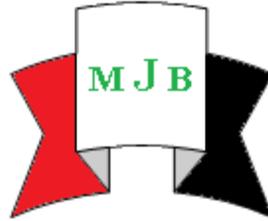


Immunohistochemical Expression of p53 in Squamous Cell Carcinoma of the Lip (A Clinicopathological Study)

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Received 11 May 2014

Accepted 18 January 2015

Abstract

Lip carcinoma is most commonly seen after skin carcinoma in the head-neck region. Mutation of the p53 gene has been reported in a variety of human malignant tumors like squamous cell carcinoma (SCC) and is frequently associated with over expression of p53 protein.

The purpose of this study was to analyze the immunohistochemical over expression of p53 in SCC of the lip in relation with the clinicopathologic pictures.

The materials used in this study consist of (40) formalin fixed, paraffin-embedded lip biopsy specimens of squamous cell carcinoma. p53 immunoreactivity was evaluated in 1000 cells examined under the microscope at 40 magnification and recorded as percentage of p53 positive tumor cells in relation to different clinical and histopathological factors.

The p53 index for squamous cell carcinoma of the lip was ranging between 0.5%-58%, and (72.5%, 29 case) showed positivity for p53 and (27.5%, 11 case) were negative. The highest number and percentages of p53 positive cases were seen associated with males (74.19%), ≥ 60 year's age group (73.33%), patients with outdoor occupations (100%), upper lip (75.0%), >2 cm size lesions (73.68%), patients with palpable lymph node (74.91%), moderately and poorly differentiated type (77.78%), and positive involvement of surgical margins (78.57%). Statistical analysis showed no significant relation of the mean labeling indices with the sex, site, tumor size, the presence or absence of palpable lymph node, grade of differentiation, and involvement of surgical margins ($p > 0.05$), but it showed significant relation with the patient age and occupation ($p < 0.05$).

The old age patients (≥ 60 years) and patients with outdoor occupations showed the highest p53 labeling index significantly.

Keywords: Apoptosis; P53; Immunohistochemistry; Squamous cell carcinoma; Malignancy.

الخلاصة

سرطان الشفة هو اكثر شيوعا بعد سرطان الجلد في منطقه الراس والرقبه. تم تسجيل طفرة الجين p53 في مختلف الاورام الخبيثة للانسان مثل سرطان الخلية الحرشفية وعادة يكون مرتبط مع زيادة التعبير للبروتين p53. تهدف هذه الدراسة الى تقييم مدى زياده تعبير البروتين p53 في حالات سرطان الخلية الحرشفية للشفة بالمقارنة مع المتغيرات السريرية المرضية المختلفة.

تضمنت هذه الدراسة ٤٠ عينة مرضيه لسرطان الخلية الحرشفية في الشفة المثبتة بمادة الفورمالين والمغمورة في البارافين. التفاعل المناعي الى p53 تم تقييمه في ١٠٠٠ خليه فحصت تحت المايكروسكوب تحت قوه تكبير ٤٠ وسجلت كنسبه منويه للخلايا السرطانيه الموجهه الى p53 في علاقته الى مختلف العوامل السريرية والنسجية المرضية.

مقياس p53 لسرطان الخلية الحرشفية للشفة يتراوح بين ٠.٥% الى ٥٨% واطهرت ٢٩ حالة (٧٢.٥%) p53 ايجابي بينما ١١ حالة (٢٧.٥%) p53 سلبي. ان اعلى نسبة لحالات p53 الايجابي كانت في الذكور (٧٤.١٩%) وفي عمر اكثر او يساوي ٦٠ سنة (٧٣.٣٣%).

