cross the basal layer and invade the underlying connective tissue.

In order to spread and disseminate in the body, malignant cells have to break tissue barriers to reach blood or lymphatic vessels, break the vasculature wall to enter the circulation (intravasation) and exit from the circulation (extravasation) in another organ. Finally the cells have to survive and proliferate in the new organ (Fidler, 1991). Intravasation is an early step of the process leading to metastasis. It has been suggested that uPA and uPAR and MMP-9 are required to complete the breaching of the vascular wall (Kim et al., 1998; for a further discussion of these proteases, see below). According to another model, tumor cells displace

endothelial cells and line the vascular bed (Warren and Shubik, 1966; Prause and Jensen, 1980; Hammersen et al., 1985; Chang et al., 2000). Tumor cells are then constitutively shed into the blood stream in large numbers (millions of cells per day).

*Mechanism underlying tumor metastasis*

Tumor metastasis is a multistep process, which includes detachment, migration and proteolysis (Fidler, 1991).

Detachment: Cadherins are a family of epithelial $Ca^{+2}$-dependent adhesion molecules, with central roles in cell-cell adhesion. Without active cadherins, cells will detach from each others even when other adhesion molecules are operating. Loss of E-cadherin is associated with an increased risk for the development of metastasis in human tumors (Shimoyama and Hirohashi, 1991; Schipper et al., 1991; Vleminckx et al., 1991; Oka et al., 1993).

Cell migration: Cell migration is defined as the locomotion of a cell on substratum of ECM proteins. Cell migration is important for many physiological and pathological processes such as embryogenesis and cancer metastasis (Lauffenburger and Horwitz, 1996). During cell migration, the leading cytoplasmic edge of the cell becomes extended. This process involves adhesion, providing traction to pull the cell body forward.

Proteolysis: ECM proteases are important determinants of invasive capacity. These proteases can be divided into four classes: metalloproteases, serine proteases, cystein proteases and aspartyl proteases. During cancer progression, protease expression is often up-regulated, protease inhibitors down-regulated, and inactive zymogen forms of proteases converted into active forms. Matrix proteases may be associated with the cell surface either by having a transmembrane domain or by binding to a specific protease receptor or to integrins (Werb, 1997; Stetler-Stevenson, 1999). In many types of carcinomas, matrix-degrading enzymes are not produced by epithelial cancer cells but rather by stromal or inflammatory cells (Basset et al., 1990; Werb, 1997). The proteolytic process is discussed more in detail below.

*Serine proteases and the plasminogen activator system*

This group of proteases has a reactive serine residue in their active site. The urokinase and tissue type plasminogen activators and plasmin belong to this group.

Plasminogen activators

The plasminogen activators (PA), urokinase type PA and tissue type PA, are serine proteases which catalyze the conversion of plasminogen to plasmin. uPA is the most common form of plasminogen activator associated with cancer. In addition, uPA is involved in various physiological and pathological processes including embryogenesis, ovulation, wound healing, inflammation and rheumatoid arthritis. uPA has an Mr of 50 kDa and consists of 2 disulfide bridge-linked polypeptide chains: a C-terminal B-chain that contains a serine protease domain (SPD) and an N-terminal A-chain, which contains a kringle (K) domain and a growth factor (GF) domain (Figure 3). uPA is initially released from cells in a one-chain zymogen form called pro-uPA. Pro-uPA becomes activated upon binding to its specific surface receptor by cleavage of the Lys158-Ile159 peptide bond by plasmin or other proteinases (Danø et al., 1985). The resulting 2-chain uPA has an activity which is 250-fold higher than that of pro-uPA. uPA

has a restricted substrate specificity with plasminogen as the main substrate (Danø et al., 1985; Sakela and Rifkin, 1988; Mignatti and Rifkin, 1993). Several studies have demonstrated that elevated levels of uPA is associated with poor prognosis of patients with breast cancer (Duffy et al., 1988; Foekens et al., 2000), colorectal cancer (Skelly, et al., 1997, Kim, et al.,1998) and esophageal cancer (Nekarda, et al., 1998). Some cancers have been reported to secrete tPA (Cajot et al., 1986). It has been reported that elevated t-PA expression is associated with a favorable prognosis in breast carcinoma, possibly due to the fibrinolytic activity of t-PA that results in the inhibition of tumor cell emboli (Yamashita et al., 1993).

Plasmin

The precursor of plasmin, plasminogen, is synthesized in the liver. Plasminogen contains five consecutive "kringle" domains followed by a serine proteinase domain (Figure 3). Urokinase or tissue-type plasminogen activator converts plasminogen to plasmin by hydrolysis of the Arg561-Val562 peptide bond. Plasmin has a broad substrate specificity and is believed to play a key role in the degradation of many extracellular matrix proteins (Shapiro et al., 1996), in the activation of pro-metalloproteases (Murphy et al., 1992) and in the activation of cytokines (Odekon et al., 1994). These processes may all result in increased metastatic potential of cancer cells. The angiogenesis-inhibitor angiostatin is a fragment of plasmin, consisting of kringles 1-4 (O'Reilly et al., 1994). Systemic administration of angiostatin has been reported to inhibit the growth of human and murine primary carcinoma cells in mice (O'Reilly et al., 1996).

Plasminogen activator inhibitors

The activities of uPA and plasmin are controlled by the plasminogen activator inhibitors 1 and 2 (PAI-1 and PAI-2), and $\alpha$2-antiplasmin, respectively. PAI-1 and PAI-2 bind to the catalytic part of uPA but do not bind to the zymogen form. PAI-1 is the main inhibitor of uPA and is able to inhibit both free and receptor bound uPA. By a process dependent on the receptor of uPA (uPAR) and the $\alpha_2$-macroglobulin receptor, the uPA/PAI-1 complexes are internalized and degraded in lysosomes (Andreasen et al., 1994, 1997). Lymph node metastases at presentation, higher frequencies of relapses, and decreased survival of breast and lung cancer patients have been associated with high PAI-1 production ( Janicke et al., 1991; Grondahl-Hansen et al., 1993; Pedersen et al., 1994). The finding that high levels of PAI-1 are associated with poor prognosis may seem counter intuitive since PAI-1 is a protease inhibitor. It has recently been demonstrated that the presence of complexes between uPA and PAI-1 is associated with longer recurrency free survival of patients with primary breast cancer, whereas elevated levels of total PAI-1 are associated with shorter survival (Pedersen et al., 2000). These data suggest that PAI-1 may exist in two distinct compartments, one where PAI-1 is inhibiting uPA activity and the proteolysis of the ECM, and another where it has a positive influence on tumor growth.

