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## Original Paper

# Urokinase-type Plasminogen Activator (uPA) and Plasminogen Activator Inhibitor Type 1 (PAI-1) in Tissue and Serum of Head and Neck Squamous Cell Carcinoma Patients

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**The aim of this study was to determine urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) concentrations in tumour and adjacent normal tissue samples from 58 patients, and in serum samples from 40 of 58 patients with squamous cell carcinoma of the head and neck obtained at diagnosis and after completion of therapy. uPA and PAI-1 serum concentrations were also measured in 28 healthy volunteers who served as controls. Measurements were made using enzyme-linked immunosorbent assay (ELISA) techniques. For both uPA and PAI-1, significantly elevated concentrations were measured in tumour tissue as compared with normal tissue (uPA: 8.89 versus 0.41 ng/mg total protein (mgp), $P < 0.0001$; PAI-1: 23.9 versus 1.47 ng/mgp, $P < 0.0001$). A statistically significant difference in uPA concentrations was found between normal laryngeal and non-laryngeal tissue (0.52 versus 0.3 ng/mgp, $P = 0.008$), and in PAI-1 concentrations between $T_{1+2}$ and $T_{3+4}$ stage of disease (17.32 versus 35.63 ng/mgp, $P = 0.04$). The uPA concentrations positively correlated with those of PAI-1 measured in both tumour ($R_S = 0.62$, $P < 0.0001$) and normal tissue ($R_S = 0.30$, $P = 0.02$). In serum samples, lower concentrations of PAI-1 were measured in the control group than in patients with cancer (412.0 versus 680.5 ng/ml serum (mls), $P = 0.0006$). The time of collection of the serum sample did not influence uPA and PAI-1 concentrations, and no association was observed between their concentrations and any clinical and histopathological prognostic factors tested. Our results indicate that both uPA and PAI-1 may play a specific role in the process of invasion and metastasis, and might also be of prognostic value in squamous cell carcinoma of the head and neck.**


**Key words: head and neck carcinoma, plasminogen activator inhibitor type 1, squamous cell carcinoma, urokinase-type plasminogen activator**
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## INTRODUCTION

SERINE PROTEINASE urokinase-type plasminogen activator (uPA) is believed to play a pivotal role in destructive events leading to local invasion and seeding of tumour cells. Mediated by uPA, a cascade-like transformation of pro-enzyme plasminogen into the fibrinolytic peptide plasmin results in the direct breakdown of the basement membrane and extracellular matrix proteins, or indirectly through the activation of latent collagenases. uPA enzymatic activity is counterbalanced by four different types of naturally occurring inhibitors, plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2) being the predominant inhibitors in plasma and tissue [1, 2].

Little is known about the role of uPA and its inhibitors in the biology of invasive growth of squamous cell carcinoma (SCC) of the head and neck. In 1987, Björlin and colleagues [3] measured considerable levels of uPA in the medium of cultures from a panel of head and neck SCCs (HNSCCs) established as xenografted tumours in nude mice. Only traces of tissue-type plasminogen activator (tPA) were secreted in the medium. Similarly, Parolini and associates [4] observed

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no tPA in neoplastic cells of human larynx immunohistochemically, but both uPA immunoreactivity and uPA mRNA expression were present in tumour cells. uPA activity and antigen concentrations were significantly higher in the tumour than in their counterparts of normal mucosa, and were more pronounced in node-positive than in non-metastatic primaries. An increase in the tumour uPA antigen level was suggested to depend, at least partially, on an increased expression of the *uPA* gene. In addition, Clayman and colleagues [5] speculated that transcriptional activation of the *uPA* gene led to elevated levels of uPA mRNA and its protein, rather than gene amplification and/or increased mRNA stability. Higher uPA concentrations have been measured in tissue samples from resected tumours than in non-malignant counterparts, as was the case in the study of Petruzzelli and associates [6] who compared uPA concentrations in cell-free supernatants from 14 established HNSCC lines and their controls. In HNSCCs, significantly higher PAI-1 concentrations in the primary and lymph node metastases, compared with the normal mucosa, were reported by Zeillinger and colleagues [7].

