Amany H.M. Kamel, Samia M. Kamal, Amira E. Fares. Evaluation of the expression of cytokeratin 5 and tumor suppressor gene p53 in the gingiva of smokers and heavy smokers. International Journal of Academic Research Part A; 2014; 6(5), 119-132.
***DOI:** 10.7813/2075-4124.2014/6-5/A.18*

Library of Congress Classification: RK301-493

EVALUATION OF THE EXPRESSION OF CYTOKERATIN 5 AND TUMOR SUPPRESSOR GENE P53 IN THE GINGIVA OF SMOKERS AND HEAVY SMOKERS

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DOI: 10.7813/2075-4124.2014/6-5/A.18

Received: 14 Feb, 2014
Accepted: 17 Aug, 2014

ABSTRACT

Background: Medical research has proved that cigarette smoking endangered body health and also delayed recovery from injuries and diseases. This study was conducted to examine the histopathological changes induced by smoking and to evaluate the expression of p53 and cytokeratin 5 in the gingiva of smokers and heavy smokers.

Methods: Gingival biopsies were obtained from systemically and periodontally healthy 45 individuals, of middle age during extraction of badly decayed teeth. Five were non smokers and represented the control group, 20 were light smokers and 20 others were heavy smokers. The specimens were subjected to histological and immunohistochemical study as well as morphometric and statistical evaluation of p53 and cytokeratin 5 expression.

Results: Hyperplastic and mild to moderate dysplastic changes were reported in the gingiva of light smokers. Whereas the heavy smokers experienced more severe pathological changes, so that carcinoma in situ and early invasive squamous cell carcinoma was detected. The immunohistochemical results revealed a highly significant increase in the area percentage level of cytokeratin5 and p53 in the gingiva of light and heavy smokers compared to the control group. The same correlation existed between heavy and light smokers with $p \leq 0.001$.In term of optical density; there was a highly significant increase in both biomarkers in light smokers compared to the control. However, p53 represented only a significant increase in heavy smokers compared to light smokers ($p<0.05$).On the other hand no significant correlation was reported for the intensity of cytokeratin5 between heavy and light smoker groups ($p>0.05$).

Conclusions: Smoking induced hyperplastic and dysplastic changes in gingiva of smokers that were severer in heavy smokers to the extent of premalignant and malignant tissue alterations.

Key words: Cytokeratin 5, Tumour suppressor gene p53, Hyperplasia, Dysplasia, Gingiva, Immunohistochemistry

1. INTRODUCTION

Tobacco use is one of the leading causes of premature death, disease and disability throughout the world. It has been proved that tobacco smoking causes coronary heart disease, respiratory distress and cancers. Tobacco has the dubitable distinction of being the world's leading cause of malignancy. Its incidence is on the rise in many regions worldwide including the Middle East. Pregnancy complications, male impotency, increased risk of osteoporosis, senile cataracts and delayed healing of peptic ulcers, all are some of the other manifestations of cigarette smoking[1].

The most serious oral condition induced by smoking is oral cancer. The National Institutes of Health, through the National Cancer Institute, determined in 1998 that smoking causes a variety of cancers including cancers of the oral cavity (lip, tongue, mouth, throat), esophagus, larynx, and lung. Roughly half cases of periodontitis or inflammation around the teeth are attributed to current or former smoking. In addition, complications may further include leukoplakia on the mucous membrane of the oral cavity, including the tongue, and a loss of taste sensation or salivary changes [2].

Pre-existing inherited mutations and/or mutation susceptibility of tumor suppressor genes such as p53, which are known to play a major role in determining cancer susceptibility, have been a subject of investigations in tobacco-related carcinogenesis**.** Mutation of p53 occurs in stressed cells leading to the loss of the protein function. The alterations of p53 impair the ability of the cells to repair or undergo apoptosis in response to DNA damage which will lead to uncontrolled cell growth [3]. Mutated p53 genes have a longer half-life than the normal or wild-type genes and have been demonstrated more frequently in oral mucosal carcinoma cells than in any other