PAI-2 has been described as a less efficient but more specific inhibitor of uPA than PAI-1 (Conese and Blasi, 1995). PAI-2 is not efficiently secreted to the extracellular matrix and is an inhibitor of intracellular processes (Kumar and Baglioni, 1991). The role of PAI-2 in malignancy is less defined and the presence of PAI-2 antigen is associated with a good prognosis for some tumor types and a poor prognosis for others (Bouchet et al., 1994; Kruithof et al., 1995). PAI-1 inhibits VN/integrin-dependent cell migration through binding to VN. This function is independent of PAI-1 inhibition of uPA activity (Stefansson and Lawrence, 1996; Kjoller et al., 1997). VN/uPAR dependent cell migration is also substantially inhibited by PAI-1 (Deng et al., 1996; Kanse et al., 1996)

Urokinase-type plasminogen activator receptor

The receptor for urokinase-type plasminogen activator (uPAR/CD87) is a cell surface protein with a molecular mass of 55-60 kDa. It consists of three homologous protein domains, known as domains 1, 2 and 3 (Behrendt et al., 1996), and it is heavily glycosylated (Ploug et al., 1998). The first domain contains the ligand-binding region, involved in high affinity binding to the epidermal growth factor-like module of uPA. This domain is also required for uPAR-mediated cell binding to VN (Sidenius and Blasi, 2000). The sequence linking the domain 1 and 2 of uPAR has chemotactic properties (Fazioli et al., 1997). It has been demonstrated that this region is highly sensitive to cleavage by uPA and other proteases (Hoyer-Hansen et al., 1997). The uPAR protein does not have any transmembrane or intracellular domain and attaches to the plasma membrane via a GPI anchor (Ploug et al., 1991). High uPAR levels are associated with a poor prognosis of patients with squamous cell lung cancer (Pedersen et al., 1994), colon cancer (Ganesh et al., 1994), and breast cancer (Grondahl-Hansen et al., 1995). Forms of uPAR that lack the glycolipid anchor have been demonstrated, and are referred to as soluble uPAR (suPAR). Recently it was shown that elevated levels of suPAR predicts the survival of patients with colorectal cancer (Stephens et al., 1999).

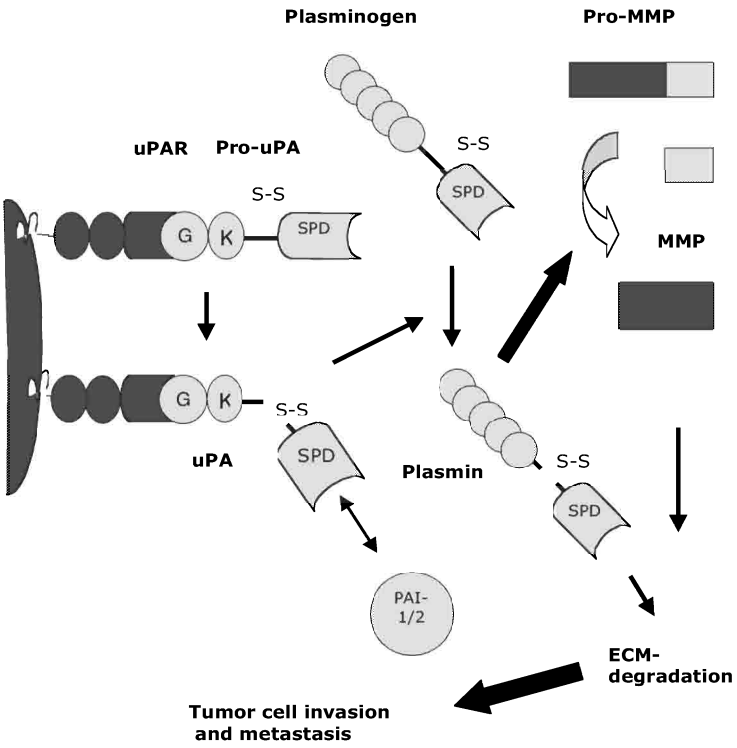


**Figure 3. The members of uPA system and their biochemical properties.** Pro-uPA becomes activated upon binding to its receptor (uPAR). PAI-1/2 are two potential inhibitors of uPA. Activated uPA catalyzes the conversion of plasminogen to plasmin. Plasmin in turn either directly degrades ECM proteins or catalyzes the conversion of pro-MMP to activated MMP. These events result in the enhancement of tumor cell invasion and finally development of metastasis. SPD, serine protease domain; K, kringle domain; G, growth factor domain.

uPAR-integrin interaction

In addition to binding uPA and facilitating cell invasion, uPAR is also involved in cellular adhesion (Nusrat and Chapman, 1991; Waltz et al., 1993), migration (Ossowski et al., 1999) and signaling (Del Rosso et al., 1993). Since uPAR is a GPI-linked protein, transmembrane adaptor proteins are required for these different biological activities. These adaptor proteins have been identified to be β1-β3 integrins.

Different models have been proposed for how uPAR and integrins interact, and how the interactions activate cellular signaling pathways. In the first scenario, integrins are thought to be active in a caveolin-dependent fashion by ligand binding before they interact with uPAR (Ghiso et al., 1999; Wei et al., 1999). Caveolin is a protein which is known to associate with Src family kinases (Chapman et al., 1999; Okamoto et al., 1998) and coimmunoprecipitates with β1 integrins (Wei et al., 1996, 1999; Wary et al., 1998). Wei *et al* (1999) proposed that activation of integrins upon ligand-binding results in oligomerization of caveolin, which leads to the activation of Src kinases. uPAR then moves to the integrin-caveolin-Src kinase complexes and enriches it with further caveolin and associated signaling molecules (Wei et al., 1999). This leads to ERK-activation, which combined with FAK activity and cytoskeleton changes, is required for migration of cells *in vitro* (Figure 4a).

In the second scenario, the need for caveolin is not universal (Figure 4b). uPA-bound uPAR interacts with α5β1 integrins to keep the integrins in an active state. This results in interaction between uPA-uPAR-integrin complexes and fibronectin and finally a powerful activation of the MEK1/ERK pathway (Ghiso et al., 1999). When ERK activity reaches a threshold level, tumor cells begin to proliferate *in vivo*.

The well established function of uPAR as an activator of the proteolytic cascade, together with its signaling functions, enables uPAR to coordinate tumor cell adhesion and migration through the extracellular matrix and also to determine whether cells will, or will not, proliferate *in vivo* (Ossowski and Aguierre-Ghiso, 2000).

