*Applied Cancer Research 2009;29(2): 83-88*

Original Article

# Immunoexpression of p53 and p16 Proteins as Biomarkers in Oral Carcinogenesis

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## Abstract

**Objective:** Defects in the cell cycle control system can lead to phenotype changes and consequently to the progression of oral cancer. Thus, the aim of this study was to analyze the immunohistochemical expression of p53 and p16 and their connection with hyperplastic and neoplastic progression. **Materials and Methods:** Sixty-four histopathologic specimens were submitted to immunohistochemical technique to anti-p53 and anti-p16. **Results:** This study showed a significant increase in the level of p53 in dysplasia and oral carcinomas. Regarding p16 immunoexpression, a decrease was observed in mild to moderate dysplasia, and remained consnt between moderate dysplasia to poorly differentiated carcinoma. **Conclusion:** The study confirmed that the higher expression of p53 protein plays a significant role in tumor development and oral cancer progression, while the loss of p16 expression seems to be related only for the progression of carcinogenesis.
**Keywords:** Proteins. Immunohistochemical. Genes, p53. Genes, p16. Mouth. Neoplasms.

## Introduction

Oral carcinogenesis is a mutifactorial and complex process related to the sequential occurrence of alterations in genetic structures, promoting inhibitory or excitatory effects of the tumor oncogenes and gene suppressors, compromising the histophysiology of the division, differentiation and cell death.[1-3] Among the tumor gene suppressors which accumulate genetic alterations, TP53 and MTS1 are among the leading.[2-3]

The p53 protein is a phosphoprotein sensitive to the damage in the DNA encoded by the gene TP53, located on 17p13.1.[4] Its primary function is to promote the interrupting of cellular cycle progression, thus allowing the repair of damaged DNA or inducing apoptosis.[4-7] Genetic mutations in TP53 and/or inactivation of its product (p53) represent common alterations in several neoplasms, affecting approximately 50% of neoplasms involving humans and plays a cooperative role in the expression of several proteins favoring the progression of cancer, among which are carcinomas of the mouth.[1, 3–10]

Multiple tumor suppressor 1 (MTS1), is a gene located on chromosome 9p21 and encodes a 16kD protein, p16, which has an inhibiting function of the cyclin-dependent kinase (CDK) 4/6.[11-13]In conditions of genotoxic stress, p16, if connected to CDKs, impede the liberation of the E2F protein, and consequently, the progression of the cellular cycle.[11-13] Nevertheless, the loss of p16 expression and the consequent inactivation of the protein have been implicated in the tumorigenesis of countless neoplasms.[12]

This work had as objective the relation of the immunoexpression of the p53 and p16 proteins as biomarkers of oral carcinogenesis, as well as to correlate them with the histopathologic parameters of neoplastic grading.

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## Materials and Methods

Sixty-four oral incisional biopsy samples embedded in paraffin were used in the study. In accordance with histopathologic evidence, the samples were classified into seven groups: normal mucosa, acanthosis (epithelial hyperplasia), dysplasias (discreet, moderate and intense) and carcinomas well and poorly differentiated. The diagnoses and casuistry are represented in Table 1.

The analysis of the samples for conventional histopathologic diagnosis followed the classification proposed by Kaugers et al. (1988),14 while for dysplasias and the differentiation among the carcinomas was carried out in accordance with the histologic grading system proposed by Anneroth et al. (1986).15

**Table 1-** Clinical – pathologic characteristics of the studied specimens

| Histopathologc Diagnosis | | Sex | | |
|---|---|---|---|---|
| | Anatomic site | Masc | Fem | Average Age(year) |
| MN | Gingiva (n=2) | 3 | 3 | 32 |
| | Tongue (n=4) | | | |
| AC | Buccal Mucosa (n=4) | 5 | 2 | 29 |
| | tongue(n=3) | | | |
| DD | Inner lip (n=4) | 4 | 6 | 47 |
| | Tongue (n=5) | | | |
| | Palate (n=1) | | | |
| DM | Inner lip (n=4) | 7 | 3 | 43 |
| | Tongue (n=6) | | | |
| DI | Lower lip (n=4) | 6 | 4 | 49 |
| | Tongue (n=5 | | | |
| | Palate (n=1) | | | |
| CEC1 | Lower lip (n=4) | 8 | 2 | 59 |
| | Tongue (n=3) | | | |
| | Palate (n=1) | | | |
| | Mouth floor (n=2) | | | |
| CEC2 | Lower lip (n=5) | 10 | 1 | 63 |
| | Tongue (n=4) | | | |
| | Palate (n=2) | | | |
| Total | 64 | 43 | 21 | |

MN = mucosa with normal characteristics; AC = acanthosis; DD = discreet dysplasia; DM = dysplasia moderate; DI = dysplasia intense/carcinoma in situ; CEC1 = squamous cell carcinoma well differentiated; CEC2 = squamous cell carcinoma poorly differentiated.

