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FIELD CANCERIZATION IN HEAD AND NECK SQUAMOUS CELL CARCINOMA: immunohistochemical expression of p53 and Ki67 proteins: clinicopathological study

Cancerização por campo em carcinoma de células escamosas: expressão imuno-histoquímica de proteínas p53 e Ki67: estudo clínico-patológico

Marcos Vinício Macedo de Oliveira[a], Carlos Alberto de Carvalho Fraga[a], Camila Santos Pereira [a], Lucas Oliveira Barros[a], Erica Silva Oliveira[a], André Luiz Sena Guimarães[b], Alfredo Maurício Batista de Paula[b]

[a] Undergraduate students, Department of Biological Sciences, Universidade Estadual de Montes Claros (Unimontes), Montes Claros, MG - Brazil, e-mail: andreluizguimaraes@gmail.com
[b] DDS, PhD, Department of Dentistry, Universidade Estadual de Montes Claros (Unimontes), Montes Claros, MG - Brazil.

Abstract

OBJECTIVE: This study is aimed to evaluate the immunostaining influence of p53 and Ki67 proteins in areas of field cancerization of head and neck squamous cell carcinoma (HNSCC). We analyzed associations of these proteins with clinicopathological parameters and the relation between their immunoexpression in HNSCC. **MATERIAL AND METHOD**: In a retrospecive analysis, 40 patients with HNSCC were selected according to the recurrence of the disease, forming two groups: recurrent and non-recurrent HNSCC. Morphological gradations and imunnohistochemical analysis of p53 and Ki67 were performed in invasive front and tumor adjacent epithelium. **RESULTS**: It was found significant associations between tumor recurrence and p53 positivity in mucosa and invasive front. However, no association was found between p53 immunostaining and the clinicopathological parameters. Ki67 was not related to any clinicopathological parameter either. The association between Ki67 and p53 expression was not significant. There was no significant influence of recurrence in the clinicopathological parameters. Individuals with T1/T2 tumor size, non-recurrent and p53-negative in tumor adjacent epithelium presented better overall survival of HNSCC. **CONCLUSION**: p53 positivity in adjacent epithelium and invasive front of recurrent HNSCC is suggested in this study.

Keywords: Squamous cell carcinoma. Head and neck. p53. Ki67. Morphological gradation.

Resumo

***OBJETIVO**: Avaliar a influência da imunoexpressão das proteínas p53 e Ki67 em áreas de campos de cancerização do carcinoma de células escamosas da cabeça e pescoço. Analisamos a associação dessas proteínas com parâmetros clínico-patológicos e a relação entre sua imunoexpressão.*

***MATERIAL E MÉTODO**: Em análise retrospectiva, 40 pacientes foram selecionados, de acordo com a recorrência da doença, formando dois grupos: com recorrência e sem recorrência da neoplasia. Gradações morfológicas e análises histoquímicas foram efetuadas na área de invasão e no epitélio adjacente ao tumor. **RESULTADOS**: Encontrou-se associações significativas entre a recorrência do tumor e positividade para p53 na mucosa e na área da lesão. Entretanto, não encontrou-se associação entre p53 e parâmetros clínico-patológicos. Ki67 não foi relacionada com qualquer parâmetro, igualmente. A associação entre expressão de Ki67 e p53 não foi significante e não houve influência significante de recorrência nos parâmetros clínico-patológicos. Indivíduos com tumores T1/T2, não recorrentes, e p53 negativos no epitélio adjacente ao tumor apresentaram melhor sobrevida à neoplasia. **CONCLUSÃO**: Positividade para p53 no epitélio adjacente e na área da neoplasia de carcinoma de células escamosas é sugerida por este estudo.*

***Palavras-chave**: Carcinoma de células escamosas. Cabeça e pescoço. p53. Ki67. Gradação morfológica.*

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth commonest kind of cancer in the world (1), comprising neoplasias in several anatomical sites as the mouth, pharynx and larynx (2). It has been suggested that factors like disorders in genes involved in carcinogenic metabolism, DNA repair and cell cycle control may contribute to interindividual variations in HNSCC susceptibility (3, 4). Abnormalities in genes which are responsible for controlling apoptosis and cell proliferation have been frequently identified and associated with clinical development of HNSCC (5-7).

