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Components of the plasminogen activator system and their complexes in renal cell and bladder cancer: comparison between normal and matched cancerous tissues

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OBJECTIVE

To analyse and compare the concentration of plasminogen activator (PA), urokinase-type PA (uPA), tissue-type PA (tPA), PA inhibitor (PAI)-1 and PAI-2, and the complexes uPA-PAI-1 and tPA-PAI-1 and calculated uPA and tPA uncomplexed with PAI-1 ('free') in urothelial cell carcinoma and matched benign urothelium, and in renal cell carcinoma (RCC) and matched benign renal tissue.

PATIENTS AND METHODS

Tissue samples were obtained during cystectomy (33 patients) and nephrectomy (55), and specific enzyme-linked immunosorbent assays were used to assess the PA components in extracts of these tissues.

RESULTS

Tissue levels of uPA-PAI-1 and tPA-PAI-1, but also PAI-1 itself, were greater in tumorous bladder and kidney tissue than in matched normal tissue (by 1.5–7.8 times). Free tPA was clearly lower in tumour tissue (by 0–0.12-fold). In bladder cancer, but not in RCC, levels of uPA (15.8-fold) and free uPA (16.4-fold) were greater in tumour tissue. Free uPA levels were less in RCC (0.41-fold). For both normal bladder and kidney tissue, there was no clear correlation between uPA-PAI-1 complex and either component. However, the formation of tPA-PAI-1 complexes in normal bladder and kidney tissue was primarily determined by PAI-1. Interestingly, in tumour tissues there was a strong, significant correlation between complex levels and both components.

CONCLUSION

RCC and bladder cancer show distinct profiles of components of the PA system. This study provides a basis for further studies into both the (patho)physiological role of the PA system in these tumours, and into a possible relation with tumour progression and prognosis, and as target for therapy.

KEYWORDS

bladder cancer, plasminogen activator system, renal cell carcinoma

INTRODUCTION

Different protease systems are involved in cancer progression, especially in invasion and the development of metastases. One of these systems is the plasminogen activator (PA) system, in which the serine protease urokinase-type PA (uPA) is central [1–3]. Generally, this protease is released from cells as an inactive single-chain pro-enzyme, and then converted to its active two-chain form by limited proteolysis [4,5]. uPA can be regarded as a multifunctional protein that is involved in both proteolysis and signal transduction, the latter via the uPA receptor. Another PA is the tissue-type PA, tPA; unlike uPA, tPA has a strong affinity for fibrin, and is considered to be primarily involved in thrombolysis [6]. The activity of uPA and tPA can be neutralized by two main specific inhibitors (I), PAI type-1 and type-2, which form inactive complexes of 1:1 stoichiometry with the PAs [6,7].

Results from many different human cancers (including breast [8,9], oesophageal [10], stomach [11], colorectal [12] and lung [13]) have shown that the level of components of the PA system in tumours is significantly higher than in corresponding normal tissue or benign tumours of the same tissue type. More importantly, high levels of uPA and PAI-1 in malignant tumours correlate with a poor prognosis for the patient. For example, uPA and PAI-1 are among the strongest prognostic factors so far described for breast cancer [8,9]. However, tPA can denote a good prognosis [14]. The complexes between uPA or tPA and PAI-1 are only formed between active forms of the PA and its inhibitor, and might have prognostic and/or predictive value [14–18]. In many other malignancies, including colorectal [12], stomach [19] and RCC [20,21], components of the PA system have some prognostic value, but for most

TABLE 1 The median (interquartile range) levels of components of the PA system in kidney and bladder tissues

