ORIGINAL ARTICLE

# PROGNOSTIC VALUE OF PLASMINOGEN ACTIVATOR INHIBITOR-1 IN HEAD AND NECK SQUAMOUS CELL CARCINOMA

**Luciënne Speleman, MD,[1] Jeroen D. F. Kerrebijn, MD, PhD,[1] Maxime P. Look, MSc,[2] Cees A. Meeuwis, MD, PhD,[1] John A. Foekens, PhD,[2] Els M. J. J. Berns, PhD[2]**

[1] Department of Otolaryngology-Head and Neck Surgery, Erasmus MC Daniel den Hoed, Rotterdam, The Netherlands
[2] Department of Medical Oncology, Erasmus MC Daniel den Hoed, Rotterdam, The Netherlands. E-mail: p.berns@erasmusmc.nl

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**Abstract:** *Background.* Tumor cell biological factors, such as urokinase plasminogen activator (uPA) and its inhibitor plasminogen activator inhibitor-1 (PAI-1), cathepsin D, and c-myc play a role in tumor invasion, metastasis, and proliferation. In this study, the prognostic importance of these factors in patients with primary head and neck squamous cell carcinoma (HNSCC) was evaluated and correlated with clinicopathologic variables.

*Methods.* In 46 paired primary tumors and normal tissues, levels of uPA, PAI-1, cathepsin D, and c-myc amplification were determined. The clinical follow-up was over 10 years. Relationships between cell biological factors and patient and tumor characteristics were studied by the Mann–Whitney test. The Cox proportional hazard model was used for univariate and multivariate analysis.

*Results.* In this study, only a high level of PAI-1 was associated with a significantly shorter disease-free survival ($p < .01$). PAI-1 levels were higher in tumors with perineural invasion ($p < .01$). Both PAI-1 and uPA levels were higher in patients who smoked ($p < .01$ and $p = .02$). In univariate analysis, smoking ($p = .04$), excessive alcohol intake ($p = .02$), perineural invasion ($p = .001$), and vaso-invasion ($p = .009$) were associated with a shorter disease-free survival. The only factor related to overall survival was perineural invasion ($p = .045$). The combination of a high PAI-1 level and perineural invasion appeared to be a significant predictor of a shorter disease-free interval ($p = .01$).

*Conclusion.* PAI-1 may present a novel prognostic factor for patients with HNSCC. Perineural invasion and PAI-1 level combined seemed to be prognostic for disease-free survival.
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**Keywords:** head and neck cancer; uPA; PAI-1; perineural; invasion

*Correspondence to:* E. M. J. J. Berns



Head and neck squamous cell carcinoma (HNSCC) represents the sixth most common cancer worldwide.[1] Annually there are approximately 500,000 new cases of HNSCC worldwide, 2500 cases in The Netherlands alone.[2,3] Despite advances in diagnosis and therapy, long-term survival of HNSCC patients has only moderately improved during the past 20 years. This is due to the relatively high local recurrence rate, metastatic spread, and secondary primary cancers. About 10% to 20% of the patients develop regional recurrences,[4,5] 25% to 30% distant metastases,[6] and 5% to 36% develop a second primary tumor in the head and neck region.[4]

Tumor status and especially cervical lymph node status are regarded as the most important prognostic factors for overall survival. It has been reported that the 5-year overall survival is reduced by approximately 50% in patients with a node

(N)-positive neck.[7] In addition, there is a strong association of perineural invasion with local recurrence and regional nodal metastases.[8–10] Prognostic factors, ie, neck node metastases and perineural invasion, have an impact on treatment strategy such as adjuvant radiotherapy. Identification of new (cell biological) prognostic markers could help to select patients with poor or good prognosis and result in more aggressive or stricter treatment strategies or, on the other hand, might spare patients the burden of aggressive treatment.

It is widely accepted that cancer arises as a result of the accumulation of (epi)genetic alterations in oncogenes and tumor suppressor genes. Apart from these genes, genes involved in angiogenesis, immunoregulation, cellular response to stress, motility, adhesion, and invasion are involved.[11] Thus, a combination of these genes may provide important prognostic information and serve as markers for disease outcome in HNSSC patients.

Cancer cell invasion and metastasis result from a coordinated interaction between proteolytic enzymes that degrade the extracellular matrix (ECM). The urokinase plasminogen activator (uPA) system plays a central role in this process. uPA facilitates the conversion of plasminogen into the serine protease plasmin. Plasmin is capable of degrading most extracellular proteins, either directly or indirectly, through the activation of other proteinases. The lysosomal enzyme cathepsin D is 1 of the enzymes that could initiate this proteolytic cascade, which leads to activation of the precursor form of uPA. Plasminogen activator inhibitor-1 (PAI-1) is thought to be the primary inhibitor of uPA, by forming a firm complex. In addition to binding to uPA, PAI-1 can also attach itself to the ECM protein vitronectin, which allows PAI-1 to modulate cellular adhesion and migration.[12,13] It also stimulates angiogenesis. Paradoxically PAI-1 promotes cancer invasiveness and metastatic spreading if one considers that the anti-proteolytic activity of PAI-1 should potentially inhibit cancer invasion.[14] This paradox was confirmed by the report that cancer invasion and angiogenic activities are abolished in PAI-1 deficient mice. When this PAI-1 deficiency was circumvented, by intravenous (IV) injection of a replication-defective adenoviral vector expressing human PAI-1, the invasion and its associated angiogenesis were restored.[15] In this prospective study, the relation of uPA, PAI-1, cathepsin D protein levels, and c-myc amplification with patient characteristics, local recurrence, regional and distant metastasis, second primary tumors, and overall survival were analyzed. The tissues were collected consecutively in 1 hospital, with the advantage that a unified therapy-protocol was used for all patients.

## MATERIALS AND METHODS

**Patients.** Forty-six consecutively collected biopsy samples were included. Patients were surgically treated at the Daniel den Hoed of the Erasmus Medical Centre Rotterdam for primary HNSCC between 1990 and 1991. Specimens of the primary tumor and normal mucosa, which were taken from the oral cavity not adjacent to the tumor, of the same patient were excised and immediately snap frozen in liquid nitrogen. Exclusion criteria were as follows: previous diagnosis of HNSCC and previous radiotherapy; histology other than squamous cell carcinoma and tumor sites other than those listed in Table 1 (oral cavity, oropharynx, larynx, hypopharynx). The median age of patients at the time of surgery was 64 years (range, 38–84 years). Thirty-nine patients (85%) were treated with adjuvant radiotherapy postoperatively according to the criteria of Fletcher and Evers.[16]

The patient and tumor characteristics, ie, established clinical and histomorphological factors including pT classification, differentiation-grade, nodal-status, perineural invasion, and vaso-invasion are listed in Table 1. Tumors were staged according to the TNM classification system as proposed by the Union Internationale Contre le Cancer.[17]

The median survival time of patients alive was 122 months after primary diagnosis (range, 48–148 months). During follow-up, 27 (58%) patients developed a relapse of disease, of whom 7 (15%) had a local recurrence, 5 of them in combination with regional metastasis, 2 patients (4%) with only regional metastasis, 5 (11%) with distant metastasis, and 13 (28%) patients with a second primary tumor. Thirty-eight patients died, 20 (43%) as a result of tumor relapse.