For the completion of immunohistochemical analysis with antibodies anti-p53 and anti-p16, histopathologic cuts with 3 μm of thickness were made and deparaffined The process of antigenic recuperation employed a citrate solution of 10 mm (pH 6.0) in immersion bath at 95°C for 30 minutes.

The incubation of the slides was carried out by diluting anti-p53 primary antibodies (clone DO-7, of DAKO/AS, Glostrup, Denmark) and anti-p16 (Ab-4, clone 16P04 Labvision, Fremont, CA, USA) in BSA in the volume proportion of 1:50, in humid chamber, at 4°C for 18 hours (overnight). The incubation with the secondary antibody and tertiary of the streptavidin-biotin complex, "kit LSAB" (DAKO - KO690, Glostrup, Denmark), was carried out during 20 minutes at room temperature, revealed by diaminobenzidine and counter-stained by Harris's haematoxylin.

The measurement of positivity of the immunopositive nuclei for anti-p53 and positive nuclei and cytoplasms for anti-p16 for each analyzed slide was carried out by selecting five independent levels, with the representative area of the evaluated groups (hot spots) diagnosed as acanthosis, dysplasias or carcinomas. The areas were observed in light microscope (21/3 QUIMIS, Brazil) with image capture (310 SDC-310-Samsung, Korea) coupled to the camera for obtaining the digital images (magnification, 100x) in clear field without image overlapping.

The histomorphometric parameters were analyzed with the help of an image analysis program (IMAGE TOOL 2.00, University of Texas Health Science Center at San Antonio, USA). After the area was selected, the "count and tag" plug-in of ImageTool was used to measure the total cellular content in each field, until reaching 1,000 cells present in the specimens. Next, the data were transferred to a spreadsheet and analyzed.

For statistical analysis, the number of immunopositive cells was computed in a total of 1,000 cells counted per studied specimen. The examples of samples were considered positive when corresponding to immunomarking above 5%, or in other words, 50 immunopositive cells.

The Kruskal-Wallis test was used to verify if there was statistical difference among groups using p53 and p16, while correlation between p53 and p16 was verified using the Mann-Whitney U-test, establishing for both tests a level of significance less than 5%.

## Results

The distribution of the samples in accordance with the variables sex, location and morphologic diagnosis are shown in Table 1. Table 2 presents the relation of the anatomical and pathological diagnoses of the incisional biopsies, the minimum and maximum presence of positivity for the diagnosed cases, as well as the medians of the immunopositivity for the anti-p53 and anti-p16

**Table 2**- Relation among histopatologic diagnosis and quantification of p53 and p16

| Histopathologc Diagnosis | p53 | | p16 | |
|---|---|---|---|---|
| | min-max | median | min-max | median |
| MN | 0-10 | 0 | 612-674 | 600 |
| AC | 0-4 | 3 | 408-833 | 695 |
| DD | 22-304 | 121 | 17-601 | 439,5 |
| DM | 402-734 | 445 | 89-265 | 193,5 |
| DI | 309-778 | 569 | 82-318 | 154 |
| CEC1 | 467-973 | 677 | 68-370 | 189 |
| CEC2 | 636-856 | 792 | 199-504 | 303 |

antibodies.

In normal mucosa (NM), the nuclear expression of p53 was shown absent in 83.34% (1/6) of the diagnosed samples and present only in 28% of the samples that contained epithelial hyperplasia, (AC), revealing a maximum number of four cells marked in 1,000 analyzed. Nevertheless, there was an immunomarking rate below 5% in these groups.

