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Expression of p16INK4a and p14ARF in the Carcinogenesis of Actinic Cheilitis and Squamous Cell Carcinoma of the Lip

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ABSTRACT

This study focused on investigating the roles of p16INK4a, p14ARF, and p53 proteins in the development of squamous cell carcinoma of the lip. A total of 46 tissue samples were analyzed, which included 19 cases of squamous cell carcinoma of the lip, 14 cases of actinic cheilitis, and 13 samples of normal lip mucosa. The study also evaluated protein expression in the epithelial tissue surrounding the tumors. Notably, p16INK4a levels were significantly higher in actinic cheilitis compared to normal mucosa (P = 0.001). p14ARF expression increased progressively from normal mucosa to actinic cheilitis (P = 0.001) but decreased significantly during the transition from actinic cheilitis to carcinoma (P = 0.003). For p53, its expression levels increased from normal mucosa to actinic cheilitis (P = 0.001) and further increased in carcinoma cases (P = 0.008). In addition, a positive correlation was observed between p14ARF and p53 in the epithelium adjacent to the carcinoma. This study concluded that while p16INK4a and p14ARF expression levels can reflect certain stages, they are not definitive markers for predicting the progression of actinic cheilitis into carcinoma. Despite the activation of the p14ARF/p53 pathway in the surrounding epithelial tissue, it seems insufficient to prevent the development of cancer.

Keywords: p14ARF, Actinic cheilitis, Lip, Carcinogenesis, p16INK4a

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Introduction

Squamous cell carcinoma (SCC) of the lip is the leading form of oral cancer, accounting for the majority of malignant lesions in the oral cavity, specifically 90-95% of cases. Actinic cheilitis (AC), a condition mainly affecting the lower lip, results from prolonged exposure to ultraviolet (UV) radiation and holds the potential to progress into SCC of the lip [1-5].

According to the Turkish Ministry of Health's Cancer Statistics for 2017, the incidence of lip cancer, age-standardized, was 0.9 per 100,000 for males and 0.2 per 100,000 for females [6]. A study from Turkey's eastern region found that men were more frequently affected by lip cancer, with SCC being the dominant histopathological type (89%). The average age of diagnosis was typically over 50 years [7]. Another investigation in western Turkey revealed similar trends, with men showing a higher prevalence and the average age surpassing 50 years. Factors such as early alcohol consumption, low education levels, poor oral hygiene, and improper dietary habits were linked to an increased risk of oral cancer development [8].

It is generally accepted that SCCs of the head and neck region arise from a shared premalignant source, with genetic mutations contributing to the progression towards malignancy [9, 10]. These mutations often involve the inactivation of tumor suppressor genes and the activation of proto-oncogenes, such as p16INK4a, p53, cyclin D1,

p14, FHIT, RASSF1A, EGFR, and Rb [11, 12]. Loss of chromosome 9p21, which is common in 70-80% of head and neck cancers, is a frequent genetic alteration seen in squamous dysplasia [9].

The CDKN2A locus on chromosome 9p21 is a critical genetic region that is often altered in human cancers, second only to p53 in frequency. This region produces two vital tumor suppressor proteins: p16INK4a and p14ARF [13]. p16INK4a plays a crucial role in regulating the retinoblastoma (Rb) pathway, and its absence leads to uncontrolled cell cycle progression, promoting tumorigenesis [14]. On the other hand, p14ARF regulates the cell cycle negatively by inhibiting the MDM2 oncoprotein, which in turn modulates p53, acting as a tumor suppressor in a p53-dependent pathway [15].

While some studies highlight the value of increased p16INK4a expression in diagnosing dysplastic lesions in the skin and oral cavity, others argue that a reduction in its expression might hold greater diagnostic significance [16]. Research on the immunohistochemical expression of p14ARF is limited [17].

While much research has focused on the effects of UV radiation on skin cancers, fewer studies have investigated the mechanisms driving the development of AC and SCC [18-22]. Given the importance of understanding these pathways, this study examines the nuclear expression of p16INK4a, p14ARF, and p53 in normal lip mucosa, actinic cheilitis, and SCC of the lip. The aim is to explore the potential role of these markers in early carcinogenesis and to evaluate their relationship with histopathological prognostic factors.