	Kidney				Bladder			
	Normal	Tumour	T/N	*P**	Normal	Tumour	T/N	*P**
uPA	0.71 (0.79)	0.50 (0.89)	0.51 (1.15)	0.081	0.39 (0.48)	7.29 (6.34)	15.8 (27.5)	<0.001
tPA	1.28 (1.38)	0.36 (0.65)	0.30 (0.53)	<0.001	8.65 (9.51)	3.53 (10.8)	0.55 (1.55)	0.313
PAI-1	0.92 (1.39)	7.26 (25.5)	7.81 (26.8)	<0.001	1.30 (1.59)	8.85 (24.5)	7.49 (20.1)	<0.001
PAI-2	0.24 (0.38)	0.74 (1.23)	3.99 (4.91)	<0.001	0.33 (0.60)	1.80 (8.77)	10.0 (28.7)	<0.001
uPA-PAI-1	0.06 (0.08)	0.43 (0.59)	4.54 (13.4)	<0.001	0.10 (0.19)	2.05 (5.41)	13.1 (48.8)	<0.001
tPA-PAI-1	0.41 (0.53)	0.61 (1.06)	1.43 (3.45)	0.030	6.66 (7.25)	10.5 (18.2)	2.54 (6.14)	<0.001
Free uPA	0.67 (0.79)	0.34 (0.81)	0.41 (1.07)	0.012	0.35 (0.48)	5.63 (6.33)	16.4 (42.1)	<0.001
Free tPA	1.15 (1.39)	0.17 (0.46)	0.12 (0.46)	<0.001	6.92 (9.43)	0 (8.65)	0 (1.44)	0.008

**Wilcoxon signed-rank test.*

malignancies data are still preliminary and conflicting, especially for urological tumours like bladder cancer [22,23].

In the present study we analysed and compared the concentration of the PA system components (uPA, tPA, PAI-1, PAI-2 and the complexes uPA-PAI-1 and tPA-PAI-1) in urothelial cell carcinoma and matched benign urothelium, and in RCC and matched benign renal tissue. By addressing all these different components of the PA system, and by assessing matched tumour and normal tissue samples from two different urological tumour types, we intended to provide a basis for further studies into both the (patho)physiological role of the PA system in these tumours and into a possible relation with tumour progression and prognosis, and as target for therapy.

PATIENTS AND METHODS

To prepare tumour cytosols, the matched normal and tumour bladder tissue samples were collected during cystectomy from 33 patients with invasive bladder cancer, and matched normal and tumour RCC samples were obtained from nephrectomy in 55 patients. All tissue samples were snap-frozen in liquid nitrogen and kept at −80 °C until analysis. Then 350 μL of Tris-buffered saline (0.02 M Tris-HCl, 0.125 M NaCl, pH 8.5) containing 1% Triton X-100 was put in PTFE capsules, each containing a tungsten carbide bullet. The capsules were cooled in liquid nitrogen. Five to 10 slices (each 40 μm thick) obtained from frozen tissues were placed in the capsule, which was cooled again in liquid nitrogen. The slides were subsequently pulverized using a microdismembrator (Braun-Melsungen, Melsungen, Germany). The resulting powders were transported to centrifuge tubes (on ice) and after thawing the homogenates were centrifuged at 105 000***g*** for 45 min at 4 °C. The supernatants (cytosolic fraction) were stored at −80 °C.

Grebenschikov *et al.* described the multi-applicable ELISA procedure in detail [24,25]. The sandwich structure used includes four different antibodies (Abs), i.e. a coating Ab (duck anti-chicken), a capture Ab (chicken anti-analyte), a trapping Ab (rabbit anti-analyte) and finally a detecting Ab (labelled goat anti-rabbit). Depending on the combination of capture and trapping Ab used, this ELISA format enables the analysis of uPA, tPA, PAI-1, PAI-2 and the complexes uPA-PAI-1 and tPA-PAI-1. Before the assays, samples were diluted 10 times for uPA, uPA-PAI-1, PAI-1 and PAI-2, and 20 times for tPA and tPA-PAI-1 in dilution buffer. In the total antigen uPA assay both uPA from Ukidan (Serono, Unterschleissheim, Germany) and uPA-PAI-1 from Dr P. Andreasen [25] were used as calibrators. The uPA-PAI-1 levels obtained in the corresponding uPA-PAI-1 ELISA are read the other way around from the standard curve in uPA assay using uPA-PAI-1 as calibrator. Thus on the ordinate the optical density signal read originates from the complex. The signals originating from the complex are subtracted from the total signal obtained from the total tPA assay. In the total antigen tPA assay both tPA from Actilyse (Boehringer, Ingelheim, Germany) and tPA-PAI-1 from Andreasen [25] are used as calibrators. Protein concentrations were measured using the Pierce (BCA) protein assay with Pierce BSA as a standard. All values are expressed as ng/mg protein.

