EUROPEAN JOURNAL OF CANCER 42 (2006) 811–819

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Position Paper

Molecular regulation of an invasion-related molecule – options for tumour staging and clinical strategies

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ARTICLE INFO

Article history:
Received 18 January 2006
Accepted 19 January 2006
Available online 17 April 2006

Keywords:
Urokinase-receptor
Transcription
In vivo
AP-1
AP-2
Sp1
EMSA

ABSTRACT

This review provides a summary of the European Association for Cancer Research Award Lecture, presented at the ECCO13 meeting in Paris in November 2005. It is a brief overview on the biological and clinical relevance of the urokinase receptor (u-PAR), an essential molecule to promote invasive and metastatic tumour phenotype and shown to be associated with early relapse and poor prognosis in many different types of cancers. The review summarizes the most important transcriptional mechanisms regulating *u-PAR* gene, and will focus on the differential binding of transcription factors to *u-PAR* promoter elements from studies in resected tumour and normal tissues of colorectal and gastric cancer patients. These studies conducted by our group may help to understand transcriptional mechanisms, which are employed to promote invasion and metastasis, in subpopulations of cancer patients. Such studies could lead to a more target-oriented patient selection and therapy against transcriptional and oncogeneic regulators in cancer.

1. Tumour-associated proteolysis, invasion and the urokinase system

Invasion and metastasis of malignant cells require the degradation of extracellular matrix components (e.g. type IV collagen, laminin, vitronectin), allowing tumour cells access to systemic circulation. These events can be achieved, at least in part, by "tumour-associated proteases" which have been classified into serine, aspartic, cysteine, threonine and metalloproteinases.[1–4] One of the proteases which has been implicated in the invasive phenotype of tumour cells is the urokinase-type plasminogen activator (u-PA), a 55 kDa serine protease which, via activation of plasminogen to active plasmin, is able to cleave several components of the extracellular matrix including fibrin, fibronectin, proteoglycans, laminin and collagen IV. [3–13] Urokinase is secreted from fibroblasts, leukocytes, urogenital cells and also tumour cells as a single-chain proform, and is proteolytically activated either extracellularly or by binding to the urokinase-receptor (u-PAR).[12–17]

2. u-PAR promotes u-PA-mediated proteolysis by specific structural and functional properties

The proteolytic efficacy of the urokinase enzyme relies on its interactions with factors that, together with u-PA, comprise

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doi:10.1016/j.ejca.2006.01.018