Materials and Methods

Study design

The research adhered to the ethical standards outlined in the Declaration of Helsinki and received approval from the Ethics Committee at Kırıkkale University School of Medicine (IRB-2009/012). The study involved three groups of Caucasian participants: i) AC group: 14 patients with Actinic Cheilitis (average age: 56.57 years, age range: 40-84 years, 4 females/10 males); ii) SCC group: nineteen individuals with lip squamous cell carcinoma (SCC) (average age: 64.3 years, age range: 50-82 years, 3 females/16 males); iii) control (C) group: 13 healthy individuals with histologically normal lip tissue (average age: 50.5 years, age range: 38-72 years, 6 females/7 males). Histological slides of all specimens were examined by 2 pathologists (ANA, ESA) to confirm the diagnosis.

For the AC diagnosis, keratinocytes in the epidermis were observed to be disorganized, showing atypical cytology and increased mitotic activity. SCC diagnosis was confirmed by the presence of invasive malignant squamous cells. In the SCC group, several factors were recorded, including histological grade, presence of ulceration, invasion of lymphatic or neural structures, tumor thickness, size, and metastasis to lymph nodes. Tumor grades were determined based on Broders' classification [23], and thickness was measured from the granular layer to the deepest tumor cell layer using an ocular micrometer.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin-biotin peroxidase method and DAB as the chromogen, with counterstaining performed with Mayer's hematoxylin. Paraffin blocks were sectioned into 5-µm slices, followed by deparaffinization, rehydration, and antigen retrieval using EDTA buffer. Sections were then incubated with primary antibodies for 1 hour: Anti-p16INK4a (p16INK4a Ab-4(16PO4), Neomarkers Ltd, dilution 1/100), Anti-p14ARF (GeneTex, GTX23642, dilution 1/50), and Anti-p53 (SP5, Neomarkers Ltd, dilution 1/100).

Immunohistochemical staining evaluation

Immunostaining for p16INK4a, p53, and p14ARF in the nuclei of keratinocytes was considered positive. For each specimen (AC, SCC, and control), the staining was classified as being located in the lower one-third, lower two-thirds, or upper one-third of the epidermis, with additional recording for superficial areas and invasive tumor fronts in SCC specimens. The H-Score system, modified for this study [24], was used to quantify staining. H-scores for each marker were calculated for all lesions, including invasive tumor islands and peritumoral dysplastic epidermis in SCC specimens. No assessment of stromal cell positivity was conducted.

Statistical analysis

Data analysis was performed using SPSS version 15.0 (Chicago, IL, USA). Descriptive statistics were used to express mean values with standard deviations. Since the H-scores for p16INK4a, p14ARF, and p53 were non-normally distributed across the study groups, non-parametric statistical tests were applied. The Kruskal-Wallis one-way ANOVA was used to compare mean H-scores among the groups. Pairwise comparisons were made using the Mann-Whitney U and Wilcoxon signed-rank tests when significant differences were found. Additionally, H-scores for peritumoral epidermis and invasive islands in SCCs were compared using the Wilcoxon signed-rank test. Spearman's rank correlation was used to evaluate relationships between the H-scores within each group and to identify correlations with histopathological features.

Results and Discussion

None of the SCC cases exhibited lymphovascular invasion, and perineural invasion was noted in only 2 cases. Of the three SCC cases with lymph node dissection, none showed evidence of metastasis. Six tumors (31.6%) were larger than two cm in diameter, and seven tumors (36.8%) were thicker than 5 mm. Tumor invasion reached the muscle tissue in ten cases, adipose tissue in 2, the entire dermis in 4, and the middle dermis in 2 cases. Tumor grading revealed that 74% of SCC tumors were classified as grade 1 (n = 13) and grade 2 (n = 2), with 26% classified as grade 3 (n = 4). According to AJCC 8th edition staging, 53% (n = 10) were T1N0M0, and 47% (n = 9) were T2N0M0.

The patterns of expression for p16INK4a, p14ARF, and p53 in the epidermis are summarized in **Tables 1 and 2**. **Table 1** shows the distribution of immunostaining patterns, and **Table 2** presents the immunostaining locations within the epithelium. Regarding p16INK4a expression, 30.8% (4/13) of control specimens exhibited staining in the lower two-thirds of the epidermis, while 64.3% (9/14) of AC lesions and 94.8% (18/19) of SCC lesions demonstrated similar staining patterns, particularly in the peritumoral epidermis.