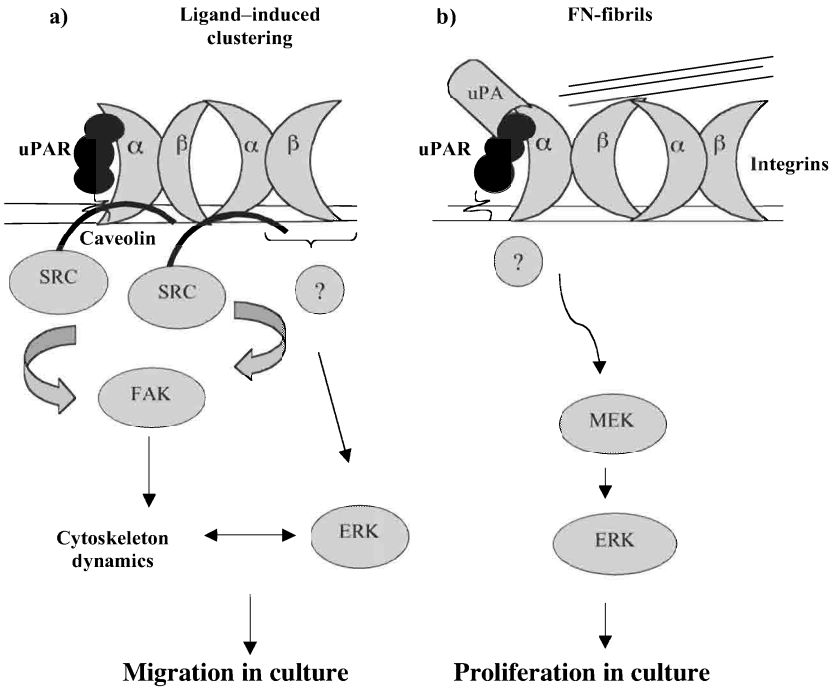


**Figure 4.** Proposed models for uPAR–integrin interaction, the activated signaling pathways and the biological outcomes of a) caveolin-dependent and b) caveolin-independent uPAR-integrin interaction.

*Metalloproteases*

Metalloproteases (MMPs) contain a $Zn^{2+}$ or $Ca^{2+}$-ion in their active site and require proteolytic cleavage to become active (Woessner, 1991). Members of this family of proteases are able to degrade ECM proteins such as fibronectin, laminin, elastin and different types of collagens (Mignatti and Rifkin, 1993; 1996). The MMP family includes: 1) Type 1 collagenases: MMP-1, MMP-8 and MMP-13; 2) Gelatinases: MMP-2 and MMP-9; 3) Stromelysins: MMP-3, MMP-7, MMP-10 and MMP-11; 4) Metalloelastase: MMP-12; 5) Membrane type metalloproteases (MT-MMPs) (Rabbani, 1998; Stamenkovic, 2000). The enzymatic activities of the metalloproteases are regulated by endogenous inhibitors such as $\alpha_2$-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs). $\alpha_2$-macroglobulins are the regulators in the fluid phase and TIMPs are the key inhibitors in tissue.

*Cystein and Aspartyl proteases*

From the perspective of tumor invasion, the most relevant protease in the groups of cysteine and aspartyl proteases is cathepsin D (an aspartyl protease). This protease normally functions at an acidic pH in the lysosome (Hasilik and Neufeld, 1980). Metastatic breast tumor cell lines secrete high levels of the pro-form of cathepsin D due to both overexpression of the cathepsin

D gene and to an altered processing of the precursor protein (Rochefort et al., 1990). Studies in estrogen receptor positive breast cancer cell lines revealed that cathepsin D expression is highly regulated by estrogens and growth factors (Westley and Rochefort, 1980; Cavailles et al., 1993). Cathepsin D expression has been most extensively studied in breast cancer, where it has been shown to be a useful prognostic marker (Spyratos et al., 1989; Thorpe et al., 1989; Fernö et al., 1994).

## Interleukin-1 and cancer

IL-1 is a multifunctional cytokine that can exert effects on nearly all cell types, often together with other cytokines. There are three members of the IL-1 gene family, IL-1α, IL-1β and the IL-1 receptor antagonist (IL-1Ra). IL-1α and IL-1β are synthesized as precursors without leader sequences and they are cleaved by specific cellular proteases to become mature cytokines. Pro-IL-1α is biologically active whereas pro-IL-1β has minimal biological activity (Mosley et al., 1987). IL-1α remains intracellular and is released by dying cells and subsequently cleaved by extracellular proteases (Kobayashi et al., 1988) (Figure 5). Pro-IL-1β can be cleaved to mature IL-1β by the IL-1β converting enzyme (ICE) and subsequently released from cells (Dinarello, 1997). Pro-IL-1α plays a role in the regulation of normal differentiation of endothelial and epithelial cells (Hauser et al., 1986).

A number of different effects of IL-1 on tumors and on tumor cell lines have been reported. Addition of IL-1 to cell cultures reduces cell proliferation either through cytocidal (Onozaki et al., 1995; Lachman et al., 1996) or cytostatic effects (Herzog and Collin, 1992). Several mechanisms have been suggested to be responsible for the anti-proliferative activity of IL-1:
1) induction of other cytokines such as IL-6 (Evans et al., 1992); 2) increased release of oxygen radicals (Hirose et al., 1993); 3) increased cell differentiation and polymerization of F-actin (Koga et al., 1993) ; 4) cell cycle arrest (Belizario and Dinarello, 1991); 5) increased generation of NO (Bonta and Ben-Efraim, 1993). It has been reported that IL-1 can synergize with cytostatic drugs or with other cytokines to inhibit the proliferation of malignant cells *in vitro* (Braunschweiger et al., 1993). Another possible mechanism for the antitumoral activity of IL-1 is the induction of immune surveillance cells (*i.e.*, natural killer cell and macrophages) and the development of a specific immune response by affecting IL-2 secretion (Muegge et al., 1989; Smith, 1992). Finally, IL-1 is anti-angiogenic and will inhibit endothelial cell proliferation *in vitro* and *in vivo* (Cozzolino et al., 1990). Antitumoral effects of IL-1 have been described in phase I and phase II clinical trials (Hornung et al., 1992; Katsanis et al., 1994; Janik et al., 1996).

IL-1α has been reported to enhance metastasis (Giavazzi et al., 1990). IL-1α produced by ER-negative breast cancer cells induces the expression of IL-6 and uPA in fibroblasts, effects mediated through activation of the transcription factor NK-κB (Bhat-Nakshatri et al., 1998). In addition, IL-1 stimulates the expression of metalloproteases in fibroblasts (McNaul et al., 1990; Emonard and Grimaud, 1990).