The aim of this study was to determine the concentrations of uPA and PAI-1 in tissue and serum of HNSCC patients. Possible correlations with established clinical and histopathological prognostic factors were calculated to estimate their potential prognostic value for this particular cancer type.

## PATIENTS AND METHODS

*Patients*

58 patients treated with curative intent for primary ($n = 47$) or recurrent ($n = 11$) HNSCC entered the study. There were 5 females and 53 males, aged between 37 and 72 years, with a median age of 60 years. All patients were operated on and 43 of 58 were postoperatively irradiated. The clinical and histopathological characteristics of the primary tumours are listed in Table 1. Because of the small number of recurrent tumours, they were not classified by their histopathological profile. The UICC TNM classification system was used for tumour staging [8], and histopathological grade was defined according to WHO criteria [9].

*Biochemical analysis of uPA and PAI-1*

uPA and PAI-1 concentrations were measured in 58 pairs of extracts, which were prepared from tumour and adjacent normal tissue samples (matched pairs) weighing 200–500 mg, obtained at surgery. These were immersed in liquid nitrogen immediately following resection; fat and necrotic parts of the tissue were carefully removed. The tissue homogenates were prepared from frozen specimens by pulverisation (Mikro-Dismembrator, Braun-Melsungen, Melsungen, Germany) and centrifugation (100 000 *g*/45 min, 4°C), using 3 ml Tris buffered saline (TBS) (0.02 M Tris–HCl, 0.125 M NaCl, pH 8.5) which contained 1% Triton X-100 (Sigma, St. Louis, Missouri, U.S.A.), under gentle shaking for 12 h at 4°C. Supernatants were divided into aliquots and stored at − 70°C until use. Protein was determined using the method of Bradford [10].

For the quantitative determination of total uPA (single chain and high molecular weight uPA forms, as are receptor-bound molecules and those complexed with PAI-1 and PAI-2) and PAI-1 (latent and active forms of PAI-1 and PAI-1 complexes) in tissue extract, commercially available enzyme-linked immunosorbent assays (ELISAs) (IMUBIND® uPA ELISA Kit and IMUBIND® Tissue PAI-1 ELISA Kit, American Diagnostica Inc., Greenwich, U.S.A.) were used. Briefly, samples were incubated in microtest wells, precoated with the murine monoclonal antibody against human uPA (PAI-1), serving as a capture antibody. Bound uPA (PAI-1) molecules were recognised with a secondary biotinylated antibody followed by the addition of streptavidin-conjugated horseradish peroxidase.

In 40 of 58 patients, the uPA and PAI-1 concentrations were measured in serum samples, representing match pairs, obtained from each of these patients at diagnosis (sample no. 1) and 7–407 days (median, 54 days) after completion of therapy (sample no. 2). Immediately after removal, venous blood samples of 5 ml were centrifuged at room temperature and stored at − 70°C until analysis. For the determination of uPA and PAI-1 serum concentrations, the same biochemical assays were used as described above.

In order to assess possible differences in uPA and PAI-1 serum concentrations between healthy individuals and head

*Table* 1. *Clinical and histopathological characteristics of primary tumours* (n = 47)

| | |
|---|---|
| Tumour localisation | |
| Oral cavity | 8 |
| Oropharynx | 15 |
| Hypopharynx | 4 |
| Larynx | 20 |
| TNM stage | |
| I | 2 |
| II | 7 |
| III | 13 |
| IV | 25 |
| TNM classification | |
| $T_1$ | 3 |
| $N_0$ | 2 |
| $N_{2B}$ | 1 |
| $T_2$ | 18 |
| $N_0$ | 6 |
| $N_1$ | 2 |
| $N_{2A}$ | 1 |
| $N_{2B}$ | 4 |
| $N_{2C}$ | 4 |
| $N_3$ | 1 |
| $T_3$ | 17 |
| $N_0$ | 10 |
| $N_1$ | 1 |
| $N_{2B}$ | 5 |
| $N_{2C}$ | 1 |
| $T_4$ | 9 |
| $N_0$ | 5 |
| $N_1$ | 2 |
| $N_{2B}$ | 1 |
| $N_{2C}$ | 1 |
| $M_0$ | 47 |
| $M_1$ | 0 |
| Tumour histology | |
| Squamous cell carcinoma | 47 |
| Histopathological grade | |
| Well differentiated | 1 |
| Moderately differentiated | 38 |
| Poorly differentiated | 4 |
| Unknown | 4 |
| Extracapsular spread | |
| Positive | 15 |
| Negative | 9 |