Table 1. Positive immunohistochemical staining percentages of the cases.

	C group (n = 13) n (%)	AC Group (n = 14) n (%)	SCC group (n = 19) n (%)	PE of SCC* (n = 19) n (%)
P16	4 (30.8)	9 (64.3)	12 (63.1)	18 (94.8)
P14	13 (100)	14 (100)	19 (100)	19 (100)
P53	13 (100)	14 (100)	16 (84.2)	19 (100)

*PE of SCC (peritumoral epidermis of SCC)

Table 2. Immunohistochemical staining localizations of cases in the C, AC, and PE of SCC groups

Location in epidermis	C group n (%)			AC group n (%)			PE of SCC n (%)		
	P16	p14	p53	P16	p14	p53	P16	p14	p53
Lower third (basal)	4 (30.8)	6 (46.1)	13 (100)	8 (57.1)	3 (21.4)	10 (71.4)	13 (68.4)	4 (21)	17 (89.5)
Middle third	0	7 (53.9)	0	1 (7.1)	4 (28.6)	4 (28.6)	5 (26.3)	11 (58)	2 (10.5)
Upper third	0	0	0	0	7 (50)	0	0	4 (21)	0
Total cases		13			14			19	

*PE of SCC (peritumoral epidermis of SCC)

In the case of p14ARF expression, half of the AC group (7 out of 14) showed positive staining in the lower two-thirds of the epidermis, with a similar staining distribution observed in the peritumoral epithelium of 15 out of 19 SCC cases (78.9%). For the control group, all samples displayed p14ARF positivity within the lower two-thirds of the epidermis. In addition, 50% of the AC samples and 21% of the peritumoral SCC samples exhibited p14ARF staining within the upper regions of the epithelium.

When examining p53, both the control and AC groups demonstrated positive staining in the lower two-thirds of the epidermis. Furthermore, this staining pattern was consistent in the peritumoral epithelium of all SCC samples **(Figure 1)**.