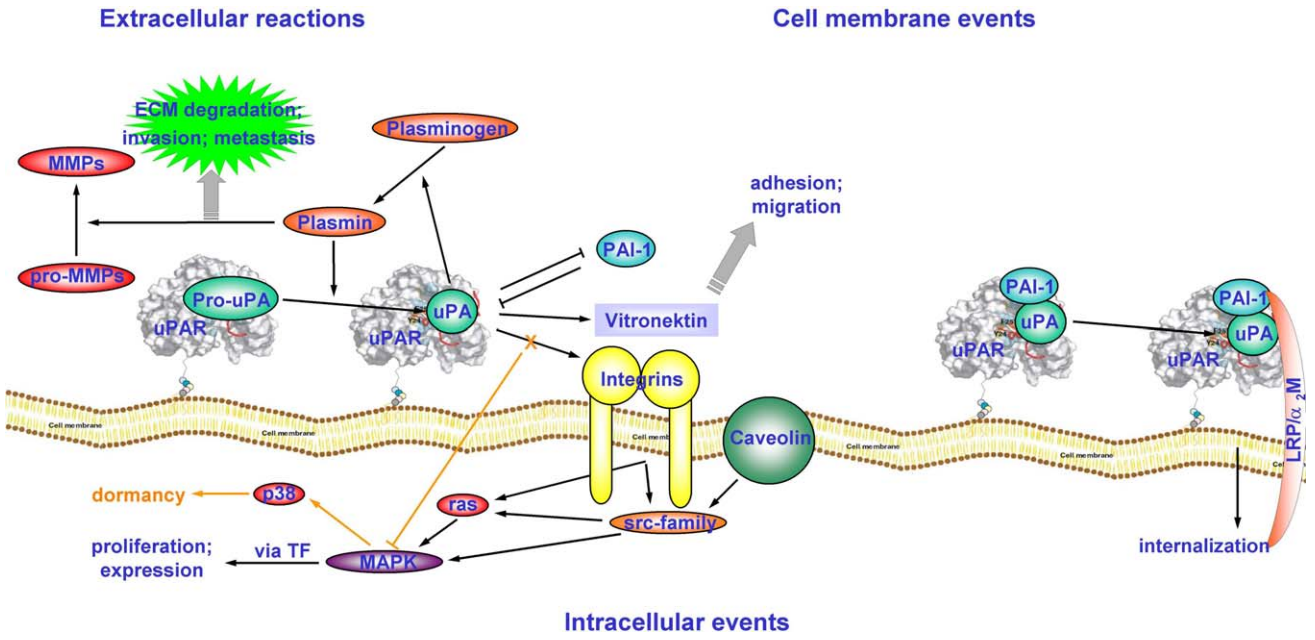

Fig. 1 – Examples of functional properties of the urokinase receptor (u-PAR). The drawing of the molecular structure of u-PAR has been adapted from crystal structure recently published.[132]

the "u-PA-system".[6] Among these, the u-PA-specific inhibitors plasminogen-activator-inhibitor (PAI)-1, PAI-2 and nexin 1, and the cell surface receptor u-PAR are essential. u-PA binds to the 55–60 kDa heavily glycosylated, disulfide-linked cell surface receptor (u-PAR) specifically and with high affinity.[18,19] Receptor-bound u-PA is inactivated by PAI-1 (-2), the trimeric complex u-PAR/u-PA/PAI is internalized into the cell, in part together with α-2-macroglobulin receptor and its ligand.[20–25] The free u-PAR is recycled to the cell surface to bind a further u-PA-molecule.

The u-PAR consists of three similar repeats approximately 90 residues each and is anchored to the cell membrane via a glycosyl-phosphatidylinositol chain.[26,27] This GPI-anchor is hypothesized to enable a high intramembrane mobility.[18–31] Furthermore, u-PAR is glycosylated at N-residues of glucosamine and sialic acid, thereby regulating its affinity for u-PA.[26] The receptor-bound u-PA, as compared to the fluid phase enzyme, activates plasminogen much more efficiently, this being reflected by a 40-fold decrease in K_m of urokinase for its substrate.[32]

u-PAR gene expression plays an important role in inflammation, tissue remodeling, wound healing, embryogenesis, chemotaxis and adhesion.[2,5,6,17,21,29,33–46] Furthermore, recent studies have implicated that an interaction of u-PAR with integrins with resulting signal transduction events can regulate a switch between cell proliferation and dormancy.[47] Further studies have implicated u-PAR in cell migration, cytoskeletal rearrangement and proliferation, since u-PAR can be found localized at cellular focal contacts, lamellipodia and in caveolae, and can induce the phosphorylation of focal adhesion kinase (FAK), cytoskeletal proteins and Src-family members.[40,45,48–54] A schematic illustration of the most important functions of u-PAR is given in Fig. 1.

3. u-PAR gene expression promotes invasion and metastasis

Numerous studies have shown overexpression of u-PAR in diverse human malignant tumours in contrast to the corresponding normal tissue and/or surrounding stromal cells.[55–63] and suggested u-PAR as a characteristic of the invasive or even the malignant phenotype.[64–67]