**Assay of uPA, PAI-1, Cathepsin D, and c-myc in Tumor Tissue Extracts.** Tumor tissues were immersed in liquid nitrogen immediately following resection and were pulverized in the frozen state with a microdismembranator as recommended by the European Organization for Research and Treatment of Cancer (EORTC).[18] The resulting tissue powder was then suspended in EORTC receptor

**Table 1.** Expression levels of uPA, PAI-1, and cathepsin D in tumor and the relation with clinico-pathologic variables.

| Patient/tumour characteristics | UPA | | | PAI-1 | | | Cathepsin D | | | c-myc | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *n* | Tumor MD*(25/75%) | *p value* | *n* | Tumor MD*(25/75%) | *p value* | *n* | Tumor MD*(25/75%) | *p value* | *n* | Tumor % amplification | *p value* |
| Overall | 46 | 1.1 (0.3–3.6) | | 46 | 55 (27–134.4) | | 46 | 26.4 (19.6–35.9) | | 40† | | |
| Sex | | | | | | | | | | | | |
| Male | 27 | 1.3 (0.3–3.7) | $p = .67$ | 27 | 65.8 (34.3–179.7) | $p = .23$ | 27 | 26.4 (19.8–34.0) | $p = .41$ | 22 | 9 | $p = .43$ |
| Female | 19 | 1.1 (0.6–3.6) | | 19 | 43.2 (18.2–84.2) | | 19 | 29.1 (19.5–46.7) | | 18 | 22 | |
| Age, y | | | | | | | | | | | | |
| <50 | 9 | 1.3 (0.4–3.7) | $p = .30$ | 9 | 72.6 (26.4–126.8) | $p = .21$ | 9 | 33.7 (20.4–35.9) | $p = .71$ | 8 | 0 | $p = .22$ |
| 50–59 | 10 | 1.2 (0.9–5.2) | | 10 | 107.2 (55.3–188.6) | | 10 | 30.1 (19.6–40.7) | | 9 | 44 | |
| ≥60 | 27 | 1.1 (0.1–2.6) | | 27 | 43.2 (18.2–84.2) | | 27 | 24.8 (18.7–35.4) | | 23 | 9 | |
| Smoking | | | | | | | | | | | | |
| No | 11 | 0.7 (0.0–1.1) | $p = .02$ | 11 | 28.8 (12.8–40.5) | $p < .01$ | 11 | 26.4 (17–42.5) | $p = .47$ | 9 | 0 | $p = .23$ |
| Yes | 35 | 1.7 (0.4–4.3) | | 35 | 72.6 (43.0–179.8) | | 35 | 26.4 (20.4–35.9) | | 31 | 19 | |
| No | 14 | 0.6 (0.1–2.6) | $p = .28$ | 14 | 39.5 (18.2–57.1) | $p = .14$ | 14 | 26.4 (18.7–46.7) | $p = .70$ | 12 | 0 | $p = .71$ |
| Alcohol | | | | | | | | | | | | |
| 1–6 per day | 21 | 1.3 (0.4–4.9) | | 21 | 55.7 (27–170.8) | | 21 | 24.4 (19.5–35.5) | | 19 | 21 | |
| >6 | 11 | 1.7 (0.8–4.2) | | 11 | 78 (45.2–188.6) | | 11 | 29.7 (20.4–35.4) | | 9 | 22 | |
| Localization | | | | | | | | | | | | |
| Oral cavity | 27 | 1.4 (0.3–4.3) | $p = .83$ | 27 | 64.3 (21.2–222.2) | $p = .87$ | 27 | 27.6 (17.8–46.7) | $p = .39$ | 23 | 17 | $p = .89$ |
| Oropharynx | 7 | 1.0 (0.7–1.3) | | 7 | 55.3 (15.7–72.6) | | 7 | 33.7 (24.8–40.7) | | 7 | 29 | |
| Larynx | 9 | 0.7 (0.1–2.6) | | 9 | 56.2 (40.5–80) | | 9 | 21.4 (20.4–27.2) | | 7 | 0 | |
| Hypopharynx | 3 | 1.7 (0.4–2.4) | | 3 | 65.8 (43.2–102.8) | | 3 | 18.7 (18.6–29.7) | | 3 | 0 | |
| T classification | | | | | | | | | | | | |
| T1 | 2 | 1.3 (0.1–2.6) | $p = .58$ | 2 | 116 (38.6–193.3) | $p = .91$ | 2 | 25.3 (21.4–29.1) | $p = .57$ | 2 | 0 | $p = .12$ |
| T2 | 12 | 1.2 (0.4–4.0) | | 12 | 73.6 (16.3–178.4) | | 12 | 19.7 (16.9–41.3) | | 11 | 0 | |
| T3 | 15 | 1.3 (0.7–4.9) | | 15 | 55.7 (28.8–126.8) | | 15 | 29.7 (24.4–34) | | 12 | 33 | |
| T4 | 17 | 0.8 (0.1–2.6) | | 17 | 56.2 (34.3–80.0) | | 17 | 27.2 (20.4–41) | | 15 | 7 | |
| N classification | | | | | | | | | | | | |
| N0 | 19 | 0.9 (0.3–4.3) | $p = .47$ | 19 | 78.0 (27.0–193.3) | $p = .31$ | 19 | 24.4 (18.7–33.9) | $p = .32$ | 15 | 7 | $p = .14$ |
| N1 | 27 | 1.4 (0.6–3.6) | | 27 | 55.3 (26.4–84.2) | | 27 | 29.7 (20.4–41.0) | | 25 | 20 | |
| Tumor grade | | | | | | | | | | | | |
| Good | 23 | 1.0 (0.1–2.6) | $p = .15$ | 23 | 45.2 (18.2–193.3) | $p = .95$ | 23 | 21.4 (18.7–34) | $p = .33$ | 19 | 5 | $p = .04$ |
| Mild | 23 | 1.5 (0.7–3.6) | | 23 | 60.7 (28.8–126.8) | | 23 | 28.7 (23.2–41) | | 21 | 24 | |
| Vaso-invasion | | | | | | | | | | | | |
| No | 25 | 1.1 (0.3–2.6) | $p = .54$ | 25 | 55.3 (14.4–126.8) | $p = .26$ | 25 | 27.2 (19.8–35.9) | $p = .89$ | 21 | 14 | $p = .47$ |
| Yes | 21 | 1.2 (0.7–3.6) | | 21 | 64.3 (40.5–170.8) | | 21 | 25.3 (19.6–35.1) | | 19 | 16 | |
| Perineural invasion | | | | | | | | | | | | |
| No | 35 | 1.0 (0.3–2.6) | $p = .20$ | 35 | 44.6 (21.2–80.0) | $p < .01$ | 35 | 25.3 (18.7–34) | $p = .07$ | 30 | 17 | $p = .81$ |
| Yes | 11 | 2.4 (0.7–4.5) | | 11 | 126.8 (64.3–255.7) | | 11 | 35.9 (21.4–51.7) | | 10 | 10 | |
| Extranodal growth‡ | | | | | | | | | | | | |
| No | 8 | 2.1 (0.7–4.1) | $p = .51$ | 8 | 68.1 (31.7–157.0) | $p = .40$ | 8 | 20.8 (16.4–31.5) | $p = .11$ | 8 | 25 | $p = .48$ |
| Yes | 19 | 1.3 (0.6–3.0) | | 19 | 43.0 (26.4–72.6) | | 19 | 33.7 (23.2–42.5) | | 17 | 18 | |