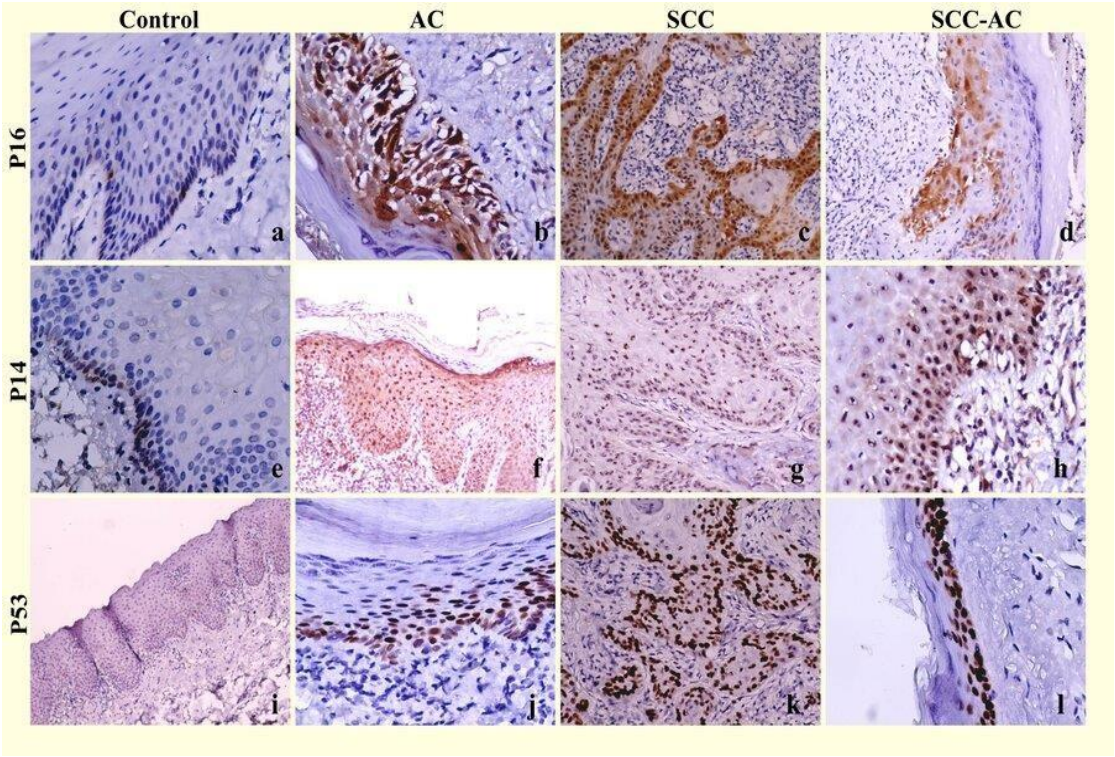

Figure 1. Representative images of p16, p14, and p53 expression patterns; nuclear expression of p16 in (a) normal mucosa, (b) actinic cheilitis, (c) squamous cell carcinoma, (d) peritumoral epidermis (a, b: x400; c,d:x200, IHC); nuclear and nucleolar expression of p14 in (e) normal mucosa, (f) actinic cheilitis, (g) squamous cell carcinoma, (h) peritumoral epidermis (e,h:x400; f,g:x200, IHC); nuclear expression of p53 in (i) normal mucosa, (j) actinic cheilitis, (k) squamous cell carcinoma, and (l) peritumoral epidermis (i:x100; j,l: x400; k:x200, IHC)

The expression patterns of p16INK4a, p14ARF, and p53 in the invasive islands of SCC are presented in **(Table 3)**. In the SCC group, four cases exhibited p16 immunostaining around the invasive islands. Among these, four cases showed scattered focal staining, one case demonstrated a diffuse pattern, and four cases had staining in the superficial regions of the islands for p16.

Table 3. Distribution of immunohistochemical stainings in the invasive areas of squamous cell carcinoma cases.

The staining pattern in invasive tumor islands	P16 n (%)	P14 n (%)	P53 n (%)
Peripheral	4 (21)	16 (84.2)	16 (84.2)
Scattered	4 (21)	3 (15.8)	0
Diffuse	1 (5)	0	0

In the SCC group, p14ARF staining was observed in 16 cases at the basal region of invasive islands, with three cases showing a scattered staining distribution. For p53 expression, 16 cases demonstrated a distinct staining pattern along one or two lines at the basal periphery of the invasive islands, while three cases lacked any immunoreactivity.

Interestingly, all SCC cases that were negative for p16INK4a showed positive staining for both p14ARF and p53. Additionally, the three p53-negative cases were found to display positivity for both p16INK4a and p14ARF.

Descriptive statistics of the H-Scores for p16INK4a, p14ARF, and p53 in all the study groups are provided in **(Table 4)**, and their distribution is illustrated in a box-and-whisker plot **(Figure 2)**.

The mean H-score for p16INK4a was significantly elevated in both the AC and SCC groups compared to the control group ($P = 0.001$, $P = 0.005$).

Table 4. H-Score values of p16, p14, and p53 expressions.

	C group H-scores (n = 13)	AC group H-scores (n = 14)	SCC group H-scores (n = 19)	PE of SCC H-scores (n = 19)
P16	5.62 (0-35)	32.79 (0-101)	45.32 (0-123)	53.89 (0-120)
P14	52.92 (10-68)	160.93 (101-205)	119.95 (64-178)	143.89 (70-186)
P53	7.54 (5-25)	59 (12-150)	109.89 (0-156)	59.84 (15-169)

*PE of SCC (peritumoral epidermis of SCC)

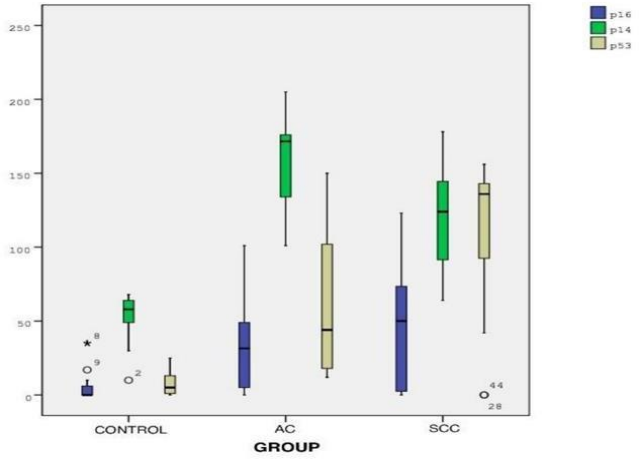

Figure 2. Box-and-whisker plot of H-Score values of p16, p14, and p53 in C, AC, and SCC groups

The p14ARF expression levels were notably higher in the AC and SCC groups than in the C group (P = 0.001 for both), with SCC showing a significantly lower mean H-score compared to AC (P = 0.003).