Several experimental studies implicated u-PAR in tumour invasion and metastasis. For example, overexpression of human u-PAR increased the ability of osteosarcoma cells to penetrate a reconstituted basement membrane,[68] and the invasive potential of tumour cells into a chicken-embryo chorioallantoic membrane.[69] In squamous carcinoma cells,[70] antisense *u-PAR* decreased invasiveness into a chorionallantoic membrane. In further in vitro models, anti-messenger strategies or antibodies reduced invasion of diverse cell lines.[71,72] In cultured lung cancer, optimum invasiveness was seen only if u-PA, PAI-1 and u-PAR were co-expressed.[73] For the step of intravasation into blood vessels, Kim and colleagues[74] have shown that the expression of u-PAR, besides u-PA and MMP-9, is required. Diverse u-PAR antagonists prevent tumour growth, angiogenesis, extracellular matrix degradation and invasion in diverse cell lines, for example colon cancer.[66,75–77] Further studies in colon cancer[57,58] postulated an in vivo paracrine interaction between tumour- and surrounding stromal cells, the former overexpressing u-PAR, the latter secreting u-PA and PAIs.

4. The relevance of u-PAR for prognostis and potential therapeutic therapy of cancer patients

The clinical relevance of u-PAR and its association with invasion and metastasis has been proposed by studies which

found a higher amount of u-PAR in metastases as compared to primary tumours.[78–80] Prospective studies on diverse cancers involving large patient numbers have demonstrated a correlation of high u-PAR (and/or u-PA-/PAI-1) expression with short survival times and advanced tumour stages. Thus, the u-PAR and/or u-PA/PAI-1 have already been shown to be significant prognostic risk factors in many cancers including breast,[79,81] lung,[42] colon,[82,83] esophageal and gastric cancer,[59,60,84–88] while some of these studies even reported an independent impact in multivariate analysis. In 203 gastric cancer patients, we showed that the u-PA-system (represented by PAI-1) together with evidence of minimal residual disease is appropriate to establish a biological staging model of gastric cancer, able to identify new subgroups of patients which are high at risk despite having early stage tumours according to the pTNM-classification.[85] In addition, we found that the u-PAR is a potential marker for a positive in vivo selection of disseminated tumour cells in the bone marrow, and a characteristic for the establishment of minimal residual disease[89–91] in solid cancer. In addition, u-PAR on disseminated cells significantly correlates with a poor survival of gastric cancer patients.[80,92,93] In recent studies on breast cancer, Harbeck demonstrated the u-PA-system as a predictor of response to chemotherapy.[94] Taken together, these studies stongly suggest a clinical relevance of u-PAR and the u-PA-system for diagnosis, systemic spread, prognosis, prediction, and response to therapy.[71,95]

5. Molecular and transcriptional regulation of *u-PAR* gene expression-results from in vitro studies

The objective to counter *u-PAR* gene expression in malignant tumours necessarily implies the question as to how it is regulated. As suggested by previous studies, *u-PAR* gene expression is controlled mainly at the transcriptional level in malignancies such as colon cancer, although further mechanisms such as mRNA stability and receptor recycling are additionally involved.[96–101] Altered transcription of the gene is the main mediator of *u-PAR* gene expression brought about by, for example, epidermal growth factor (EGF), basic fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), transforming growth factor β type 1 (TGF-β1), phorbol 12-myristate 13-acetate (PMA), IFN-α- or IFN-γ, protein kinase C (PKC), protein kinase A (PKA)/c-AMP, the MAPK- and the JNK-pathway.[96,99,110,111,37,97,112,113,108]

The *u-PAR* gene spans 7 exons and is located on chromosome 19q13 [102] Transcription from the gene yields a 1.4 kb mRNA or an alternatively spliced variant lacking the membrane attachment peptide sequence.[103,104] The human *u-PAR* promoter sequence was first described by Wang et al.[64] and Soravia et al.[105] Like classical "housekeeping genes", it lacks TATA and CAAT boxes and contains a GC-rich proximal sequence with multiple Sp1 consensus elements. Initial studies[105] reported three potential transcriptional start sites, the most upstream of which – an A following a C – appeared to be the main transcriptional initiation site.[106,107]