Values are given as the median (interquartile range) in ng/mg protein. Differences between normal and tumour tissue levels were assessed for statistical significance using the paired nonparametric Wilcoxon signed-rank test. Correlations between complexes and their components were assessed using a Spearman rank correlation test; in all tests $P < 0.05$ was considered to indicate statistical significance.

RESULTS

The levels of the components of the PA system were measured in normal kidney tissue and matched RCC samples (Table 1, Fig. 1), and in normal bladder and bladder cancer tissues (Table 1, Fig. 2). In kidney tissues, of all components measured, only uPA did not differ significantly between normal and tumorous tissue (ratio tumour/normal 0.51, $P = 0.081$). However, PAI-1 (7.81, $P < 0.001$), PAI-2 (3.99, $P < 0.001$), uPA-PAI-1 (4.54, $P < 0.001$) and tPA-PAI-1 (1.43, $P = 0.030$) levels were significantly higher in tumour tissue, whereas both uPA not complexed to PAI-1 ('free' uPA, 0.41, $P = 0.012$), and total tPA (0.30, $P < 0.001$) and free tPA (0.12, $P < 0.001$) were lower in RCC tissue than in matched normal tissue (Table 1, Fig. 1). In bladder tissue, the pattern was similar to that in kidney tissue, except for uPA (15.8, $P < 0.001$) and free uPA (16.4, $P < 0.001$), that were significantly higher in bladder cancer tissue than in matched normal bladder tissue (Table 1, Fig. 2).

Considering differences between the urological tissues, the levels of tPA (8.65 ng/mg protein) and tPA-PAI-1 complex (6.66) were markedly higher in normal bladder tissue than in normal kidney (1.28 and 0.41, respectively). Also, levels of uPA (7.29 vs 0.50),

FIG. 1. Levels of components of the PA system in normal kidney tissue and matched RCC tissue from the same patient. Significance of differences (Wilcoxon signed-rank test) are shown above the subfigures.