human cancer. Its occurrence in oral dysplasias suggests that its alteration is an event which occurs early in carcinogenesis[4]. Cytokeratins (CKs) including CK5 are important protectors of epithelial structural integrity under conditions of stress, but have also been recognized as regulators of other cellular functions, including motility, signaling, growth and protein synthesis. Cytokeratin filaments and associated proteins conform a complex network which extends from the surface of the nucleus to the cell membrane, thus retain the integrity of epithelium. In cancer, keratins have traditionally been used as diagnostic tools, but accumulating evidence points to their importance as prognostic markers and, more interestingly, as active regulators of epithelial tumorigenesis and treatment responsiveness [5]. Accordingly, the present investigation aimed to determine the effect of cigarette smoking on the structural integrity of gingival tissue in smokers and heavy smokers and to evaluate the immunohistochemical expression of CK5 and the tumor suppressor gene p53 as well.

2. MATERIALS AND METHODS:

Study population

The study was conducted at the faculty of Oral and Dental Medicine, Cairo University. Forty five male individuals of middle age (ranging from thirty to forty years) were included in the study. The patients were visiting the out-patient department for extraction of badly decayed teeth and their oral examination had revealed clinically healthy gingiva. The study population was clinically healthy, free from systemic disease and receiving no medication (proper case history).

The patients were classified into three groups, the control group (C) consisted of five healthy nonsmoker patients with clinically healthy gingiva. The smoker or light smoker group (LS) consisted of twenty patients who smoked ~ 10 cigarettes per day for ~ three years [6]. The third group was the heavy smoker group (HS) and consisted of twenty heavy smoker patients who smoked ≥ 20 cigarettes per day for five years or more[7].

Sample collection

Gingival tissue biopsies obtained from the three groups were of 2mm average size and were excised from the extraction sites at the interdental papilla. An informed consent was obtained from every patient included in the study after explaining the purpose of the work. The study protocol was approved by the institutional ethical committee of the faculty of Oral and Dental medicine, Cairo University, Egypt.

Experimental procedures

All the gingival specimens were washed in sterile saline solution o.15M and fixed in 10% neutral formalin solution, dehydrated in ascending grades of ethyl alcohol and then cleared in xylene. The processed tissue was then embedded in fresh paraffin wax and sectioned at four-micron thickness.

The sections were subjected to the following investigations:

i- Histopathological study

After the tissue has been paraffin embedded, sectioned, mounted on glass slides, the slides were then stained with the nuclear dye (Hematoxylin) and rinsed, then stained with Eosin (H and E stain) and cover slipped[8]. The procedure was carried out to examine the histological features of gingival tissues and the structural changes induced by smoking.

ii- Immunohistochemical investigation

The samples were subjected to immunohistochemical (IHC) analysis using the Mouse monoclonal antibody against CK 5 (keratin 5 Ab-1 (Clone XM26)) to assess the integrity of gingival tissue.Also anti-p53 monoclonal Mouse IgG2a [Bp53-12-1] antibody was used to study the expression of p53 antigen in the gingival specimens and to correlate it with the histopathological changes.

Preparation of the specimen

Four micron- thick sections were put on positively charged slides (optiplus) to provide a strong adhesive surface for tissues and cells. Steps of staining procedures were carried out according to the manufacturer's directions. Deparaffinization was carried out in Xylene followed by rehydration in descending grades of ethyl alcohol and washed in phosphate buffered saline (PBS).To block the endogenous peroxidase activity, the sections were incubated in 3% hydrogen peroxidase and then rinsed with PBS. Antigen retrieval was applied before adding the primary antibody and then sufficient linking of biotinylated secondary antibody was added. Finally incubation with peroxidase labeled streptavidin was performed. In order to develop a color, the chromogen diamino-benzidine (DAB)was used and counter staining was achirved in Mayer's haematoxylin. Appropriate positive controls as well as non immune serum for negative controls were run concurrently.

