Expression of $p21^{\text{WAF}}$ in Salivary Gland Mucoepidermoid Carcinoma and its Relation to Histologic Grade

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The biologic behavior and factors influencing the development of salivary gland mucoepidermoid carcinoma are not fully understood. Alteration of the cyclin-dependant kinase inhibitor $p21^{\text{WAF}}$ could cause uncontrolled proliferation leading to cancer. Thirty-five mucoepidermoid carcinomas were graded and immunohistochemically stained for $p21^{\text{WAF}}$. The percentage of positive tumor cells was determined using an eyepiece graticule and a computer-assisted image analyzer, which revealed 8.6% and 22.9% of the cases to be positive for $p21^{\text{WAF}}$, respectively. A statistically significant correlation was not observed between $p21^{\text{WAF}}$ and grading. Considering the absence of $p21^{\text{WAF}}$ expression in most mucoepidermoid carcinomas, it appears that the inhibitory effect of $p21^{\text{WAF}}$ on cell growth is removed in most cases. Given the lack of correlation with tumor grade, it is possible that the impact of $p21^{\text{WAF}}$ is in the earlier stages of tumorigenesis. A $p53$-independent pathway of $p21^{\text{WAF}}$ induction may exist for the small proportion of tumors that showed positivity.

**Keywords:** mucoepidermoid carcinoma; $p21^{\text{WAF}}$; grading; immunohistochemistry; salivary glands

Mucoepidermoid carcinoma (MEC) is the most common malignant neoplasm observed in the major and minor salivary glands.\(^1,2\) Its widely variable biologic behavior, ranging from relatively indolent to highly aggressive, generally correlates with tumor grade and stage.\(^3,4\) Several clinical and histologic prognostic factors such as age, sex, and site of origin have been assessed for MEC, but clinical stage and histologic grade of the tumor have evolved as the most consistent prognostic factors.\(^5,6\)

Numerous methods have been proposed for classifying this tumor into 2 or 3 histologic grades.\(^1,3\) One of the most widely accepted is a point-scoring scheme proposed by Ellis et al.\(^1,6,7\) at the Armed Forces Institute of Pathology (AFIP), which has been applied in many previous studies.\(^8-10\) Numerous surveys have confirmed the correlation between the grading system proposed by Ellis et al and the prognosis of minor and major salivary gland MEC (excluding the submandibular gland).\(^5,11-13\)

The orderly progression of cells through different phases of the cell cycle is orchestrated by various molecules and proteins, including $p21^{\text{WAF}}$, which is a cyclin-dependant kinase (CDK) inhibitor.\(^14,15\) It has been shown that $p21^{\text{WAF}}$ levels determine the activity of kinases required for cell cycle progression.\(^16\) $P21^{\text{WAF}}$ inhibits the cyclin/CDK complexes and thus prevents the phosphorylation of pRb necessary for cells to enter the S phase in which DNA is synthesized.\(^14\) Considering the fact that tumors and malignancies result from uncontrolled cell growth, the importance of the inhibitory role of $p21^{\text{WAF}}$ is obvious. The prognostic significance of $p21^{\text{WAF}}$ has been investigated in numerous human tumors, including...
gastric cancer, \textsuperscript{17,18} rectal cancer, \textsuperscript{19,20} and squamous cell carcinoma of the tongue. \textsuperscript{21}

The potential role of p21\textsuperscript{WAF} in the development and progression of MEC is not fully understood. The aim of this study was to assess the expression of p21\textsuperscript{WAF} in major and minor salivary gland MEC and its possible correlation with histopathologic grading to gain a better understanding of the nature and tumorigenesis of this neoplasm and also to evaluate the possible correlation between histopathologic grade and p21\textsuperscript{WAF} expression. To our knowledge, this is the first study in the English literature focusing on the expression of p21\textsuperscript{WAF} in major and minor salivary gland MEC.

Materials and Methods

Thirty-five formalin-fixed paraffin-embedded blocks of MEC were retrieved from the pathology archive of Cancer Institute, Tehran University of Medical Sciences, during 1981 to 1991. All specimens consisted of completely excised primary tumors from the major and minor salivary glands (excluding submandibular gland). The clinical data were reviewed to record the age and sex of the patients and the site of the lesions. The youngest patient was a 9-year-old boy, presenting with an asymptomatic mass in the left parotid gland, and the oldest was a 71-year-old man with a painful mass, also in the left parotid gland. Twenty-one patients (60\%) were men and 14 (40\%) were women. None of the patients had received any chemotherapy or radiotherapy before surgical resection.