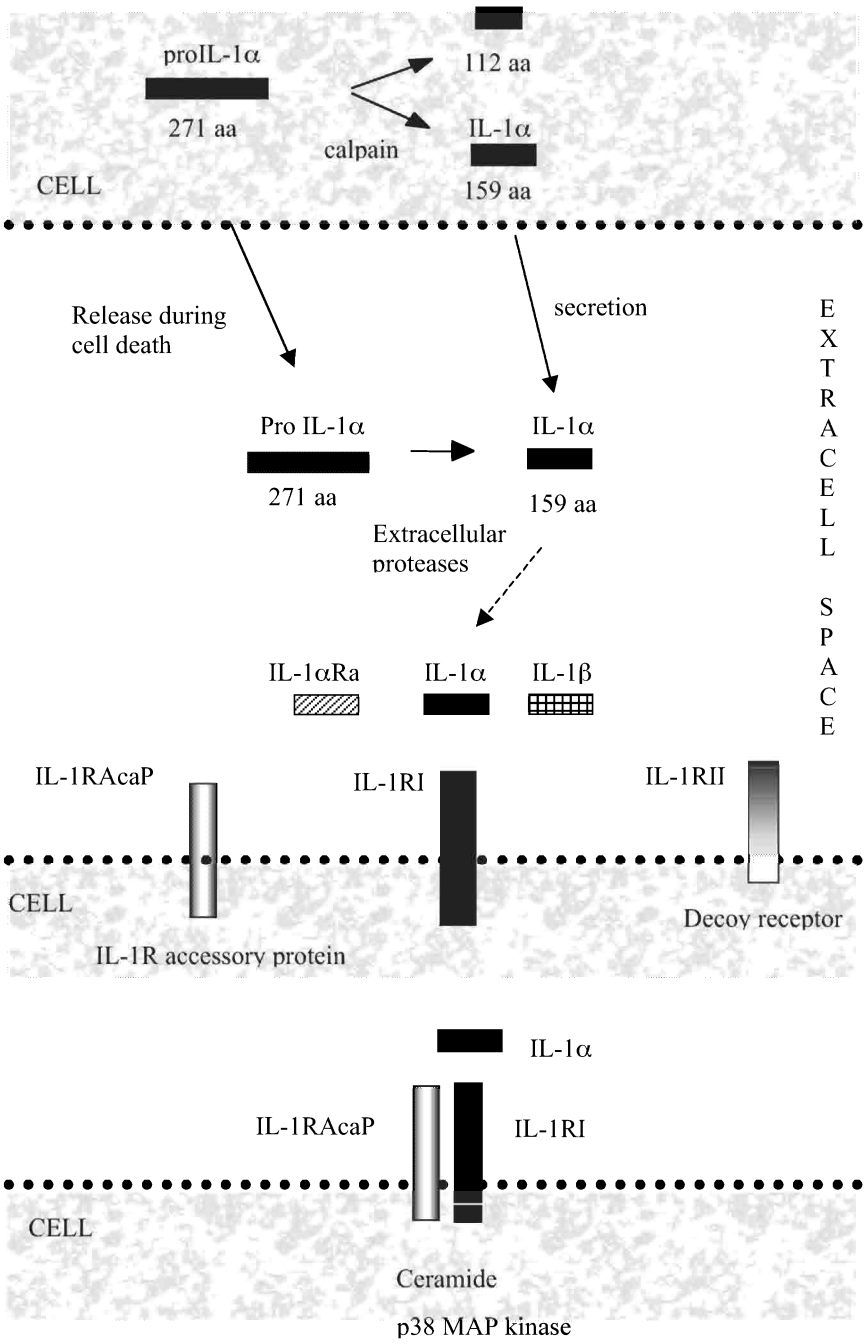


**Figure 5**. Pro IL-1α remains intracellularly until it is activated by calpain. Alternatively, IL-1α is released from dying cells and activated by extracellular proteases. Pro IL-1β becomes activated by the interleukin-converting enzyme (ICE). IL-1α competes with IL-1α receptor antagonist for binding to the IL-1R1.

## Urinary bladder carcinoma

*Classification*

Almost all bladder cancers in Western countries are transitional cell carcinomas (TCC). Tumors are classified based on the degree of tissue invasion where Ta tumors are limited to the mucosa, T1 tumors are limited to submucosa (lamina propria) and T2+ tumors invade the main muscle layer (Figure 6) (Hall and Prout, 1990). The system of grading used in the present studies is that of Bergkvist et al. (1965), distinguishing G2a from G2b tumors. Recent studies have shown that mutations of the p53 gene are more frequent in G2b tumors compared to G2a tumors (Berggren et al., 2001).

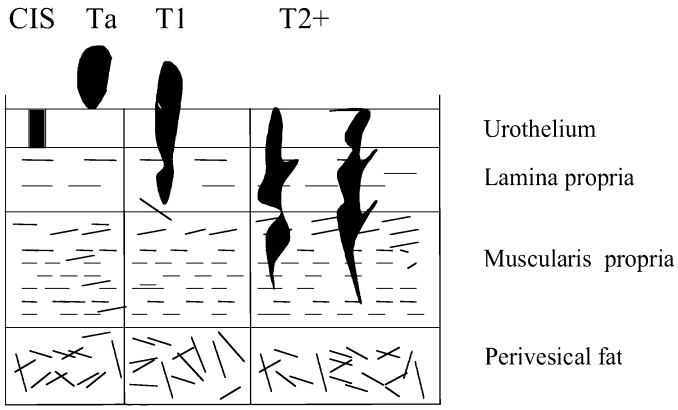


**Figure 6. Stage classification of urinary bladder carcinoma.** The urinary bladder consists of the transitional cell epithelium, the lamina propria, the muscular layer and the perivesical fat. Urinary bladder tumors can be distinguished as carcinoma *in situ (CIS),* as tumors limited to the urothelial cell layer (Ta), as tumors limited to the lamina propria (T1) or as tumors invading the muscle layer (T2+).

*Incidence and Etiology*

Bladder cancer is the fourth most common cancer in men in the Western world with a rising incidence (Knowles, 1999). Bladder cancer has a strong relation to industry related carcinogens. The aromatic amines 4-aminobiophenyl and benzidine are bladder carcinogens (Johansson and Cohen, 1997). 4-aminobiophenyl is one of many aromatic amines in tobacco smoke. The relative risk of bladder cancer increases 2.0 to 4.0 fold for smokers compared to non-smokers (Silverman et al., 1989).

*Diagnosis*

The diagnosis of patients with urinary bladder carcinoma can be problematic due to the unspecific nature of the most common symptoms like irritative voiding and painless hematuria. Bladder tumors are classically diagnosed using cystoscopy. This method is sensitive and can be used to determine tumor size and multifocality (Kausch and Bohle, 2001). Cytology on cells sedimented from urine is also a common, but is less sensitive method

*Prognosis*

Bladder cancer has two major clinical phenotypes. Approximately 75% of the bladder tumors are not muscle invasive (CIS, Ta, T1). Ta and T1 tumors show a high recurrency rate (50%-70%), but the risk for progression is only 10-15%. About 25% of all bladder tumors are muscle-invasive at the time of diagnosis and have a less favorable prognosis (Knowles, 1999). At presentation, 5% of all patients have metastases. Approximately 50% of the patients with muscle invasive tumors develop lethal distant metastasis after radical cystectomy. Carcinoma i*n situ* (CIS) is a flat, high-grade tumor that initially respects mucosal boundaries (Figure 6) but is believed to be the precursor of more lethal invasive cancers. Patients with this type of tumor have a poor prognosis in up to 60% of the cases (Althausen et al., 1976). Histopathological studies have suggested that muscle invasive bladder carcinomas are not derived from superficial lesions but from CIS, or can arise *de novo* (Schalken et al., 1992).