iii- Morphometric study

The immunohistochemical sections were examined by the image analyzer computer system using the software Leica Qwin. The immunoreactivity for p53 and CK5 were measured in the form of area percentage in a standard measuring frame per ten non overlapping fields for every specimen using a magnification (x400) by light microscopy transferred to the monitor's screen. Areas containing the most uniformly stained tissues were chosen for evaluation. These areas were masked by a blue binary colour using the computer system for measurement. The intensity of the immunereaction was measured by the optical density in a measuring frame per ten fields using a magnification (x400) after transforming the image into the gray mode. Areas with maximum gray were

masked by a blue binary color. The intensity of the gray color was then measured by the computer in the form of maximum gray, minimum gray, sum of gray and average gray.

iv- Statistical analysis:

The image analyzer recorded data were obtained and statistically analyzed and summarized as means and standard deviations using the Student t-test and ANOVA test, where p-value ≤ 0.05 was considered to be significant and p-value ≤ 0.001 was considered to be highly significant.

3. RESULTS

I-Haematoxylin & Eosin results

The gingiva of the control group appeared consisting of keratinized stratified squamous epithelium overlying the lamina propria (Fig. 1A). Most of gingival specimens of (LS) group revealed hyperplastic changes or mild to moderate degree of dysplasia. There were almost all features of cytological atypia along with loss of normal architecture, stratification and cellular orientation. Moreover, in 20% of gingival specimens, cell nests were clearly observed in the prickle cells at the deep ends of rete pegs without any evidence of invasion or loss of basement membrane integrity (Fig1B). There were some degree of loss of normal configuration of epithelial ridges; they became broad and flattened or tortuous. Acanthosis was also a common observation (Fig1C). Dense cellularity was evident in basal and suprabasal layers of some specimens with incidence of binucleated cell and less distinct intercellular spaces in comparison to the control group. Moreover, pleomorphism and anisonucleosis together with improper orientation on basement membrane were observed. Round, oval, elongated, flattened, curved and angular nuclei were observed all over the epithelial layers along with atypical mitosis in upper prickle cells (Fig1D). Mild to moderate basilar hyperplasia was observed in 25% of gingival specimens (Fig. 1E). Loss of keratinization was evident in many specimens (Fig1. D & E) and a picture of koilocytes was a common observation in the superficial epithelial layers of the gingiva of LS. The granular or superficial cells and some of the upper prickle cells appeared with intracellular vacuolization or edema forming a sharply demarcated perinuclear halo surrounding deeply stained nuclei of variable size (Fig.1 C & E).

Unlike the control group, the lamina propria of most of the gingival specimens showed a variable degree of increased vascularity and dilatation of the blood vessels (Fig.1C & D). At several sites blood vessels appeared in close proximity to the basement membrane, juxtaposed to and seemed projecting into the hyperplastic epithelium and disrupting the integrity of basement membrane. Moreover, the widely dilated engorged blood vessels were seen to compress the basal cells and might lead to their degeneration. Furthermore, extravasated RBCs appeared scattered among the basal cells. Marked increase in the degree of inflammatory cell infiltration which may encompass wide blood vessel, and sometimes aggregate in the form of clusters of hyperchromatic cells was clearly demonstrated. Moreover, diffuse infiltration was also observed (Fig.1 B& E). Most of the gingival specimens of this group showed a conspicuous increase in the number of intraepithelial inflammatory cells that appeared to fit in the intercellular spaces. They were seen as flattened curved nuclei with no visible cytoplasm adapting themselves to the contour of intercellular spaces. They have been referred to as squiggle cells (Fig.1 C & D).