A general pathologist (F.T.) and 3 oral pathologists (S.E., M.K., P.M.) reevaluated all cases by light microscopy using sections stained with hematoxylin and eosin and mucicarmine. An average of 8 slides were available for each patient (range, 2 to 16 slides). The tumors were histologically graded according to the grading system advocated for MEC by Ellis et al.\textsuperscript{1,6,7}

For immunohistochemical assessment, a representative block was selected for each case. Staining procedure was strictly performed according to the manufacturer's instruction. Briefly, 4-\textmu m sections cut from formalin-fixed, paraffin-embedded blocks were dewaxed in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked by immersion of the slides in 70% methanol with 3\% hydrogen peroxide. For antigen retrieval, the sections were soaked in 10 mM citrate buffer (pH 6) and processed in a microwave oven for 10 minutes. The sections were rinsed in distilled water and thereafter in phosphate-buffered saline. They were incubated with p21\textsuperscript{WAF} monoclonal antibody (DAKO, Glostrup, Denmark) at a working dilution of 1:25 overnight at 4\degree C.

Immunohistochemistry analysis was performed by the standard avidin-biotin complex method. Diaminobenzidine was used as the substrate for localizing antibody binding. Meyer hematoxylin was used for counterstaining. Positive and negative controls were performed at the same time for each section. The primary antibody was replaced by nonimmune mouse serum as negative control, and tonsillar tissue was used as positive control.

All immunohistochemistry sections were examined for positive staining by light microscopy. Tumor cells were considered positive if there was any staining in the nuclei, regardless of the staining intensity. According to previous studies,\textsuperscript{22-24} a tumor is considered immunoreactive if more than 10\% of the cells are positive. The areas representative of p21\textsuperscript{WAF} expression in each section were selected, and a minimum of 1000 cells was counted in 10 high-power fields (HPF) (\times400) under a light microscope, using an eyepiece graticule (conventional counting method). The percentage of positive nuclei was recorded as the labeling index. The sections were also analyzed using a computer-assisted image analyzer system (CAS200, Becton-Dickinson, Franklin Lakes, NJ) and the percentage of positively stained nuclei (computer-assisted labeling index) per 1000 cells, assessed in 10HPF, was determined. Scoring and interpretation of immunohistochemical results were performed by one of the authors (S.E.M.) without any knowledge of clinical data and histologic grade.

The association between p21\textsuperscript{WAF} expression and tumor grade was assessed with the nonparametric Mann-Whitney test. Statistical correlation between age, sex, and site of tumor with p21\textsuperscript{WAF} expression and with tumor grade was analyzed using the Spearman correlation coefficient and Mann-Whitney U tests. A value of $P < .05$ was considered statistically significant. Data are expressed \pm standard deviation (SD).

Results

Clinicopathologic Aspects

Thirty-five primary MECs of major and minor salivary glands were subjected to immunohistochemical reaction with p21\textsuperscript{WAF}. The mean age of the patients
was 42.1 years (range, 9 to 71 years), with 21 males (60%) and 14 females (40%). The site of the primary tumor was the parotid gland in 23 patients (65.7%) and minor salivary glands in the palate, floor of the mouth, lip, and maxilla in 12 (34.3%). Sixteen patients complained of a painless mass being present for 21.6 months (range, 1 to 60 months); other commonly reported symptoms were pain in 12 and paresthesia in 9. The entire gland was excised in 63% of the patients; but the rest had subtotal excision to preserve the facial nerve and prevent damage to vital structures.

**Histologic Aspects**

According to the criteria proposed by Ellis et al., the tumors were subdivided into 19 low-grade (54.3%), 6 intermediate-grade (17.1%), and 10 high-grade (28.6%) cases (Fig. 1).

**Immunohistochemical Findings**

In specimens that contained normal oral squamous mucosa, parabasal staining with p21WAF was observed (Fig. 2). It was also noted that neither the normal salivary gland acini nor the normal ducts stained with p21WAF.

In both conventional and computer-assisted counting methods, 8 of the 35 tumors showed reactivity with p21WAF (Fig. 3). Conventional p21WAF labeling index was 2.0 ± 5.2 (range, 0 to 22.64; median, 0) and computer assisted p21WAF labeling index was 5.0 ± 10.1 (range, 0 to 37.09). The remaining 27 cases (77.1%) displayed a labeling index of 0. By both counting methods (conventional and computer-assisted), immunoreactivity was observed in all grades: of the 8 cases with p21WAF staining, 3 were low grade, 2 were intermediate, and 1 was high grade.

According to Shariat et al., Osman et al., and Kapranos et al., positive nuclear protein expression was assessed at the 10% level. Consequently, 3 cases (8.6%) and 8 cases (22.9%), which revealed reactivity in 10% or more tumor cells, were considered positive in the conventional and computerized counting groups, respectively. Of the 3 positive specimens in the conventional group, 1 was low grade, 1 was intermediate grade, and 1 was high grade; and of the 8 positive cases in the computerized group, 3, 2, and 3 were low, intermediate, and high grade, respectively.