Ki67 phosphoprotein is present in the cell nucleus during all the cellular division phases at different tissues of different species. Its absence in G_0 phase results in an excelent marker of mitosis. The specific role of this phosphoprotein remains unknown, mainly due to the loss of homology with other proteins, but some functions have been suggested: organization and maintenance of DNA architecture and ribosome synthesis during the cell division (8). p53 protein mediates some cellular processes like cell cycle induction; apoptosis, when DNA is damaged; it helps in genetic recombination; it induces DNA repair and improves the genetic stability (9). p53 is the proteic product of TP53 gene and it controls the normal cell development, preventing, thereby, the cell proliferation containing harmed DNA (10). The loss of p53 or its mutation allows an increase in replication of damaged DNA, leading to a potential cancer susceptibility (11, 12).

The field cancerization concept was introduced in 1953 in analysis verifying the presence of histologically abnormal tissue in the margins of oral squamous cell carcinoma (13, 14). This concept is applied no only to the independent development of a second primary tumor, but also the occurence of precancerous lesions (15). Some studies have established an association among genetic alterations as loss of heterozygosity (16), microsatellite instability (17), cromossomal instability (18) and p53 gene mutations (19) in macroscopically normal tumor adjacent areas. The presence of a field containing genetically altered cells represents a continuous risk factor to the growth of secondary cancers (20).

The aim of this study is to evaluate the influence of the p53 and Ki67 immunostaining in the field cancerization of HNSCC. We also verify associations of the immunoexpression of both proteins with clinicopathological parameters. We still analyzed the relation between these proteins in HNSCC.

MATERIAL AND METHOD

Patients

The studied group was a series of 40 fully reviewed and followed-up primary HNSCC of patients submitted to surgical resection for treatment of the disease between 1997 and 2007, in Montes Claros - Minas Gerais, Brazil. All cases

were histologically confirmed as HNSCC. Health records of these patients were retrieved and epidemiological, and clinicopathologic data were analyzed. In order to verify the prognostic value of the field cancerization with recurrence of HNSCC, patients were selected in two groups according to the recurrence of lesion (non-recurrent and recurrent). Ethical approval for the study was obtained from the relevant local ethic committees (Unimontes/COEP, protocol 299/2006). All patients were staged according to the UICC TNM Classification of Malignant Tumors (1997) (21). Clinicopathological data were also categorized: presence or absence of clinically detected cervical mestastasis (N0 and N1/N2/N3); clinical tumor size of the lesions (T1/T2 and T3/T4); morphological gradation of the invasive front (22) (F1, F2 and F3); morphological gradation according to World Health Organization (WHO) criteria (I: well differentiated, II: mildly differentiated and III: poorly differentiated) (23).

Histopathological and immunohistochemical analyses

Samples were formalin fixed, paraffin embedded, serially sectioned at a thickness of 5μm, performed on hematoxylin and eosin (H&E) stained sections and evaluated under conventional light microscopy. Only HNSCC samples that had sufficient adjacent non-malignant epithelium under microscopic evaluation were selected. These areas were collected at sites at least 2 cm away from the edge of tumor mass, with the best effort of avoiding presence of malignant cells. The malignancy gradation system of invasive front is based on four morphological parameters identified in the malignant parenchyma and in its relation to the surrounding stroma. These parameters are: 1) degree of keratinization; 2) nuclear polymorphism; 3) pattern of invasion; and 4) host response (degree of lymphoid infiltration). Histopathological and immunohistochemical analyses were independently performed by an oral pathologist (De-Paula, AMB).