*Abbreviations: uPA, urokinase plasminogen activator; PAI-1, plasminogen activation inhibitor-1; MD, median values.*
**Median values and interquartile range in ng/mL.*
*†Does not add up to 46 because of missing values.*
*‡Does not add up to 46 because 19 tumors did not have nodal metastasis.*

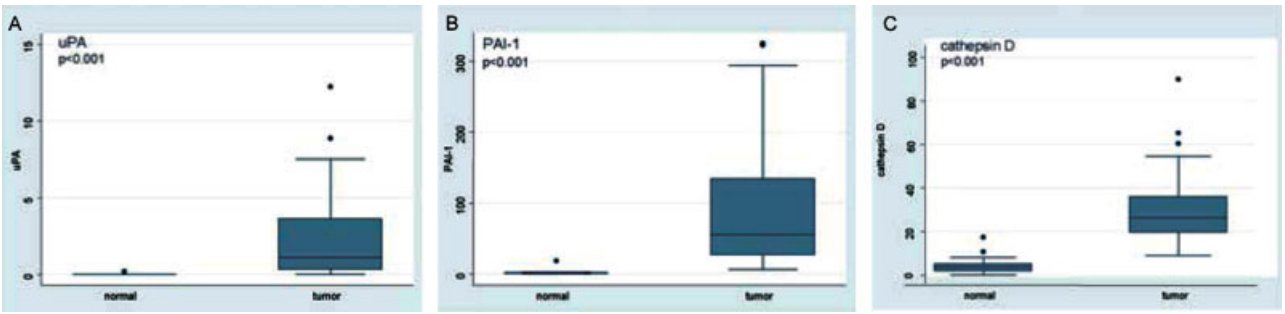


**FIGURE 1.** Comparison of levels of urokinase plasminogen activator (uPA) (**A**), plasminogen activation inhibitor-1 (PAI-1) (**B**), and cathepsin D (**C**) in primary tumor specimens and normal mucosa, which was taken from the oral cavity not adjacent to the tumor of the same patient. The Box-Whisker graphs present the amount of protein as measured by IRMA or ELISAs, respectively (as described in the Materials and Methods section). Box shows the range between 25th and 75th percentiles, with a horizontal line at the median value. Whiskers extend to 5th and 95th percentiles of values. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

buffer (10 m*M* $K_2HPO_4$, containing 1.5 m*M* dipotassium chloride EDTA, 3 m*M* $NaN_3$, 10 m*M* monothioglycerol, and 10% v/v glycerol, pH 7.4). The suspension was centrifuged for 30 minutes at 100,000*g* at 4°C to obtain the supernatant fraction (cytosol). uPA and PAI-1 were analyzed with enzyme-linked immunosorbent assays (ELISAs) in tissue cytosols. For the estimation of the cathepsin D protein levels, we used a radiometric immunoassay (ELSA-CATH-D; CIS bio international, Gif-sur-Yvette, France). The details of the assay procedures have been described previously.[19–21] For c-myc amplification, high molecular weight chromosomal DNA was isolated and analyzed as described by Berns et al.[22]

**Statistics.** The strength of the association of uPA, PAI-1, and cathepsin D was determined with Spearman rank correlation. The association of these factors (as a continuous variable) with other patient and tumor characteristics was tested with the nonparametric Mann–Whitney *U* test with patient and tumor characteristics as grouping variables. Levels of factors measured in both tumor and normal tissue samples were compared using the Wilcoxon matched-pairs signed-rank test. A significant trend between the log transformed PAI-1 concentration and the rate of relapse was observed in univariate Cox regression. This justified the search for a cut-off value to determine whether a dichotomy or a more or less continuous trend was present. For this purpose, we applied isotonic regression analysis[23] with disease-free survival as endpoint. Survival curves were generated using the method of Kaplan–Meier[24] and the log-rank test was used to test for differences in (disease-free) survival. Although the Kaplan–Meier curves, to visualize the disease-free survival, were shown until 60 months, the complete follow-up time (of 120 months) has been used for all analyses.

The likelihood ratio test was used to test for differences between models with variables included and excluded. PAI-1, uPA, and cathepsin D were analyzed as log-transformed variables.

## RESULTS

**Relationships of Expression Levels of uPA, PAI-1, Cathepsin D, and c-myc Amplification with Patient and Tumor Characteristics: Normal Versus Tumor Tissue.** The levels of uPA, PAI-1, and cathepsin D, and c-myc amplification were studied in 46 tumor and normal tissue samples. In all cases, the uPA, PAI-1, and cathepsin D levels were higher in tumor tissue than in their normal counterparts (shown in Figure 1). uPA was not expressed in normal tissue. The median level in tumor was 1.1 (interquartile range, 0.3–3.6; $p < .001$). The median level of PAI-1 was 45 times higher in tumor tissue than in their normal counterparts, 55 (27–134.4) versus 1.2 (0.6–1.9) ($p < .001$), and the median level of cathepsin D was 7 times higher in tumor tissue than in their normal counterparts, 26.4 (19.6–35.9) versus 3.7 (1.8–5.1) ($p < .001$) (see Figure 1). As expected, c-myc amplification was only observed in tumor tissue. Myc amplification (more than 3 gene copies) was observed in 15% of all tumor samples. The levels of each marker in tumor tissue are shown in Table 1.

