P21WAF1/CIP1 Protein and Tongue Cancer Prognosis

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Abstract. The expression of the cell cycle regulatory protein p21waf1/cip1 was analyzed immunohistochemically in tissue from the tumor and adjacent non-tumor epithelia of 54 patients with tongue cancer in order to determine the proportion of tongue carcinomas with altered p21waf1/cip1 expression, establish whether this alteration is an early event in tongue carcinogenesis and to investigate whether p21waf1/cip1 expression has predictive prognostic value in these tumors. The percentage of p21waf1/cip1-positive neoplastic cells was calculated. Adjacent non-tumor epithelium was classified as normal, hyperplastic or dysplastic, and the p21waf1/cip1 expression was only considered normal in suprabasal layers. P21waf1/cip1 expression was negative in 57.4% (31/54) of the tumors and was aberrant in the non-tumor adjacent tissue of all patients studied. Neither the absence nor the degree of p21waf1/cip1 expression influenced the survival of patients in the present series. P21waf1/cip1 system alteration may be an early and frequent event in tongue carcinogenesis.

Oral squamous cell carcinomas (OSCCs) account for approximately 4% and 2% of neoplasms in men and women, respectively (1). Although the oral cavity is readily accessible and frequently examined, late diagnosis of OSCC is frequent (2) and is associated with a low five-year survival rate. Poor outcomes in these patients have prompted researchers to investigate the prognostic value of various clinical and pathologic parameters (3, 4). Several studies have pointed out the inadequacy of the TNM system for prognosis in OSCCs (5-9) because some large tumors progress well, whereas some smaller tumors may lead to death. With this background, many research groups have been involved in the

search for clinical, histopathological and molecular factors related to the prognosis of OSCCs (10-12). The p21waf1/cip1 gene is located on chromosome 6p21.2 (13) and encodes the synthesis of the p21waf1/cip1 protein, which binds to cyclins, Cdk, and the proliferation cell nuclear antigen (PCNA), forming quaternary complexes (14). This association inhibits DNA polymerase delta, preventing DNA synthesis (15, 16). Through the action of this protein, the cell cycle is arrested, preventing G1- to S-phase transition. The p21waf1/cip1 protein has been demonstrated to induce cell differentiation (17, 18), senescence and apoptosis (19, 20). These functions suggested that p21waf1/cip1 expression might behave as a marker of tumor prognosis. However, contradictory results have been published by studies which addressed this issue (21-25). There has been scant study of the prognostic significance and role of p21waf1/cip1 in carcinogenesis of the tongue (14, 26, 27), a tumor that often carries a poor prognosis (28).

In the present study, the expression of the cell cycle regulatory protein p21waf1/cip1 was analyzed in tissue from the tumor and adjacent non-tumor epithelia of patients with tongue cancer, in order to determine the proportion of tongue carcinomas with altered p21waf1/cip1 expression, establish whether this alteration is an early event in tongue carcinogenesis, and investigate whether p21waf1/cip1 expression has predictive prognostic value in these tumors.

Materials and Methods

A study was performed on 81 patients with squamous carcinoma of the tongue treated at our university hospital before 1996. The patients’ clinical data were obtained from the hospital medical records, including: the values of the T parameter; the increase in cervical lymph node involvement, determined by clinical methods (N); and the presence of distance metastasis, according to IUAC and AJCC criteria (29).

Locoregional tumor recurrence and the time period between treatment and recurrence were recorded. Survival after the surgery was recorded in months. The status of the patient at the time of the study was registered as "alive without disease", "alive with disease" and "death from tongue cancer".
Figure 1. Positive (A) (arrows) and negative (B) p21\textsuperscript{waf1/cip1} expression in tongue carcinoma (40X).
The pathologic T measurement was taken from the pathology report in the patient’s medical record. The histopathological data were obtained by studying tissue sections of the operative specimen, using hematoxylin-eosin staining. The involvement of cervical lymph nodes by the tumor (pathologic N) was evaluated following IUCC and AJCC criteria (29). Adjacent non-tumor epithelium was classified as normal, hyperplastic or dysplastic.