and neck cancer patients, enzyme and inhibitor concentrations were also measured in serum samples obtained from a group of 28 healthy volunteers (4 females, 24 males), aged 36–61 years (median 50 years), free of any type of cancer or infectious disease (control group). Serum collection, preparation, and uPA and PAI-1 measurements were the same as in the group of cancer patients.

The concentrations of uPA and PAI-1 in tissue extracts were expressed as ng/mg of total protein (ng/mgp), and their serum concentrations in ng/ml of serum (ng/mls).

*Statistical analysis*

Median concentrations of uPA and PAI-1, and their relative increases (ratios between tumour and normal tissue concentrations, as well as between serum sample concentrations obtained before and after therapy) were calculated. Median concentrations of uPA and PAI-1 in match pairs were compared by Wilcoxon signed rank test. The Mann–Whitney *U* test was used for calculating their relationship to various clinical and histopathological prognostic factors, and the difference between serum samples collected from healthy volunteers and patients with cancer. Spearman's rank correlation was used to test the relationship between tissue and serum uPA and PAI-1 concentrations. All tests were two-sided and results were considered significant at the $P < 0.05$ level.

## RESULTS

Both uPA and PAI-1 concentrations were significantly higher in tumour tissue than in their normal counterparts (uPA: 8.89 versus 0.41 ng/mgp, $P < 0.0001$; PAI-1: 23.9 versus 1.47 ng/mgp, $P < 0.0001$) (Table 2). The uPA concentrations differed significantly between laryngeal and non-laryngeal (i.e. upper digestive tract organs) normal tissue samples (0.52 versus 0.3 ng/mgp, $P = 0.008$), and were lower in the tissue of recurrent tumours than of primaries (7.52 versus 9.21 ng/mgp, $P = 0.09$). Significantly higher PAI-1 concentrations were measured in locally advanced ($T_3$ and $T_4$) tumours than in early ($T_1$ and $T_2$) tumours (35.63 versus 17.32 ng/mgp, $P = 0.04$).

Median relative increases of uPA and PAI-1 in tissue samples were 23.2 and 14, respectively. In tissue samples from patients with laryngeal tumours, a relative increase in uPA was significantly lower than that observed in other head and neck tumours (14.56 versus 33.34, $P = 0.004$), and in recurrent tumours the uPA relative increase was lower than in primary tumours (9.79 versus 25.97, $P = 0.07$). A relative increase of PAI-1 did not correlate with any clinical or histopathological prognostic factors under investigation.