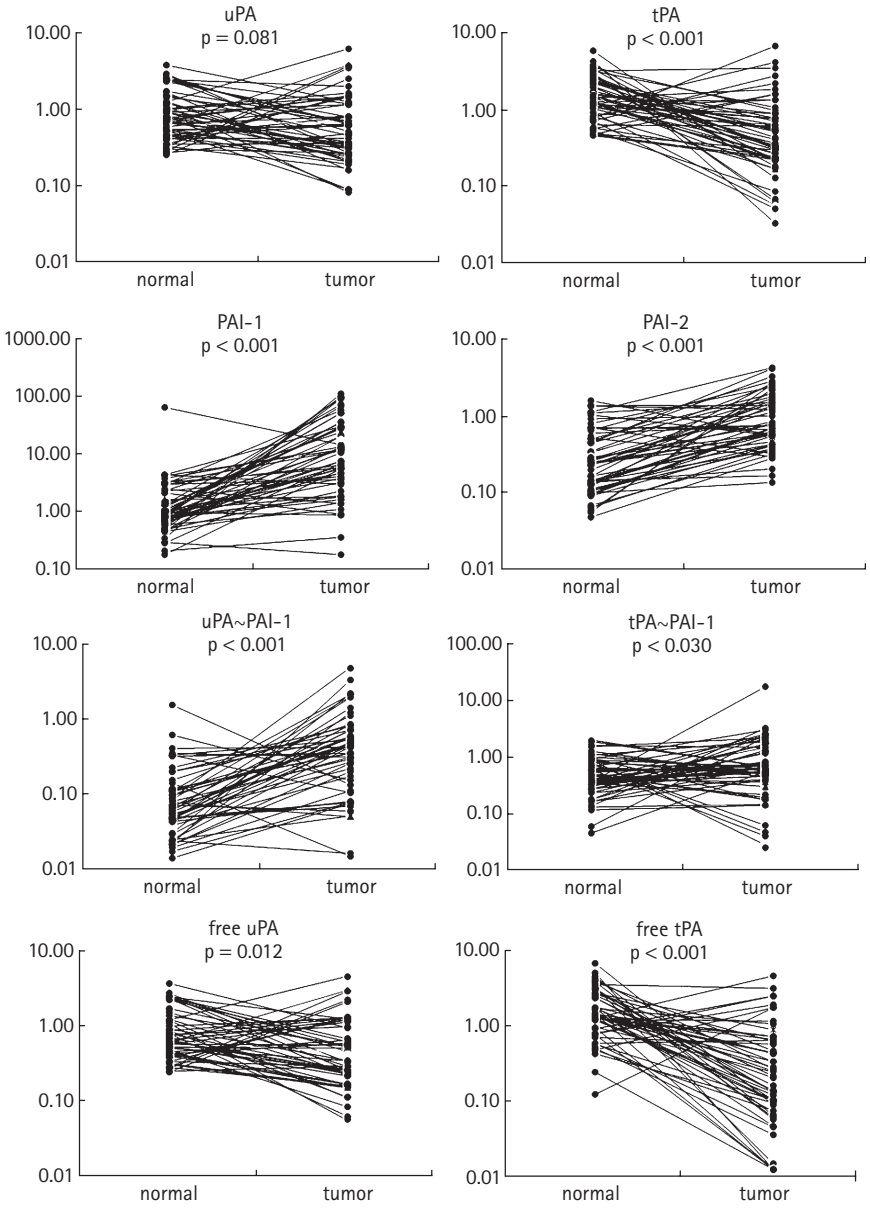


TABLE 2 Correlations between complexes and individual components

Sample	uPA	tPA	PAI-1
RCC, normal tissue			
uPA-PAI-1			
R	0.208		0.237
P	0.127		0.082
tPA-PAI-1			
R		0.182	0.521
P		0.183	<0.001
RCC, tumour tissue			
uPA-PAI-1			
R	0.752		0.738
P	<0.001		<0.001
tPA-PAI-1			
R		0.580	0.392
P		<0.001	0.003
Bladder cancer, normal tissue			
uPA-PAI-1			
R	0.249		0.357
P	0.163		0.041
tPA-PAI-1			
R		0.024	0.713
P		0.894	<0.001
Bladder cancer tumour tissue			
uPA-PAI-1			
R	0.549		0.796
P	0.001		<0.001
tPA-PAI-1			
R		0.543	0.542
P		0.001	0.001

tPA (3.53 vs 0.36), tPA-PAI-1 complex (10.5 vs 0.61) and free uPA (5.63 vs 0.34) were all notably higher in bladder cancer tissue than in RCC tissue. Thus, overall the PA system is more active in bladder tissue, both normal and cancerous, than in kidney tissue.

The degree of complex formation between uPA or tPA with PAI-1 is limited by the component with the lowest active level. This component will determine the levels of uPA-PAI-1 or tPA-PAI-1 complex. To establish this component, we compared the level of uPA-PAI-1 and tPA-PAI-1 complex with uPA and PAI-1, and tPA and PAI-1, respectively (Table 2). In normal renal tissue, the uPA-PAI-1 level did not correlate with its constituting components, but the tPA-PAI-1 level strongly correlated with PAI-1 levels ($R_s = 0.521$, $P < 0.001$). In RCC (Fig. 3a), uPA-PAI-1 levels correlated strongly with both uPA (0.752, $P < 0.001$) and PAI-1 (0.738, $P < 0.001$) levels. Similarly, tPA-PAI-1 levels correlated with both of its constituting components, although most strongly with tPA (0.580, $P < 0.001$).

In normal bladder tissue, uPA-PAI-1 levels correlated marginally with both components, although only significantly with PAI-1 (0.357, $P = 0.041$). For tPA-PAI-1 levels, there was a strong correlation only with PAI-1 (0.713, $P < 0.001$). In bladder tumour tissue (Fig. 3b), uPA-PAI-1 correlated with both components, but most strongly with PAI-1 (0.796, $P < 0.001$). tPA-PAI-1 correlated with tPA (0.543, $P = 0.001$) and PAI-1 (0.542, $P = 0.001$).

Thus, for both normal bladder and kidney tissue, there was no clear correlation between uPA-PAI-1 and either constituting component uPA or PAI-1. However, the formation of tPA-PAI-1 complexes in normal bladder and kidney tissue is primarily determined by PAI-1 levels. Interestingly, in tumour tissues there was a strong, significant correlation between complex levels and both components (Fig. 3).