*Treatment*

The choice of treatment strategy is based on different factors such as tumor size, involvement of lymph nodes, presence of metastases, tumor stage and grade, and the physical conditions of the patient. Low grade tumors without muscle invasion are usually treated with transurethral resection, sometimes supplemented with intravesical chemotherapy or radiation therapy, especially in the case of recurrent or multiple tumors (Soloway, 1980; Huland et al., 1984; Quilty and Duncan, 1986; Kaufman et al., 1993). Bacillus Calmette-Guerin (BCG) immunotherapy is also used for treatment of patients with non-invasive tumors and has shown to lead to prolonged protection from tumor recurrency. In the cohort studied here (patients in Stockholm during 1995-1996), about 60% of the patients diagnosed for muscle invasive urinary bladder cancer received treatment aimed to cure the patient (radical cystectomy or radiotherapy).

*Prognostic factors for bladder carcinoma*

Staging and Grading

Stage and grade are the most reliable parameters for progression and recurrency of urinary bladder carcinoma.. Patients with Ta and T1 tumors have a progression rate of 4% and 30%, respectively (Burchardt et al., 2000). The progression rate is 2-10% for patients with grade 1 tumors, 11-19% for patients with grade 2 tumors and 33-45% for patients with grade 3 tumors (de Vere White and Stapp, 1998).

DNA-ploidy and S-phase fraction

Flow cytometry can be used to determine DNA ploidy and to estimate proliferation (S-phase fraction) of tumor cells. High grade carcinomas and carcinomas *in situ* (CIS) are frequently aneuploid and show a high S-phase fraction. Diploid tumors are generally of low grade and stage and are associated with a better prognosis (Koss et al., 1989; Schapers et al., 1993). Ki-67 and proliferation cell nuclear antigen (PCNA) are two immunohistochemical markers of cellular proliferation, and Ki-67 correlates to progression and reduced survival rate in TCC (Norming et al., 1992).

Blood group- related antigens (ABH and Lewis antigen)

The ABH and Lewis blood group-related antigens are present on the surface of healthy urothelial cells. Loss of the ABH-antigen (Coon et al., 1982; Hakomori, 1985) and enhanced Lewis X antigen expression is associated with malignant transformation (Cordon-Cardo et al., 1988). Immunocytological evaluation of two consecutive voided urine specimens for the Lewis X antigen is the most sensitive method currently available for noninvasive detection of transitional cell tumors (Pode et al., 1998).

Proto-oncogenes

Among the *ras* gene family members, mutations of the H-*ras* gene are the most commonly reported in bladder cancer. Recent results from a transgenic mouse model demonstrated that Ha-*ras* activation can induce urothelial proliferation *in vivo* (Zhang et al., 2001). However, H-*ras* mutations are infrequent in bladder cancer, and there is a lack of correlation between *ras* oncogene mutations and the clinical behavior of TCC of the bladder (Knowles and Williamson, 1993).

EGF-R is a transmembrane glycoprotein that is activated by binding of either EGF or TGF-$\alpha$. EGF-R is expressed in the basal cell layer of the healthy transitional epithelium but is expressed in all cell layers in the malignant tissue. EGF-R expression has been reported to be a prognostic factor for patients with bladder cancer (Mellon et al., 1995).

The proto-oncogene CCDN1 (the gene encoding the cyclin-D1 protein) is amplified and over-expressed in a subset of TCCs (Proctor et al., 1991; Bringuier et al., 1996; Oya et al., 1998). Over-expression of cyclin-D1 has been associated with early recurrency of superficial bladder cancers, but not with poor overall survival (Shin et al., 1997).

Tumor suppressor genes

p53 mutations are found in a larger proportion of muscle invasive bladder tumors compared to superficial tumors (Sidransky et al., 1991; Williamson et al., 1994) and in a higher frequency of CIS tumors compared to Ta tumors (Spruck et al., 1994). Several studies have reported that p53 is an independent predictor for outcome of patients with bladder cancer (Sarkis et al., 1993, Soini et al., 1993).

Invasive bladder tumors show a higher frequency of alterations at the Rb-locus, and Rb alterations are associated with a significantly shorter 5-years survival (Cordon-Cardo et al., 1992; Logothetis et al., 1992). Rb alterations are also found more frequently in tumors of high grade and stage (Cairns et al., 1991; Ishikawa et al., 1991; Cordon-Cardo et al., 1992; Wada et al., 2000).

Deletions of chromosome 9 occur in more than 60% of all bladder cancers across all grades and stages, and are likely to be initiating events (Tsai et al., 1990). These deletions focus on the *CDKN2A/ARF* and the *CDKN2B* loci at 9p21 (Cairns et al., 1991; Devlin et al., 1994). This complex region encodes three distinct proteins $p16^{INK4A}$, p14ARF and $p15^{INK4B}$, all of which are negative regulators of the cell cycle (Chin et al., 1998).

# DISCUSSION OF THE METHODS USED

*Quantitative PCR*

We have in the present work measured the abundance of RNA transcripts in tumors. RNA expression levels are likely to better reflect the level of gene expression compared to protein levels, in particular for genes encoding secreted proteins.

Five methods are commonly used for the quantitation of RNA expression: Northern blotting (Thomas, 1980), *in situ* hybridization (Parker and Barnes, 1999), RNAase protection assay (Melton et al., 1984), S1 protection assay (Berk and Sharp, 1977), and the reverse transcription polymerase reaction (RT-PCR)(Weis et al., 1992). The main limitation of the first four methods is their comparatively low sensitivity. RT-PCR is the most sensitive and flexible of method for quantitation of RNA expression (Rappolee et al., 1988; Wang and Brown, 1999). In conventional RT-PCR, the reverse transcribed RNA is amplified and the PCR products are separated by gel electrophoresis. The resulting bands are then quantified. The TaqMan technique is based on the use of a dually labeled, non-extendable oligonucleotide hybridization probe (Heid et al., 1996). This probe contains FAM (6-carboxy-fluorescein) as fluorescent reporter dye covalently linked to the 5′end. The quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) is usually covalently linked to the 3′end. During PCR, the probe hybridizes to the template of interest and is subsequently cleaved by the 5′ to 3′exonuclease activity of Taq DNA polymerase (Holland et al., 1991). This results in an increase of the fluorescent emission of the reporter dye, which is proportional to the amount of specific PCR products. This sequence of events occurs during each PCR cycle and does not interfere with the enzymatic reaction and the accumulation of PCR products (Heid et al., 1996). Real-time PCR offers several advantages over other quantitative PCR methods: a) high specificity: specific hybridization of both the primers and the probe are necessary to generate a signal, b) reliable results: the Ct value used for real-time PCR is measured when the amplification is in the log phase of PCR product accumulation, which means that the concentrations of reaction components are still not limiting, c) a wide dynamic range: samples do not have to be serially diluted to achieve a dynamic range of five order of magnitude, d) no post PCR handling: the reaction and the measurements are performed in a closed system.