وللمرضى الذين يعملون تحت اشعة الشمس (١٠٠٪) وللشفه العليا (٧٥٪) ولحجم ورم اكبر من ٢ سم (٧٣,٦٨٪) وللمرضى الذين لديهم الغدة اللعابية مملوثة (٧٤,٩١٪) و لنوع التمييز النسيجي في حالات الوسط والضعيف (٧٧,٧٨٪) وشمول الحافة الجراحية (٧٨,٥٧٪). اظهرت التحليلات الاحصائيات عدم وجود علاقة معنوية بين معدل مقاييس p53 والمتغيرات مثل: الجنس, الموقع, حجم الورم, اصابة الغدة اللعابية ودرجة التمييز النسيجي وشمول الحافة الجراحية (p>٠.٠٥), ولكن اظهرت علاقة معنوية مع العمر والوظيفة (p<٠.٠٥). المرضى كبار السن (اكبر او يساوى ستون سنة) والمرضى اصحاب الوظائف التى هى فى العراء أظهروا اعلى معدل معنوى لمقاييس p53 .

Introduction

SCC may be defined as “An invasive epithelial neoplasm with varying degrees of squamous differentiation and a propensity to early and extensive lymph node metastases” [1], occurring predominantly in alcohol and tobacco-using adults in the 5th and 6th decades of life [2]. Lip carcinoma is a common malignancy, and cancer of the lower lip is the most frequent cancer of the oral cavity in the elderly. Talabani *et al* [3] found that the lip was the most commonly affected site in oral cancer in Sulaimani/Iraq, and the most frequent histopathological type was squamous cell carcinoma (43.84%). The etiologic factors are long-term sunshine exposure, smoking, alcohol intake, chronic immune suppression, and chronic infection with HPV. Thermal injuries with cigarette and pipe smoking have been widely reported as factors leading to development of lip cancer. People who develop lip cancer are mostly farmers, seamen, outdoor workers, and similar [4]. Lower lip squamous cell carcinoma is not perceived as an aggressive carcinoma since it grows slowly, easily diagnosed and can usually be treated effectively, has a good prognosis if the cervical lymph nodes remain uninvolved [5].

Alterations in tumor suppressor genes, like p53, are frequently found in various types of cancer and have been considered as molecular markers of cancer, and p53 over expression protein detection by immunohistochemistry has shown to be a reliable tool as indicative of alterations at the gene level, since wild-type p53 has a

short half-life and is theoretically not detectable by this method [6].

The immunoexpression of oral squamous cell carcinoma (OSCC) in different sites of the oral cavity was studied by Claudia *et al* [7]. They found that it was positive in (33) case of oral squamous cell carcinoma OSCC (76.8%) out of (43), with 77.8% in the tongue, 87.5% in the floor of the mouth, 66.7% in the alveolar ridge/gingiva, 75.0% in the retro molar region, and 71.5% in the lip and buccal mucosa. While Crosthwaite *et al* [8] found that all SCCs from lip lesions were immuno-positive for p53.

This study attempted to investigate the immunohistochemical localization of p53 in SCC of the lip in relation to sex, age, site, lymph node involvement, tumor size, grade of squamous cell carcinoma differentiation, and involvement of surgical margins.

Materials and Methods

The materials used in this study consist of 40 formalin fixed, paraffin-embedded lip biopsy specimens of squamous cell carcinoma, 29 were retrieved from the archives of Department of Histopathology, Rizgary Teaching Hospital, Erbil, Kurdistan region of Iraq, and the other (11) blocks were obtained from the private laboratories, in the period between January/2008 and January/2011. Demographic data and clinical aspects of the tumors, as reported in the forms, were analyzed. Sections were made and stained with hematoxylin and eosin, and additional sections were made for immunohistochemical study. The positive tissue control included in this study was breast ductal

carcinoma tissue section, and the negative tissue controls indicates a tissues specimen, processed using a non immune serum and applying the antibody diluents alone, this was done under the same test conditions throughout the work time and run with each batch of stain.

