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Expression of urokinase plasminogen activator and receptor in conjunction with the *ets* family and AP-1 complex transcription factors in high grade prostate cancers

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Abstract

Expression of the urokinase plasminogen activator (uPA) and its receptor (uPAR) correlates with tumour cell invasiveness and helps to determine the prognosis of prostate and other cancers. The purpose of this study was to establish in prostate cancer, the *ets* family and AP-1 complex transcription factors that might activate the inducible AP-1 and AP-1/PEA3 elements of the uPA enhancer. uPA and uPAR were expressed preferentially in adenocarcinoma cells, but not the stroma of high grade prostate cancers. The *ets* family paralogues Fli-1 and Elf-1 were also highly expressed in adenocarcinoma cells of the majority of cancers, while Erg 1,2 and Ets-2 were expressed in a minority of cancers and Elk-1, PEA3 and PU.1 were minimally expressed. A minority of cancers expressed high levels of cytoplasmic and/or nuclear c-Jun and c-Fos transcription factors. We speculate as to the molecular basis for such expression. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The expression of the urokinase plasminogen activator (uPA) and its receptor (uPAR) are prognostic indicators for the clinical outcome of many cancers [1,2], including adenocarcinoma of the prostate [3]. uPA activates numerous matrix metalloproteinases and concomitantly promotes proteolysis of the extracellular matrix, tumour cell migration, intra- and extravasation, and also activation of mitogens and effectors of angiogenesis and of the osteoblastic reaction important for bone metastases [2,4,5]. Tumour cell invasiveness and metastatic capacity are highly correlated with the expression of uPA and uPAR and are blocked by their inhibitors.

Expression of uPA is controlled by an inducible enhancer region bound by AP-1 complex proteins (Jun, Fos and activating transcription factor (ATF) family members and partners), *ets* family proteins, homeodomain proteins, the nuclear factor (NF)κB transcription factor [6]; also, the turnover of uPA mRNA is regulated [7]. The levels and activities of these transcription factors and the proteins determining uPA mRNA stability are modulated by, among others, the mitogen- and stress-activated protein kinase pathways [8]. In particular, prostate cancer progression correlates with activation of the mitogen-activated protein kinase (MAPK) pathway [9].

While AP-1 complex and NFκB proteins are expressed in many tissues, *ets* family proteins can be specific to particular types of tissues and cells and to cancers derived from them [10]. The over two dozen paralogous members of this ancient family of transcription factors can be organised into five subfamilies and 13 groups, based upon sequence conservation of their DNA binding domains [11]. Activation of the uPA enhancer also requires homeodomain proteins [12], which like *ets*

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proteins, can be specific to the development and maintenance of epithelial cells and the cancers derived from them. As the requirement for such tissue-specific proteins for expression of uPA may provide targets to selectively block tumour cell invasion, we seek to identify those particular *ets* family, AP-1 complex and homeodomain proteins that are highly expressed in prostate cancer, and then plan to analyse whether they are involved in uPA expression.

2. Materials and methods

2.1. Tissue samples

Archival tissue samples of 25 high-grade prostate cancers with an average Gleason score greater than 7 and 4 tissue samples of benign prostate hyperplasia (BPH) were provided by the Pathology Service of the Medical Academy, Sofia, Bulgaria. The tissues had been fixed in 10% buffered formalin and embedded in paraffin. Sections were cut at 4–8 μm, placed onto Superfrost glass slides (Fisher, USA), stained with haematoxylin and eosin, and independently evaluated by two pathologists.

2.2. In situ *hybridisation and immunohistochemistry*

uPA and uPAR cDNAs were prepared from human U937 cell mRNAs, cloned and transcribed by T7 RNA polymerase using the Genius IV kit (Boehringer, USA) in the sense and antisense orientation to prepare riboprobes labelled with digoxigenin-bio 11-d uridine triphosphate (UTP). For *in situ* hybridisation, the slides were deparaffinised, rehydrated and microwaved twice for 5 min in Antigen Retrieval Solution (Vector Laboratories, USA). Hybridisation was carried out in a humidified chamber at 37°C overnight with 300 ng/ml digoxigenin-labelled probe. After incubation with polyclonal sheep antidigoxigenin Fab fragments (750 U/ml, 1:200 dilution) signals were developed with 0.2 mg/ml nitroblue tetrazolium, 0.3 mg/ml 5-bromo-4-chloro-3-indoyl phosphate and 300 μg/ml levamisole (Sigma, USA) as recommended by the manufacturer, and the tissues counterstained with nuclear fast red. Hybridisation specificity was assessed by RNAse pretreatment, which abolished the signal, and by the lack of signal when a sense probe was used for the hybridisation.