**Tumor Grade and Expression of p21WAF**

Tables 1 and 2 demonstrate the distribution of conventional and computerized labeling index in different grades, which were analyzed by the Mann-Whitney test. A significant difference was not observed between the
groups (P < .05). The correlation between the conventional and computerized labeling index and histologic grade was also determined by Spearman correlation coefficient, which was 0.152 (P = .6) for the conventional labeling index and histologic grading and 0.157 (P = .4) for the computerized labeling index and histologic grading. These results indicated that the p21WAF labeling index was independent of tumor grade among MECs of the major and minor salivary glands.

Clinical Variables
The p21WAF labeling index. No statistically significant correlation was found for an association between the p21WAF labeling index and age, sex, and tumor site (P < .05).

**Histopathologic grading.** Because of the limited number of cases, assessment of a possible correlation between the 3 grades and clinical variables was not feasible. According to Okabe et al,8 the samples in this study were divided into 2 histologic grades: high grade (consisting of intermediate and high-grade) versus low-grade. No statistically significant correlation was found between the 2 histologic grades and age, sex, and tumor site (P < .05).

Discussion
Mucoepidermoid carcinoma is most frequently seen in the third to fifth decades of life,4 with a slight female predilection. About 53% occur in the major salivary glands, 21% originate from the minor glands of the palate, 19% occur in other intraoral minor glands, and 7% are observed in different sites of the aerodigestive tract.1 With respect to age and site of occurrence, our series confirms the data previously reported in the literature.1-3,8-25 In the present study, women accounted for 40% of the cases, which is less than the proportion noted in some reports1,3,8,26-27 but is in agreement with others.5,10-11 Statistical significance was not observed in the correlation between p21WAF labeling index with age, sex, and site of origin.
Disorders of cell cycle control are one of the major causes of cancer. The defective function of regulatory cell cycle elements leads to increased cell proliferation and expansion of the damaged cells’ genome. Among the positive regulators of the cell cycle is the CDK family of kinases. The activity of this family can be negatively modified by p21WAF, which binds to and can inhibit these CDKs. Wild-type p53 transcriptionally induces p21WAF, but p21WAF may be induced independent of p53. Differentiation-inducing agents such as trans-retinoic acid, growth factors, or prostaglandin A2 can also initiate p21WAF transcription. As a negative regulator of the cell cycle, p21WAF is a potential tumor suppressor gene. Mice lacking p21WAF display accelerated breast tumor development after expression of Ras but not Myc. Nonetheless, the role of p21WAF and its impact on breast cancer development and outcome is not clear, with conflicting data based on series with limited number of cases. The issue is further complicated by functional interactions between p21WAF and other known prognostic markers such as p53.

Immunohistochemical expression of several cell cycle, proliferation, and growth factor markers have been described in MEC; however, we were not able to find a published report on the investigation of p21WAF in this tumor. Our findings on the pattern of immunohistochemical expression in normal epithelial tissue were in agreement with a study conducted by Regezi et al. that in normal control tissues, nuclear staining of p21WAF was suprabasal and limited to keratinocytes in the lower half of the epithelium. Also, salivary glands and normal ducts did not stain with p21WAF. The absence of p21WAF staining has been reported by Gohring et al. in benign lobular and ductal epithelia adjacent to infiltrating breast carcinomas as well. Considering some similarities between the salivary glands and the mammary duct system, it is noteworthy that similar confusions remain about the role of p21 in tumorigenesis at both sites.

In the present study, 22.5% (8/35) of the samples expressed p21WAF. Similar values were reported in non-Hodgkin lymphoma, early cervical carcinoma, and oral squamous cell carcinoma. Analogous to the findings in the present investigation, a statistically significant correlation between the p21WAF labeling index and histologic grading has not been observed in adenocarcinomas of the uterine cervix, invasive ductal carcinoma of the breast with and without nodal involvement, and clear cell renal carcinomas. Considering the low expression of p21WAF in all 3 grades and the lack of correlation between its expression and the histologic grade of MEC, it may be postulated that the impact of p21WAF is in the earlier stages of tumorigenesis and that loss of p21 develops in the early but not the later stages of this process; therefore, other factors related to the cell cycle (dependent or independent of p21WAF) should be explored for their possible role in MEC carcinogenesis.

The p53 gene is the most common target for genetic alterations in human tumors and is closely associated with p21WAF during the p53-dependent G1 arrest; therefore, it may be considered as one of the elements responsible in the tumorigenesis of MEC.