Soravia et al.[105] reported that the basal expression of the gene was regulated via Sp1 motifs proximal and upstream of the transcriptional start site. In colon cancer, constitutive and PMA-inducible expression of the gene required a footprinted region located at basepairs −190/−171 of the promoter containing an AP-1 consensus motif bound with Jun-D, c-Jun, c-Fos and Fra-1,[96] this motif mediated the induction of *u-PAR* gene expression via the MAPK- and the JNK-pathway.[97,108]

As shown by one of our previous studies, this AP-1 consensus motif was also required for the induction of *u-PAR* gene expression brought about by the K-ras oncogene.[116] A substantial reduction of endogenous u-PAR protein and u-PAR-mediated proteolysis was observed in HCT116 clones in which activated K-ras had been deleted. In gelshift- and CAT-reporter analysis, we detected a decrease of the binding of c-Jun, Jun-D, c-Fos and Fra-1 in the K-ras-knockout-clones, and this was paralleled by a severe reduction of promoter activity when the AP-1-consensus motif within promoter region −190/−171 was deleted. These results suggest that activated K-ras regulates u-PAR and u-PAR-mediated proteolysis in colon cancer, at least in part via region −190/−171 of the promoter bound with AP-1-transcription factors. Muller et al. showed that constitutively active V12 H-Ras and Rho-A produced an increase in transcription from the *u-PAR* promoter.[114] In addition, Okan et al.[115] showed that a constitutively activated RalA mutant can activate u-PAR transcription through an AP-1 dependent mechanism.

Further studies demonstrated another footprinted region (−152/−135) of the *u-PAR*-promoter containing putative binding sites for (mismatched) Sp1, AP-2 and PEA3 binding motifs.[96] We showed that this motif is bound by an AP-2α-like protein being closely related to, however not identical with, authentic AP-2α, Sp1 and Sp3 transcription factors.[109] Binding of the AP-2α-like protein was found to be important for a constitutively high *u-PAR*-promoter activity in a highly invasive colon cancer cell line, and for PMA-stimulated u-PAR expression in a cell line with low constitutive u-PAR expression. Interestingly, a dominant negative AP-2 expression construct not only reduced *u-PAR* promoter activity and *u-PAR* gene expression, but also substantially inhibited u-PAR-mediated proteolysis. These results suggest that an inhibition at the transcriptional level can be applied to suppress u-PAR-mediated proteolysis, thereby potentially invasion and metastasis.

The binding of Sp1 to region −152/−135 of the *u-PAR* promoter was shown to be important in part for PMA-induced *u-PAR* promoter activity, but, more interestingly, for the induction of *u-PAR* gene expression by the *c-src*-oncogene in colon cancer.[127] In SW480 colon cancer clones stably expressing a constitutively active Src (Y-c-src527F), increased u-PAR protein and laminin degradation paralleling elevated Src activity was evident as compared to parental cells. Nuclear run-on experiments indicated that the increased u-PAR protein was due largely to transcriptional activation. While transient transfection of SW480 cells with Y-c-src527F induced a u-PAR-CAT-reporter, mutations preventing Sp1-binding to promoter region −152/−135 abolished this induction. Mobility shift assays revealed increased Sp1 binding to region −152/−135 with nuclear extracts of Src-transfected SW480 cells. Finally, the amounts of endogenous u-PAR in resected colon cancers significantly correlated with Src-activity, a high endogenous Src-activity in a first prognostic analysis being an independent poor prognostic factor in colorectal cancer.[117] These data suggest that *u-PAR* gene expression

and u-PAR mediated proteolysis are regulated by Src, and require the promoter region (−152/−135) bound with Sp1, thus, demonstrating that transcription factor Sp1 is a downstream effector of Src. They further suggest a clinical relevance of Src-regulated *u-PAR* gene expression, a notion which was recently supported by evidence that specific Src- and u-PAR-inhibition leads to a significant reduction of invasion of colon cancer cells.[118]

