Research Article

Assessment Profile of Biomarker Indexes with *FHIT* Gene Methylation in Oral Epithelium Tissues among Smokers and Non-smokers in Duhok Province, Iraq

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Abstract

Background and objectives: Different pathological and molecular changes can occur in oral epithelium after exposure to cigarette smoking. Management of these changes which may predispose to dysplasia and subsequently invasive cancer depends on the ability to predict malignant transformation like assessment of proliferative index and tumor related genomic markers. Fragile Histidine Triad (*FHIT*) gene is a tumor suppressor gene that is frequently inactivated by methylation, and this inactivation has been linked to cancer development in many tissues. The present study is aimed to investigate ki67-labeling index, p16 immunoexpression, and the methylation status of the *FHIT* gene in oral epithelium among smokers. **Materials and methods:** Tissue samples from 150 oral epithelial were taken from smokers and nonsmokers with different epithelial pathologies. The samples were processed, categorized histologically and studied immunohistochemically for ki67 and p16 expression using the automated immunostainer technique. The methylation status of the *FHIT* was analyzed by methylation-specific PCR using primer specific for both methylated and unmethylated DNA. **Results:** Among smokers, both oral squamous cell carcinoma and non-neoplastic oral epithelium (particularly hyperplasia) showed a significant high ki67 expression, while p16 was overexpressed in hyperplastic and inflammatory oral epithelium with a trend toward low grade squamous carcinoma. *FHIT* methylation was detected among smokers in both non neoplastic oral epithelium and squamous cell carcinoma. **Conclusion:** Smoking implies a great deal on the degree of ki67 labeling index in both squamous carcinoma and non-neoplastic oral epithelium, while smoking causes p16 overexpression in non-neoplastic epithelium and variable among squamous carcinoma (SSC). Smoking also induced *FHIT* methylation in neoplastic and non-neoplastic oral epithelium (NNOE).

Keywords: Smoking; Molecular detection; Biomarkers index; Cancer correlation

Introduction

Recently, the concept of smoking related tumorigenesis in oral mucosa is well-established. For predicting malignant transformation of oral lesions, there is a substantial need to improve the technologic assessments of epithelial alterations that seem to be invariably associated with cancer development rather than depending on clinical or histologic criteria. [1]

Cell proliferation is a vital biologic process for all living cells in which its abnormality is correlated with development and progression of cancer. [2] During active phases of cell cycle, the non-histone nuclear protein (Ki67) is expressed in normal and tumor cells. Its labeling index (Ki67-LI) is an excellent indicator of cell proliferation among smokers. It harbors diagnostic and prognostic values in malignant transformation. [3]

Tumor suppressor gene, p16 is a strong and specific inhibitor of cyclin-dependent kinases 4 and 6. It also down-regulates cyclin D-dependent phosphorylation of the retinoblastoma (Rb) protein and thus blocking the cell-cycle progression from the G1 to the S-phase on need. Inactivation of this gene has been demonstrated in dysplastic and malignant lesions. [4,5] Cigarette smoking can alter both Ki-LI and p16 immunoexpression, which have been reported even in healthy looking mucosa of smokers. [6]

Inactivation of methylated Fragile Histidine Triad (*FHIT*), the tumor suppressor gene has been reported in previous studies. *FHIT* is specifically found to be methylated in lung cancer of smokers that indicates a strong correlation between smoking and gene methylation. [7]

It has been hypothesized that tobacco-induced molecular alterations in oral epithelium are similar to those in the bronchial epithelium. Therefore, oral epithelial biopsy may reflect the

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deep and more risky pulmonary tissue sampling for assessing different molecular alterations.

Therefore, the rising prevalence of tobacco smoking among younger people worldwide prompted us to investigate the Ki67-LI, p16 immunoexpression and methylation status of the *FHIT* gene in oral mucosa among smokers in Duhok City, Iraq.

Materials and Methods

Sampling and patient consent

Clinically, unremarkable epithelial samples of gingiva were obtained from 81 smoker patients and 44 nonsmoker patients (Control group) attended specialized Duhok Dental Centers for routine tooth extraction and surgical extraction of third molar. After taking approval from Research Ethics Committee at Duhok General Directorate of Health for interference, written consents have been signed by the clients. History of smoking was obtained from all sampled individuals, and patients with more than 10 cigarettes a day were considered as smokers. Additionally, archival patient paraffin blocks of 25 oral squamous cell carcinoma (SCC), from which 19 with a history of smoking and 6 with history of nonsmoking were retrieved.