Tissue immunostaining was performed with the primary antibodies listed in Table 1. Briefly, tissues were fixed to glass slides by heating for 30 min at 50°C. After deparaffination and rehydration with graded alcohols, the tissues were rinsed with phosphate-buffered solution (PBS) and incubated with 0.1% (v/v) hydrogen peroxide, and then with non-immune serum (from the same source as the secondary antibody) for 30 min at room temperature. Excess serum was removed and the tissues were incubated with the primary antibody overnight at 4°C in a humidified chamber. The working dilution for each antibody was determined empirically as the lowest concentration required to produce unequivocal staining (Table 1). After four washes with PBS for 15 min, secondary antibodies were applied for 30 min at room temperature, and colour developed using the ABC Elite kit (Vector Laboratories, USA) according to the manufacturer's recommendations with 3′-3′-diaminobenzidine as a substrate, with a peroxidase concentration of 0.3% (w/v) followed by 0.1% (w/v) osmium intensification. The tissues were dehydrated and mounted. Staining of at least 10% of the tissue with moderate or great intensity was considered to be a positive test. Control slides in which PBS was substituted for the primary antibody were included in each experiment.

Table 1
Antibodies

Antibody	Supplier	Catalogue no.	Working dilution	Antigen retrieval
uPA	Am. Diag.	#3689	1:100	–
uPAR	Am. Diag.	#3937	1:50	–
Ets-1	Santa Cruz	sc-350	1:2000	+
Ets-2	Santa Cruz	sc-351	1:2000	+
Fli-1	Santa Cruz	sc-356	1:2000	+
Elf-1	Santa Cruz	sc-361	1:2000	+
Erg1,2	Santa Cruz	sc-353	1:2000	+
Elk-1	Santa Cruz	sc-355	1:2000	+
PEA3	Santa Cruz	sc-113	1:2000	+
c-Jun	Santa Cruz	sc-45	1:300	+
c-Jun	Novo Castra	NCL-cJUN	1:70	+
c-Fos	Santa Cruz	sc-52	1:300	+
p53	DAKO	M 7001	1:100	+
E-cadherin	Zymed	13-1700	1:100	–

To confirm the specificity of c-Jun staining, sections were incubated with two different anti c-Jun antibodies — mouse monoclonal (Novo Castra, USA) and rabbit polyclonal (Santa Cruz, USA), and consecutive slides were pretreated with bacterially expressed c-Jun protein (for the monoclonal antibody) or with synthetic peptide (for the polyclonal antibody) used for immunisation. Reactivities of both antibodies were blocked by this procedure.

3. Results

3.1. uPA and uPAR mRNAs and proteins are expressed by adenocarcinoma cells of high grade prostate cancers

Immunohistochemical staining for uPA was observed in the vast majority (over 80%) of tissue sections of predominantly high grade prostate cancers, where uPA protein was predominantly localised in adenocarcinoma cells, and not the stroma (Fig. 1a). The few cancers in