The gingival of HS showed architectural and cytological changes ranging from mild dysplasia to actual early invasive squamous cell carcinoma (SCC). A mild to moderate epithelial dysplastic changes were reported in about 80% of specimens (Fig.2A&B) even in the case of invasive SCC. Few cases of severe epithelial dysplasia (carcinoma in situ) were reported but without any evidence of connective tissue invasion (Fig.2C&D). Loss of normal stratification is a common finding and is manifested in the form of basilar hyperplasia and acanthosis. Basal cell hyperplasia was observed in most of the gingival specimens of HS. Moreover, disorganization of basal layer and loss of polarity of basal cells were also evident and were highly obvious in many specimens.The rete pegs configuration was usually altered and appeared as bulbous enlargement (tear drop shaped) and were clearly seen in about 50% of gingival specimens. Other specimens revealed irregular nodular budding of the basal layers which appeared as secondary projections or nodules to arise and branch at indifferent angulations into the lamina propria (Fig. 3A). Disruption of basement membrane integrity was also realized in HS group and was found associated with massive infiltration of chronic mononuclear inflammatory cells that seemed to disrupt the integrity of basement membrane to extend intraepithelialy and lodge intercellulary as squiggle cells (Fig.2B). Actual loss of basement membrane integrity was identified in the gingival specimen for a heavy smoker individual where the dysplastic epithelial nest cells disrupt the basement membrane and invade the underlying connective tissue and settle as early invasive SCC. They appeared surrounded by an eosinophilic homogenous band and the nearby connective tissue showed massive infiltration with hyperchromatic pleomorphic mononuclear inflammatory cells (Fig 3B). With respect to acanthosis, loss of normal cellular arrangement and inconspicuous intercellular spaces were evident. Moreover, cell nests were identified in stratum spinosum of some gingival specimens without loss of basement membrane integrity (Fig 9) and at the deepest end of retepegs where spinous cells proliferation disrupted basement membrane and invaded the lamina propria (Fig 3B).

Abnormal keratinization was also identified. Some specimens appeared non keratinized (Fig.2A) and may present irregular surface composed of swollen faint cells with pyknotic nuclei. Other specimens presented a relative increase in keratinization which invaginated and lined the surface epithelium (Fig.2B).Few cases appeared with continous layers of koilocytic atypia that reached a deep level in the stratum spinosum (Fig 3A). The cytological alterations associated with the dysplastic epithelium were mainly hyperchromatism particularly in the basal and superficial nuclei (Fig.2A&B). Increased N/C ratio was also detected and was usually associated with prominent nucleoli (Fig.2C). Multiple nucleoli were not infrequent together with incomplete cytokinesis

(Fig.3D). Atypical mitotic figures were identified in many specimens and were numerous throughout the whole epithelial thickness in case of severe dysplasia. Furthermore, pleomorphic bizarre-shaped nuclei were also detected. Karyorrhexis and karyolysis were detected associated with severe dysplasia (Fig. 2C).Squiggle cells were also observed in all gingival specimens of this group and were seen extensively infiltrating the gingival epithelium in about 50% of the cases (Fig.2B). They were clearly identified in the intercellular spaces of the basal and suprabasal layers. The lamina propria showed a variable degree of vascular changes in the form of vasodilatation and extravasations of RBCs . However, a rich capillary network was identified subepithelially in the papillary lamina of most cases. Inflammatory cell infiltration was reported in almost all gingival specimens and hyperchromatic dense diffuse mononuclear inflammatory cells were evident (Figs. 2A & 3B).