In the immunohistochemical method, samples were submitted to sections of 3μm, mounted on aminopropyltriethoxysilane coated slides, deparaffinized and hydrated in alcohol. Antigen retrieval was performed with microwave (700W) for three cycles of 8 minutes in citrate buffer (10mM), pH = 6.0. Then they were washed in 3% hydrogen peroxide for 30 minutes to inhibit endogenous peroxidase. After blocking non-specific binding with normal goat serum (1:30), serial sections were incubated with mouse monoclonal anti-p53 (1:75, clone DO7, Novocastra™, New Castle, UK) and anti-Ki67 (1:100, Clone MIB1, DakoCytomation™, Glostrup, Denmark), overnight at 4°C. After incubation with the primary antibodies, sections were thoroughly rinsed and newly incubated with a streptavidin-biotin-peroxidase supersensitive kit method (LSAB Kit, ready to use; DakoCytomation, Glostrup, Denmark) for 30 minutes. After washing, samples were revealed with 3,32-diaminobenzidine-tetrahydrochloride (DAB) in 20mM Tris-HCL (pH = 7.4) containing 0.001% H_2O_2 for 15 min, and then lightly counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted with Entellan (Merck™, Darmstadt, Germany). Negative controls were performed by replacing the primary antibody with 20mM Tris-HCL buffer. Only neoplastic cells that presented a nuclear brown-colored staining were considered positive.

Cell counts were done in 20 representative and consecutive microscopic high-power fields (X400) and, at this magnification, each field (integration graticule) had an area of 0.096 mm^2. Samples were categorized in relation to the immunopositivity of p53 protein where the lesions were classified as positive ($p53^+$) and negative ($p53^-$). $p53^+$ cases presented more than 5% of immunostained cells. Then, the immunohistochemical evaluation of the proliferative activity of tumor cells was done using the Ki67 marker. Similarly to p53, proliferation indexes (PI) were measured as the percentage of immunostained neoplastic cells. Median value of PI was used to classify the samples in $Ki67^+$ (PI > median) and $Ki67^-$ (PI £ median). Negative controls were used by omitting the primary antibodies. The immunohistochemical analyses of these proteins was performed in the invasive front and in the tumor adjacent epithelium.

Statistical analysis

Statistical analysis comprehended χ^2-test with Fisher exact test to evaluate the association of immunostaining of the proteins and the influence of tumor recurrence in clinicopathological parameters. Kaplan-Meier analyses were compared

by the log-rank test. Variables with $p \leq 0.25$ were included in the Cox proportional hazards regression to estimate predictive factors of crude survival. Cox's proportional-hazard regression analysis was used to assess the simultaneous association between multiple factors and disease-free progression. In all statistical analyses, a confidence above 95% ($p < 0.05$) was considered significant. Computer calculations were performed with the statistical pack SPSS™ (SPSS Inc., Chicago, IL, USA), version 17.0 for Windows®.

RESULTS

Epidemiological, clinical and morpholo-gical parameters are described in Table 1. The group of patients with recurrent tumor had 24 individuals. Figure 1 shows the immunostaining of the studied proteins. Proliferation index expressed by Ki67 in invasive front varied between 9.900% and 61.700%, with a mean value of 29.316% (± 11.902) and median of 27.14%. In adjacent mucosa, proliferative activity had a mean of 42.460% (± 13.561) of cells, ranging from 19.700% to 77.900% with the median of 40.25%.