DISCUSSION

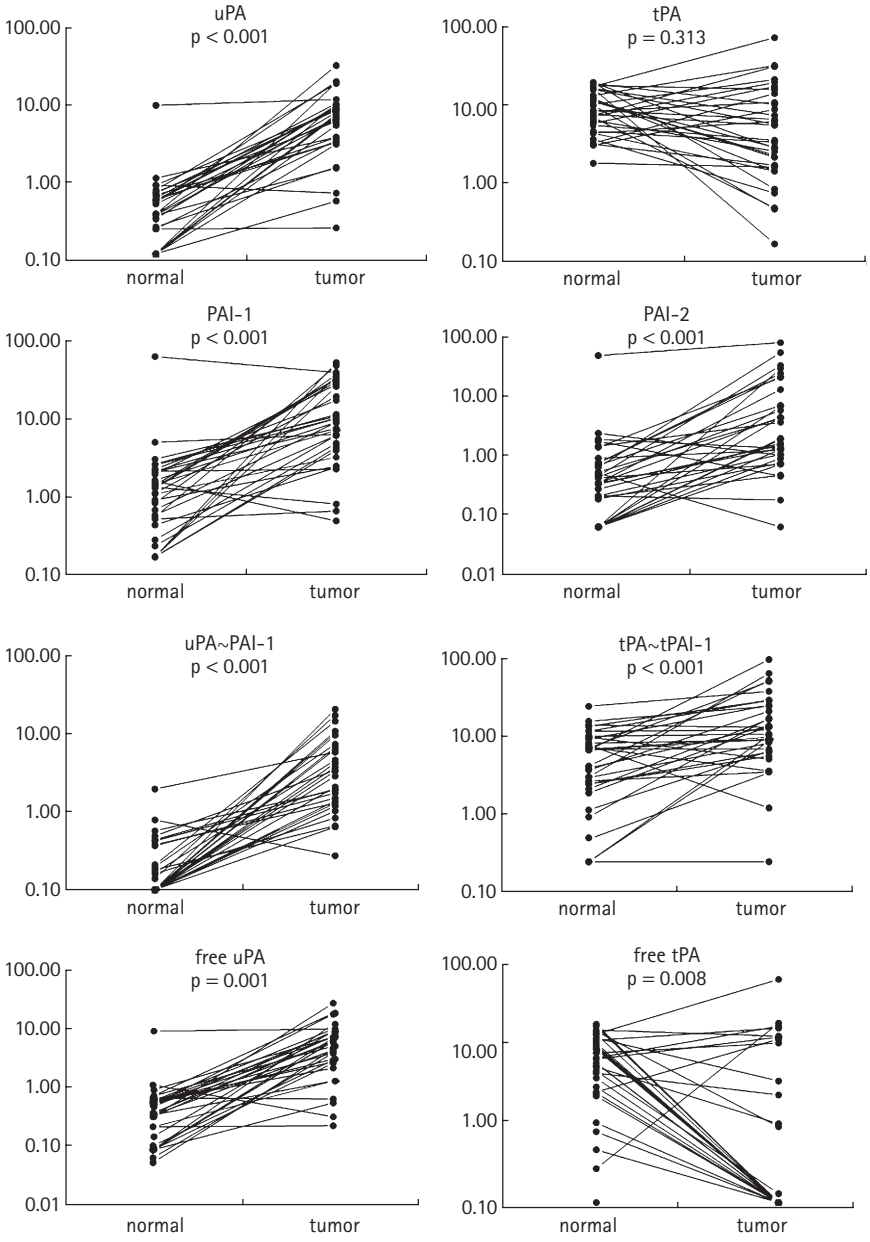
We show that tissue levels of in particular the complexes of uPA and tPA with their inhibitor, PAI-1, but also PAI-1 itself, are greater in tumorous bladder and kidney tissue than in matched normal tissue. By contrast, free, uncomplexed levels of tPA are clearly lower in tumour tissue. In bladder cancer, but not in RCC, level of uPA and free uncomplexed uPA are greater in tumour than in normal tissue. Indeed, free uPA levels are attenuated in RCC. These results underscore the complexity of the PA system and denote interesting differences between these urological solid tumours.