A significant correlation was observed between increased PAI-1 level and perineural invasion

and between increased levels of uPA and PAI-1 levels and smoking habits of patients, ie, tumors without perineural invasion had low levels of PAI-1 ($p < .01$); and nonsmoking patients showed similar trends for uPA and PAI-1 ($p = .02$ and $p < .01$, respectively). A trend toward significance was found between cathepsin D and perineural invasion ($p = .07$) (Table 1). A significant higher amplification rate of c-myc was seen in mildly differentiated tumors ($p = .04$).

Furthermore, a trend was seen toward high levels of PAI-1, uPA, c-myc, and alcohol abuse (more than 6 units a day) since levels of all 3 factors were at least doubled, although these differences were not statistically significant. The median uPA levels in patients with lymph node metastasis were higher when compared with patients without nodal metastasis. Higher levels of PAI-1 were observed in smaller tumors (T1–T2), whereas larger tumors (T3–T4) showed c-myc amplification.

**Univariate Analysis for Disease-Free Survival and Overall Survival.** All clinical and biological tumor variables potentially associated with relapse of disease (local recurrence, regional recurrence, or second primary tumor) and overall survival (OS) were tested individually in a univariate model. The prognostic factors for disease-free survival are shown in Table 2. In Cox univariate regression analysis, perineural invasion (hazard ratio [HR] 4.3; 95% confidence interval [CI] 1.8–10.4; $p = .009$), high alcohol intake (HR 5.5; 95% CI 1.3–22.5; $p = .02$), smoking (HR 4.4; 95% CI 1–19; $p = .04$), and vaso-invasion (HR 3.2; 95% CI 1.3–7.7; $p = .009$) were significantly related to relapse of disease. Perineural invasion was the only parameter that was significantly less favorable for overall survival (HR 2.3; 95% CI 1.1–4.8; $p = .045$) (not shown).

The only cell biological factor that was associated with relapse of disease in a test for trend was PAI-1 (HR 1.7; 95% CI 1.1–2.5; $p = .009$). This justified the search for a cutpoint for PAI-1 to enable in an exploratory analysis its prognostic value as a dichotomized variable. With isotonic regression analysis, 14 ng/mg protein was chosen as cutpoint to classify tumors as PAI-1 high and PAI-1 low. Patients with high levels of PAI-1 experienced a significantly earlier relapse of disease ($p = .01$, Figure 2A) and a shorter overall survival (n.s.) compared with patients with low levels of PAI-1 (Figure 2B). The complete follow-up time has been used for all analyses.

**Table 2.** Relation of patient and tumor characteristics and cell biological factors with disease-free survival: univariate analysis.

| Patient and tumor characteristics | *n* | HR | 95% CI | *p* value |
|---|---|---|---|---|
| All | 46 | | | |
| Sex | | | | |
| Male | 27 | 1 | | |
| Female | 19 | 0.8 | 0.3–1.8 | .56 |
| Age, y | | | | |
| <50 | 9 | 1 | | |
| 50–59 | 10 | 1.0 | 0.3–2.7 | .93 |
| ≥60 | 27 | 0.5 | 0.2–1.4 | .19 |
| Smoking | | | | |
| No | 11 | 1 | | |
| Yes | 35 | 4.4 | 1.0–19.0 | .04 |
| Alcohol | | | | |
| non | 14 | 1 | | |
| 1–6 per day | 21 | 2.7 | 0.8–9.6 | .11 |
| >6 | 11 | 5.5 | 1.3–22.5 | .02 |
| Localization | | | | |
| oral cavity | 27 | 1 | | |
| oropharynx | 7 | 1.7 | 0.6–4.7 | .34 |
| larynx | 9 | 1.4 | 0.5–4.1 | .56 |
| hypopharynx | 3 | 1.0 | 0.1–7.8 | 1.00 |
| T classification | | | | |
| T1 | 2 | 1 | | |
| T2 | 12 | 0.6 | 0.1–5.4 | .69 |
| T3 | 15 | 0.4 | 0.1–3.5 | .41 |
| T4 | 17 | 0.5 | 0.1–3.7 | .46 |
| N classification | | | | |
| N0 | 19 | 1 | | |
| N+ | 27 | 0.9 | 0.4–2.0 | .71 |
| Tumor grade | | | | |
| Good | 23 | 1 | | |
| Mild-poor* | 23 | 1.8 | 0.8–4.3 | .16 |
| Radical surgery | | | | |
| Radical | 28 | 1 | | |
| Poorly radical | 8 | 0.6 | 0.2–2.0 | .38 |
| Irradical | 10 | 1.5 | 0.6–4.0 | .39 |
| Vaso-invasion | | | | |
| No | 25 | 1 | | |
| Yes | 21 | 3.2 | 1.3–7.7 | .009 |
| Perineural invasion | | | | |
| No | 35 | 1 | | |
| Yes | 11 | 4.3 | 1.8–10.4 | .001 |
| Extranodal growth† | | | | |
| No | 8 | 1 | | |
| Yes | 19 | 1.0 | 0.9–1.2 | .85 |
| uPA‡ | 46 | 1.4 | 1.0–2.0 | .06 |
| PAI-1‡ | 46 | 1.7 | 1.1–2.5 | .009 |
| cathepsin D‡ | 46 | 1.9 | 0.8–4.8 | .15 |
| c-Myc | 40 | 2.3 | 0.8–6.7 | .12 |

*Abbreviations: HR, hazard ratio; CI, confidence interval; uPA, urokinase plasminogen activator; PAI-1, plasminogen activator inhibitor-1.*
**Taken together because only 1 tumor showed poor differentiation.*
*†Does not add up to 46, because 19 patients had a node-negative neck, not applicable.*
*‡All log transformed.*

Although there was a trend toward a poor disease-free survival in tumors with c-myc amplification, this difference was not statistically signifi-