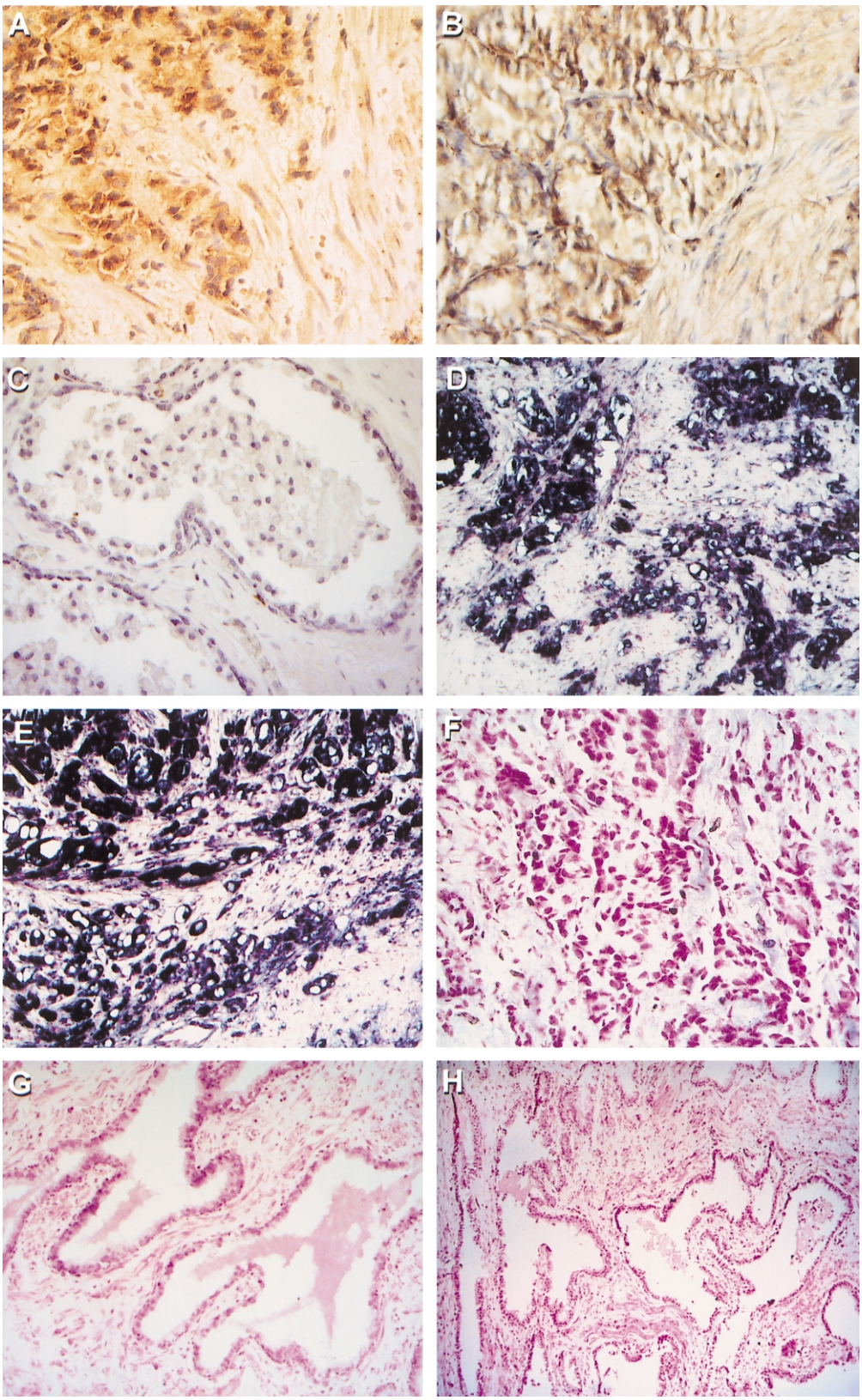

Fig. 1. Immunohistochemical localisation of uPA and uPAR proteins and transcripts in prostate cancer and benign prostate hyperplasia (BPH) tissues. (a) uPA protein expression in adenocarcinoma cells of high-grade prostate cancer. Note the lack of stromal involvement. (b) uPAR protein expression in adenocarcinoma cells of high grade prostate cancer. (c) Weak or lack of uPAR expression in BPH tissues. (d) uPAR mRNA *in situ* hybridisation signals in adenocarcinoma cells of high grade prostate cancer. (e) uPA mRNA *in situ* hybridisation signals in adenocarcinoma cells and infrequent fibroblast-like or inflammatory cells in the stroma of high grade prostate cancer. (f) Lack of *in situ* hybridisation signals by sense uPA riboprobe. (g) Lack of signals for uPA mRNA in BPH tissues. (h) Lack of signals for uPAR mRNA in BPH tissues.