Microscopical assessment

Tissues obtained from biopsies were immediately fixed in 10% formalin, processed by automatic tissue processor and then embedded in paraffin blocks. Sections of about 4.0 µm thick were cut from each block, deparaffinized, held on labelled glass slides and then stained by the hematoxylin and eosin using autostainer technique. All cases were examined microscopically for any histological change. On the other hands, immunohistochemical (IHC) staining (Envision TM FLEX mini kit, Dako, Denmark) was performed by Autostainer Link instrument-open system, using monoclonal anti-human ki67 antibody (Clone MIB-1, Dako, Denmark) and purified mouse anti-human p16 antibody (BD PharminogenTM, USA). Appropriate positive and negative controls were used with each run.

Lesion scoring

Ki67 expression was considered positive when the cell nuclei are stained. [8] Semi quantitative assessment was carried out for Ki67-LI as follows: LI= number of positive cells/total number of cells × 100. Then two scores were adopted, Ki-67: Low (<50%), and high (≥ 50%), as previously described. [9,10] For p16 expression, cases with ≥ 5% nuclear and/or cytoplasmic expressions were considered positive. [11]

Molecular detection

In this study, only 100 cases (70 smokers and 30 nonsmokers) were involved because of time and equipment limitations. DNA was extracted from formalin fixed paraffin embedded tissues according to the manufacturer's instructions (QIAamp DNA FFPE Tissue Kit, Qiagen, Germany). Sodium bisulfite conversion for DNA was done using EpiTect Bisulfite Kit (Qiagen, Germany), and then only 2µg gDNA from each sample were subjected to sodium bisulfite modification.

Moreover, the methylation status of the *FHIT* was analyzed by methylation-specific PCR (MSP) (EpiTect MSP kit, Qiagen, Germany) in which 2 sets of primers were designed to amplify methylated and unmethylated DNA for *FHIT* gene including promoter region for *FHIT* gene. Primer sequences [12] used were as follows:

FHIT-M F: 5'-TTGGGGCGCGGGTTTGGGTTTTTACGC-

FHIT-M R: 5'-CGTAAACGACGCCGACCCCACTA-3'

FHIT-U F: 5'-TTGGGGTGTGGGTTTGGGTTTTTATG-3'

FHIT-U R: 5'-CATAAACAACACCAACCCCACTA-3'

PCR reactions were carried out in a volume of 25 µl and the condition used were initial denaturation at 95 °C for 10 minutes, followed by 40 steps cycles of denaturation at 94 °C for 15 seconds, 55 °C annealing for 30 seconds, 72 °C for 30 seconds, and final extension at 72 °C for 10 minutes. Negative control without DNA and positive control for methylated and unmethylated DNA (Qiagen, Germany) were used for each set of PCR. Later on, products were analyzed on 2% agarose gels, stained with EtBr, visualized under UV illumination and the amplified product was visualized under a source of UV light. Observed bands were either methylated (M) or unmethylated (U). Bands with both methylated and unmethylated appearance considered as partially methylated (PM). [13]

Statistical analysis

Data were analyzed using IBM SPSS Statistics Software version 25. Chi-square test was applied for statistical associations and differences. Cases with violated Chi-square assumption, Fisher's Exact test results were reported. The level of significance was set at 0.05 and a p value equal or less than 0.05 was considered statistically significant. When cell number was zero value, 0.5 was added to all cells in the same table to correct for undefined findings. [14] Then data were re-analyzed using this correction factor OpenEpi Program. [15]

Results

Histopathological analysis

Among 150 studied cases, 100 were smokers including 81 (54%) NNOE and 19 (12%) SCC. The remaining 50 cases were nonsmokers, from which 44 (29%) were NNOE and 6 (4%) were SCC [Figure 1].