Table 1 - Distribution of the patients according to epidemiological, and clinicpathological parameters

Parameters	n	%
Recurrence		
No	16	40.0
Yes	24	60.0
Age		
≤ 46 years	13	32.5
> 46 years	27	67.5
Gender		
Female	6	15.0
Male	34	85.0
Tumor site		
Oral cavity	22	55.0
Oropharynx	10	25.0
Larynx	5	12.5
Hypopharynx	3	7.5
Tumor size		
T1/T2	12	30.0
T3/T4	28	70.0
Nodal metastasis		
Absent	17	42.5
Present	23	57.5
TNM		
I/II	7	17.5
III/IV	33	82.5
Invasive front gradation		
F1	3	7.5
F2	20	50.0
F3	17	42.5
Who gradation		
I	15	37.5
II	10	25.0
III	15	37.5
Tobacco use		
No	10	25.0
Low	2	5.0
Moderate	9	22.5
Severe	19	47.5
Alcohol use		
No	16	40.0
Low	9	22.5
Moderate	2	5.0
Severe	13	32.5

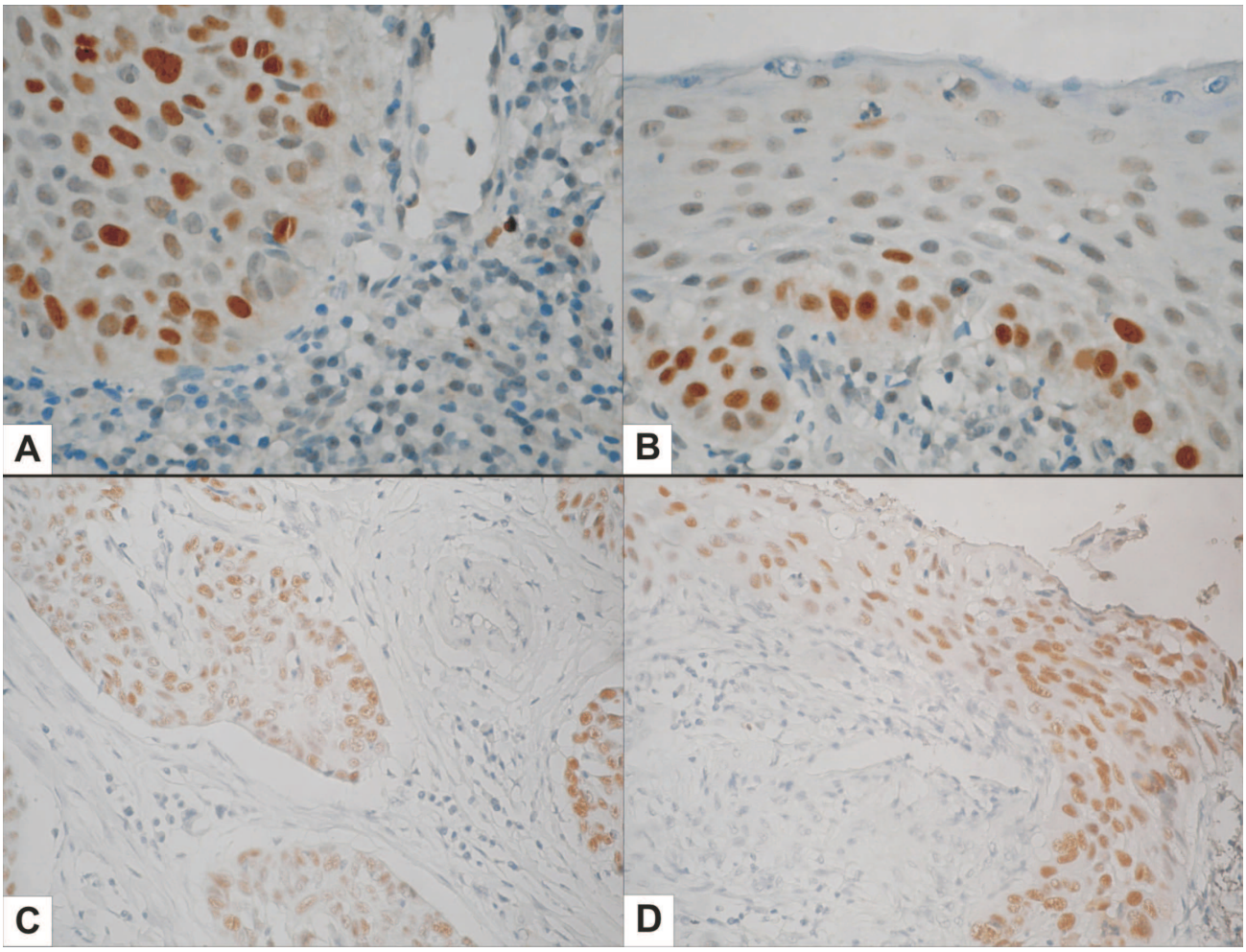

Figure 1 - A: Ki67^{+} immunostaining in invasive front area of HNSCC; B: Ki67^{-} immunostaining in adjacent epithelium of HNSCC; C: p53^{+} immunostaining in invasive front of HNSCC; D: p53^{+} immunostaining in adjacent epithelium of HNSCC (Coloration: Diaminobenzidine; Counterstained: Mayer's hematoxylin; Figures A and B, magnification 400X; Figures C and D, magnification 100X)

χ^2 and Fisher exact tests show the relation of the clinicopathological parameters with p53 immunopositivity and PI in adjacent epithelium and invasive front of HNSCC (Table 2). This analysis revealed only a significant association of tumor recurrence and p53 positivity in mucosa ($p = 0.007$) and invasive front ($p = 0.005$). No significance was found in the comparison between the analyzed proteins. Table 3 demonstrates there was not any statistically significant value in the evaluation of clinicopathological parameters between the two studied groups: tumor recurrence and non-recurrent tumor.