*Normalization*

Internal standards are used to compensate for sample-to-sample variation during quantitative RT-PCR,. The ideal standard should be expressed at a constant level among different tissues of an organism, at all stages of development, and should be unaffected by experimental treatments. The three most commonly used RNA transcripts to normalize the patterns of gene expression are: glyceraldehyde-3-phosphate-dehydrogenase (GAPDH), β-actin and ribosomal RNAs (rRNAs).

β-actin is a commonly used internal standard for Northern blots and RT-PCR. However, the levels of β-actin can vary in response to experimental treatments (Ryseck et al., 1989; Spanakis, 1993). Another disadvantage of using β-actin as an internal standard is the presence of pseudogenes in the genome, which may interfere with the interpretation of the results (Dirnhofer et al., 1995; Raff et al., 1997; Mutimer et al., 1998).

The RNA encoding GAPDH is a moderately abundant transcript that is frequently used as an endogenous control for quantitative RT-PCR analysis. However, GAPDH levels vary significantly between different individuals (Bustin et al., 1999) and vary during the cell cycle (Mansur et al., 1993). GAPDH expression has been shown to be up-regulated in tumors (Ripple and Wilding, 1995; Chang et al., 1998). Finally, a number of GAPDH pseudogenes are present in the human genome (Arcari et al., 1989).

rRNAs have been shown to be a reliable internal standards in rat liver (de Leeuw et al., 1989) and in human skin fibroblasts (Mansur et al., 1993). There are, however two drawbacks to its use: rRNA transcription can be affected by various pathological conditions (Alon et al., 1999; Pogue-Geile et al., 1991; Spanakis, 1993) and rRNA transcripts are abundantly expressed in relation to target mRNAs.

*The tumor material*

Tumor samples, representing all newly diagnosed cases of urinary bladder neoplasms in the Stockholm area, were collected from four different hospitals between 1995 and 1996. Four samples per patients were taken using cold cup biopsy and snap frozen in –80°C. rRNA sequences could not be adequately amplified from about one third of all tumors. The success rate of rRNA amplification was unrelated to the size of the tumors and was similar between different stages. RNA degradation was therefore considered as the most likely reason for unsuccessful amplification. One possible explanation for RNA degradation could be suboptimal handling and freezing procedures. Alternatively, RNA may be degraded due to slow penetration of the denaturing agents into the tissue or during homogenization, or may be degraded during subsequent purification steps. In future studies, the number of samples which cannot be analyzed, must be decreased.

# AIMS

- To achieve a better understanding of the basis for the constitutive over-expression of uPA by a human carcinoma cell line (paper I).

- To evaluate the prognostic significance of elevated expression of urokinase and its receptor in the progression of the disease of patients with urinary bladder carcinoma (paper II).

- To investigate the relationship between uPA and IL-1$\alpha$ expression and also to study the role of IL-1$\alpha$ in the outcome of patients with urinary bladder carcinoma (paper III and IV).

# RESULTS AND DISCUSSION

***Paper I***

The mechanism underlying constitutive uPA expression by tumor cells or by tumor stroma is not well understood. It has been reported that hypomethylation of the uPA promotor causes over-expression of this gene in the MDA-MB-231 breast cancer cell line (Xing and Rabbani, 1999). Results from other studies have shown that uPA expression levels are induced by growth factors, phorbol ester and UV-light (D'Orazio et al., 1997) and that constitutive uPA expression in tumor cells is dependent on the ERK signaling pathway (Lengyel et al., 1995; 1996).

A large number of genes are regulated by the ERK signaling pathway (Seddighzadeh et al., 2000). Studies have indicated that some of these genes are induced by a relatively low signaling strength of the pathway, whereas others (i.e. p21Cip1) require a higher signaling strength (Sewing et al., 1997). The goal of this study was to investigate the relationship between the strength of ERK signaling and uPA expression levels. As a model, we used the highly malignant breast cancer cell line MDA-MB-231. The endogenous levels of uPA expression in MDA-MB-231 cells was 13 fold higher compared to a less malignant breast cell line, MCF-7. A 10-fold decrease in the levels of phosphorylated ERK was observed after treatment of MDA-MB-231 cells with the MEK-1 inhibitor PD 98059, in parallel with a reduction of uPA mRNA and protein levels. These data are consistent with a role of the MEK-ERK pathway for constitutive expression of uPA in these cells.

The effect of MEK-1 inhibition on cell proliferation and cyclin-D1 mRNA levels was also examined. Cyclin-D1 expression is induced by Ras (Liu et al., 1995) and Raf (Lloyd et al., 1997). The levels of cyclin-D1 expression in MDA-MB-231 cells were reduced by treatment with PD 98059 to levels similar to those observed in MCF-7 cells, and the rate of cell proliferation was inhibited.

In order to investigate how increased ERK signaling affects the expression of uPA and cyclin-D1, cells were treated with the phosphatase inhibitor okadaic acid (OA).

In order to examine whether ERK phosphorylation could be further increased in MDA-MB-231 cells, cells were treated with the phosphatase inhibitor okadaic acid (OA). ERK phosphorylation was strongly induced by OA, demonstrating that only a fraction of ERK1/2 is phosphorylated (and active) in MDA-MB-231. Somewhat surprisingly, the increase in ERK phosphorylation induced by OA was not paralleled by increases in cyclin-D1 and uPA expression. In contrast, the mRNA levels of Jun-B, a gene previously shown to be regulated by ERK and not by JNK in breast cancer cells (Alblas et al., 1998), were induced by OA. Similarly, the expression of the cyclin kinase inhibitor p21Cip1, known to be induced by strong ERK signaling (Sewing et al., 1997), was strongly induced.

All together, these results demonstrate that the level of ERK activity in MDA-MB-231 cells is sufficient to maintain a high level of uPA expression and rapid cell proliferation, and that further increases in signaling do not increase these levels, but may increase expression of the cdk inhibitor p21Cip1. The uPA and cyclin-D1 genes appear to respond to a lower threshold of ERK signaling than the Jun-B and p21Cip1 genes.