Recently, a new tumour suppressor gene (*pdcd4*) has been identified inhibiting neoplastic transformation of epithelial cells.[121–123] Our own preliminary studies indicate that Pdcd4 downregulates *u-PAR* gene expression by inhibiting *u-PAR* gene transcription, this in part being mediated by Sp3 bound to the same *u-PAR* promoter region −152/−135, which mediates constitutive PMA- and Src-inducible *u-PAR* gene expression.[124] Therefore, the two promoter elements −152/−135 (AP-2/Sp1/Sp3) and −190/−171 (AP-1) appear as two key *cis*-elements regulating diverse means of u-PAR-control, a notion which motivated us to select these two motifs for in vivo screening of resected patient tumours (see below). An overview on key mechanisms mediated by these two motifs can be seen in Fig. 2.

Further transcription factors have been implicated in the regulation of *u-PAR*, for example NFκB[119] bound to a non-consensus NFκB motif (−51/−30), and KLF4[120] bound to multiple regions of the proximal 200 bp of the *u-PAR* promoter. PEA3 bound to a PEA3/ets motif at −248 bp, via β3-integrin, acts as a transcriptional repressor.[121]

6. In vivo evidence for u-PAR regulation, and overview on own studies differentiating transcriptional regulation of u-PAR in resected patient tissues

All data in the aforementioned studies have been gained from in vitro models and mostly cell lines. However, there is a big difference between artificial cell line models, and "real" patient tumours, or the in vivo situation in general. Recently, first reports on the transcriptional regulation of u-PAR in vivo have been published, implicating a differential functional relevance of different *u-PAR* promoter motifs for diverse tissues, and our own recent studies clearly implicated a differential impact of *u-PAR* promoter motifs in resected tumour and normal tissues.

In an elegant study, Wang et al.[125] defined *u-PAR* promoter regions required for expression of this gene in transgenic mice bearing a LacZ reporter regulated by varying amounts (0.4, 1.5, and 8.5 kb) of upstream sequence. The 0.4-kb *u-PAR* promoter directed weak and strong LacZ expression in the placenta and epididymis, respectively, both of which are tissues that express endogenous u-PAR. Conversely, transgene expression in the apical cells of the colon positive for endogenous u-PAR protein required 1.5 kb of upstream sequence for optimal expression. Furthermore, a new regulatory region spanning −1295/−1192 driving u-PAR expression in colonic cells, was suggested in this work. Placental transgene expression was augmented with the 8.5 kb upstream fragment compared with