For immunohistochemical staining, a thin tissue sections (4 μ m) were cut from paraffin blocks and mounted on silanized slides and placed in oven over night at 55 °C. The sections were deparaffinized in xylene for 5 minutes, then hydrated in 100% ethanol, 90% ethanol, 70% ethanol each for 5 minutes respectively, and then rinsed by distilled water for 5 minutes. Slides placed in antigen retrieval and placed in pressure cooker for 50 minutes at 75 °C. Then the container with the slides was removed from the steamer and allowed to cool slowly for 10-20 minutes at room temperature. The slides were rinsed in PBS solution. The excess buffer was tapped off gently and the sections are wiped around by gauze pad and a circle around the section was made by pap pen. Enough hydrogen peroxidase block was applied to cover all the tissue and was incubated for 10 minutes in order to block endogenous peroxidase activity. The slides were rinsed in PBS for 5 minutes, incubated with protein block for 5 minutes, washed in PBS for 5 minutes, incubated with primary antibody for 30 minutes at room temperature, washed in PBS for 5 minutes, incubated with post primary block for 30 minutes, washed in PBS for 5 minutes, incubated with NovoLink™ Polymer (UK) for 30 minutes, then rinsed in 2 jars of Tris-Buffer Solution (TBS) for 5 minutes each respectively with gentle rocking.

Diaminobenzidine (DAB) working solution was prepared by adding 50 μ l of DAB Chromogen to 1ml of NovoLink™ DAB substrate buffer, the sections were incubated with this solution for 5 minutes which resulted in a brown colored

precipitate at the antigen sites. Slides were rinsed with tap water, hematoxylin was used as nuclear counter stain for 30 seconds, the slides were washed in running water gently, sections then were dehydrated in graded ethanol (70%, 90%, 100%) for 2 minutes each respectively, then were transferred to xylene, then slides were mounted, dried and examined under light microscope.

Evaluation of the results:

All hematoxylin and eosin stained slides were examined to find the grade of squamous cell carcinoma differentiation: well, moderately, or poorly differentiated types. Positive expression of p53 gives clear cut nuclear staining of brown color. Random selection of the field was used for analysis of all cases. In all cases that show variable staining, the areas of greatest nuclear staining were chosen. In cases show patchy distribution of p53, the areas that show the highest staining were chosen for assessment.

For quantitative analysis of p53 positive cells, the cells were counted under a light microscope, then the counting was repeated by two independent pathologists and the average of the readings was calculated. Only the number of cells showing nuclear over expression of p53 was quantified by counting at least 1000 cells in five representative fields at 40X objective in each case. Calculation of the labeling index is based on the ratio of the number of immunopositive cells per 1000 counted cells per case studied, and then divided by 10 to express the index in percentage. Then the absolute labeling indices were transformed into the following scores (Lee *et al*, 2005):

1. (-) negative, $\leq 5\%$.
2. (+) weak, $>5 - \leq 25\%$.
3. (++) moderate, $>25 - \leq 50\%$.
4. (+++) strong, $>50\%$.

Results

Most of the cases of SCC in the lip were common among the males (31) and comprising (77.5%) of the total patients, and only nine cases were associated with the females and comprising (22.5%), with a male to female ratio equal to 3.44:1. The age of the individuals ranged between (30-81) years with a mean age of (65.95±15.14) years for the total sample (61.87±15.81 years for males and 67.77±11.51 years for females). The age group (60-74) years showed the highest number of cases of SCC of the lip (19 cases), with (47.5%) prevalence, followed by the age group (≥75) years (11 cases) with (27.5%) prevalence. This mean that the age group ≥60 years constitutes (75%), and the age group <60 years constitutes (25%). Regarding the occupations, most of the patient's occupations were outdoor (20 cases) and constitutes (68.97%), only nine cases (31.03%) were associated with indoor occupations, and the occupations were not recorded in 11 cases. The result also showed that the lower lip (36 cases) was mostly affected by SCC (90%), and only four cases seen associated with the upper lip (10%).

In addition to that 21 case (52.5%) have a size equal or less than 2cm in its maximum length, 18 cases (45%) have a size more than 2cm to 4cm, and only one case (2.5%) had more than 4cm size. Nine cases with no clinically palpable lymph node (22.5%) and 31 cases with clinically palpable lymph node (77.5%). Table 1 shows the clinical characteristic of SCC in the lip.