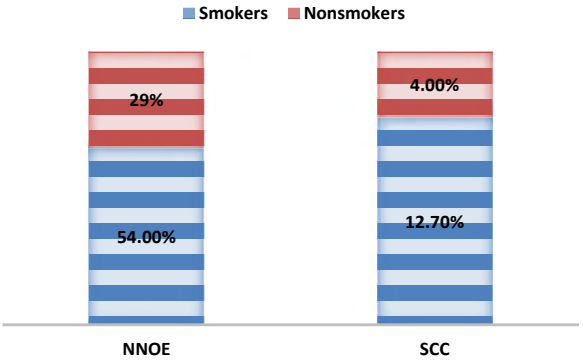


Figure 1: Histpathological detection of investigated cases. (NNOE: Non Neoplastic Oral Epithelium, SCC: Squamous Cell Carcinoma).

Immunohistochemical assessment

Ki67-labeling index (Ki67-LI): As shown in Table 1, a high Ki67-LI was demonstrated in smokers with NNOE (40.8%) and SCC (68%). Compared with nonsmokers, the difference was significantly high (p<0.001) among NNOE. Out of 125 NNOE cases, 117 were associated with epithelial hyperplasia of which 42.7% were smokers with highly significant (p<0.001) Ki67-LI expression. In contrast, no significant difference of this index was noted among the remaining 8 negative hyperplastic cases regardless of the smoking status [Table 2]. Regarding inflammation, Ki67-LI was significantly high in smokers with inflammatory NNOE (p= 0.005), particularly among the chronic phases with p= 0.001 [Table 3]. Ki67 immunostaining in both SCC and NNOE were demonstrated in Figure 2 (A, B, and C) for well differentiated, moderately differentiated and poorly differentiated SCC, respectively, while Figure 3 shows Ki67 immunostaining in NNOE.

p16 expression: Among 40 (32%) positive NNOE cases, smokers showed a significantly high p16 immunoexpression (24%) compared to nonsmokers (8%) with a p value= 0.041. Regarding SSC, although there was a trend of smokers to have p16 overexpression, the difference was not significant [Table 4]. Among the NNOE, smokers with oral epithelial hyperplasia showed a significantly high p16 immunoexpression compared to non-hyperplastic cases, p= 0.014 and 0.25 respectively [Table 5]. As shown in Table 6, a significantly high p16 immunoexpression was demonstrated in smokers with inflammatory NNOE (p= 0.043). No difference was observed in association with severity of inflammation [Table 6].

In SCC, as shown in Table 7, low grade cases showed a significantly higher p16 immunoexpression than the high grade tumors (p= 0.056). Given the smoking in consideration, differences of p16 immunoexpression in both low and high grade SCC cases were not significant [Table 8]. Figure 4 (A and B) demonstrates p16 immunostaining in NNOE, while Figure 5

Table 1: Smoking status in relation with Ki67-LI expression.

Epithelial status	Smoking Status	Ki67-LI			p-value
		Low No. (%)	High No. (%)	Total No. (%)	
Non-neoplastic Epithelium	Nonsmokers	31 (24.8)	13 (10.4)	44 (35.2)	<0.001*
	Smokers	30 (24)	51 (40.8)	81 (64.8)	
	Total	61 (48.8)	64 (51.2)	125 (100)	
Squamous cell carcinoma	Nonsmokers	3 (12)	3 (12)	6 (24)	0.070**
	Smokers	2 (8)	17 (68)	19 (76)	
	Total	5 (20)	20 (80)	25 (100)	

*Chi-square test, **Exact test

Table 2. Smoking status and Ki76-LI expression in non-neoplastic oral epithelium (NNOE).

NNOE	Smoking Status	Ki67-LI			p-value
		Low No. (%)	High No. (%)	Total No. (%)	
With hyperplasia	Nonsmokers	29 (24.8)	13 (11.1)	42 (35.9)	< 0.001*
	Smokers	25 (21.4)	50 (42.7)	75 (64.1)	
	Total	54 (46.2)	63 (53.8)	117 (100)	
Without hyperplasia	Nonsmokers	2 (25)	0	2 (25)	1.000 **
	Smokers	5 (62.5)	1 (12.5)	6 (75)	
	Total	7 (87.5)	1 (12.5)	8 (100)	

*Chi-square test, **Fisher Exact test

Table 3: Smoking status, Ki76-LI and inflammation in non-neoplastic oral epithelium (NNOE).