The immunolocation for the p53 antibody appeared heterogeneous in the specimens diagnosed as dysplasia discreet (DD), moderate (DM) and intense (DI). There was greater positive cellular concentration restricted to areas with loss of stratification of the epithelium, especially when the cellular content exhibited alteration of the volumetric relation between nucleus and cytoplasm and/or acantholysis, establishing in this manner an increase in number, be it absolute and /or median, in accordance with the morphologic grade established by Kaugers et al. (1988)[14] as highlighted in Table 2, showing significant statistical differences ($p < 0.05$) among the analyzed groups and summarized in Figure 1.

For the specimens diagnosed as CEC1 (squamous-cell carcinoma well differentiated), the immunopositivity parameters of p53 revealed a greater concentration in the

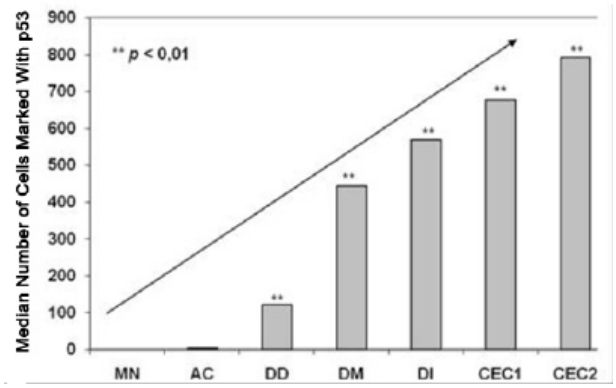


**Figure 1-**Median Number ofr Cells Marked With p53

peripheral areas of the invasion nests (Figure 4A), or in cells arranged in the external portions of the plexiform invasions adjacent to the conjunctive. The phenotype of p53 immunoexpression in the cases of CEC2 appeared diffused in areas of invasion independent of the atypical cell morphology (Figure 4B).

The immunoexpression of p16 was similar between the groups NM and AC (Figure 4C). There

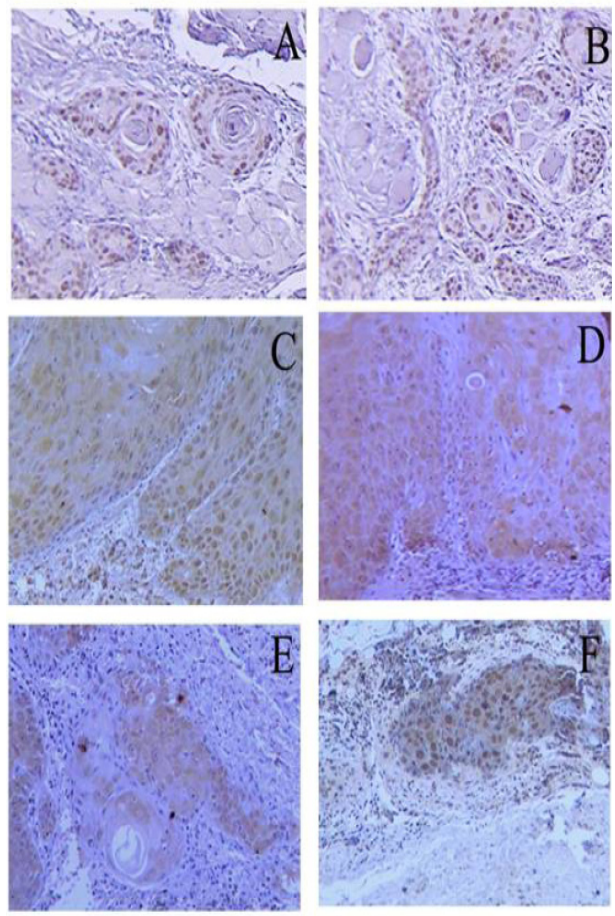


**Figure 4**

was predominance of immunomarking in the region of cellular cytoplasm, principally in squamous stratum cells. Significant loss of p16 expression occurred in the processes of carcinogenesis (DD, DM and DI). In the group DD (Table 2), median cells immunomarked was less than the control ($p < 0.05$), and this decrease was accented in the group DM (Figure 2). Nevertheless, the median of cells p16 positive among DM, DI, CEC1 and CEC2 remained similar (Figure 2). These findings suggest that the loss of p16 expression is an early event in the process of carcinogenesis, but not a crucial factor in the progression of mouth cancer, as occurred with p53.