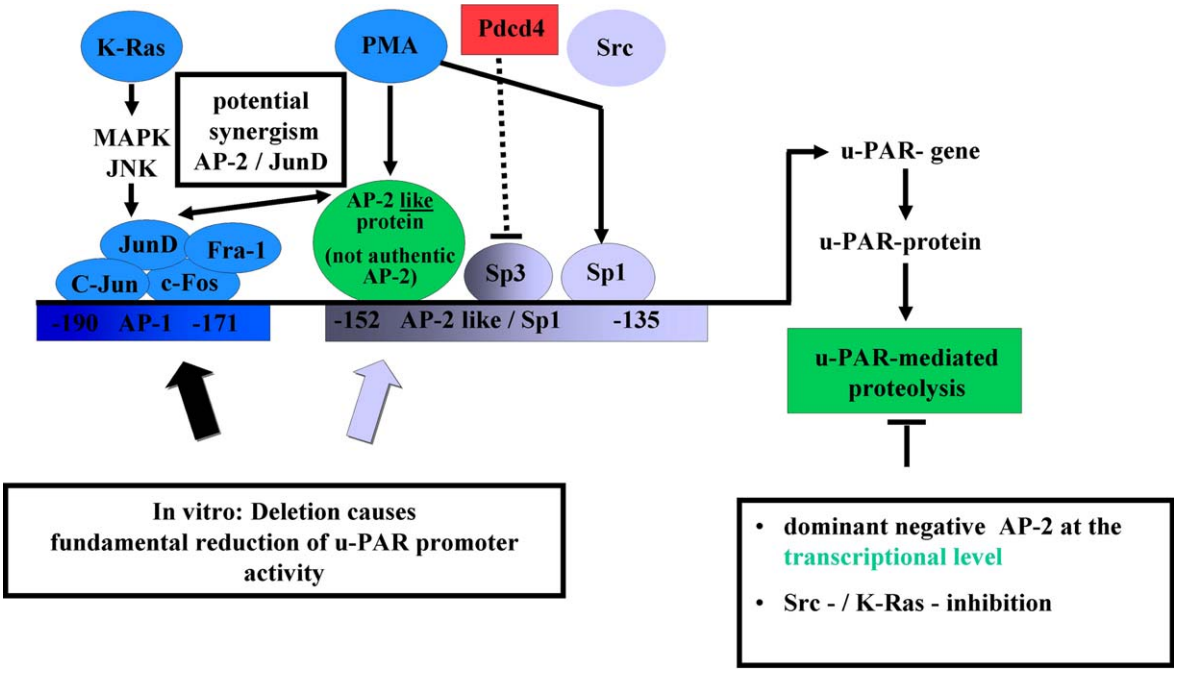

Fig. 2 – Schematic overview of the main transcription factors that binds to *u-PAR* promoter motifs, which has been studied by our group for their relevance in vivo in resected patient tissues. Both elements are highly relevant for *u-PAR* gene expression in cancer, since their deletion causes a dramatic reduction of *u-PAR* promoter activity in vitro. Both elements mediate diverse means of u-PAR control brought about by important oncogeneic molecules such as K-Ras or Src, in addition to tumour suppressor Pdcd4. Previous in vitro data indicated a potential synergism between both promoter motifs, especially between the AP-2-like protein and JunD. The figure further indicates different possibilities of inhibiting *u-PAR* gene expression or u-PAR mediated proteolysis, for example by a natural tumour suppressor, inhibition of signalling cascades (Src/Ras), or inhibition directly at the transcriptional level.

the shorter 1.5 kb fragment, indicating additional element(s) between −1.5 and −8.5 kb for optimal expression. This data suggested new upstream sequences for tissue-specific gene expression from a transgenic in vivo model, over and above previous in vitro studies.

In our recent studies, we were interested to ask if transcription factor binding to the most relevant *u-PAR* promoter elements, which had been detected previously in our cell line models, could not only be verified in resected tumour tissues of cancer patients, but also if differences in transcriptional regulation via these promoter elements could be detected between resected tumour and corresponding normal tissues of cancer patients. Thus, potential patient populations, who could profit from molecular therapies targeted against such promoter regions, or against signalling cascades being mediated by these promoter regions, were sought. In the first clinical study comparing transcription factor binding to *u-PAR* promoter region −152/−135 (the AP-2/Sp1/Sp3-motif, see above and Fig. 2) in 145 patients with resected colorectal or gastric cancers, we found an almost tumour-specific transcription factor binding to this important *u-PAR* promoter motif in almost 60% of cases when comparing primary tumours and corresponding normal mucosae,[126] which correlated with high endogenous u-PAR protein amounts in these tumour tissues. This concerned especially the AP-2-like protein and Sp1. This first study suggested that, in a subpopulation of as high as 60% of patients with colorectal or gastric cancer, a rather tumour-specific transactivation of *u-PAR* gene expression by this combined AP-2/Sp1/Sp3 promoter motif could be speculated. Since this combined AP-2/Sp1/Sp3 motif is a crucial *u-PAR* promoter element (see chapter above) mediating diverse means of u-PAR regulation (Fig. 2), the deletion of which caused a dramatic reduction of spontaneous *u-PAR* promoter activity,[96] a differential activation of this motif between tumour and normal tissues in patients would be highly attractive for potential targeting strategies.