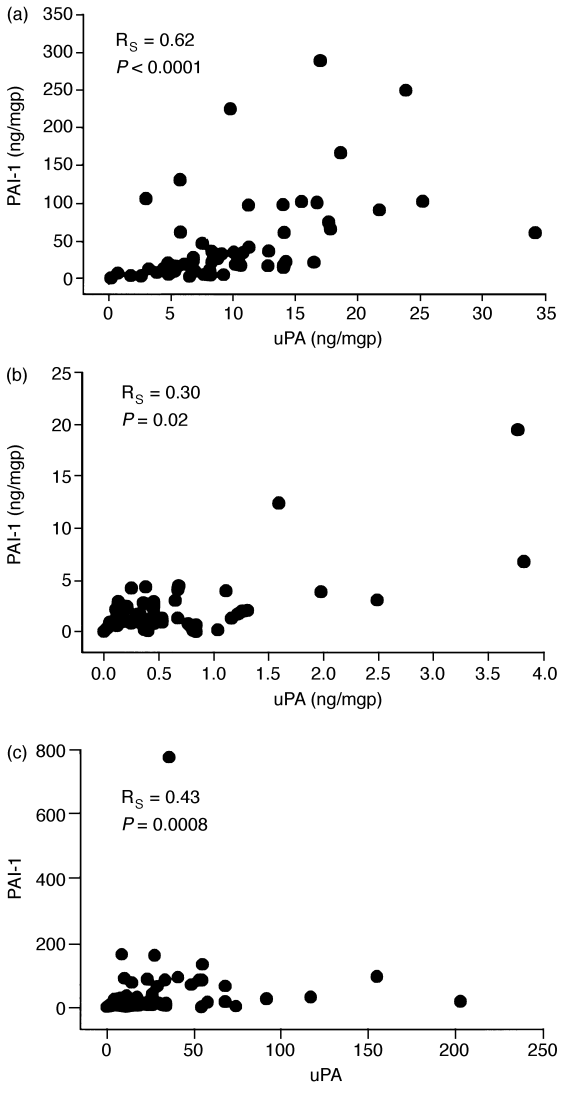


**Figure 1. Relationship between urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) concentrations measured in tumour samples (a) and normal tissue samples (b), and between their relative increases (c).**

There was a strong positive correlation between uPA and PAI-1 concentrations measured in tumour as well as in normal tissue samples (tumour tissue: $R_S = 0.62$, $P < 0.0001$; normal tissue: $R_S = 0.30$, $P = 0.02$). The same was observed in the case of their relative increases ($R_S = 0.43$, $P = 0.0008$) (Figure 1).

*Table 2. Urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) concentrations in tissue and serum samples*

| | | uPA | | PAI-1 | |
|---|---|---|---|---|---|
| Sample | *n* | Median (range) | *P* value | Median (range) | *P* value |
| Tissue (ng/mg total protein) | | | | | |
| Tumour | 58 | 8.89 (0.2–34.18) | < 0.0001 | 23.9 (1.18–290.2) | < 0.0001 |
| Normal | 58 | 0.41 (0.0–3.82) | | 1.47 (0.0–19.64) | |
| Serum (ng/ml serum) | | | | | |
| At diagnosis | 40 | 0.48 (0.24–1.92) | NS | 680.5 (85.0–1243.0) | NS |
| After therapy | 40 | 0.6 (0.3–1.86) | | 600.5 (5.9–1251.0) | |

NS, not significant.

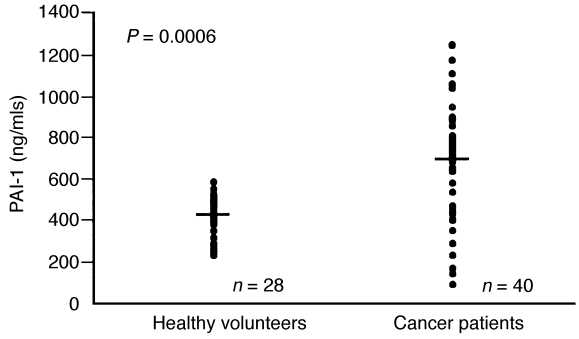


**Figure 2. Plasminogen activator inhibitor type 1 (PAI-1) concentrations in the sera of healthy volunteers ($n = 28$) and head and neck cancer patients ($n = 40$). The bold line indicates the median value.**

In a group of healthy volunteers, lower PAI-1 serum sample concentrations were measured than in HNSCC patients (412.0 versus 680.5 ng/mls, $P = 0.0006$) (Figure 2). In the case of uPA, no difference was observed between the groups (data not shown).

There were no differences in uPA or PAI-1 concentrations measured in serum samples obtained at diagnosis and after therapy (Table 2), and there was no correlation observed with any of the clinical or histopathological prognostic factors tested. However, when only the patients in whom the second serum sample was taken $\geq 30$ days after completion of therapy were considered, a borderline significant difference between sample nos 1 and 2 for uPA concentrations was observed (0.48 versus 0.60 ng/mls, $P = 0.08$).

Median relative increases in uPA and PAI-1 in serum samples were 0.9 and 1.05, respectively. There was no correlation observed between the established clinical or histopathological prognostic factors and uPA or PAI-1 relative increases calculated from their serum concentrations. In addition, there was no statistically significant difference between serum concentrations of either uPA or PAI-1 measured before and after therapy as well as between their relative increases.