Immunohistochemical analysis was performed using the avidin-biotin method. Slides were deparaffinized in xylene, hydrated and incubated with 0.5% (v/v) H2O2 in methanol for 20 min, to block the endogenous peroxidase activity. The slides were then washed with Tris-buffered saline (TBS) and heated for 15 min at 100°C in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Non-specific binding was blocked by incubation with 1% BSA for 1h. Sections were incubated with primary antibody at 1: 100 dilution (0.1 μg/ml) overnight at 4°C. The anti- p21waf1/cip1 antibody used in this study was obtained from Santa Cruz Biotechnology Inc. (187, monoclonal IgG antibody). The color was developed using diaminobenzidine (DAB) as the chromogen. The slides were extensively washed with TBS after each step. Finally, the slides were counter-stained with Mayer’s hematoxylin and mounted with DPX mountant. For the negative control, the primary antibody was replaced by phosphate buffer saline (PBS). For the positive control, tissue was used from an oral carcinoma known to intensely express p21waf1/cip1 protein.

The immunohistochemical analysis was performed without knowledge of the clinical and pathological characteristics of each case: at least 1000 neoplastic cells were examined in random fields, counting the percentage of positively-marked nuclei using a high-power (x40) objective lens. The tumors were assigned to one of four categories (−, <25% of nuclei positive; +, 25-50%; ++, >50-75% and ++++, >75% positive) (Figure 1). In the assessment of non-tumor adjacent epithelia, p21waf1/cip1 protein expression was only considered normal in suprabasal layers and was considered aberrant if it was basal, basal and suprabasal, or absent (Figure 2).

Statistical analysis. The disease-specific survival rate was determined with the Kaplan-Meier product-limit actuarial method. Comparison of two or more survival curves was performed with the log rank test. Prognostic factors were evaluated by univariate analysis and by multivariate analysis using the Cox proportional hazards regression model.

Results

We studied 81 patients with cancer of the tongue, 64 males and 17 females, with a mean age of 58 years (range, 37-87 years). The T and pathologic T results are shown in Table I.
Table II lists the results for lymph node involvement and presence of distance metastasis.

Forty-six patients (67.6%) showed no recurrence of the tumor. The mean time period between surgery and locoregional recurrence was 15.74 months (range, 1-48 months).

The five-year survival rate of the series was 68.5% (50 patients alive after 5 years). The mean survival of the patients who died from oral cancer was 30.5 months (range, 1-84 months). Eight patients were lost to the follow-up for survival analysis.

Adequate tissue samples for the immunohistochemical analysis of p21\textsuperscript{wafl/cip1} expression were only available for 54 patients of the initial study population of 81. The p21\textsuperscript{wafl/cip1} expression in the tongue carcinomas is exhibited in Table III. Out of the 54 patients with tongue cancer analyzed by immunohistochemistry, adjacent non-tumor tissue was available for only 43 (79.62%) of them. No adjacent non-tumor tissue sample was classified as normal; in two cases (4.65%) it was classified as hyperplastic and in 41 cases (95.34%) as dysplastic. P21\textsuperscript{wafl/cip1} expression in the adjacent non-tumor epithelia is shown in Table IV. Out of the 38 cases of p21\textsuperscript{wafl/cip1}, negative non-tumor epithelium tissue, the adjacent tumor was also p21\textsuperscript{wafl/cip1}-negative in 25 of them. In the remaining 13 cases, there was p21\textsuperscript{wafl/cip1} expression in the tumor but none in the adjacent epithelium.

According to the statistical multivariate analysis, the five-year survival of the patients was influenced by the following variables: T ($p<0.01$), N ($p<0.05$), pathologic N ($p<0.05$), extracapsular spread ($p<0.05$) and locoregional recurrence ($p<0.01$). Neither the absence nor degree of p21\textsuperscript{wafl/cip1} expression influenced the survival of patients in the present series.