As indicated in previous sections a further motif important for *u-PAR* gene regulation in cancer had been implicated by previous in vitro studies, which was a consensus AP-1 region (−190/−171). In addition, our previous in vitro studies had suggested a synergism between this AP-1 motif, and the combined AP-2/Sp1/Sp3 motif in cell lines.[96,109] For this second AP-1 motif, we performed an additional translational study on 103 patients with colorectal cancer and compared resected tumour and normal tissues for tumour-specificity of this motif and in vivo evidence of synergism.[133]

Tumour-specific AP-1-binding to region −190/−171 of the *u-PAR*-promoter was found in 40% of patients, which is less than for the AP-2/Sp1/Sp3 motif. Subgroup analysis showed tumour-specific binding for c-Fos in 58%, for c-Jun in 50%, for JunD in 39%, and for Fra-1 in 4% of cases. AP-1-binding correlated significantly with u-PAR protein amounts in both normal and tumour tissues ($P < 0.001$), this being in contrast to a tumour-specific correlation with u-PAR of the AP-2/Sp1-region. In analysis for both promoter regions, 62% of cancers showed simultaneous binding for AP-1, AP-2 and Sp1, 11% for AP-1 and AP-2, 16% for AP-2 and Sp1, a minority of cases for binding of one factor only. The binding of AP-1, AP-2 and Sp1 correlated significantly with each other ($P < 0.001$), the combination of AP-1 and AP-2 showing the highest correlation with u-PAR ($P = 0.008$). Preliminary survival analysis indicated a trend for poorer prognosis for binding of all three factors. Therefore, the AP-1 site −190/−171 appeared as a less tumour-specific regulator than the Sp1/AP-2-motif −152/−135, which is again interesting for future potential clinical consequences. Data furthermore corroborated the hypothesis of synergism between both elements in resected tumours. These studies with resected patient tissues has demonstrated for the first time, that certain promoter elements seem to be employed differentially in different human tissues to promote the expression of a gene, or might even be specifically employed by a tumour to upregulate gene products that are relevant for invasion/metastasis.

7. Conclusions

Taken together, the u-PAR is a key factor in promoting tumour-progression, invasion and metastasis. Therefore, downregulation of its expression would be a clinically promising strategy to inhibit cancer invasion and metastasis. Potential strategies that could be hypothesized to achieve down-regulation of *u-PAR* gene expression and proteolysis may be the inhibition of Src and K-Ras (e.g. by small molecular compounds, see Fig. 2) or even a direct targeting of transcriptional mechanisms or the promoter itself (e.g. as shown in Fig. 2 with dominant negative AP-2, or as triplex-forming oligonucleotides). However, for applying such therapeutic strategies, appropriate patient selection will be crucial. Therefore, we consider our first in vivo studies comparing resected tumour and normal tissues of cancer patients, for the differential activity of transcription factors at different promoter elements of the *u-PAR* gene, as a first step in defining appropriate patient sub-populations. Similar studies may lead to a much more precise molecular classification of patient sub-groups, who may profit from certain therapeutic strategies available at the molecular level. Considering further therapeutic approaches targeting u-PAR and the uPA-system which are already at hand,[118,128–131] in addition to more concerted efforts to provide precise molecular diagnostics in patient specimen, it is to be expected that u-PAR, the u-PA-system or its molecular regulators might add significantly to cancer diagnosis and therapy in the future.

Taken together, a promising clinical potential in targeting u-PAR, the u-PA-system or its molecular regulators, is expected in the treatment of invasion, progression, and metastasis of cancer.

Conflict of interest statement

None declared.

Acknowledgments

H.A. is supported by the Alfried Krupp von Bohlen und Halbach-Stiftung, Essen, Germany (Awardee of the Alfried Krupp Prize for Young University Teachers of the Krupp Foundation), the Wilhelm Sander Stiftung, Munich, the Auguste Schaedel-Dantscher-Stiftung, Garmisch, and the National Cancer Institutes (NCI), USA, in collaboration with Verein zur Foerderung

der Krebsforschung, Hamburg. I thank Dr. Jens Schumacher, Dr. Jörg Leupold and Mrs. Erika Hillerich for helpful comments and support with the manuscript.

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