NNOE	Ki67-LI	Smoking status			p-value
		Non Smokers No. (%)	Smokers No. (%)	Total No. (%)	
With inflammation	Low	26 (26.8)	23 (23.7)	49 (50.5)	0.005*
	High	12 (12.3)	36 (37.2)	48 (49.5)	
	Total	38 (39.1)	59 (60.9)	97 (100)	
Without inflammation	Low	5 (17.9)	7 (25)	12 (42.9)	0.057*
	High	1 (3.6)	15 (53.5)	16 (57.1)	
	Total	6 (21.5)	22 (78.5)	28 (100)	
Acute Inflammation #	Low	5 (19.2)	6 (23.1)	11 (42.3)	1**
	High	6 (23.1)	9 (34.6)	15 (57.7)	
	Total	11 (42.3)	15 (57.7)	26 (100)	
Chronic Inflammation #	Low	21 (29.6)	17 (23.9)	38 (53.5)	0.001*
	High	6 (8.4)	27 (38.1)	33 (46.5)	
	Total	27 (38)	44 (62)	71 (100)	

*Chi-square test, **Fisher Exact Test
(Analysis done for cases with inflammation, in relation to severity)

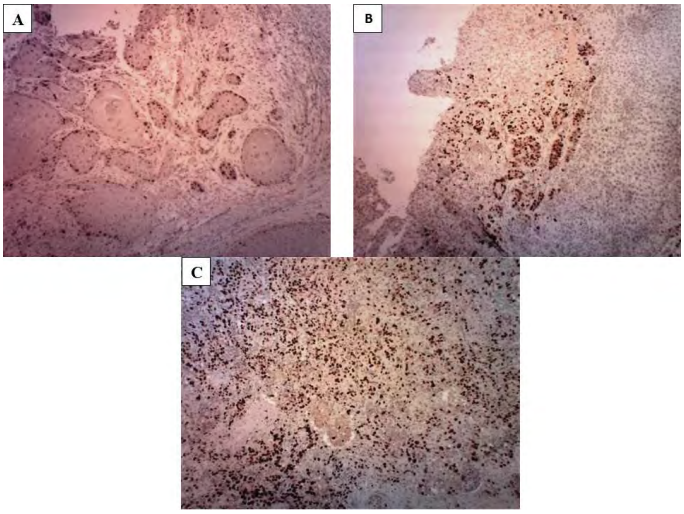


Figure 2: Ki67 immunohistochemical expression in (A): Well differentiated; (B) Moderately differentiated; and (C) Poorly differentiated oral squamous cell carcinoma (10x).

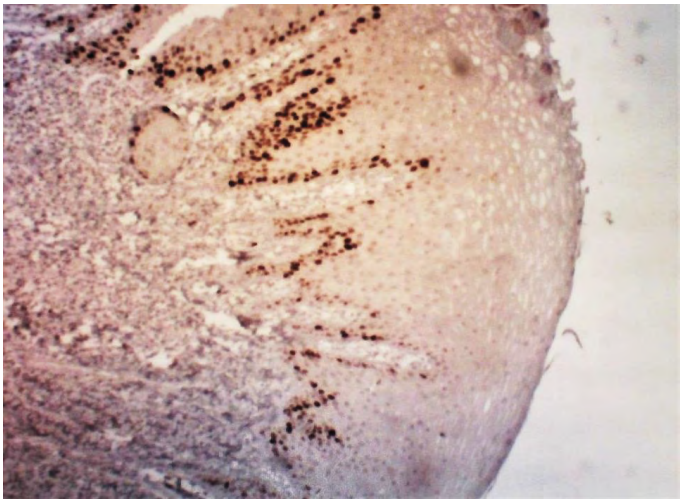


Figure 3: Ki67 immunohistochemical expression in non-neoplastic oral epithelium with hyperplastic changes (10x).

Table 4: Smoking status and p16 expression.

Epithelial status	p16 expression	Smoking status			p-value
		Nonsmokers No. (%)	Smokers No. (%)	Total No. (%)	
Non-neoplastic oral epithelium	Negative	35 (28)	50 (40)	85 (68)	0.041*
	Positive	9 (8)	31 (24)	40 (32)	
	Total	44 (36)	81 (64)	125 (100)	
Squamous cell carcinoma	Negative	3 (12)	8 (32)	11 (44)	1.00**
	Positive	3 (12)	11 (44)	14 (56)	
	Total	6 (24)	19 (76)	25 (100)	

*Chi-square test, **Fisher exact test

Table 5: Smoking status and p16 expression in non-neoplastic oral epithelium (NNOE).