The p53 expression levels were found to be elevated in both the AC and SCC groups, with SCC showing significantly greater p53 expression than AC (P = 0.008).

When comparing the expression of p16INK4a, p14ARF, and p53 between the peritumoral epidermis and the tumor tissue in SCC cases, no significant differences were noted for p16INK4a. However, p14ARF expression was lower in the tumor area compared to the peritumoral region, while p53 expression was higher in the tumor (P > 0.05, P = 0.007, P = 0.005).

No significant differences were observed between the peritumoral epithelium and AC cases without tumors in terms of the H-scores for the three markers (P > 0.05).

Spearman's correlation analysis revealed no significant association between the clinicopathological features and the expression of p16INK4a, p14ARF, or p53. A positive correlation between p14ARF and p53 expression in the peritumoral epithelium of SCC cases was observed (P = 0.011).

While much of the existing research on head and neck cancers and oral SCCs has focused on tumors from different locations, there has been an emphasis on studying SCC of the lip as a subgroup [12, 25, 26], with particular attention to markers like p53/Ki67 and CD44/VEGF, which have been used to predict tumor progression and prognosis [27]. Previous studies have identified genetic changes such as homozygous deletions and mutations in the INK4A-ARF genes in both the tumor and tumor-free margins of oral cancers [28]. Our research is the first to analyze the expression of p16INK4a, p14ARF, and p53 specifically in AC and SCC cases of the lip.

Inactivation of p16INK4a is a common event in head and neck SCCs, typically caused by homozygous deletion, DNA methylation, or point mutations [28-31]. There is ongoing debate over the role of p16INK4a expression in immunohistochemistry. Sanchez-Cespedes *et al*. [32] argued that the absence of p16INK4a expression serves as a marker of gene inactivation. Reduced expression of p16INK4a has also been linked to premalignant oral lesions and oral cancers [30].

The relationship between mutations in the p16INK4a gene and its protein expression remains complex, with some studies reporting increased expression while others report a loss of the p16INK4a protein despite gene inactivation [33, 34].

In their investigation of the transition from normal skin to SCC, Hodges and Smoller [34] noted that p16INK4a expression was more prominent in SCC in situ compared to actinic keratosis, with full-thickness staining patterns

in the former, suggesting its role in the progression of skin SCC. Our study observed similar findings, with higher p16INK4a expression in AC compared to normal mucosa, supporting the idea that actinic cheilitis on the lips may resemble actinic keratosis on the skin in terms of p16INK4a expression.

Tokman *et al*. [26] examined p16INK4a and p53 expression in oral SCC and found that 58% of lip SCC cases exhibited positive p16INK4a staining. Our results corroborate these findings, with 63% of lip SCC cases showing positive p16INK4a expression.

The patterns of p16INK4a expression observed in AC and SCC can be attributed to the accumulation of inactive p16INK4a proteins, preventing cells from halting the cell cycle, or the dysfunction of other components of the Rb pathway, leading to excessive expression of p16INK4a. This supports the hypothesis that UV exposure may impair p16INK4a tumor suppressor activity, in line with previous research [35].

Few studies have investigated the expression of p14ARF in head and neck cancers, including AC, although its role in regulating p53 has been documented [33, 36]. Weber *et al*. [36] found that p14ARF expression was absent in malignant and benign head and neck tumors with methylation in the p16INK4a and p14ARF genes, while tumors without methylation exhibited moderate to strong expression.

Our study revealed an important increase in p14ARF expression as normal mucosa transitioned to AC, although its expression decreased in SCC compared to AC. This suggests that p14ARF may act as a protective factor during the formation of AC but fails to prevent progression to SCC. Despite higher p14ARF levels in SCC compared to normal mucosa, its role in halting carcinoma development appears limited.

TP53 mutations are among the most common genetic alterations in head and neck SCC, and the mutational patterns of TP53 in lip cancer differ from those in oral cavity cancers [37, 38].