***Papers II***

About half of the patients diagnosed with muscle invasive bladder tumors die from their disease. An increased understanding of mechanisms that are associated with disease progression is essential for improved prediction of patient outcome and for development of novel therapeutic strategies. The urokinase plasminogen activator system is one of the most important proteolytic systems active in the extracellular matrix, and it was of interest to study the impact of tumoral uPA and uPAR expression on the out-come of patients with bladder carcinoma**..**

The tumor biopsies included in this study were sampled from a population-based material of 600 tumors. uPA and uPAR mRNA levels were determined in 291 samples using Taqman PCR, but due to RNA degradation adequate amplification could only be achieved from 194 tumors.

A significant correlation between uPA and uPAR expression was observed. The mean levels of uPA expression were similar in Ta and T1 tumors but were elevated in T2+ tumors. A similar pattern was observed for uPAR expression levels. The median uPAR expression level was significantly increased in T2+ tumors, whereas the median values for uPA expression were similar between different tumor stages. Based on the distribution of expression between tumors of different stages, we chose the median value as the cut-off for uPAR and the upper quartile value as the cut-off for uPA. Since classification of the cause of death was difficult for some of the patients, both overall death and death classified as caused by cancer was chosen as outcome variables.

The results from Cox multivariate analysis after adjustment for stage, grade, age and gender demonstrated that both elevated expression of uPA and uPAR were associated with an increased risk for overall death and death in cancer in the entire material. In addition, elevated levels of uPA were associated with an increased relative hazard ratio (RHR) for death from cancer in the T2+ group of patients. Using univariate analysis, high uPA and uPAR expression levels were associated with increased an RHR for death in cancer in the group of patients with G2b tumors (RHRs being 1.53 and 2.05 respectively) and G3 tumors (RHR being 1.64 and 2.17 respectively).

It has been reported that the co-expression of uPA and uPAR is important for the invasive behavior of bladder cancer cells *in vitro* (Hudson and McReynolds, 1997). Combining uPA and uPAR expression in the statistical analysis showed an increased RHRs for death from cancer in the entire group of patients and in the subgroup of patients with T2+ tumors, compared to the RHRs associated with these factors alone. Kaplan-Meier plots showed that after 18 months of follow-up, patients with high uPA and uPAR mRNA levels in their tumors had a survival rate of 60 % compared to a survival rate of 91% for patients with low uPA and uPAR expressing tumors.

Another interesting finding was that high uPAR expression was associated with an increased risk for development of metastasis during follow-up of all patients and in the subgroup of patients with muscle invasive tumors. We found that elevated uPAR expression was associated with a high risk of death in cancer in patients subjected to radical cystectomy (Figure 7). We speculate that this finding reflects a high propensity for micrometastasis, present at diagnosis, of tumors expressing uPAR. Patients with manifest metastatic disease are not expected to be cured by cystectomy. The decreased risk associated with elevated uPAR in the non-cystectomized patients may be due to a weaker importance of uPAR in this group of patients.

Conversely, a lower relative hazard ratio (1.5) was associated with high uPA expression in the group of patients subjected to cystectomy compared to patients that had not been cystectomized (RHR: 2.5). This could mean that some of the patients with high uPA expressing tumors actually had been cured by the cystectomy (Figure 7).

The data obtained by stratifying patients into cystectomized/non-cystectomized groups were generally not statistically significant. They do generate the hypothesis, however, that tumors expressing high levels of uPA and uPAR may have somewhat different properties; high uPAR being relatively more important for micrometastasis and high uPA more important for local progression. These hypotheses should be tested in future studies.

This is the first time uPAR expression has been studied in human bladder carcinomas. Previous studies have investigated the role of uPA in predicting the outcome of patients with this disease (Hasui et al., 1992). The main difference between the present study and previous studies is the larger number of tumors that were analyzed. In addition, in this study cancer specific death was used as the outcome variable together with overall death.

The results from this project strongly suggest that uPA and uPAR are useful prognostic markers for urinary bladder cancer, and that these markers can be used to refine the staging of T2+ tumors. Furthermore, the results suggest strategies for therapeutic intervention in this disease. Inhibitors of uPA and uPAR have been described (Magdolen et al., 2000). Future clinical trials will show if uPA and uPAR inhibitors/antagonists could be combined with already established curative methods.