Table 2 - Associations between epidemiological, clinical and morphological parameters and p53 immunostaining and PI in adjacent mucosa and invasive front of HNSCC. Values were calculated by c^2 and Fisher exact tests

	Invasive front						Adjacent epithelium					
Parameters	**p53⁻**	**p53⁺**	**p**	**Ki67⁻**	**Ki67⁺**	**P**	**p53⁻**	**p53⁺**	**P**	**Ki67⁻**	**Ki67⁺**	**P**
Age												
? 45 years	3	10	0.391	11	2	0.460	7	6	0.592	8	5	0.408
> 45 years	9	18		14	13		15	12		14	13	
Recurrence												
No	9	7	**0.005**	10	6	0.632	13	3	**0.007**	8	8	0.422
Yes	3	21		15	9		9	15		14	10	
Tumor size												
T1/T2	2	10	0.207	7	5	0.495	4	8	0.073	5	7	0.223
T3/T4	10	18		18	10		18	10		17	11	
Nodal metastasis												
Absent	5	12	0.612	10	7	0.466	9	8	0.538	9	8	0.538
Present	7	16		15	8		13	10		13	10	
TNM												
I/II	1	6	0.306	3	4	0.224	3	4	0.383	3	4	0.383
III/IV	11	22		22	11		19	14		19	14	
Invasive front gradation												
F1	0	3		1	2		1	2		1	2	
F2	7	13	0.466	13	7	0.555	11	9	0.716	8	12	0.062
F3	5	12		11	6		10	7		13	4	
WHO gradation												
I	2	13		7	8		6	9		7	8	
II	3	7	0.138	7	3	0.273	6	4	0.318	6	4	0.714
III	7	8		11	4		10	5		9	6	
Tumor site												
Oral cavity	9	13		12	10		12	10		12	10	
Oropharynx	2	8	0.355	7	3	0.449	5	5	0.586	5	5	0.586
Larynx	1	4		3	2		4	1		4	1	
Hypopharynx	0	3		3	0		1	2		1	2	
Ki67 (invasive front)												
Ki67⁻	7	18	0.495	-	-		-	-		-	-	
Ki67⁺	5	10		-	-		-	-		-	-	
Ki67 (adjacent epithelium)												
Ki67⁻	-	-		-	-		6	16	0.471	-	-	
Ki67⁺	-	-		-	-		6	12		-	-	