Previously the expression of components of the PA system were investigated extensively in many types of malignant tumours. Most of these studies investigated the level of the components in tumour extracts by ELISA assays. In particular for breast cancer, the prognostic values of uPA and PAI-1 levels were studied and validated extensively [26,27]. Thereby, uPA and PAI-1 have achieved a 'level of evidence I' as a tumour biomarker [28]. Indeed, uPA and PAI-1 levels (as assessed by ELISA) are being used to prospectively assign patients with breast cancer to a high-risk or low-risk group, after which adjuvant treatment is scheduled accordingly in a multicentre European trial [29]. By contrast, high tPA levels in breast tumour cytosols are significantly associated with prolonged survival, but when complexed with PAI-1 denote a poor prognosis [14]. This is in line with the present results that (free) tPA levels are lower, whereas the complex levels are significantly higher, in tumour tissue.

Each of these components exists in chemically different forms, some of them biologically active and some inactive. The complexes uPA-PAI-1 and tPA-PAI-1 can be considered as special manifestations of the inactive forms of the activator uPA and tPA. As most ELISAs presently available are unable to distinguish among the different forms of the activators (active, inactive, free or complexed), the signals obtained are the sum of the signals produced by the separate forms. Therefore, the clinical significance of each component of the PA system to the overall value is unknown. In the present study we selectively measured the concentrations of the complexes in addition to the overall immunoreactive signal, to assess their significance.

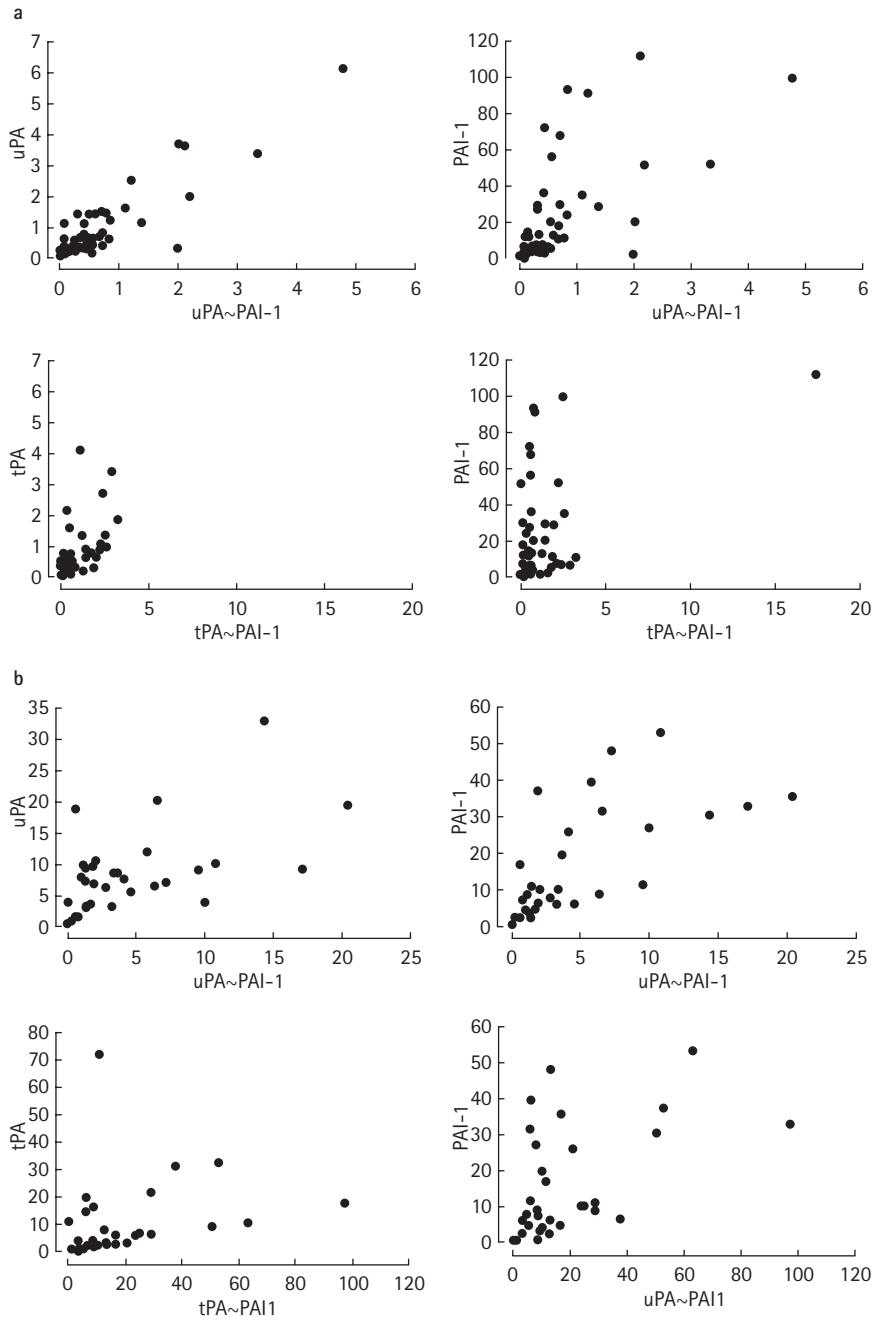
Our data on uPA and tPA are in accordance with those of Hasui *et al.* [30], who found a significantly higher level of uPA in bladder cancer extracts than in normal bladder tissue. These authors also found no correlation between tissue tPA level and malignancy. In the present study we found a significantly higher concentration of the inhibitors and their complexes in urothelial carcinoma than in normal bladder tissue. There were high levels of tPA-PAI-1 and most of the tPA in bladder tissue was complexed to the type-1 inhibitor and not present in another form.