The histopathological results showed that 31 case of SCC of the lip were well differentiated (77.5), eight cases (20%) were moderately differentiated, and only one case (2.5%) was poorly differentiated. In addition to that 26 cases (65%) showed negative involvement of surgical margins, and 14 cases (35%) were positive (Table 2).

Immunohistochemical staining for p53, when observed, was found exclusively in the nuclei of epithelial cells. None of the negative controls displayed brown staining in epithelial or in any other cells. Sections of the breast ductal carcinoma included as a positive control were consistently positive, indicating a successful immunohistochemical results.

The highest percentages of p53 positive cases were seen associated with males (74.19%), ≥60 year's age group (73.33%), patients with outdoor occupations (100%), upper lip (75.0%), >2 cm size lesions (73.68%), and patients with clinically palpable lymph node (74.91%) as seen in Table-3. Statistical analysis showed no significant relation of the mean labeling indices ± standard deviation with the gender (p=0.160), site (p=0.424), tumor size (0.854), and the presence or absence of palpable lymph node (p=0.961), but it showed significant relation with the age (p=0.014) and occupation (p=0.001) as seen in Table-5. In addition to that the highest percentages of p53 positive cases were seen associated with moderately and poorly differentiated type (77.78%) and positive involvement of surgical margins (78.57%) as seen in Table-4. Statistical analysis showed no significant relation of the mean labeling indices ± standard deviation with the grade of differentiation (p=0.950), and involvement of surgical margins (p=0.864) as seen in Table-6. Nuclear immunostaining in well, moderately, and poorly differentiated squamous cell carcinoma of the lip are seen in Figure 1, Figure 2 and Figure 3 respectively.

The p53 index for SCC of the lip was ranging between 0.5%-58%, and 72.5% (29 case) showed positivity for p53 (2 strong positive, 9 moderate positive, and 11 weak positive in well differentiated type, one strong positive, 4 moderate positive, and one weak positive in moderately

differentiated type, and one moderate positive in poorly differentiated type), and 27.5% (11 cases) were negative (9 associated with well differentiated type, and 2 cases were associated with moderately differentiated type). Figure- 4 shows the relative frequency of different categories of positive and negative tumor cells for p53 immunostaining in the study sample.

Discussion

The clinicopathological characteristic of SCC in the lip:

The study found that (77.5%) of the patients were males, and (22.5%) were females with a male to female ratio equal to 3.44:1, this might attributed to the more cigarette smoking and the outdoor occupations among the males. Most of the patients were ≥ 60 years of age, the sharp and linear increase of oral cancer associated with aging may be explained by the decline in immune response and accumulation of environmental carcinogen due to prolong exposure throughout the life of the patient. In addition to that 68.97 % of the patients had outdoor occupations, this comes in agreement with the study of Demathe *et al* [9], they found that the highest prevalence of lip SCC occurs in males, between the sixth and seventh decades of life, and 76.7% of them are out door patients.

The study also showed that (90%) of the cases involved the lower lip, this comes in agreement with the results of Demathe *et al* [9], this may be due to greater effect of direct ultraviolet radiation on a vermilion border of the lower lip anatomically, which is main etiologic factor in development of squamous cell carcinoma [4].

Regarding the size of the tumor, in the present study 21 case (52.5%) have a size equal or less than 2cm in its maximum length, 18 case (45%) have a size more than 2cm to 4cm, and only one case (2.5%) had more than 4cm size. This result nearly

comes in agreement with that of Ostwald *et al* [10], they found that the percentage of cases with tumor size less than 2cm in diameters were (61.76%), while the larger tumors constitute (38.23 %), but these results disagree with that of Demathe *et al* [9].