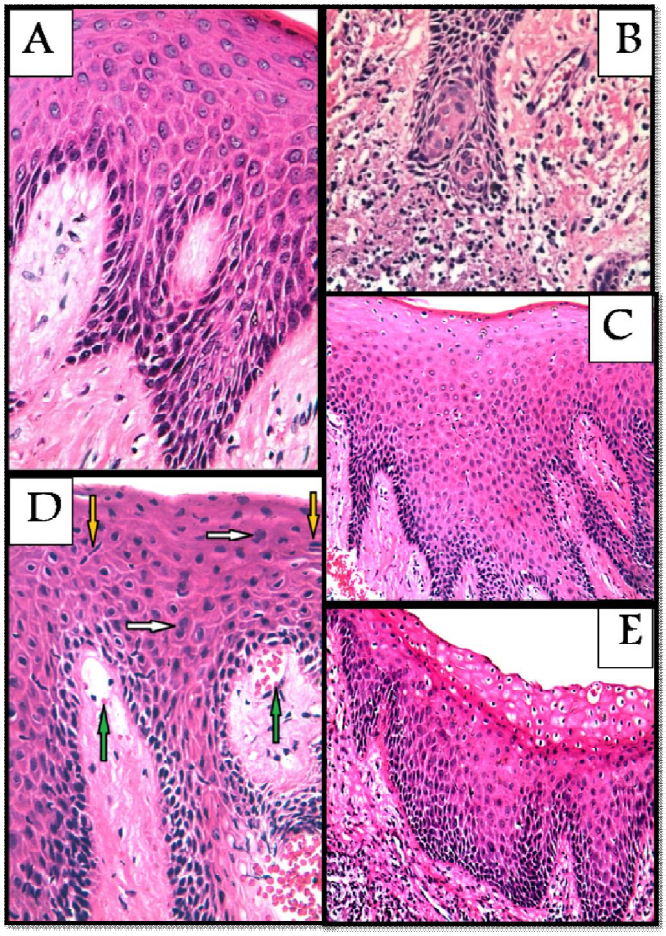


Fig.1-(A) Photomicrograph of the gingiva of the control group, showing normal histological features of keratinized stratified squamous epithelium.(B) Photomicrograph of the gingiva of LS showing two cell nests at the deep end of rete peg with intact basement membrane and mononuclear hyperchromatic inflammatory cells in adjacent connective tissue **(H & E Stain, Orig. Mag. 400). (C)** Another case of LS showing acanthosis, loss of normal configuration of epithelial ridges , hyperchromatism and widely dilated engorged blood vessel indenting the basal cells . Squiggle cells and koilocytic atypia were also seen **(H & E Stain, Orig. Mag. 200).(D)** Photomicrograph of the gingiva of LS showing cellular & nuclear pleomorphism, anisonucleosis, mitosis at high level in the prickle cell layer (yellow arrows), binuclear cells (white arrows), multiple dilated blood vessels (green arrows) and enormous blood vessel with extravasation compressing and projecting into the epithelium leading to loss of basement membrane integrity.Squiggle cells were clearly identified **(H & E Stain, Orig. Mag. 400).(E)** Mild to moderate degree of basilar hyperplasia and a picture of koilocytic atypia at the superficial squamous cells and some upper prickle cells were clear in gingiva of LS **(H & E Stain, Orig. Mag. 200)**