which little or no uPA protein was detected were of intermediate grade. By comparison, no or very weak expression of uPA protein was observed in BPH tissues (data not shown). *In situ* hybridisation revealed high levels of uPA mRNAs in prostate adenocarcinoma cells (Fig 1e; compared with control hybridisation using the sense probe in Fig. 1f) and little or no expression in BPH tissues (Fig. 1g). Some fibroblast-like cells expressed uPA mRNAs in the tumour periglandular areas, but glandular and periglandular cells in the non-tumour tissues contained little or no uPA mRNAs.

uPAR was also detected in the majority of high-grade cancers, in the cytoplasm and membranes of adenocarcinoma cells (Fig. 1b), with little or no expression in BPH tissues (Fig. 1c). *In situ* hybridisation localised uPAR mRNA expression to the adenocarcinoma cells (Fig. 1d). Weak or no signals for uPAR mRNA were detected in BPH tissues (Fig. 1h).

These observations indicate that uPA and uPAR are expressed primarily in adenocarcinoma cells and only minimally in the stroma of high grade prostate cancers. This pattern of expression differs significantly from several other cancers, including lung, breast and colon, where predominantly the stromal cells are reported to express uPA [1,2].

3.2. *Expression of* ets *family proteins in high grade prostate cancers*

We stained high-grade prostate cancers with antibodies against representative members of seven *ets* subfamilies and groups [11] that we considered to be potential activators of uPA expression in prostate adenocarcinoma cells (Table 1). The basis for our selection was either that these *ets* paralogues have been reported to interact with AP-1 complex (a likely prerequisite for induction of the uPA enhancer), or that they have been reported to be capable of inducing uPA expression in cultured cells or preferentially expressed in epithelial cells or cancers.

Of the antibodies utilised, two directed against the *ets* family proteins Elf-1 and Fli-1 caused intense nuclear staining of adenocarcinoma cells of the majority of high grade prostate cancers (Fig. 2, panels a and b; Elf-1: 16/25 and Fli-1: 20/25 tissues; Table 2), with the few negative tissues generally having intermediate Gleason scores. In contrast, antibody staining against several other candidate *ets* family members occurred only in a minority of high grade cancers: nuclear staining by anti-Erg-1,2 antibody was detected in adenocarcinoma cells in 7/25 of the high grade cancers and in the endothelial cells of small caliber blood vessels in the stroma in most tissues (Fig. 2 panel c; Table 2); nuclear staining by anti-Ets-2 antibody was observed in adenocarcinoma cells in only 4/25 tissues; staining by anti-Ets-1 antibody occurred almost exclusively in the stroma of the tumours; and weak staining by anti-PU.1 antibody occurred throughout all the cells of most tissues, including BPH (data not shown). No staining was detected with anti-Elk-1 or anti-PEA3 antibodies, indicating these proteins are not highly expressed in advanced prostate cancers.

Weak signals were detected in the stroma and glandular epithelium in several BPH tissues when stained with anti-Ets-1 and Ets-2 antibodies. Anti-Elf-1 antibody stained several BPH tissues mainly in the glandular epithelium. Anti-Elk-1 antibody stained one BPH tissue in the basal cell layer of the acini in several BPH tissues. Erg-1/2 staining occurred in the microvasculature and basal cell layers, and weak Fli-1 staining occurred in the basal cell layers of BPH tissues (data not shown).

3.3. *Expression of AP-1 complex proteins in high grade prostate cancers*

Strong staining of prostate adenocarcinoma cells by anti-c-Jun or anti-c-Fos antibodies occurred in only 8/25 and 9/25 cancers, respectively, did not correlate with the grade of the cancer, and was not detected in BPH tissues (Table 2; Fig. 2 panels d–f). This indicates that high level expression of these particular components of the AP-1 complex is not required for uPA or uPAR expression. Remarkably, c-Jun and c-Fos staining was frequently localised to the cytoplasm of adenocarcinoma cells of prostate cancers (and as well, in PC-3 prostate adenocarcinoma cells; data not shown). Cytoplasmic immunolocalisation of c-Jun was observed with two separate antibodies, a mouse monoclonal antibody and a rabbit polyclonal antibody, which were claimed by commercial suppliers not to react with JunB or JunD (Table 1). Reactivity of the respective antibodies was blocked by preincubation with the peptide utilised for immunisation (Fig. 2, compare panels g and h), and by bacterially expressed and purified Jun protein (data not shown), supporting claims by the suppliers that the antibodies are specific for c-Jun.