In NM, AC, DD and DM (Figure 4D) a predominance of immunopositivity was noted in areas of the cellular cytoplasm, while the areas of DI, CEC1 (Figure 4E) and CEC2 (Figure 4F) the immunoexpression manifested itself as much in cytoplasm as in the nucleus. In short, the numbers of cells showing positive immunophenotype p16 and the median values showed a tendency to fall, conforming if held to dysplastic criteria. Comparatively, the values of p for Kruskal-Wallis are presented in Figure 2, and do not reveal statistical differences between NM and AC, and show significant differences ($p < 0.05$) to DD when compared to MN and AC and significant differences for the cases of DM, DI, CEC1 and CEC2 ($p < 0.01$).

Though p53 and p16 are related to oral carcinogenesis, the correlation analysis of Mann Whitney

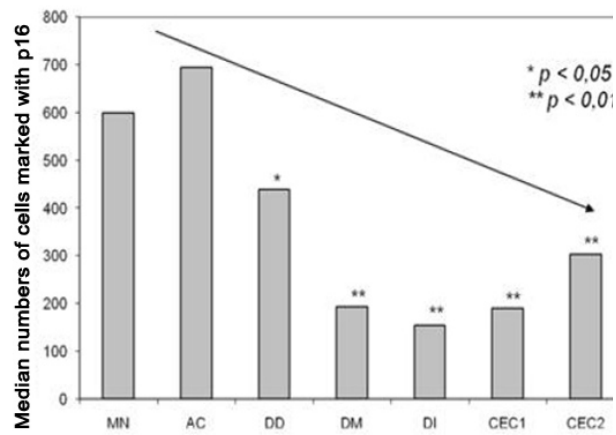


**Figure 2**-Median numbers of cells marked with p16

U-test showed an absence of correlation between the increase of p53 immunoexpression and loss of p16 expression ($p < 0.05$) as highlighted in Figure 3 . This result suggests that the pathways of the gene suppressors of the tumor can take place simultaneously in the development of oral cancer, but as distinct pathways.

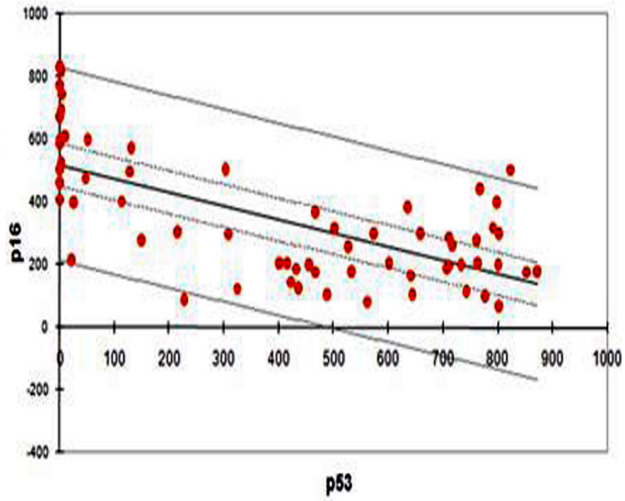


**Figure 3-** p53 and p16 are related to oral carcinogenesis, the correlation analysis of Mann Whitney U-test showed an absence of correlation between the increase of p53 immunoexpression and loss of p16 expression ($p < 0.05$)

## Discussion

Countless works have been looking to demonstrate and relate alterations in the proteins p53 and p16 as criteria of neoplastic development and progression. In this work, the proteins p16 and p53 were investigated in samples of different neoplastic and hyperplastic specimens employing the monoclonal antibodies anti-p53 and anti-p16. The results revealed that the marking for p53 was observed in all the processes showing dysplasias, but that they are absent (number of immunomarked cells less than the signification level of 5%) in NM and AC. We suggested that few immunomarked cells in AC refer to wild-type p53 encountered in the process of cellular renovation or in cells that suffer any genotoxic stress with apoptotic finality.