NNOE	p16 expression	Smoking status			p-value
		Nonsmokers No. (%)	Smokers No. (%)	Total No. (%)	
Without epithelial hyperplasia	Negative	1 (12.5)	6 (75)	7 (87.5)	0.25 **
	Positive	1 (12.5)	0	1 (12.5)	
	Total	2 (25)	6 (75)	8 (100)	
With epithelial hyperplasia	Negative	34 (29.1)	44 (37.6)	78 (66.7)	0.014*
	positive	8 (6.9)	31 (26.4)	39 (33.3)	
	Total	42 (36)	75 (64)	117 (100)	

*Chi-square test, **Fisher exact test

Table 6. Smoking status, p16 and inflammation in non-neoplastic oral epithelium (NNOE).

Non-neoplastic oral epithelium	p16 immuno-expression	Smoking status			p-value
		Non Smokers No. (%)	Smokers No. (%)	Total No. (%)	
With inflammation	Negative	31 (32)	36 (37.1)	67 (69.1)	
	Positive	7 (7.2)	23 (23.7)	30 (30.9)	0.043*
	Total	38 (39.2)	59 (60.8)	97 (100)	
Without Inflammation	Negative	4 (14.2)	14 (50)	18 (64.2)	
	Positive	2 (7.8)	8 (28)	10 (35.8)	1.0 **
	Total	6 (22)	22 (78)	28 (100)	
Acute Inflammation#	Negative	10 (38.4)	11 (42.3)	21 (80.7)	
	Positive	1 (3.8)	4 (15.5)	5 (19.3)	0.356 **
	Total	11 (42.2)	15 (57.8)	26 (100)	
Chronic Inflammation #	Negative	21 (29.6)	25 (35.2)	46 (64.8)	
	Positive	6 (7.5)	19 (27.7)	25 (35.2)	0.073*
	Total	27 (37.1)	44 (62.9)	71 (100)	

*Chi-square test, **Fisher Exact test

Table 7: p16 expression and squamous cell carcinoma (SCC) grade.

Squamous cell carcinoma	p16 expression		Total No. (%)	p- value
	Negative No (%)	Positive No (%)		
Low grade	6 (24)	13 (52)	19 (76)	
High grade	5 (20)	1 (4)	6 (24)	0.056*
Total	11 (44)	14 (56)	25 (100)	

*Chi-square test

Table 8: p16 expression among smokers and nonsmokers in squamous cell carcinoma (SSC) in relation to tumor grade.

Squamous cell carcinoma grade	p16 expression	Smoking status			p-value
		Non Smokers No. (%)	Smokers No. (%)	Total No. (%)	
Low grade	Negative	3 (15.8)	3 (15.8)	6 (31.6)	
	Positive	3 (15.8)	10 (52.6)	13 (68.4)	0.320 **
	Total	6 (31.6)	13 (68.4)	19 (100)	
High grade	Negative	0	5 (83.3)	5 (83.3)	
	Positive	0	1 (16.7)	1 (16.7)	0.5*#
	Total	0	6 (100)	6 (100)	

*Chi-square test
*Fisher exact test, # Cells are corrected for statistical purposes