The present study showed that 77.5 % were well differentiated, 20% were moderately differentiated, and 2.5 % were poorly differentiated. This results disagree with that of Demathe *et al* [9], they found that the well differentiated type of SCC of the lip constituted 30%, the moderate type constituted 66.7%, while the poorly differentiated type constituted 3.3%, undoubtedly such discrepancy is due to variation in the method of data collection, criteria of analysis and the sample used in addition of visual judgment of the pathologist.

The negative involvement of the surgical margins in the cases of SCC of the lip constituted 65%. This may be due to the high percentage of well differentiated type of the SCC of the lip in this study, which shows less mitotic activity than the moderate or poor types. This result disagree with that of Demathe *et al* [9] study, they found that the tumor that invade the nearby tissue constituted 63.3%, while the lesion that not invade the nearby tissue constituted 36.7%. Caly *et al* [11] found that for surgical margins, 94.23% were negative and 5.77% positive.

p53 immunostaining distribution in SCC of the lip:

In the present study the p53 index for SCC of the lip was ranging between 0.5%-58%, and (72.5%) showed positivity for p53 and (27.5%) were negative. Perea *et al* [12] found that immunostaining p53 positive was in 70.6% of cases in SCC of the lip studied, but Fabbrocini *et al* [13] found that the p53 protein was positive in approximately 50% of SCC cases of the lip. In normal cells, wild type p53 protein has a

very short half life (6-20 min) and is present in such small quantities that it cannot be detected by immunohistochemical methods. However, missense mutations in the p53 gene often result in a more stable gene product and prolong the half life of the p53 protein, causing it to accumulate within cell nuclei to the extent that it can be easily detected by means of immunohistochemistry, mutated p53 genes and stabilization of the mutated p53 allows IHC to be routinely used to demonstrate it in tissue sample [14].

The study also showed that the highest percentages of p53 positive cases were seen associated with males (74.19%), ≥ 60 year's age group (73.33%), patients with outdoor occupations (100%), upper lip (75.0%), $>2\text{cm}$ size lesions (73.68%), patients with clinically palpable lymph node (74.91%), moderately and poorly differentiated type (77.78%), and positive involvement of surgical margins (78.57%) Ostwald et al [10] study 34 case of SCC in the lip and found that 50% of the cases were p53 positive in the males, while in the females 62.5% of the cases were p53 positive, 45.45% of the cases were p53 positive in patients bellow 60 years of age, 56.52% of the cases were p53 positive in patients above or equal to 60 years of age, 42.85% of the cases were p53 positive with a tumor size less than 2cm in diameters, while in the larger tumors 61.53% of the these cases were p53 positive, 55.56% of the well differentiated cases were p53 positive, 50% of the moderately differentiated type were p53 positive, and 66.67% of the poorly differentiated type were p53 positive.

Statistical analysis in the present study showed no significant relation of the mean labeling indices \pm standard deviation with the gender, site, tumor size, palpable lymph node, grade of differentiation, and involvement of surgical margins ($p > 0.05$), but it showed significant relation with the

patient age and occupation ($p < 0.05$). Several studies considering the immune reactivity of p53 were done in OSCC from different anatomical sites in the oral cavity including the lip, like Claudia *et al* [7], Panjwani and Sadiq [15], and Motta et al [16].

Claudia et al [7] found that no statistically significant difference observed in p53 immunoexpression mean indexes with respect to sex, age of patients, and the anatomical site. Panjwani and Sadiq [15] found that the p53 immunopositivity was decreased further as the tumor became poorly differentiated. Motta *et al* [16] studied the relationship of p53 immunopositivity versus local involvement and histological grade; it did not yield statistical meaning in any of the cases. In addition they studied the relationship with tumor volume, the relationship proved significant.

The differences in mean index results could be due to the methodology employed, type and dilution of primary antibody, or due to the type of tissue analyzed (The formalin- fixed tissues are less reactive to antibodies than frozen sections), its pretreatment, antibody applied, different p53 antibodies have different sensitivities and specificities. The p53 negative cases may result from very low levels of mutant p53, a nonsense mutation or a truncated p53 protein in its N-terminal portion, which would not be recognized by antibody, or due to an accumulation of Mdm2 protein caused by gene amplification, promoting p53 degradation and non-detection by immunohistochemistry [14,17].