We examined these tissues for other proteins having well-characterised subcellular localisations so as to ascertain if the tissue preparation caused the unexpected localisation of the c-Jun and c-Fos proteins. The prostate adenocarcinoma cells exhibited intense nuclear p53 staining, and adjacent non-tumour tissue or BPH tissues exhibited no such staining; strong E-cadherin staining of the cell membranes was observed in BPH tissues and weak or no staining for E-cadherin was observed in prostate cancer cells; nuclear staining was detected for androgen receptor in the majority of tissues (data not shown). These results suggest the cytoplasmic staining of c-Jun and c-Fos was not due to faulty specimen preparation.

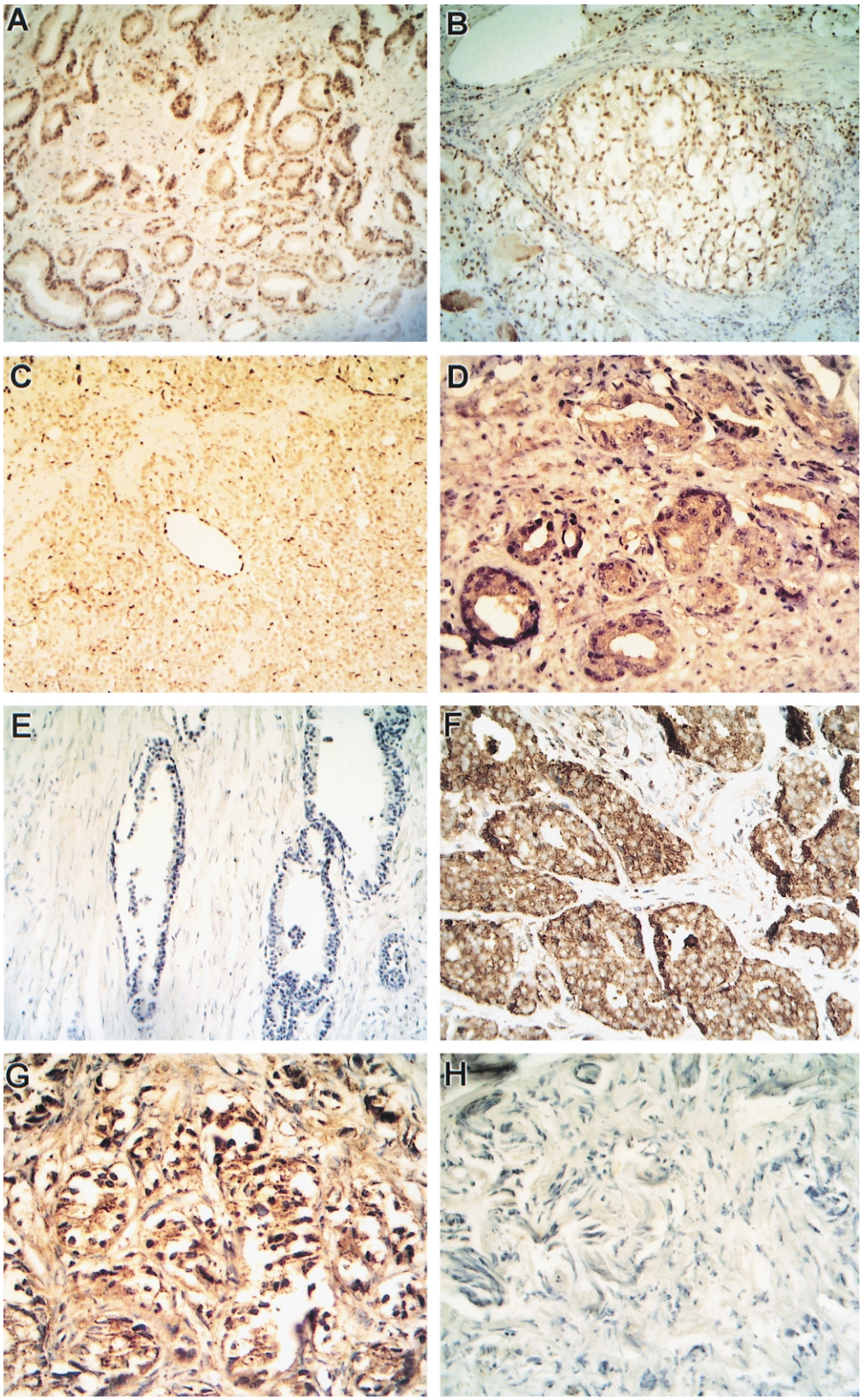

Fig. 2. Immunohistochemical localisation of transcription factors in prostate cancer and benign prostate hyperplasia (BPH) tissues: (a) staining by anti-Elf-1 in adenocarcinoma cells of high grade prostate cancers; (b) staining by anti-Fli-1 in adenocarcinoma cells of high grade prostate cancers; (c) anti-Erg-1,2 staining in the endothelial cells of the stromal blood vessels; (d) predominantly cytoplasmic staining by anti-c-Fos in high grade prostate cancers; (e) lack of c-Fos staining in BPH tissues; (f) predominantly cytoplasmic and nuclear staining by anti-c-Jun in high grade prostate cancers; (g), (h) blocking of cytoplasmic and nuclear staining by the peptide used to prepare the anti-c-Jun antibody ((g) no preincubation with peptide compared with (h) preincubation of peptide).