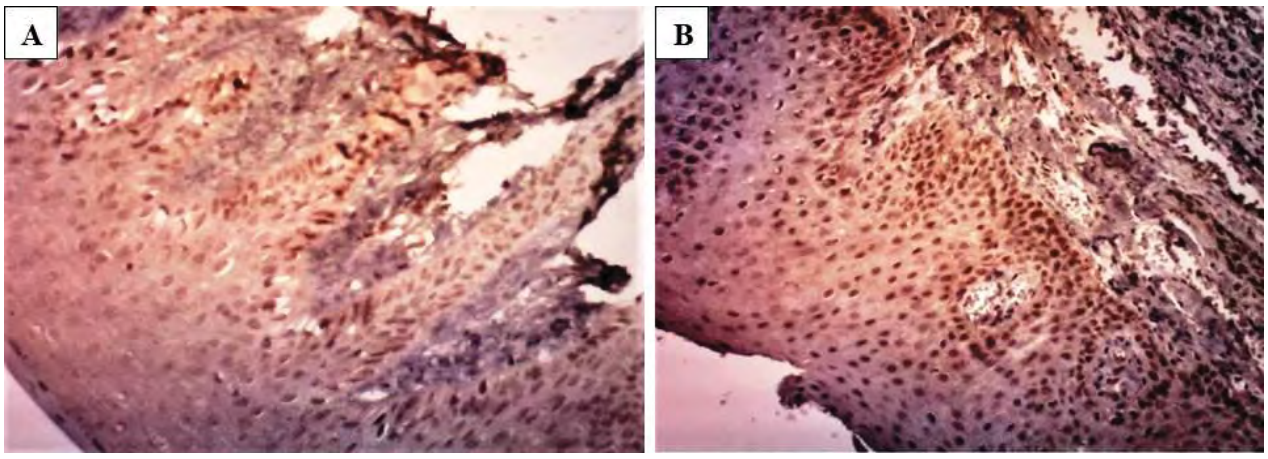


Figure 4: p16 immunohistochemical expression in non-neoplastic oral epithelium (10x).

(A, B, and C) shows p16 immunostaining in well differentiated, moderately differentiated and poorly differentiated SCC, respectively.

Molecular study analysis: As demonstrated in Table 9, out of 70 smokers, 34 were methylated, and 18 were partially methylated. The remaining 18 cases were unmethylated. Comparing with nonsmokers, the difference was not significant (p= 0.196). Oral SCC cases showed a significantly high *FHIT* methylation compared to NNOE with p= 0.049 [Table 10]. As illustrated in Table 11, compared with nonsmokers, most methylated SCC cases were smokers, although doesn't match the significant value (p= 0.29).

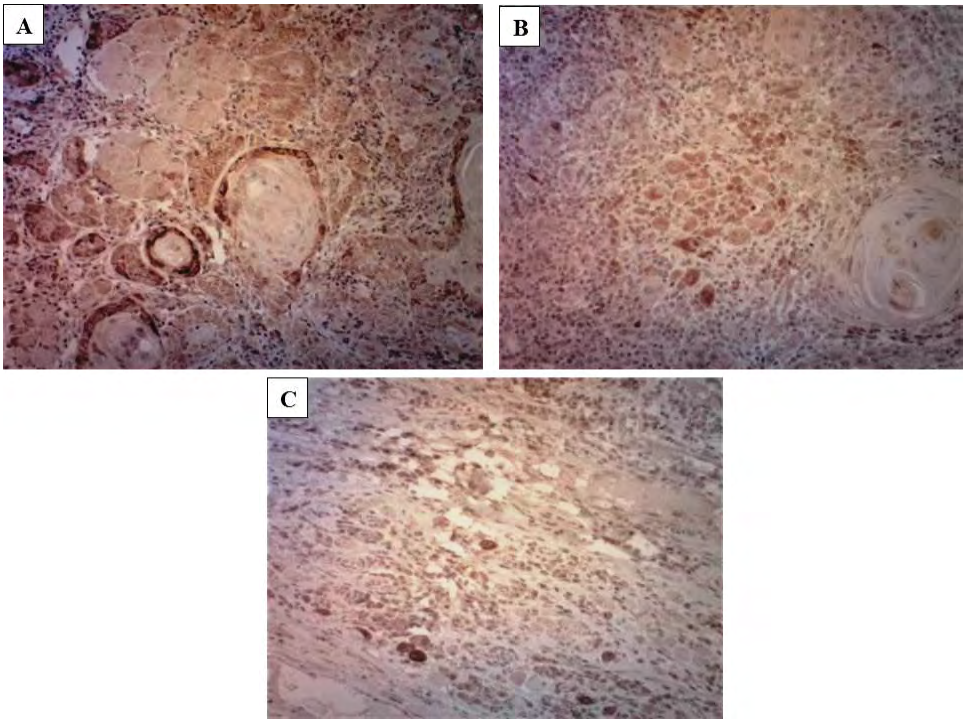


Figure 5: p16 immunohistochemical expression in (A) well differentiated; (B) moderately differentiated; and (C) poorly differentiated oral Squamous cell carcinoma (10x).

Table 9: FHIT Methylation status in relation to investigated cases.

Smoking status	Methylated No. (%)	Partially methylated No. (%)	Unmethylated No. (%)	Total No. (%)	p-value
Smokers	34 (34)	18 (18)	18 (18)	70 (70)	
Non-smokers	9 (9)	9 (9)	12 (12)	30 (30)	0.196*
Total	43 (43)	27 (27)	30 (30)	100 (100)	

*Chi square test

Table 10: FHIT Methylation in relation to cancer type.