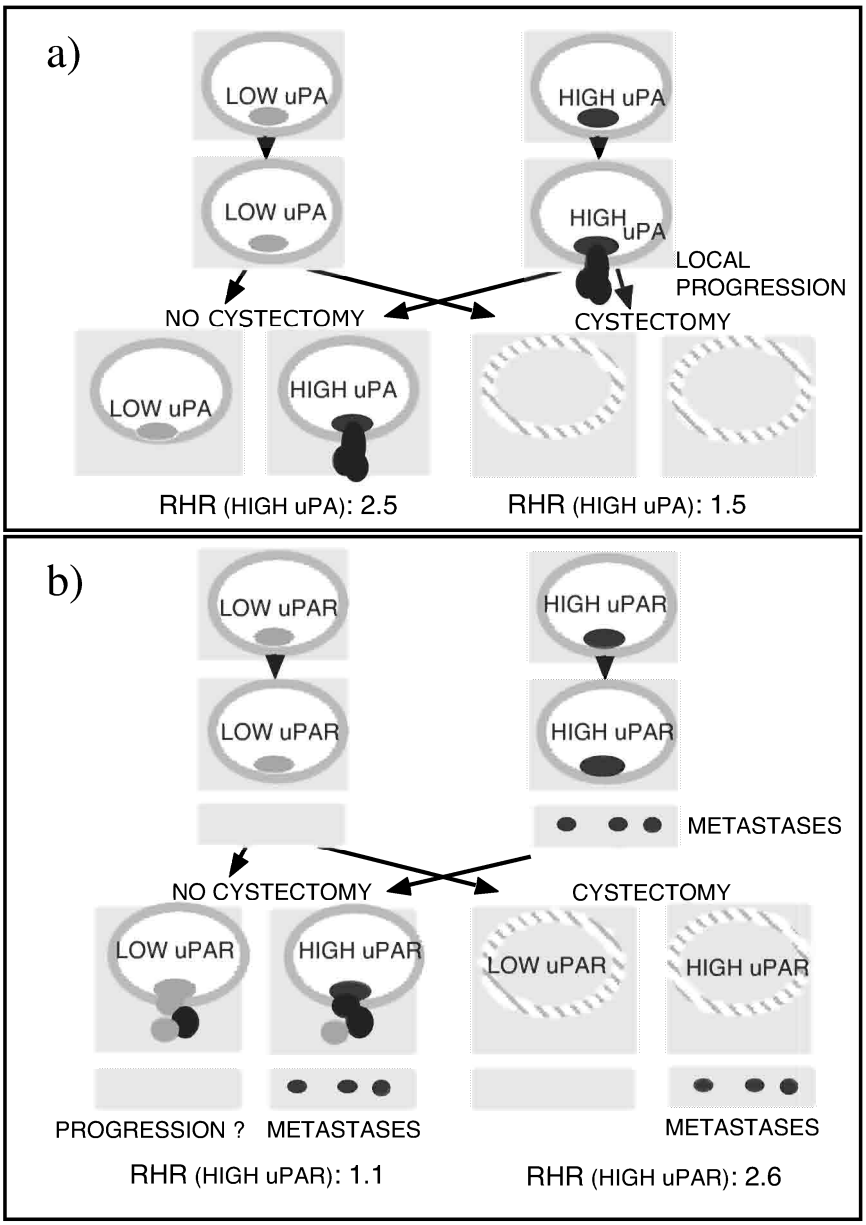


**Figure 6. A hypothetical model for the effects of high tumor expression of uPA and uPAR in cystectomized and non-cystectomized patients.** a) Patients with high uPA expressing tumors may benefit from treatment with radical-cystectomy whereas b) patients with high uPAR expressing tumors do not. These findings suggest a role for uPA in local progression and uPAR in the development of metastasis.

*Paper III and IV*

IL-1α is considered to be the major proinflammatory cytokine (Dinarello, 1991). Different effects of this cytokine on tumors and tumor cell lines have been reported. In addition, IL-1α produced by ER negative breast cancer cells has been reported to induce the expression of uPA in fibroblasts *in vitro* (Bhat-Nakshatri et al., 1998) and IL-1α strongly induces MMP (stromelysin and collagenase) expression in fibroblasts (MacNaul et al., 1990). These finding that made us hypothesize that IL-1α may stimulate uPA expression in stromal cells of bladder carcinomas, and that IL-1α and uPA expression may be co-expressed in many tumors.

The association between uPA and IL-1α expression was investigated in a material of 73 tumor biopsies using real-time quantitative PCR. IL-1α and uPA mRNA levels did not show any correlation to each others. The finding that IL-1α expression did not correlate to uPA expression does not support a role for this cytokine in the regulation of uPA expression in bladder tumors. It should be noted, however, that although uPA expression has been demonstrated in stromal cells of breast tumors (Vissher et al., 1995), it has not been demonstrated in bladder tumors, and therefore remains to be investigated.

IL-1α mRNA levels did not show any statistically significant correlation to tumor stage and grade. High mRNA levels of IL-1α were associated with longer overall survival in the entire material, in the group of patients with highly malignant tumors (G2b+G3), and in the group of patients with muscle invasive (T2+) tumors. Using Cox regression, adjusting for age, stage and grade, the RHR for low versus high IL-1α expression was 3.3. These results were quite interesting in the light of the previously reported anti-tumoral activity of IL-1α (Onozaki et al., 1985), the ability of this cytokine to inhibit angiogenesis (Norioka et al., 1987), and the anti-tumorigenic effects of IL-1α obtained after experimentally induced over-expression, most likely due to triggering of the immune system (Apte et al., 2000).

In this material, the uPA mRNA levels was 4 times higher in poorly differentiated tumors, and high uPA expression was associated with a relative risk of 3.1 for overall death.

In paper III, we extended our previous study to 157 tumors, and examined cancer-specific survival. Furthermore, using immunohistochemistry, we showed that tumor cells are the source of IL-1α expression in bladder tumors. This result is in agreement with studies showing that IL-1α is expressed by bladder cancer cell lines (Hayashi et al., 1994) and bladder cancer tissues (Sander et al., 1996).

In the extended material, IL-1α was associated with a RHR of 1.73 (n=157) and 1,76 (n=63) for death in cancer in all patients and in the group of patients with muscle invasive (T2+) tumors, respectively. The RHR for death from cancer associated with low IL-1α expression was approximately the same in the group of cystectomized and not cystectomized patients.

A number of attempts were made to better understand the association between IL-1α expression and poor outcome. We did not observe any association between low IL-1α expression and development of metastatic disease. Furthermore, since it has been shown that IL-1α expression inhibits cell proliferation *in vitro* (Onozaki et al., 1985), a correlation between IL-1α expression and the fraction of tumor cells in the S-phase might be expected. S-phase fraction data was available for approximately half of the tumors, but no correlation was observed between high IL-1α expression and low S-phase fraction. The RHR associated with

low IL-1α expression was adjusted for various established (stage, grade, and S-phase fraction, p53 abnormalities) and candidate markers (uPA and uPAR, Rb abnormalities) using bivariate analysis. Adjusting for age and stage resulted in a decrease of the RHR for cancer specific death. The lack of a mechanism to explain the association between IL-1α expression and outcome is of some concern. Future studies should be directed to investigate whether IL-1α secreted by bladder tumor cells is involved in the recruitment of immune cells, such as macrophages.

The RHRs for death in cancer associated with different parameters was examined in this material. The results demonstrated that p53 mutations were associated with the highest relative risk, followed by S-phase fraction. Loss of heterozygosity at p53 and the Rb locus were not associated with a significant risk for death in cancer.

The main result of our study, demonstrating an increased risk for death in cancer for patients with low IL-1α expressing tumors, needs to be confirmed in an independent patient cohort. Moreover, the mechanism underlying the antitumoral effect of IL-1α must be clarified.

# CONCLUSIONS

**Paper I:** Our data show that a relatively low level of ERK signaling is sufficient for constitutive overexpression of uPA in MDA-MB-231 breast carcinoma cells. Increases in ERK activity did not result in further increased uPA levels, whereas Jun-B and p21Cip1 expression was induced. In conclusion, the levels of activated ERK in MDA-MB-231 cells were sufficient to allow rapid cell proliferation and high uPA expression.

**Paper II**: High uPA and uPAR levels were associated with an increased risk for death from cancer in a material consisting of 194 bladder tumors. Moreover, high uPA expression was significantly associated with an increased risk for death from cancer in the group of patients with T2+ tumors. Elevated uPAR levels were associated with an increased risk for development of metastasis. Coexpression of uPA and uPAR was associated with a further increased RHR for death in cancer, which may reflect the involvement of both of these markers during disease progression.

**Paper III and IV:** No association was observed between IL-1α and uPA expression, suggesting that other mechanisms are responsible for the induction of uPA expression in bladder tumors. Low IL-1α expression was associated with an increased risk for death in cancer in the group of patients with T2+ tumors. Low IL-1α expression did not correlate to an increased risk for development of metastasis or to high S-phase fraction.

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