In research focused on p53 protein expression across actinic cheilitis (AC), actinic keratosis, and squamous cell carcinomas (SCC), the use of immunohistochemical techniques to detect both wild-type p53 (wt-p53) and mutant p53 has been common. However, the overexpression of p53 in these studies does not necessarily point to mutations in p53. Although TP53 mutations are often observed in cancers, their presence is not a consistent feature in all skin cancers showing high p53 expression [38, 39]. Wild-type p53 tends to rise in response to UV radiation, whereas mutated p53 accumulates due to slower degradation. Consequently, an increase in p53 expression seen in these studies might be a result of either the accumulation of mutated p53 during tumor progression or the upregulation of wild-type p53 triggered by genetic instability and DNA damage caused by other genetic factors. Studies examining skin cancers have shown that p53 mutations do not necessarily correlate with immunohistochemical overexpression of the p53 protein [39, 40].

Our investigation found p53 expression at lower levels in normal epithelial cells, which aligns with findings from other studies [41]. We identified an increase in p53 levels as normal mucosa progressed to AC and SCC, suggesting that the wild-type p53 might contribute to the formation of mutant p53, as has been suggested in other literature [41, 42]. However, we argue that this hypothesis requires further confirmation through molecular studies specifically detecting the mutated form of p53. We observed p53 expression in 84.21% of our SCC cases and found an increase in p53 expression in lip carcinogenesis.

Other studies have similarly reported increasing p53 expression as lesions progress from AC to SCC [37, 43-45]. Notably, no differences in p53 expression were found between AC cases with and without SCC association [37]. Some treatments, such as ingenol mebutate, were found to not affect p53 expression or histopathological responses in AC, despite improvements seen clinically [46-48]. Our comparison of p53 expression in the peritumoral epidermis of SCC cases and AC cases without SCC revealed no significant differences, indicating that p53 immunohistochemical staining alone is not a reliable marker for differentiating between AC and SCC.

Few studies have focused on protein expression in the epithelium adjacent to the SCC of the lip [37, 49, 50]. Our findings show a positive correlation between p14ARF and p53 expression in the peritumoral epithelium of SCC cases, while no such correlation was observed in AC cases not linked to SCC. This observation suggests that p14ARF-mediated p53 overexpression may act as a protective mechanism during the early stages of carcinoma development in AC, but is insufficient to prevent progression to SCC. Furthermore, the concurrent overexpression of p14ARF and p53 could imply that the activation of p14ARF plays a crucial role in both UV-induced carcinogenesis and the early stages of tumor formation. We also observed that, although p14ARF expression decreases after the onset of SCC, it continues to be expressed, potentially aiding in maintaining wild-type p53 activation. This may help explain why SCC of the lip tends to have a better prognosis compared to other types of oral cancers.

The p53 and Rb pathways are critical cell cycle regulators, and alterations in these pathways are frequent in various human cancers. In our study, all cases that were negative for p16INK4a displayed positive staining for both p14ARF and p53. This suggests that inactivation of p16INK4a and the Rb pathway may lead to the activation of p14ARF and wild-type p53, which could account for the higher expression of these proteins in the observed cases.

Conclusion

Our analysis found no statistically important differences in the expression of antibodies between actinic cheilitis (AC) cases and the surrounding epithelium in squamous cell carcinoma (SCC) samples. This indicates that the expressions of p16INK4a, p14ARF, and p53, when considered individually, are not reliable markers for predicting the transformation of AC into SCC. A notable positive correlation was observed between p14ARF and p53 in the peritumoral epithelium of SCC, suggesting that these two proteins, although their combined action may not be entirely sufficient, might work in concert to prevent the initiation of carcinogenesis. It is likely that additional molecular mechanisms also contribute to carcinogenesis, triggering the ARF/p53 pathway.
Although the sample size of our study is limited, our findings suggest that p16INK4a and p14ARF, both found on the same locus, are influenced by separate regulatory mechanisms in the progression from AC to SCC of the lip. These results provide preliminary evidence for future research exploring how the CDKN2A locus interacts with other molecules involved in lip squamous carcinogenesis.

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Conflict of Interest: None

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Ethics Statement: This research was reviewed and approved by the Ethics Committee of Kırıkkale University School of Medicine (IRB-2009/012).

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