Cancer	Methylated No. (%)	Partially methylated No. (%)	Unmethylated No. (%)	Total No. (%)	p-value
SCC	16 (16)	4 (4)	5 (5)	25 (25)	
NNOE	27 (27)	23 (23)	25 (25)	75 (75)	0.049*
Total	43 (43)	27 (27)	30 (30)	100 (100)	

*Chi square test

Table 11: FHIT methylation and smoking status in SCC.

SCC	Methylated No. (%)	Partially methylated No. (%)	Unmethylated No. (%)	Total No. (%)	p-value
Smokers	12 (48)	2 (8)	5 (20)	19 (76)	
Non-smokers	4 (16)	2 (8)	0 (0)	6 (24)	0.298**#
Total	16 (64)	4 (16)	5 (20)	25 (100)	

*Fisher exact test, # Cells are corrected for statistical purposes

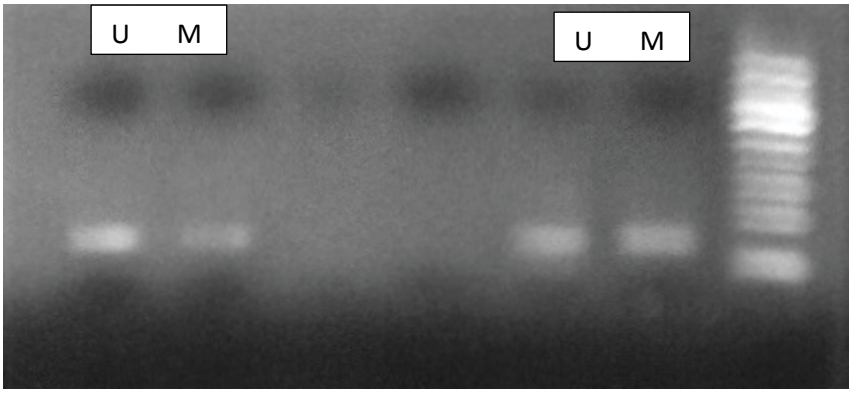
Of the tested NNOE (n= 75), 50 cases were smokers and 25 were nonsmokers. Although there were trends of smoker cases to have *FHIT* methylation while nonsmokers to be unmethylated, no significant difference was demonstrated between the two groups [Table 12].

Results of gel electrophoresis for *FHIT* gene methylation were demonstrated in Figure 6.

Discussion

We investigated immunohistochemically oral epithelial cell proliferation markers including labeling index (ki67-LI), and tumor suppressor gene protein (p16) among smokers. Compared with nonsmokers, ki67-LI was obviously high in both cancerous and non-cancerous cells, which strengthens the concept that smoking products exaggerate epithelial cell division. This exaggeration was demonstrated in both groups of SCC and NNOE (more evident among the non-neoplastic hyperplastic epithelium).

Our observation further strengthens what has been mentioned by previous studies among Switzerland people. [16] Although smoking was not taken in consideration, previous 2 studies performed in the same locality in addition to other experiments



Lane 1: Positive control for both methylated and unmethylated (74-bp PCR product); **Lane 2:** Negative control without DNA, **Lane 3:** Partially methylated sample (both methylated and unmethylated bands appeared). M stands for methylated allele, U is for unmethylated allele.

Figure 6: Gel electrophoresis of the FHIT gene methylation.

Table 12: FHIT Methylation in NNOE in relation to smoking status.

NNOE	Methylated No. (%)	Partially methylated No. (%)	Un-methylated No. (%)	Total No. (%)	P-value
Smokers	21 (28)	16 (21.4)	13 (17.3)	50 (66.7)	
Non-smokers	6 (8)	7 (9.3)	12 (16)	25 (33.3)	0.135*
Total	27 (36)	23 (30.7)	25 (33.3)	75 (100)	

*Chi square test

implied a definitive ascending ki67 index from normal/hyperplastic oral mucosa to dysplasia then SCC and the level of elevated Ki-67 proliferative index has been found to be related to the degree of epithelial neoplasia rather than hyperplastic changes. [3,9,10] However, a higher index was demonstrated in smokers in a study among Turkish people. [17]

Regarding the cell regulating protein (p16), it was nearly absent in non-neoplastic/non-hyperplastic oral lesions regardless of smoking. Lack or decrease of p16 immunostaining in non-neoplastic/non-dysplastic oral epithelium raises the argument that normal p16 protein in these tissues is unaffected or below the detection levels. [18] The concept justifies this finding that p16 is mostly undetectable in normal/non-hyperplastic oral mucosa. [4] On the other hand, the apparent positivity of p16 among hyperplastic epithelium in the current study could be attributed to the overexpression of p16 protein secondarily to other factors like smoking and inflammation irrelevant to the inflammation phase.