Table 3 - Association of epidemiological and clinicopathological parameters with recurrence of HNSCC. Values were calculated by χ^2 and Fisher exact tests

Parameters	Non-recurrent	Recurrent	P
Age			
? 45 years	5	8	0.585
> 45 years	11	16	
Tumor size			
T1/T2	4	8	0.420
T3/T4	12	16	
Nodal metastasis			
Absent	6	11	0.424
Present	10	13	
TNM			
I/II	2	5	0.408
III/IV	12	19	
Invasive Front gradation			
F1	0	3	
F2	8	12	0.308
F3	8	9	
WHO gradation			
I	3	12	
II	4	6	0.082
III	9	6	

Cox proportional hazards regression showed correlation with p53 in the adjacent epithelium (p = 0.036, OR = 4.813), tumor recurrence (p = 0.030, OR = 4.461) and tumor size (p = 0.009, OR = 9.035) (Tabela 4).

Table 4 - Best significant model of Cox regression analysis in HNSCC patients involving clinicopathological and immunohistochemical parameters

Parameters	N	P	OR	95% IC p OR	
				Lower	Upper
p53 (adjacent epithelium)					
$p53^-$	22			Referent	
$p53^+$	18	**0.036**	4.813	1.105	20.971
Recurrence					
No	16			Referent	
Yes	24	**0.030**	4.461	1.153	17.263
Tumor size					
T1/T2	12			Referent	
T3/T4	28	**0.009**	9.035	1.742	46.866

Individuals presenting T1/T2 tumor size, without tumor recurrence and negative for p53 immunohistochemistry in the adjacent epithelium were associated to a better disease-free progression. Kaplan-Meier univariate test showed an increased survival when tumor size is smaller than 4cm, T1/T2 (p = 0.052) (Figure 2). In this analysis, the other clinicopathological parameters did not have influence in overall survival. The mean of follow-up was 2.22 years (± 1.71).

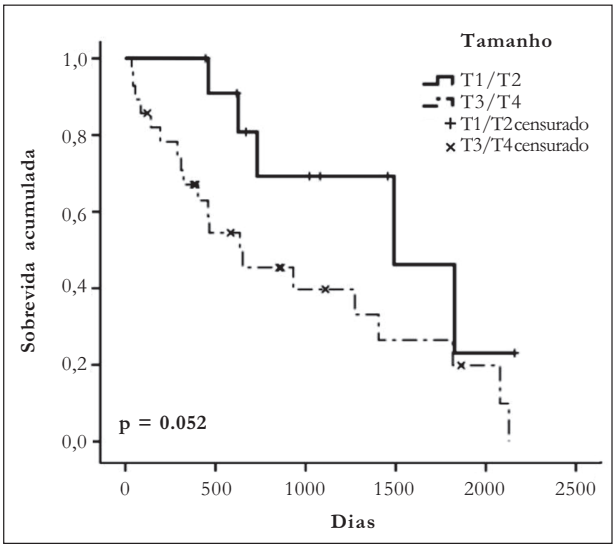

Figure 2 - Survival of HNSCC patients according to tumor size by Kaplan-Meier univariate test. Group differences compared by log-rank test

DISCUSSION

Field cancerization comprises a histologically abnormal area around tumor. This condition advantages tumor recurrence after surgical resection, constituting another cancer risk factor. Therefore, the recognition of these areas may have important clinical implications. Many works have also used epithelium to verify the presence of functional disorders in several kinds of lesions related to cancer (11, 24-26).