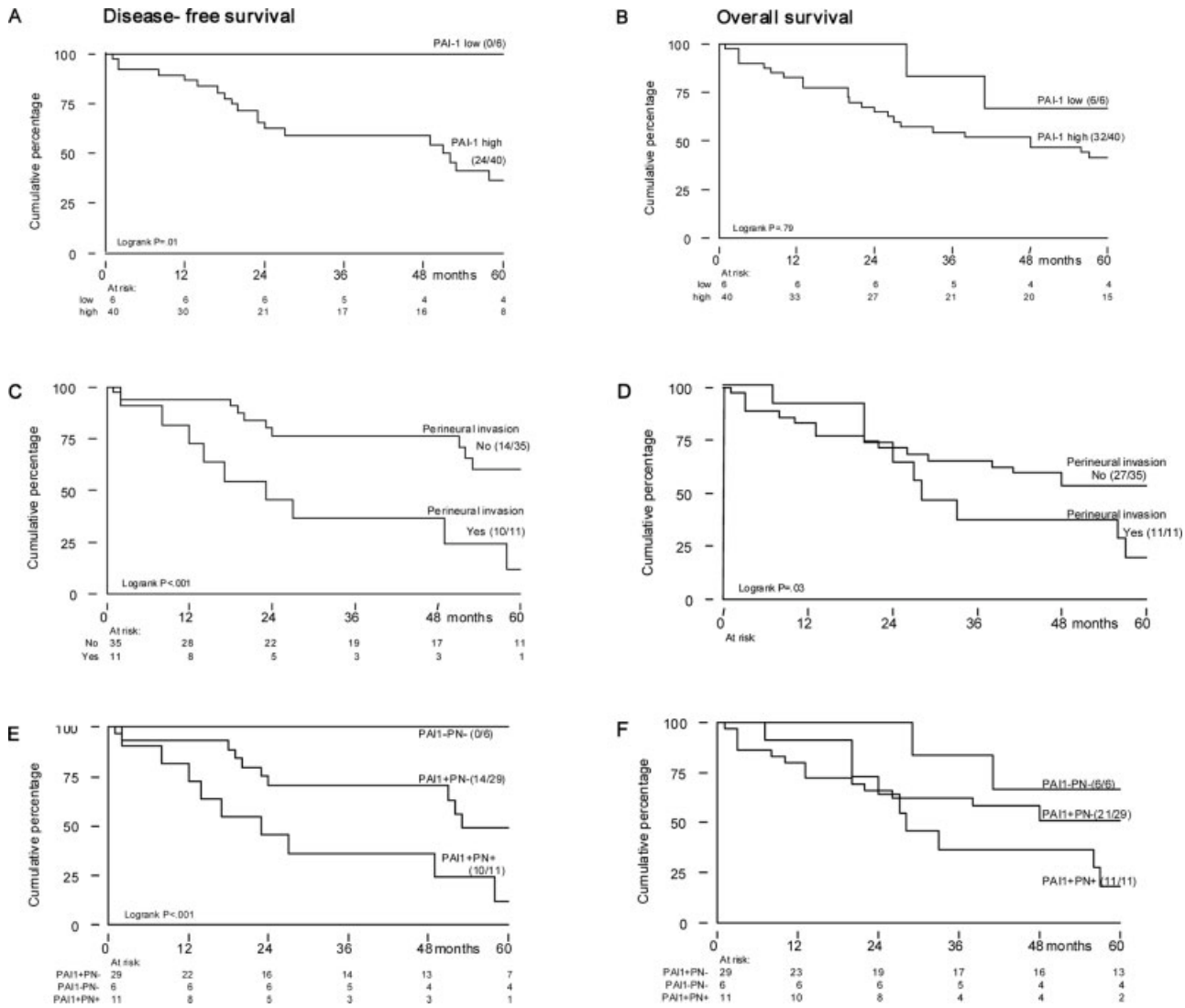


**FIGURE 2.** Disease-free and overall survival as a function of PAI-1 (**A** and **B**), peri-neural invasion (**C** and **D**), and the combination of PAI-1 and perineural invasion (**E** and **F**) in all 46 patients. The cut-off point used for PAI-1 was 14 ng/mg (as described in the Materials and Methods section). Numbers in parentheses in A,C,E indicate number of relapse/total number of patients per group and in B,C,F indicate number of deaths/total in each group.

cant (HR 2.3; 95% CI 0.8–6.7; $p = .1$). Patients with tumors with less than 3 copies of c-myc showed a more favorable disease-free survival than those with more than 3 gene copies of c-myc.

Neither uPA nor cathepsin D predicted the length of disease-free survival and overall survival.

In Figures 2C and 2D, the significantly higher relapse rate for tumors with perineural invasion ($p < .001$) are depicted. In addition, a less favorable overall survival ($p = .03$) is shown. Interestingly, the result of perineural invasion, when combined with PAI-1 on the disease-free survival, was even more discriminatory, as shown in Figures 2E and 2F.

Tumors with a low PAI-1 level and without signs of perineural invasion remained free from events, while tumors with a high PAI-1 level in combination with perineural invasion had multiple events, resulting in a poorer disease-free survival ($p < .001$).

## DISCUSSION

Besides the clinical prognostic factors, it would be beneficial for patients with HNSCC if there were tumor-associated markers available that could more reliably predict disease-progression. At present, clinical predictors for locoregional control and survival in HNSCC is mainly based on TNM

classification. However, clinical experience learns that tumor behavior of identical tumors may differ widely. Therefore, insight into tumor biology, especially related to metastasis and recurrence, would give us tremendous important information, which could finally attribute to achieve tailored therapy and follow-up protocols for each individual. The aim of our study was to identify markers, associated with metastasis, that could be used routinely to distinguish patients at risk of recurrence or aggressive disease.

The present study is the first in which the clinical follow-up is more than 10 years. Concerning the disease-free survival with this type of cancer, a long follow-up is important since secondary primary tumors mainly develop after 5 years, or even after 8 years as shown in our study. We showed that PAI-1 content, as determined by ELISA, proved to be a prognostic factor for poor disease-free survival. Besides PAI-1 content, perineural invasion was an important histologic sign for early relapse of disease. Interestingly, the combination of high PAI-1 level and perineural invasion seem to be the most powerful prognostic marker for short disease-free survival.

We observed higher levels of uPA and PAI-1 in tumor tissues when compared with their normal counterparts. This is in accordance with other studies concerning HNSCC that reported significantly higher uPA and PAI-1 levels in tumor specimens than in their normal counterparts.[25–33] Recently, using expression profiling, Chin et al[34] noted that PAI-1 was listed in the top 5% of genes that differed at a level of statistical significance between mucosa and tumor. Novel in our study is that uPA and PAI-1 levels were significantly higher in tumors from patients who had a history of smoking, and this trend, although not significant, was seen in excessive alcohol consumption as well. Both factors are the main accepted risk factors for HNSCC. Interestingly, PAI-1 levels were also significantly increased in tumors with perineural invasion. In these tumors, the levels of uPA were increased as well, although not significantly. No other relations between levels of tumor markers and patients or tumor characteristics were observed.