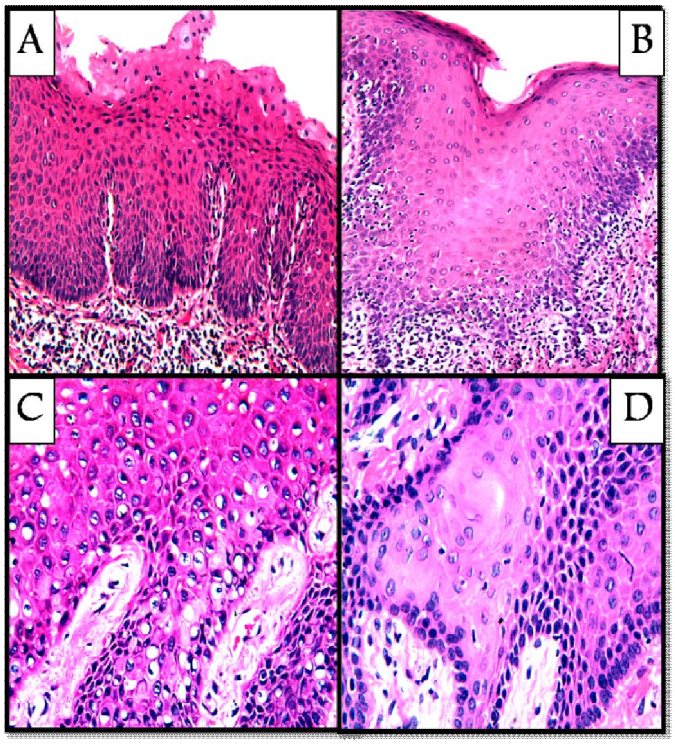


Fig. 2-(A) Photomicrograph of the gingiva of HS showing clubbing of the epithelial ridges, acanthosis and basilar hyperplasia occupying the basal third , irregular nonkeratinized epithelial surface, heavy inflammatory cell infiltrate and extavasated RBCs in lamina propria .**(B))** Photomicrograph showing a parakeratin lined furrow, acanthosis, numerous squiggle cells and massive inflammatory cell infiltration disrupting the basement membrane in gingiva of HS. **H & E Stain, Orig. Mag. 200.(C)** High magnification for severe dysplasia showing increased mitosis with excessive number of atypical mitotic figures, loss of basal polarity and nuclear pleomorphism , nuclear edema, karyorrhexis and karyolysis.**(D)** Photomicrograph showing cell nests, loss of polarity in basal cells and loss of normal stratification of the prickle cells of the gingiva of HS. **H & E Stain, Orig. Mg. 400.**

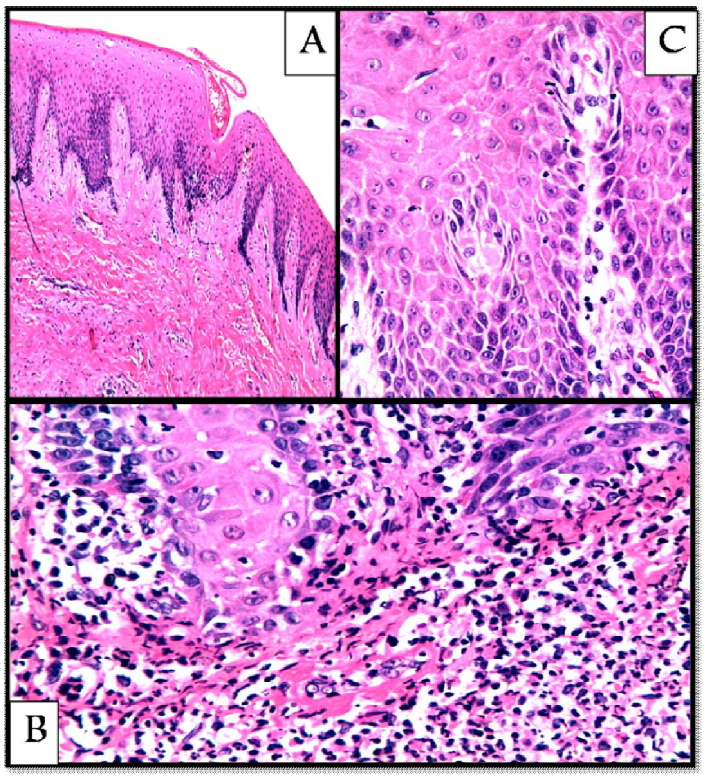


Fig.3-(A): Photomicrograph of the gingiva of HS showing secondary branches arising from altered rete pegs , keratin lining epithelial invagination, continuous layer of koilocytic atypia and fibrosis of the lamina propria **(H & E Stain, Orig. Mag. 100). (B)** High magnification of the gingiva of HS showing discontinuity in basement membrane with early invasion of epithelial cells in connective tissue which showed intense diffuse inflammatory cells infiltration displaying deep hyperchromatism, nuclear pleomorphism and anisonucleosis **(C)** Photomicrograph showing nuclear division with incomplete cytokinesis in the gingiva of HS **(H & E Stain, Orig. Mag. 400).**

Table 1. Dysplastic Features And Histopathological Changes In Gingiva Of Light Smoker Group

Dysplastic change	Percentage
Alteration of the rete pegs	70%
Basilar hyperplasia	25%
Acanthosis	60%
Hyperkeratosis	--
Loss of keratinization	40%
Cell vacuolization (koilocytes)	75%
Hyperchromatism	60%
Increased mitotic figures	50%
Anisonucleosis, anisocytosis & pleomorphism	60%
Multiple nucleoli	25%
Increased density of collagen bundles	75%
Increased vascularity	75%
Increased inflammatory cell infiltrate	75%
Blood vessels closely approaching the hyperplastic epithelium	75%
Intraepithelial inflammatory cells (squiggle cells)	60%
Cell nest	20%

Table 2. Grades Of Dysplasia In Gingiva Of Heavy Smoker Group

Degree of dysplasia	Percentage
Hyperplasia	15%
Mild dysplasia	40%
Moderate dysplasia	20%
Severe dysplasia	20%
Early invasion	5% (single case)