Table 2
Tissues[a]

Tissue no.	Gleason score	uPA		uPAR		Elf−1	Erg1,2	Fli−1	c−Jun	c−Fos
		Protein	RNA	Protein	RNA					
1	9	+	+	+	+	+	e	+	n	n
2	9	+	+	+	+	+	e	+	c	n
3	9	+	+	+	+	+	+	+	c	c
4	9	+	+	+	+	+	−	+	c	c
5	9	+	+	+	+	+	+	+	c	c
6	9	+	+	+	+	+	e	+	c	c
7	8	+	+	+	+	+	e	+	c	c
8	8	+	+	+	+	+	e	+	n	n
9	8	+	+	+	+	+	+	+	n	n
10	8	+	+	+	+	−	+	+	c	c
11	8	+	+	+	+	+	$+_f$, e	+	c	c
12	8	+	+	+	+	+	+	+	c	c
13	8	+	+	+	+	+	−	+	n	n
14	7	+	+	+	−	+	e	+	c	c
15	7	+	+	+	+	+	e	+	c	c
16	7	+	+	+	−	+	−	+	n	n
17	7	+	+	+	+	−	+	+	c	c
18	6	+	−	+	−	−	−	−	c	c
19	6	+	+	+	+	−	$+_f$, e	$+_f$	?	?
20	6	+	+	+	+	−	$+_f$, e	−	c	c
21	6	+	−	+	−	+	−	−	n	n
22	5	+	+	+	+	−	$+_f$, e	+	n	n
23	5	+	−	+	−	−	+	+	c	c
24	4	+	−	+	−	−	−	−	−	−
25	4	+	−	+	+	−	−	−	n	n

[a] Symbols: +, positive staining; −, negative staining; n, nuclear staining; c, cytoplasmic staining; $+_f$, focal positive staining in cancer cells; indistinct staining pattern.

4. Discussion

Clinical studies indicate that expression of uPA and uPAR is very important for the metastatic behaviour of many cancers. We have documented high level expression of uPA and uPAR mRNAs and proteins in adenocarcinoma cells of advanced prostate cancer, but not BPH tissues, extending an earlier study of uPA expression in prostate tissue sections [13]. As elevated expression of uPA and uPAR is of considerable prognostic value, elucidating the molecular basis for their expression may improve diagnosis and provide new therapeutic targets to block prostate cancer metastasis and invasion.