Conflicting data are published about PAI expression levels and the relation with clinicopathologic parameters. Yasuda et al[25] and Strojan et al[27] reported significantly higher PAI-1 levels in advanced (T3–T4) tumors in 28 and 58 tumors, respectively, while others did not find such associations.[30,35] High uPA antigen expression and activity have been previously associated with aggressiveness in a few reports.[28,36] In contrast, Pacheco et al[31] did not find a correlation between uPA level and clinicopathologic parameters in a smaller sample size consisting of 38 tumors. At present, only Hundsdorfer et al[35,37] reported a relationship between PAI-1 and poor prognosis in 79 HNSCC patients. In contrast to our study, though, they also observed a relationship between uPA level and tumor relapse.[37]

In the late 1980s, Duffy et al[38] were the first who showed that breast cancer patients with high uPA levels in their primary tumors had a worse disease-free survival than patients in whom uPA levels were low. These results were confirmed in a variety of other cancer types, such as ovarian, esophageal, gastric, colorectal, lung, or liver cancers.[39–44] Because PAI-1 is an inhibitor of uPA, it was initially expected to prevent cancer cell invasion and metastatic spreading. However, PAI-1 proved to play a role in promoting cancer cell invasion. This paradox was substantiated in PAI-deficient mice.[15] When this PAI-1 deficiency was circumvented, for example by intravenous injection of human PAI-1, the invasion and its associated angiogenesis were restored. Janicke et al[45] reported in 1991 that high PAI-1 concentrations predicted an adverse outcome in patients with breast cancer, which was confirmed later by many other investigators in other types of cancer. Recently, clinical validation was carried out using a level-1 evidence (LOE-1) study,[46] which showed that both uPA and PAI-1 were strong predictors of outcome in node-negative breast cancer patients, which led to the routine use of both markers in newly diagnosed breast cancer. Although there is a clear relationship between PAI-1 level and prognosis, the exact tumor biological functions of PAI-1 remain uncertain. PAI-1 seems to be multifunctional, as PAI-1 is expressed by multiple cell types and has multiple interactions. Possible mechanisms by which PAI-1 contributes to cancer dissemination include preventing excess degradation of the ECM, modulating cell adhesion and migration by binding to vitronectin,[47] playing a role in angiogenesis[15] and stimulating cell proliferation.[48] By immunohistolochemical (IHC) analysis, more understanding of the role of PAI-1 could be achieved. In carcinomas of breast,[49] colon,[50] and prostate,[51] PAI-1 is predominantly seen in the stromal compartment, while PAI-1 in HNSCC is mainly seen in cancer cells. Since we only used ELISA for measuring PAI-1 levels in our study, no information was obtained about the function of

PAI-1 in the plasminogen system in HNSCC. The combination of IHC and ELISA would have given better information, especially to understand the role of PAI-1 in the perineural invasion. On the other hand, while the quality of IHC results are disputed, ELISA has undergone the most detailed evaluation, and a study performed by Benraad et al[52] in which 6 different ELISA systems were compared showed adequate sensitivity for detecting uPA and PAI, and good correlation was found between different assays.

Besides PAI-1 content, perineural invasion was an important histologic sign for early relapse of disease. In our study, perineural invasion was the most consistent prognostic factor and was diagnosed in 29% of the tumors, compared with 6% to 52% in different other studies.[9,53–55]

In the literature, perineural invasion in HNSCC, as studied in larger groups of patients ($n$ = 74–239), is independent of tumor classification and related to an increased incidence in concurrent cervical nodal metastasis and local recurrence.[9,10,56] We found a clear relationship between perineural invasion and disease-free survival as well as overall survival. Therefore, and in accordance with the literature,[10] we conclude that perineural invasion is an important prognostic factor for HNSCC.

As expected, we observed high cathepsin D expression levels in primary carcinomas when compared with normal tissue. This is in accordance with the study of Zeilinger et al.[26] In our study, no significant associations were seen between tumor, patient characteristics, and cathepsin D expression levels. Strojan et al[36] also described a relationship between uPA, and cathepsin D levels, and disease-free survival. Interestingly, they showed that serum levels of uPA and cathepsin D could be of prognostic value. With respect to cathepsin D, Gandour-Edwards et al[8] described a predictive value of cathepsin D for cervical lymph node metastasis, using IHC, and Maurizi et al[57] using immunoradiometric assay. Our study, however, did not confirm these findings.

Although genetic alterations in the c-myc oncogene play an important role in the induction and progression of human cancer,[58–60] limited and controversial information is available on the role of c-myc in HNSCC. The present report revealed c-myc amplification in tumors of 15% of the patients, which is consistent with the described frequencies of amplification and expression of 6% to 25% in the literature.[61–67] We only observed a relationship of c-myc amplification with tumor grade. Similar to Waitzberg et al,[68] no correlation was found between c-myc amplification and clinical data in our series. In contrast to our data, Gapany et al[69] observed a negative correlation between c-myc and tumor size or nodal involvement whereas de la Guardia et al[66] and Rodrigo et al[64] observed a positive significant correlation between c-myc and T classification.

The relationship of c-myc and prognosis was analyzed in a limited number of studies. In our series, c-myc might be of value for disease-free survival, which is in agreement with 2 studies[70,71] but contradicts 2 other studies.[72,73] The reason for these conflicting results is not evident, but could reflect differences in assay methodology, small sample sizes (between 28 and 79 patients for prognostic marker studies), heterogeneity in the localization of tumors studied, and different treatments and endpoints included in the studies.

In summary, predicting the prognosis and clinical outcomes in patients is very important for the individual management of patients with HNSCC. In our prospective clinicopathologic study of 46 patients with a long follow-up over 10 years, we showed that PAI-1 protein level, as determined by ELISA, was a prognostic factor for poor disease-free survival in univariate analysis. Cathepsin D and uPA contents, however, were not prognostic. Interestingly, the combination of high PAI-1 level and evidence of perineural invasion in the tumor was associated with the shortest disease-free interval. It should be emphasized that our study was exploratory and that independent confirmation in larger patient groups is needed.

## REFERENCES


1. Quon H, Liu FF, Cummings BJ. Potential molecular prognostic markers in head and neck squamous cell carcinomas. Head Neck 2001;23:147–159.
2. Vokes EE, Weichselbaum RR, Lippman SM, Hong WK. Head and neck cancer. N Engl J Med 1993;328:184–194.
3. Visser. Feiten en fables over kanker in Nederland. Vereniging van integraal kanker centra. 2000.
4. Vaamonde P, Martin C, del Rio M, LaBella T. Second primary malignancies in patients with cancer of the head and neck. Otolaryngol Head Neck Surg 2003;129:65–70.
5. Dhooge IJ, De Vos M, Van Cauwenberge PB. Multiple primary malignant tumors in patients with head and neck cancer: results of a prospective study and future perspectives. Laryngoscope 1998;108:250–256.
6. Leemans CR, Tiwari R, Nauta JJ, van der Waal I, Snow GB. Regional lymph node involvement and its significance in the development of distant metastases in head and neck carcinoma. Cancer 1993;71:452–456.
7. Johnson NW, Warnakulasuriy S, Tavassoli M. Hereditary and environmental risk factors; clinical and laboratory risk matters for head and neck, especially oral, cancer and precancer. Eur J Cancer Prev 1996;5:5–17.