In order to evaluate such regions, we used primary tumor adjacent epithelium of patients and compared the results between the groups according to tumor recurrence. Recently, in a similar study analyzing p53, it was not found association between the presence of this protein at the histologically normal mucosa and recurrence of HNSCC (27). However, in our work, it has been identified p53 positivity in tumor recurrent patients. TP53 gene mutations are very frequent in carcinomas, representing one of the commonest genetic disturbances in the disease development. As mutant p53 protein is more stable than its own wild type, it can be detected by immunohistochemical technics (28). Thereby the significant increase of this protein in cells is considered to be consequence of its gene mutations. Such alterations have been related to disorders in molecular mechanisms that control proliferation processes, differentiation, growth, cellular migration and apoptosis (29). Consequently, TP53 gene status and proliferative activity of cells have been often related to tumor development. Thus the presence of modified p53 in tumor adjacent epithelium and invasive front of recurrent patients represents a risk marker for neoplastic recurrence in the analyzed individuals of this study.

In spite of that, it was not detected significant difference of proliferation activity in the studied groups. This result is also followed by the lack of association between the immunoexpression of p53 and Ki67 in mucosa and invasive front, which possibly indicates independent roles of both proteins. So not only changes in p53 protein promote altered cell proliferation, but other disturbances in cellular metabolic pathways should also happen to trigger the disordered mitotic activity (30, 31).

Invasive front areas represent the most infiltra-tive sites of HNSCC and reveal high incidence of neoplastic cells with poor differentiated status and an increased dissociation degree (32). p53 expression in this regions only had association with tumor recurrence; no statistically significant result occurred with the other clinicopathological parameters. In a similar study, frequent detection of higher p53 immunostaining in invasive front of oral squamous cell carcinoma did not present direct relation with clinical tumor size of the lesions, regional metastasis, local recurrence, morphological aggressiveness and patients' prognosis (33).

PI in invasive front did not have association with any clinicopathological parameter. It is possible that neoplastic cells have passed through similar malignant transformations, which did not allow us to evidence differences between their mitotic activity. Prognostic value of Ki67 has been shown in several kinds of cancer (34-36), however, it has been suggested that PI might not be the only influential factor in the different

biological behavior of the disease and more studies with other cell cycle control markers should be accomplished (37).

The similar proliferative activity in the two patients' groups may have also collaborated with no existence of clinicopathological differences between them. Tumor recurrence did not seem to have influenced over the disease course in the studied individuals. However, a work that analyzed recurrence in thyroid cancer showed strong association with clinical parameters like tumor size (> 4cm) and presence of regional metastatic lymph nodes (38). So, disease recurrence may be associated with poor prognosis, being important the development of therapeutic methods for treatment of this risk group (39). More studies should be performed in order to clarify which interventions would have better results in these patients.

Multivariate analysis shows that the group of non-recurrent patients, p53 negative in adjacent epithelium and tumor size smaller than 4cm presented better survival. According to univariate analysis, the smaller tumor size was associated with prolonged survival. Early detection of cancer has been related to good survival (40-42). Unfortunately, this situation may be influenced by socio-demographic issues related to different group of populations, evidenced by difficulties in diagnosis and treatment in some regions.

CONCLUSION

The current study described p53 positivity in the tumor adjacent epithelium and invasive front of patients with recurrent HNSCC, suggesting this protein as an important marker for identification of individuals of this risk group. No association was found between p53 and the other clinicopathological parameters. Proliferation index detected by Ki67 did not have any relation with clinicopathological parameters and tumor recurrence. This protein was not related to p53 immunostaining either. In the analysis between the group of HNSCC recurrent patients and the non-recurrent group, there was no significant difference according to clinicopathological parameters. Multivariate analysis showed that individuals presenting T1/T2 tumor size, without tumor recurrence and negative for p53 immunohistochemistry in the adjacent epithelium were associated factors to prolongation of survival.

CONFLICT OF INTEREST STATEMENT

The authors declared no conflict of interest in the present manuscript.

INFORMED CONSENT STATEMENT

The patients signed an informed consent, kept in the records, in the archives of the UNIMONTES University.

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