Various investigations of the expression of p53 in MEC are reported, and several of them indicate that its correlation with tumor grade, size, regional metastasis, and poor prognosis is statistically significant. The data from the current study indicate that expression of p21WAF is minimal in MEC, thus the possibility arises that p53 may act independent of p21WAF and other pathways should be considered; for example, when p53 is activated owing to an assault on genetic material, it binds to DNA and stimulates transcription of several genes, namely p21WAF, GADD45, MDM2, bax, and IGF-BP3. The p21WAF and GADD45 (growth arrest and DNA damage) genes are involved in G1 arrest; MDM2 down-regulates p53 after successful repair of DNA damage, and finally, bax and IGF-BP3 carry out the cell death commands of p53, if the DNA damage could not be repaired. Therefore, if p53 is considered as a possible factor in MEC carcinogenesis, any of the above-mentioned transcriptionally activated genes, excluding p21WAF, may be involved.

In addition, p53 can act in other ways unrelated to its transcriptional activity. Kourea et al. proposed a possible course of action by conducting an investigation on the correlation between p53 and p34cdc2, and their relation with p21WAF. Cyclin B1 and p34cdc2 constitute the “mitosis or maturation promoting factor” that controls the progression of the cell cycle from the G2 to the M phase. P53 causes G2 arrest by reducing the expression of p34cdc2. Kourea et al. suggested that given the association between p34cdc2 and p53, and lack of p21WAF interaction with these proteins, p53 may control the G2-M cell cycle checkpoint through mediators unrelated to p21WAF.

In the present study, 8 of 35 of the MEC cases stained positive for p21WAF. To propose a possible
explanation for the 8 cases that stained positive for p21\textsuperscript{WAF}, further investigation using additional markers, especially p53, is required. We did not examine the expression of p53 but speculate that if p53 is expressed and a significant correlation between p21\textsuperscript{WAF} and p53 is identified, 3 situations could be considered:

A p53-independent pathway may exist for p21\textsuperscript{WAF},\textsuperscript{45} as has been noted for BRCA,\textsuperscript{14,28} transforming growth factor β,\textsuperscript{46} trans-retinoic acid, growth factors, prostaglandin A2,\textsuperscript{28} and a variety of transcription factors that are induced by a number of different signaling pathways, including Sp1, Sp3, Ap2, STATs, C/EBPa, C/EBPβ, and the bHLH proteins BETA2 and MyoD.\textsuperscript{47}

P21\textsuperscript{WAF} may be successfully induced by p53, but other factors related to p21\textsuperscript{WAF} in the cell cycle, such as cyclin/CDK complexes, prRb,\textsuperscript{14} and degradation-inducing factors such as ubiquitin, proteasome, and mdm2,\textsuperscript{47,48} may be functionally altered. Therefore, even though p21\textsuperscript{WAF} is expressed, it cannot carry out its inhibitory functions.\textsuperscript{48,49}

The p21\textsuperscript{WAF} gene may be mutated; consequently, the expressed protein would be nonfunctional and would not be able to fulfill its inhibitory role on cyclin/CDK complexes and cell cycle arrest. Of course, it has been shown that such mutation is very infrequent in human tumors.\textsuperscript{50}

If, on the other hand, the tumors show total absence or, according to Kiyoshima et al,\textsuperscript{9} low expression, other factors such as ras and prRb mutations or mdm2 gene amplification should be taken into account.

**Conclusion**

Tumors use a diversity of genetic mechanisms to escape immune surveillance for survival advantage. The most effective modifications are found in dismantling the expression/function of various tumor suppressors that are involved in the regulation of cell cycle progression.\textsuperscript{51} Of the various factors involved in the cell cycle we have shown that p21\textsuperscript{WAF} is expressed only at low levels in a few salivary gland MECs. It therefore appears that the inhibitory effect of p21\textsuperscript{WAF} on cell growth is removed in most MECs. Considering the absence of any correlation of p21\textsuperscript{WAF} with tumor grade, and the even distribution of this marker in all 3 grades, it is possible that the impact of p21\textsuperscript{WAF} is in the earlier stages of tumorigenesis and that loss of p21 is critical in the early, but not the later, events of this process. Further investigations with a larger number of cases and additional immunohistochemical markers are necessary.

Also it is noteworthy that because of the complex interactions of p21\textsuperscript{WAF}, in addition to its stoichiometry (induction of cyclin/cyclin dependent kinase complex formation at low concentration and inhibition of the complex at higher concentrations),\textsuperscript{38} direct or possibly simplistic conclusions on the role of p21\textsuperscript{WAF} in tumorigenesis demand further exploration.

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**References**