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Table 1: The clinical patterns of the SCC of the lip.

Clinical parameter		No.	%
Gender	Male	31	77.5
	Female	9	22.5
Age group	<60	10	25
	≥60	30	75
Occupation	Outdoor	20	68.97
	Indoor	9	31.03
	Occupation not recorded	11	27.5
Site	Upper lip	4	10
	Lower lip	36	90
Tumor size in greatest dimension	≤2cm	21	52.5
	>2-4cm	18	45
	>4cm	1	2.5
Palpable lymph node	No	9	22.5
	yes	31	77.5

Table 2: The histopathological patterns of SCC in the lip.

Histopathological parameters		No.	%
Grade of differentiation	Well	31	77.5
	Moderate	8	20
	Poor	1	2.5
Involvement of surgical margins	Negative	26	65
	Positive	14	35

Table 3: p53 immunostaining distribution in SCC of the lip in relation to different clinical factors

Clinical parameter		p53 immunostaining distribution		
		p53 negative cases (No.&%)	p53 positive cases (No.&%)	Total No.(%)
Gender	Male	8(25.81%)	23(74.19%)	31(77.5%)
	Female	3(33.33%)	6(66.67%)	9(22.5%)
Age group (years)	<60	3(30%)	7(70%)	10(25%)
	≥60	8(26.67%)	22(73.33%)	30(75%)
Occupation	Outdoor	0(0%)	20(100%)	20(69%)
	Indoor	6(66.67%)	3(33.33%)	9(31%)
Site	Upper lip	1(25.0%)	3(75.0%)	4(10%)
	Lower lip	10(27.78%)	26(72.22%)	36(90%)
Tumor Size (cm)	≤2cm	6(28.58%)	15(71.42%)	21(52.5%)
	>2cm	5(26.32%)	14(73.68%)	19(47.5%)
Palpable lymph node	No	3(33.33%)	6(66.67%)	9(22.5%)
	yes	8(25.81%)	23(74.19%)	31(77.5%)

Table 4: p53 immunostaining distribution in SCC of the lip in relation to different histopathological factors

Histopathological parameters		p53 immunostaining distribution		
		p53 negative cases (No.&%)	p53 positive cases (No.&%)	Total No. (%)
Grade of differentiation	Well	9(29.03%)	22(70.97%)	31(77.5%)
	Moderate &poor	2(22.22%)	7(77.78%)	9(22.5%)
Involvement of surgical margins	Negative	8(30.77%)	18(69.23%)	26(65%)
	Positive	3(21.43%)	11(78.57%)	14(35%)