8. Gandour-Edwards R, Trock B, Donald PJ. Predictive value of cathepsin-D for cervical lymph node metastasis in head and neck squamous cell carcinoma. Head Neck 1999;21:718–722.
9. Fagan JJ, Collins B, Barnes L, D'Amico F, Myers EN, Johnson JT. Perineural invasion in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 1998;124:637–640.
10. Rahima B, Shingaki S, Nagata M, Saito C. Prognostic significance of perineural invasion in oral and oropharyngeal carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004;97:423–431.
11. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70.
12. Barbareschi M, Gasparini G, Morelli L, Forti S, Dalla Palma P. Novel methods for the determination of the angiogenic activity of human tumors. Breast Cancer Res Treat 1995;36:181–192.
13. Bajou K, Lewalle JM, Martinez CR, et al. Human breast adenocarcinoma cell lines promote angiogenesis by providing cells with uPA-PAI-1 and by enhancing their expression. Int J Cancer 2002;100:501–506.
14. Sier CF, Vloedgraven HJ, Ganesh S, et al. Inactive urokinase and increased levels of its inhibitor type 1 in colorectal cancer liver metastasis. Gastroenterology 1994;107: 1449–1456.
15. Bajou K, Noel A, Gerard RD, et al. Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. Nat Med 1998;4:923–928.
16. Fletcher GH, Evers WT. Radiotherapeutic management of surgical recurrences and postoperative residuals in tumors of the head and neck. Radiology 1970;95:185–188.
17. Wittekind C, Greene FL, Hutter RVP, Klimpfinger M, Sobin LH, editors. Illustrated guide to TNM/ pTNM classification of malignant tumours. Berlin: Springer Verlag; 2005.
18. Breast Cancer Cooperative Group. Revision of standards of for the assessment of hormone receptors in human breast cancer. Eur J Cancer 1980;16:1513–1515.
19. Ronne E, Hoyer-Hansen G, Brunner N, et al. Urokinase receptor in breast cancer tissue extracts. Enzyme-linked immunosorbent assay with a combination of mono- and polyclonal antibodies. Breast Cancer Res Treat 1995;33: 199–207.
20. Grondahl-Hansen J, Peters HA, van Putten WL, et al. Prognostic significance of the receptor for urokinase plasminogen activator in breast cancer. Clin Cancer Res 1995;1:1079–1087.
21. Foekens JA, Buessecker F, Peters HA, et al. Plasminogen activator inhibitor-2: prognostic relevance in 1012 patients with primary breast cancer. Cancer Res 1995;55: 1423–1427.
22. Berns EM, Klijn JG, van Staveren IL, Portengen H, Noordegraaf E, Foekens JA. Prevalence of amplification of the oncogenes c-myc, HER2/neu, and int-2 in one thousand human breast tumours: correlation with steroid receptors. Eur J Cancer 1992;28:697–700.
23. Barlow RE, Batholomew D, Bremner J. Statistical Inference Under Order Restrictions. London: Wiley; 1972.
24. Kaplan E, Meier P. Non-parametric estimation from incomplete observations. J Am Stat Assoc 1958;53:457–481.
25. Yasuda T, Sakata Y, Kitamura K, Morita M, Ishida T. Localization of plasminogen activators and their inhibitor in squamous cell carcinomas of the head and neck. Head Neck 1997;19:611–616.
26. Zeillinger R, Eder S, Schneeberger C, Ullrich R, Speiser P, Swoboda H. Cathepsin D and PAI-1 expression in human head and neck cancer. Anticancer Res 1996;16: 449–453.
27. Strojan P, Budihna M, Smid L, Vrhovec I, Skrk J. 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. Eur J Cancer 1998;34:1193–1197.
28. Parolini S, Flagiello D, Cinquetti A, et al. Up-regulation of urokinase-type plasminogen activator in squamous cell carcinoma of human larynx. Br J Cancer 1996;74: 1168–1174.
29. Itaya T, Suzuki K, Takagi I, Motai H, Baba S. Relationship between head and neck squamous cell carcinomas and fibrinolytic factors. Immunohistological study. Acta Otolaryngol Suppl 1996;525:113–119.
30. Nozaki S, Endo Y, Kawashiri S, et al. Immunohistochemical localization of a urokinase-type plasminogen activator system in squamous cell carcinoma of the oral cavity: association with mode of invasion and lymph node metastasis. Oral Oncol 1998;34:58–62.
31. Pacheco MM, Kowalski LP, Nishimoto IN, Brentani MM. Differential expression of c-jun and c-fos mRNAs in squamous cell carcinoma of the head and neck: associations with uPA, gelatinase B, and matrilysin mRNAs. Head Neck 2002;24:24–32.
32. Petruzzelli GJ, Snyderman CH, Johnson JT. In vitro urokinase type plasminogen activator levels and total plasminogen activator activity in squamous cell carcinomas of the head and neck. Arch Otolaryngol Head Neck Surg 1994;120:989–992.
33. Clayman G, Wang SW, Nicolson GL, et al. Regulation of urokinase-type plasminogen activator expression in squamous-cell carcinoma of the oral cavity. Int J Cancer 1993; 54:73–80.
34. Chin D, Boyle GM, Williams RM, et al. Novel markers for poor prognosis in head and neck cancer. Int J Cancer 2005;113:789–797.
35. Hundsdorfer B. The prognostic importance of uPA and PAI-1 in the primary resection of oral squamous cell carcinoma. Mund Kiefer Gesichtschir 2004;8:173–179.
36. Strojan P, Budihna M, Smid L, Vrhovec I, Skrk J. Urokinase-type plasminogen activator, plasminogen activator inhibitor type 1 and cathepsin D: analysis of their prognostic significance in squamous cell carcinoma of the head and neck. Anticancer Res 2000;20:3975–3981.
37. Hundsdorfer B, Zeilhofer HF, Bock KP, et al. Tumour-associated urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1 in normal and neoplastic tissues of patients with squamous cell cancer of the oral cavity—clinical relevance and prognostic value. J Craniomaxillofac Surg 2005;33:191–196.
38. Duffy MJ, Duggan C, Mulcahy HE, McDermott EW, O'Higgins NJ. Urokinase plasminogen activator: a prognostic marker in breast cancer including patients with axillary node-negative disease. Clin Chem 1998;44:1177–1183.
39. Schmitt M. The plasminogen activation system as a novel target for therapeutic strategies. Fibrinolysis 2000;14: 114–132.
40. Pedersen H, Grondahl-Hansen J, Francis D, et al. Urokinase and plasminogen activator inhibitor type 1 in pulmonary adenocarcinoma. Cancer Res 1994;54:120–123.
41. Andreasen PA, Kjoller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 1997;72:1–22.
42. Schmitt M, Harbeck N, Thomssen C, et al. Clinical impact of the plasminogen activation system in tumor invasion and metastasis: prognostic relevance and target for therapy. Thromb Haemost 1997;78:285–296.
43. Duffy MJ, Maguire TM, McDermott EW, O'Higgins N. Urokinase plasminogen activator: a prognostic marker in multiple types of cancer. J Surg Oncol 1999;71:130–135.
44. Foekens JA, Peters HA, Look MP, et al. The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. Cancer Res 2000;60:636–643.