II- Immunohistochemical results

Expression of p53: In the gingival sections of control group, p53 was mainly localized in the basal cell layer of the epithelium with strong intensity in the nuclei and mild expression in the cytoplasm. Regarding the lamina propria, p53 was also localized in fibroblastic nuclei and endothelial lining of blood vessels (Fig.4A). In comparison to the control group, the sections obtained from LS revealed a slight increase in p53 immunostain in the gingival sections displaying mild architectural and cytoplasmic alterations. However, in the specimens exhibiting obvious hyperplastic or dysplastic features, the p53 immunoreactivity was markedly increased. It appeared to include the basal and suprabasal cell layers and also to involve wide areas of stratum spinosum but in a relatively weaker intensity. Moreover, strong p53 immunostaining was found to be localized in the nuclei of the superficial squamous cells. Epithelial cell nests showed a relatively weaker immunostain for p53 especially in their centers. However, strong immunoreactivity appeared in the peripheral nuclei. Slight increase in immunoreactivity appeared in the lamina propria of the gingival specimens of this group (Fig 4B).

In HS group, a much more increase in intensity and prevalence of p53 immunostain appeared in the gingival specimens when compared to the control and LS groups. Almost all the gingival specimens revealed the expression of the protein p53 to involve all the epithelial layers but with variable labeling intensities. The strongest immunostain was usually demonstrated in the nuclei of basal and suprabasal cells and also in the superficial squamous cells. Cytoplasmic immunoreactivity with weaker intensity appeared basally and sometimes manifested in all the epithelial layers (Fig.4C). However, the epithelial cell nests at the deep end of rete pegs presented a relatively weaker immunostain particularly at their central portion. Regarding the lamina propria, the most striking change appeared in the masses of inflammatory cells that reacted positively to the immunostain with strong intensity (Fig.4D).

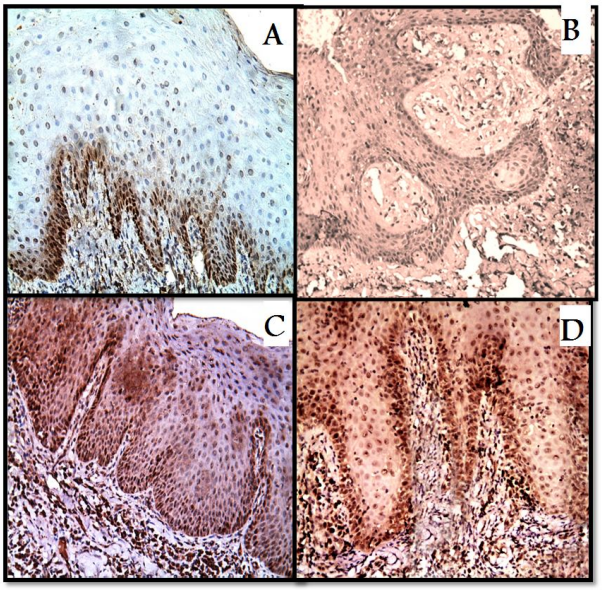


Fig.4 (A): Photomicrograph of the gingiva of (Control group) showing strong nuclear reactivity for p53 mostly in the basal cell layer and negative expression in the rest of epithelium.**(B)**Photomicrograph of the gingiva of LS showing strong nuclear expression of p53 in the basal cell layer, moderate to mild expression in the rest of epithelium and weaker expression in the center of cell nests.**(C)**Photomicrograph of the gingiva of HS showing strong nuclear expression of p53 in the basal , suprabasal and superficial spinous cell layers as well as in the masses of inflammatory cells. **(D)**Photomicrograph of the gingiva of HS showing milder expression of p53 in cell nests than the rest of epithelium . **(DAB (p53), Orig. Mag. 200).**

CK5 expression: Specimens from the control group denotes a strong membranous expression of CK5 in basal cell layer that decreased gradually in its intensity in the suprabasal layers and virtually disappeared in the remaining strata of the epithelium.. On the other hand, the basement membrane appeared with negative or weak immunostain. With respect to the lamina propria, minimal traces of CK5 might be seen confined to the wall of blood vessels (Fig.5A). Noticeable increase in the prevalence and intensity of CK5 reactivity appeared in all the examined gingival specimens of LS. The basal, suprabasal and most of the deep spinous cells of acanthotic epithelium displayed strong membranous reactivity to CK5. The subsequent more superficial prickle cell along with the granular cells presented a mild to weak immunostain. Moreover, examination of basement membrane revealed a more distinct immunostain than the control specimens (Fig.5B).