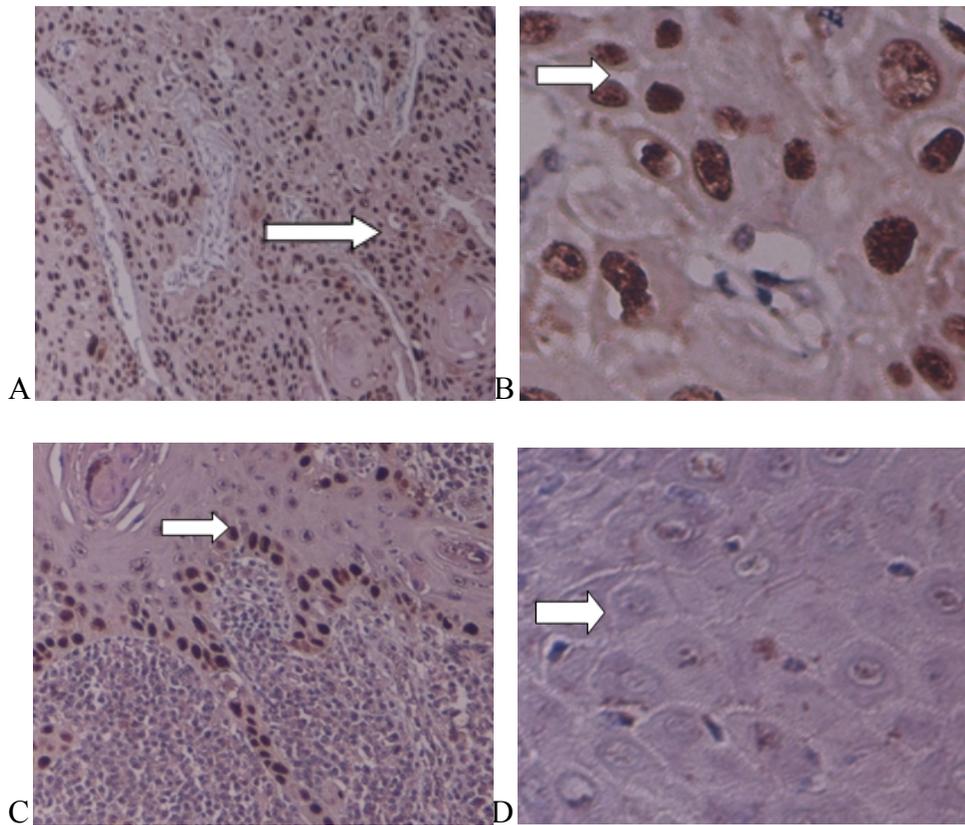


Figure 1: Well differentiated SCC of the lip. Strong positive p53 (arrows) nuclear immunostaining (A: immunohistochemistry x10; B: immunohistochemistryx40). Weak positive p53 (arrow) nuclear immunostaining (C: immunohistochemistryx10). Negative p53 (arrow) nuclear immunostaining (D: immunohistochemistry x40).

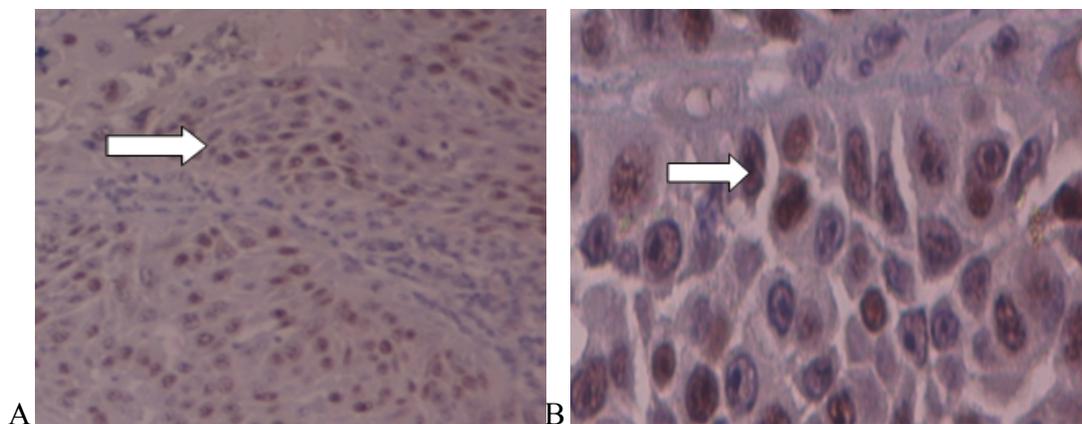


Figure 2: Moderately differentiated SCC of the lip. Moderate positive p53 (arrows) nuclear immunostaining (A: immunohistochemistry x10; B: immunohistochemistry x40).