45. Janicke F, Schmitt M, Graeff H. Clinical relevence of urokinase type and tissue-type plasminogen activators and their inhibitor PAI in breast cancer. Semin Thromb Hemost 1991;17:303–312.
46. Janicke F, Prechtl A, Thomssen C, et al. Randomized adjuvant chemotherapy trial in high-risk, lymph node-negative breast cancer patients identified by urokinase-type plasminogen activator and plasminogen activator inhibitor type 1. J Natl Cancer Inst 2001;93:913–920.
47. Loskutoff DJ, Curriden SA, Hu G, Deng G. Regulation of cell adhesion by PAI-1. APMIS 1999;107:54–61.
48. Webb DJ, Thomas KS, Gonias SL. Plasminogen activator inhibitor 1 functions as a urokinase response modifier at the level of cell signaling and thereby promotes MCF-7 cell growth. J Cell Biol 2001;152:741–752.
49. Offersen BV, Nielsen BS, Hoyer-Hansen G, et al. The myofibroblast is the predominant plasminogen activator inhibitor-1-expressing cell type in human breast carcinomas. Am J Pathol 2003;163:1887–1899.
50. Illemann M, Hansen U, Nielsen HJ, et al. Leading-edge myofibroblasts in human colon cancer express plasminogen activator inhibitor-1. Am J Clin Pathol 2004;122: 256–265.
51. Usher PA, Thomsen OF, Iversen P, et al. Expression of urokinase plasminogen activator, its receptor and type-1 inhibitor in malignant and benign prostate tissue. Int J Cancer 2005;113:870–880.
52. Benraad TJ, Geurts-Moespot J, Grondahl-Hansen J, et al. Immunoassays (ELISA) of urokinase-type plasminogen activator (uPA): report of an EORTC/BIOMED-1 workshop. Eur J Cancer A 1996;32:1371–1381.
53. O'Brien CJ, Lahr CJ, Soong SJ, et al. Surgical treatment of early-stage carcinoma of the oral tongue—would adjuvant treatment be beneficial? Head Neck Surg 1986;8:401–408.
54. Soo KC, Carter RL, O'Brien CJ, Barr L, Bliss JM, Shaw HJ. Prognostic implications of perineural spread in squamous carcinomas of the head and neck. Laryngoscope 1986;96:1145–1148.
55. Close LG, Brown PM, Vuitch MF, Reisch J, Schaefer SD. Microvascular invasion and survival in cancer of the oral cavity and oropharynx. Arch Otolaryngol Head Neck Surg 1989;115:1304–1309.
56. Lydiatt DD, Robbins KT, Byers RM, Wolf PF. Treatment of stage I and II oral tongue cancer. Head Neck 1993;15: 308–312.
57. Maurizi M, Almadori G, Cadoni G, et al. Cathepsin D concentration in primary laryngeal cancer: correlation with clinico-pathologic parameters, EGFR status and prognosis. Int J Cancer 1996;69:105–109.
58. Grandori C, Eisenman RN. Myc target genes. Trends Biochem Sci 1997;22:177–181.
59. Thompson EB. The many roles of c-Myc in apoptosis. Annu Rev Physiol 1998;60:575–600.
60. Facchini LM, Penn LZ. The molecular role of Myc in growth and transformation: recent discoveries lead to new insights. FASEB J 1998;12:633–651.
61. Merritt WD, Weissler MC, Turk BF, Gilmer TM. Oncogene amplification in squamous cell carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg 1990;116:1394–1398.
62. Leonard JH, Kearsley JH, Chenevix-Trench G, Hayward NK. Analysis of gene amplification in head-and-neck squamous-cell carcinoma. Int J Cancer 1991;48:511–515.
63. Haughey BH, von Hoff DD, Windle BE, Wahl GM, Mock PM. c-myc oncogene copy number in squamous carcinoma of the head and neck. Am J Otolaryngol 1992;13: 168–171.
64. Rodrigo JP, Lazo PS, Ramos S, Alvarez I, Suarez C. MYC amplification in squamous cell carcinomas of the head and neck. Arch Otolaryngol Head Neck Surg 1996; 122:504–507.
65. Schraml P, Kononen J, Bubendorf L, et al. Tissue microarrays for gene amplification surveys in many different tumor types. Clin Cancer Res 1999;5:1966–1975.
66. de la Guardia C, Casiano CA, Trinidad-Pinedo J, Baez A. *CENP-F* gene amplification and overexpression in head and neck squamous cell carcinomas. Head Neck 2001;23:104–112.
67. Freier K, Joos S, Flechtenmacher C, et al. Tissue microarray analysis reveals site-specific prevalence of oncogene amplifications in head and neck squamous cell carcinoma. Cancer Res 2003;63:1179–1182.
68. Waitzberg AF, Nonogaki S, Nishimoto IN, et al. Clinical significance of c-myc and p53 expression in head and neck squamous cell carcinomas. Cancer Detect Prev 2004;28:178–186.
69. Gapany M, Pavelic ZP, Kelley DJ, et al. Immunohistochemical detection of c-myc protein in head and neck tumors. Arch Otolaryngol Head Neck Surg 1994;120: 255–259.
70. Field JK, Spandidos DA, Stell PM, Vaughan ED, Evan GI, Moore JP. Elevated expression of the c-myc oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma. Oncogene 1989;4:1463–1468.
71. Porter MJ, Field JK, Leung SF, et al. The detection of the c-myc and ras oncogenes in nasopharyngeal carcinoma by immunohistochemistry. Acta Otolaryngol 1994;114: 105–109.
72. Yu Y, Dong W, Li X, Yu E, Zhou X, Li S. Significance of c-Myc and Bcl-2 protein expression in nasopharyngeal carcinoma. Arch Otolaryngol Head Neck Surg 2003;129: 1322–1326.
73. Riva C, Lavieille JP, Reyt E, Brambilla E, Lunardi J, Brambilla C. Differential c-myc, c-jun, c-raf and p53 expression in squamous cell carcinoma of the head and neck: implication in drug and radioresistance. Eur J Cancer B Oral Oncol 1995;31:384–391.