Nevertheless, the frequencies of positive p53 in a gradual and growing form found in dysplastic and carcinomatous injuries were interpreted as protein expressions of mutated p53. This interpretation is due to the number of atypical cells encountered, as well as for the constancy in which they appear in the histopathologic regions studied. These considerations are plausible for the fact that the clone DO-7 used to recognize the epitope sequence corresponding to the N-Terminal region of the molecule corresponds to a region that presents a low number of mutations, approximately not more than 5%, when compared with the central hydrophobic domain.[3,9]

In this manner, the numerical and median variables of p53 among the different studied specimens show a directly proportional degree of dysplasia, indicating

a positive correlation between positivity for p53 and initial alterations of the process of cancer.[16-19] In addition, immunopositive areas coincident with the front of invasion of the nests and plexiform areas suggest that p53 can be directly related not only to a cellular phenotype and consequent histopathologic grading but also reveals strong evidence of protein p53 to be an important factor of tumor progression.[1-3]

MTS1, gene encoder of p16, is classified as a gene tumor suppressor and it is important to maintain the homeostasis of the proliferation and cellular differentiation.[11-13]Genetic evidence shows that alterations as methylation, deletion or point mutations in MTS1 participate in the early events of carcinogenesis in several tumor types, among them the carcinomas of the head and neck.[11,20-23] However, the participation and detection of the p16 protein remains contradictory once the report of an absence or loss of protein expression, indicating mutation of the gene encoder,[21,24-25] but also highlights that the constant presence in front of the invasion of poorly differentiated carcinomas could bring about the stoppage of pathways of the cellular cycle in G1-S checkpoints, or the same senescence of neoplastic cells.[24]

The results of the present study showed positivity with cytoplasmatic predominance in the cases of NM and AC in the supra basal and stratum spinosum layers. The absence of dysplasias cells suggests that protein detection is the fruit of strong protein interaction to the messenger RNA, forming a complex that has effective participation in the processes of cellular senescence and in the differentiation of the epithelium, avoiding disordered proliferation, those functions induced by the negative feedback of genes like c-Myc and RAS, which are important in the maintenance of cellular homeostasis.[25-27]

In dysplastic processes, the number of immunopositive cells lessens. When comparing discreet, moderate and intense dysplasias, there was significant loss in numbers of p16 only between discreet dysplasia and normal mucosa or hyperplasia, and significant loss of expression also was important when comparing discreet dysplasia with moderated and intense.

Part of the results presented in this work corroborate with Mäkitie et al. (2003),[28] which describes that the loss of p16 expression can be involved in the development or progression of epithelial cancer, result of the mechanism of hypermethylation, deletion or mutation of the gene encoder of protein p16. However, the same correlations were not found in the cases of DM, DI and CEC1 and CEC2 (Figure 2).

The squamous cell carcinomas (well differentiated and poorly differentiated) analyzed in this work presented numbers of immunomarked cells for protein p16 similar to the groups of moderate and intense dysplasia. The fundamental difference is that in DI, CEC1 and CEC2 there existed immunomarking as much in cytoplasm as in the nucleus (Figures 4E and 4F).

The results presented here do not corroborate with Kommoss et al.,[29](2007) that emphasized not only efficiency of the neoplastic progression of ovary carcinomas in the absence of p16, but also described that this absence, or even, low expression of p16, is directly connected to a poor prognosis when the neoplasms were compared in patients whose tumor presented p16 positive. These results suggest that different than what takes place in ovarian carcinomas, p16 constitutes an important mechanism only in the development of oral carcinogenesis, not constituting a de facto biomarker of neoplastic progression and grading.

The interpretation of the results for p16 becomes conflicting not only for the absence of p16, but also for the complexity in several functional interpretations that positive p16 might exercise.

Among the several interpretations, analogously to what happens in the groups of NM and AC, the presence of p16 evidenced in DI, CEC1 and CEC2 could be interpreted as the perpetuation of neoplastic cellular cloning, especially when the marking is concomitant to the cytoplasm and nucleus.[30-32]

Other important interpretations of the presence of p16 occur on mutation not coincident to a protein epitope of the clone used in this study, or still to the loss of p16 interaction with the ligand portion to Cdk4/cyclin D/pRb mutant, which would indicate an intense proliferative rate of the neoplasm.

One other important fact in the interpretation of p16 which cannot be discarded is the evidence of p16 overexpression in neoplasm might be the result of the inhibition of VEGF translation, if regulated in this way, receptiveness of the neoplasm to angiogenesis, and consequently, metastasis.[32-33]

## Conclusion

The immunoexpression of the p53 protein is an efficient biomarker of the neoplastic development and progression of mouth cancer, whereas, p16 can be able to be considered an important neoplastic progression,

but does not constitute an adequate biomarker of neoplastic progression in mouth cancer.

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