In inflammation, p16 overexpression may be attributed to the activation of its protein by cytokines and inflammatory factors with the accumulation of p16 protein. Actually, p16 immunopositivity is considered as a surrogate marker for the oncogenic HPV infection. [1,19] As presumed by Danielsson et al., on their evaluating p16 expression in oral and genital lichen planus and their corresponding healthy mucosa, this overex¬pression may act as a protection against malignant trans¬formation. Negative immunoexpression in tumors with loss of p16 protein function/expression is frequently identified during early carcinogenesis and thus is often observed in non-dysplastic oral mucosa and early neoplastic cells. [20,21]

In the ongoing studied oral SCC cases, although there was p16 overexpression trend toward smokers with low grade cancer, we failed to demonstrate any significant association of p16 expression in oral SCC. Dragomir et al., [22] reported that p16 is a specific marker for the degree of differentiation and a marker of prognosis in SCC. In lung cancer, smoking has been reported to be contributed to p16 gene inactivation. [23] Oral premalignant and malignant lesions may increase or decrease expressions of p16 because of mutation with increased expression or deletion with a lack or reduced expression. [24] However, our small cancer sample size doesn't reflect the actual p16 immunoexpression, or smoking really acts as independent complete carcinogenic factor in oral epithelium and works through a different pathway independent of histologic parameters. Yet one shall put into consideration that, as in cervical squamous intraepithelial lesions and cancers, [25-27] the possible implication of human papillomavirus in the malignant transformation of oral pre-malignant lesions with or without p16 overexpression. [1,6] Dysregulation of the E6/E7 oncoprotein following viral integration into the host genome may lead to overexpression of p16. [1] This helps promote us to investigate HPV infection with smoke effects on p16 immunoexpression among both neoplastic and hyperplastic oral epithelium in this particular locality.

The substantial contribution of *FHIT* methylation in oral squamous cell carcinoma is now increasingly realized and investigated. In the studied SCC cases, a significantly high methylation rate was detected even after ignoring the partially methylated cases as the latter event may be partially effective or completely non-effective. [13] Similar consequences have been tested in a study accomplished amongst the Indian population with OSCC. [28] Methylation seems to be notably contributed to the silencing of the *FHIT* gene in head and neck SCC and is closely associated with OSCC tumorigenesis. [29,30] In this particular study, there was a trend of *FHIT* methylation and partial methylation toward smokers, although it doesn't match

the significant value. Nicotine exposure has an effect on promoter methylation; the frequency of this methylation is significantly higher among smokers compared with nonsmokers. [31]

The relatively high *FHIT* methylation in non-neoplastic oral epithelium among smokers and NNOE/nonsmokers shown in this study can be explained by the fact that tobacco smoke and/or other environmental factors like aging, dietary factors, and chemotherapeutic agents may induce *FHIT* epigenetic damage even in non-neoplastic, healthy-looking oral epithelium. [32] So genes silencing can occur through at least two mechanisms. Adding to this, loss of heterozygosity and the healthy control tissues used vary with different studies. All these can substantially affect the genetic reports and help explain the variable results of methylation status. [29,30]

Conclusion

In conclusion, smoking implies a great deal on the degree of ki67-LI in both SCC and non-neoplastic (hyperplastic and inflamed) oral epithelium. For p16 immunoexpression, smoking causes overexpression in NNOE and low-grade SCC. Smokers showed higher methylation and partial methylation rates comparing to nonsmokers.

Author contributions

Conceptualization, C.S. and I.P.; Methodology, C.S.; Formal Analysis, C.S.; Resources, C.S.; Data Curation, C.S.; Writing – Original Draft Preparation, C.S.; Writing – Review and Editing, I.P.; Supervision, I.P.

Competing Interests

The authors declare that they have no competing interests.

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