We focused upon the *ets* family and AP-1 complex proteins, as they are required for the induction of the uPA enhancer and well-characterised antibodies against them are commercially available. Detection of their high level expression in adenocarcinoma cells would support their being involved in the activation of the uPA enhancer. Immunocytochemical detection of the proteins, rather than the mRNAs, guarantees there is no block to mRNA translation in prostate adenocarcinoma cells, as has been reported for the prostate epithelial cell-specific *ets* family paralogue *hPSE* mRNA [14].

Ets-2 expression elicits a transformed phenotype in prostate cancer cells in culture and prior studies have documented elevated expression of *Ets-2* mRNAs in prostate cancer [15,16]. However, we did not detect elevated levels of the Ets-2 protein in many advanced prostate cancers. We did detect elevated expression of another member of the ETS subfamily of *ets* proteins, Fli-1, in the majority of prostate adenocarcinoma cells, and as well, expression of the closely related Erg-1,2 protein. Expression of FEV, another ETS subfamily member closely related to Fli-1 and Erg-1/2, has been reported to occur preferentially in human prostate and intestine [17], and might be an additional *ets* family paralogue that activates uPA expression in prostate adenocarcinoma cells.

Elevated expression of Elf-1 also occurred in the majority of the prostate adenocarcinoma cells, together with expression of uPA and its receptor. Elf-1 and members of the related ESE group (Elf-3/ESE-1, Elf-5/ESE-2 and ESE-3) are highly expressed in epithelial cells of the prostate and other tissues [10]. Elf-3/ESE-1 is expressed in PC-3 and DU-145 prostate cells, in the involuting prostate, and its gene is localised to 1q32, a region that is amplified in prostate cancer [18,19]. These proteins are candidates for having significant roles in uPA expression in prostate adenocarcinoma cells.

Members of the PEA3 group (PEA3, ERM, E81) have been reported to be highly expressed in mammary carcinomas [10], however we did not detect elevated expression of PEA3 in any high grade prostate cancers.

In summary, *ets* family paralogues that are highly expressed and consequently are candidate activators of uPA expression in prostate adenocarcinoma cells include members of the ERG and ETS groups of the ETS subfamily and members of the ELF subfamily. The participation of *ets* family and homeobox proteins in the regulation of uPA expression is noteworthy because the tissue and temporal expression of these transcription factors can be highly specific and may provide a means to intervene and alter the expression of uPA, so as to moderate the invasive and metastatic behaviour of tumour cells. Additional biochemical and genetic analyses are in progress to identify those members of these families that interact to activate the uPA enhancer in prostate adenocarcinoma cells.

We detected c-Jun and c-Fos staining in a minority of adenocarcinoma cells, indicating their elevated expression is not required for uPA expression. Alternatively, other members of the AP-1 complex may serve to activate the inducible uPA enhancer [20], or c-Fos and c-Jun may be activated by phosphorylation or by association with co-activators [21].

The frequent cytoplasmic localisation of c-Jun and c-Fos, however, was unexpected. Its occurence in prostate adenocarcinoma cells, but not in BPH tissues, makes this particularly interesting. Occasional cytoplasmic staining of c-Fos and c-Jun in tumour cells has been noted for other malignancies including lung [22], ovarian [23], bladder [24] and breast cancers and MCF-7 and MDA-MB-231 cells [25,26] (and our unpublished results).

c-Jun and c-Fos contain nuclear localisation sequences that direct their import into the nucleus, but their trafficking can be modulated by signal transduction pathways responsive to the cell cycle and the environment [27,28]. Oxidative-stress, acting through the redox sensor Ref-1 protein that binds c-Jun and c-Fos, determines the subcellular localisation of a yeast homologue of c-Jun [29]. We speculate that mammalian proteins that bind c-Jun and c-Fos, such as other bZIP transcription factors with which they can heterodimerise, pRb family members, androgen and oestrogen receptors and their coactivators, Jun activation domain binding protein, modulators of their redox and phosphorylation (including Ref-1), and members of the c-Jun amino-terminal kinase (JNK), MAPK and p38 protein kinase families may act in a similar fashion to modulate c-Jun and c-Fos subcellular localisation in adenocarcinoma cells. As c-Jun and c-Fos profoundly affect cell proliferation, differentiation and apoptosis better understanding of the significance of their cytoplasmic localisation in cancer cells is warranted.

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