## DISCUSSION

There are few data on the role of serine proteinase uPA and its inhibitors in the invasive behaviour of HNSCC. In our study, the concentrations of uPA and PAI-1 differed significantly between tumour and adjacent normal tissue, and were found to be higher in tumour tissue samples, as has already been observed by other authors [4, 5, 7]. The reported levels of both uPA and PAI-1 do not concur between studies, at least in part, due to differences in sample preparation and/or the ELISAs used for their determination.

In tumour as well as in normal tissue, a strong positive correlation was found between the uPA concentrations and the corresponding PAI-1 concentrations measured in the same samples. The same was observed in the case of their relative increases, which means that a higher increase in uPA concentration in a tumour compared with its normal counterpart is accompanied by a more pronounced difference in PAI-1 content in the same direction. This is an unexpected finding, since PAI-1 is known to block uPA activity, and, therefore, a decrease in PAI-1 concentration would be expected in a tumour. However, the same relationships between tumour and normal tissue PAI-1 concentrations have been found in various other malignant tumours, and elevated tumour tissue PAI-1 levels appear to be associated with poor prognosis, particularly of breast cancer patients [11]. Yet, we observed that higher inhibitor concentrations were associated with locally advanced ($T_3$ and $T_4$) tumours, which had worse prognosis than early ($T_1$ and $T_2$) tumours. There are various speculative explanations of the role of excessive PAI-1 production in tumour tissue [2, 12, 13]: (1) PAI-1 represents a self-protection mechanism against the uPA-directed tissue destruction; (2) PAI-1 may be of importance for the re-implanting of circulating tumour cells; and (3) since PAI-1 may play a role in angiogenesis, its levels in tumour extracts could reflect the extent of neovascularisation.

We found higher concentrations of uPA in normal laryngeal tissue compared with non-laryngeal tissue, as well as in tissue samples of primary tumours than in recurrent ones. Calculating the relative increase, it appears that the increase in uPA concentrations of normal versus tumour tissue of the larynx was considerably lower than that of the upper digestive tract organs, as was the case with recurrent tumours compared with primaries. Because of the small number of patients included in the study, the value of the above-mentioned correlations and their possible interpretations remains questionable. However, it is interesting that the same relationship was observed between normal laryngeal and non-laryngeal tissue with respect to the concentrations of cysteine proteinases, cathepsins B, H and L, as well as aspartic proteinase cathepsin D [14].

To our knowledge, this is the first time that uPA and PAI-1 concentrations have been measured in the sera of HNSCC patients. In healthy volunteers, significantly lower PAI-1 concentrations were measured than in HNSCC patients, which could be a systemic reflection of a localised increase in proteolytic activity in the affected individuals. The uPA serum concentrations did not differ between the groups. Both uPA and PAI-1 concentrations in serum samples from our patients were basically the same, regardless of the time of their collection. Also, the concentrations of uPA and PAI-1 in serum samples obtained at diagnosis were found not to be correlated with any clinical or histopathological prognostic factors tested. Only in the group of patients in whom the second serum sample was collected $\geq 30$ days after completion of therapy, was a slight increase in uPA concentrations observed between sample nos 1 and 2, although the difference did not reach statistical significance. Since the prolongation of the time interval between the end of therapy and sample no. 2 collection should lead to normalisation of proteolytic activity in the microenvironment of a previously treated area, uPA concentrations would be expected to decrease or, at least, not change. More experimental and clinical data are required for a relevant answer.

uPA and PAI-1 have already been considered as prognostic factors for relapse-free and overall survival, particularly of breast cancer patients [11]. Their prognostic value in HNSCC has not yet been investigated, which is not the case for other members of the proteolytic cascade. Budihna and colleagues [14] demonstrated low cathepsin B and L tumour concentrations to be associated with better disease-free and disease-specific survival. The opposite was true for cathepsin H and cysteine proteinase inhibitors stefins A and B. Similarly, cathepsin D turned out to be of prognostic importance in laryngeal carcinoma patients in the study of Maurizi and associates [15].

To conclude, our results and critical review of the literature indicate that both uPA and PAI-1 may play a specific role in the process of invasion and metastasis, and might also be of prognostic importance in HNSCC. The significance of these findings needs to be confirmed in further studies, especially in the light of their relationship to patient survival.


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