Discussion

In our series of patients with squamous carcinoma of the tongue, p21\textsuperscript{wafl/cip1} expression in the non-tumor adjacent tissue was aberrant in all cases studied. In common with other researchers (27, 30, 31), we regarded the labelling of the nuclei of suprabasal epithelial layers as normal p21\textsuperscript{wafl/cip1} expression. Because p21\textsuperscript{wafl/cip1} arrests the cell cycle in G1 and induces differentiation and senescence (17, 18, 32), subrabasal expression should be interpreted as a physiological molecular mechanism that allows suprabasal cells to differentiate and mature without replication. Indeed, given that the basal epithelial layer is the only site where cell replication is physiological, no mechanism that arrests the cell cycle should function there. It is therefore appropriate to consider the absence of p21\textsuperscript{wafl/cip1}
expression in the basal layer of normal epithelia as normal. In our series, basal and suprabasal expression was observed in five cases of dysplastic non-tumor adjacent tissue. In our view, this finding demonstrates a failed attempt by the p21waf1/cip1 system to control the cell cycle in cells that were very probably firmly on the pathway to malignancy. However, other interpretations are also possible. It has been shown that p21waf1/cip1 is required for the survival of some tumor cells types, which it endows with resistance to apoptosis (33-35). In line with this observation and our own results, Schoelch et al. (31) found extensive labelling through the whole thickness of the epithelium in cases of severe dysplasia and they postulated that p21waf1/cip1 may aid the survival of dysregulated oral keratinocytes, allowing them to develop to malignancy. Our series also included 38 cases (36 dysplastic and 2 hyperplastic) of non-tumor adjacent tissue with complete absence of p21waf1/cip1 expression. In our view, this finding reflects a loss of the p21waf1/cip1 control mechanism at very early stages of tongue carcinogenesis. Similar results have been reported by other authors (30, 36-39). Moreover, because it is highly likely that the dysplasia of adjacent epithelia precedes the development of the tumor (40-44), any aberrant expression of epithelial p21waf1/cip1 should be regarded as a marker for cancer risk. This hypothesis is supported by a report by Chang et al. (30) where 75% of their cases of oral verrucous leukoplakia (with a very strong tendency to malignant transformation) presented aberrant p21waf1/cip1 expression, also significantly associated with progression to oral squamous cell carcinoma and recurrence of the lesion.

More than 25% of neoplastic cells expressed p21waf1/cip1 in 42.60% (23/54) of our patients with squamous cell carcinoma of the tongue, slightly below the 54%-93% range reported in other studies on tongue cancer (14, 26, 27, 31, 38, 45-47). Although p21waf1/cip1 overexpression is a frequent genetic abnormality in tongue carcinomas, its biological significance is difficult to establish. The fact that p21waf1/cip1 arrests the cell cycle does not imply that overexpression of this protein necessarily enhances the control of tumor growth. Increased p21waf1/cip1 expression has been observed in breast cancer cells under conditions of nutritional restriction (35). It has also been shown that p21waf1/cip1 is essential for the survival of differentiating neuroblastoma cells (33) and confers resistance to apoptosis on differentiating myoblasts (34). These observations suggest that p21waf1/cip1 may act as a survival mechanism for neoplastic cells in adverse conditions. Thus, when p21waf1/cip1 arrests the cell cycle, it may reduce the energy requirements of the cell and increase its resistance to apoptosis in a hostile tissue environment with poor vascularization, as frequently occurs in many oral tumors. This may exemplify how neoplastic cells take advantage of a physiological molecular mechanism designed for the maturation and differentiation of normal epithelial cells.

The prognostic significance of p21waf1/cip1 expression is also controversial. Some studies on tongue (14, 26) and oral (38) cancer have demonstrated an association between p21waf1/cip1 expression and clinico-pathological parameters of proven prognostic value, such as the degree of tumor differentiation, presence/absence of subclinical lymph node metastasis, tumor size and clinical stage. However, other authors did not observe these correlations (14, 26, 27). Studies on the relationship between p21waf1/cip1 overexpression and survival in patients with tongue, oral or head and neck cancer have also shown conflicting results. P21waf1/cip1 overexpression has been described variously as a favorable and unfavorable prognostic factor (14, 26, 48), whereas in other studies, including the present one, p21waf1/cip1 expression appeared to have no prognostic value (45, 49-51).

The above discordance in results may derive from the varied inclusion criteria applied in the above series, which included tumors of different localization and biological behavior. In addition, the various monoclonal antibodies used in the analyses may have had different sensitivities for p21waf1/cip1 detection. Finally, the overexpression of this protein was variously defined as more than 1% (47), 5% (26), 10% (38, 50), 25% (27), 33% (31) or more than 50%
(45) of cells being p21waf1/cip1-positive, with evident impact on the results of these studies.

To summarize, p21waf1/cip1 system alteration may be an early and frequent event in tongue carcinogenesis. Further wide studies using agreed inclusion criteria and methodology are required to elucidate its biological and prognostic significance

References


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