FIG. 2. Levels of components of the PA system in normal bladder tissue and bladder cancer tissue from the same patient. Significance of differences (Wilcoxon signed-rank test) are shown above the subfigures.



Swiercz *et al.* [31] reported significantly higher uPA, uPA receptor and PAI-1 levels in RCC tissue than in normal kidney tissue. They also reported that PAI-1 is over-expressed in kidney cancer, with a 10–20 times higher molar ratio than uPA in some cases. In the present study the level of the inhibitors and their complexes, PAI-1, PAI-2, uPA-PAI-1 and tPA-PAI-1, was significantly higher in RCC than in normal renal tissue. Indeed, the expression of PAI-1 was 15 times higher than the expression of uPA in RCC, in accordance with Swiercz *et al.*

FIG. 3. Correlations between complexes and their components in tissue from (a) RCC and (b) bladder cancer. R_s, Spearman correlation coefficient with significance (P).



[31]. However, they found a higher level of uPA in RCC than in normal kidney tissue, which was not confirmed by our data, where there was an (insignificantly) lower concentration of uPA in RCC than in normal kidney tissue. More recently, Ohba *et al.* [20] found an association between the components of the PA system and clinicopathological features in patients with RCC, with PAI-1 being the most significant factor for predicting survival.

The role of the PAIs in tumour biology has not been defined clearly. Results from different studies suggest that a high score for the PAIs might correlate with tumour aggressiveness, as it does in breast cancer [26–28]. It is interesting that PAI-1 is thought to protect the tumour tissue against the proteolytic degradation that the tumour imposes upon the surrounding normal tissue [32]. Besides this antiproteolytic function, PAI-1 is necessary for focused and optimal invasiveness [7], associated with angiogenic activity [33], and is essential for tumour cell invasion and tumour vascularization in PAI-1-deficient mice [34]. Therefore, high uPA and high PAI-1 levels in breast tumours have the same clinical relevance, i.e. a worse prognosis. Thus the influence of PAI-1 on angiogenesis is more important than its inhibiting effect on uPA. Together these observations strongly imply that PAI-1 has a primary role in tumour progression.

In conclusion, RCC and bladder cancer show distinct profiles of components of the PA system. Overall, the levels of complexed forms of uPA and tPA with PAI-1 are higher than in normal tissue. This might signify that the active forms of these components, which are necessary to form such a complex, are instrumental in tumour formation. The decrease in free, uncomplexed levels of tPA, especially in bladder cancer, is in line with an association of this PA with less aggressive tumours. These results provide a basis for further studies into both the (patho)physiological role of the PA system in these tumours, and into a possible relation with tumour progression and prognosis, and as a target for therapy.

CONFLICT OF INTEREST

None declared.

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Abbreviations: **(u)(t)PA(I)**, (urokinase) (tissue) plasminogen activator (inhibitor); **Ab**, antibody.