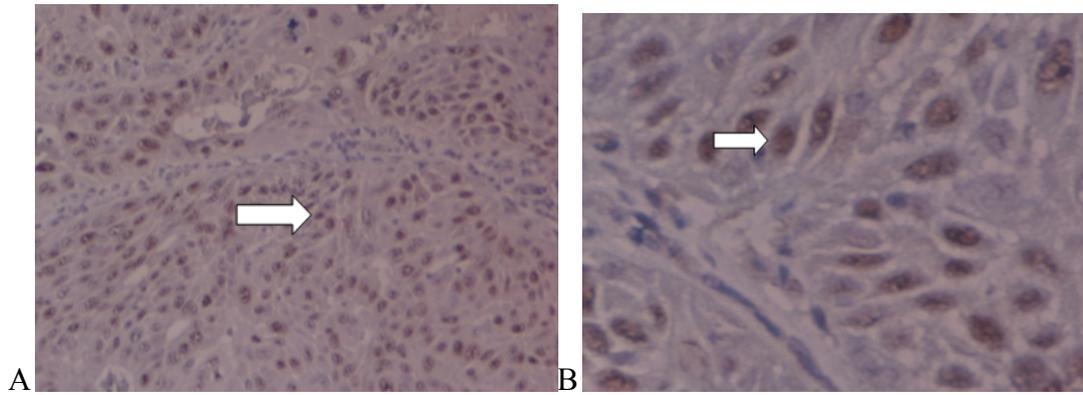


Figure 3: Poorly differentiated SCC of the lip. Moderate positive p53 (arrows) nuclear immunostaining (A: immunohistochemistry x10; B: immunohistochemistry x40).

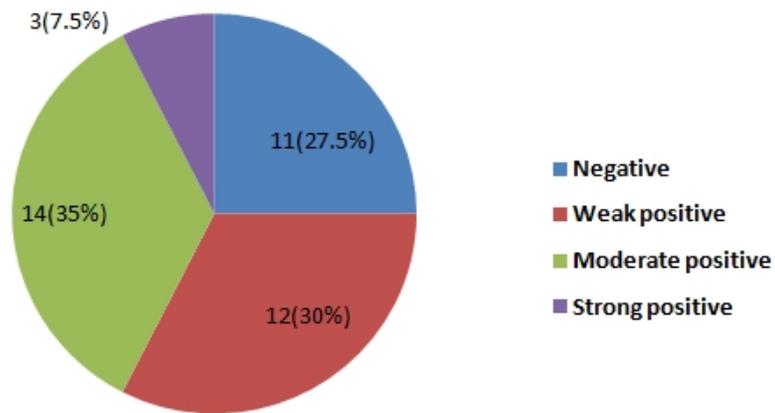


Figure 4: The relative frequency of different categories of positive and negative tumor cells for p53 immunostaining in the study sample.

Table 5: Frequency distribution of the mean, standard deviation, minimum, and maximum values of the percentages of p53-positive nuclei in the study in relation to the clinical characteristic of the SCC of the lip

Clinical parameter		Mean p53% labeling indices				
		p53 mean	± SD	Min.	Max.	P –value
Gender	Male	23.45	±16.98	1	58	0.160
	Female	15.40	±13.605	0.6	42	
Age group (years)	<60	13.24	±9.088	1	27	0.014
	≥60	24.44	±17.517	0.5	58	
Occupation	Outdoor	35.37	±10.597	20	58	0.001
	Indoor	7.74	±8.246	0.6	20	
Site	Upper lip	30.48	±20.972	27	51	0.424
	Lower lip	20.66	±15.960	0.5	58	
Tumor size	≤2cm	21.96	±16.656	0.6	58	0.854
	>2cm	21.16	±17.02	1	50	
Palpable lymph node	No	21.43	±12.559	3	42	0.961
	Yes	21.70	±17.626	0.5	58	

Table 6: Frequency distribution of the mean, standard deviation, minimum, and maximum values of the percentages of p53-positive nuclei in the study in relation to the histopathological picture of the SCC of the lip.

Histopathological parameter		Mean p53% labeling indices				
		p53 mean	± SD	Min	Max.	P -value
Grade of differentiation	Well	21.64	±17.342	0.5	58	0.950
	Moderate & poor	23.81	±14.315	3	42	
Involvement of surgical margins	Negative	21.31	±17.193	0.5	58	0.864
	Positive	22.24	±15.650	0.6	50	