Sections obtained from specimens of HS revealed an obvious increase in the intensity and prevalence of expression of CK5 all over the epithelial layers. However, the strong expression of CK5 in basal and spinous cell layers was not as sharp demarcating lines delineating cell boundaries or cell-cell junctions as had been found in control or light smoker groups. Instead, it became thicker and hazy probably involving more of the subjacent cytoplasm (Fig.5C). The epithelial cell nests immunoreactivity revealed a weaker CK5 immunostain particularly in their center as compared to the neighbouring spinous cells. With respect to the lamina propria, the inflammatory cells appeared with intensive immunoexpression. Also, a strong reactivity appeared in the endothelial lining of blood vessels that was minimal in the control (Fig.5D)

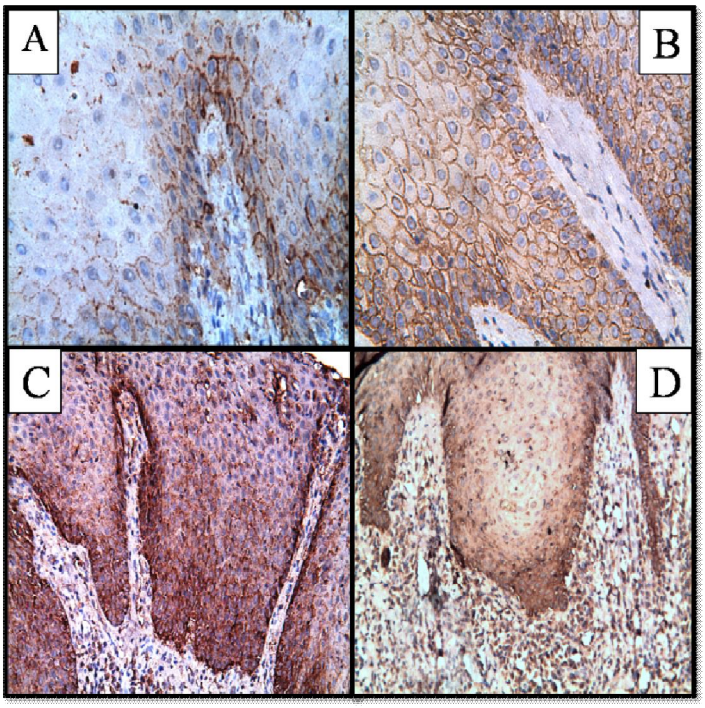


Fig. 5(A): Photomicrograph of the gingiva of (Control group) showing strong to moderate membranous expression of CK5 in the basal and suprabasal layers, negative expression in the rest of epithelium and ill-distinctive immunostain in the basement membrane **(B)** Photomicrograph of the gingiva of LS showing increased CK5 immunoreactivity all over the epithelial layers and more distinctive immunoexpression in the basement membrane. **DAB (CK5), Orig. Mag. 400. (C)**Photomicrograph of the gingiva of HS showing the stronger hazy irregular membranous expression of CK5 in the epithelium and the strong reactivity of inflammatory cells **(D)**Photomicrograph of the gingiva of HS showing obvious reduction in CK5 expression in the center of a large cell nest and strong immunostain in the endothelial lining of blood vessels. **DAB (CK5), Orig. Mag. 200.**

III- Statistical analysis of immunohistochemical results using Student t-test

1- Area percentage: The immuno-expression of p53 protein in the gingiva of LS showed a highly significant increased immunoreactivity when compared to those of control with p=0.001 using t-test. Also, the gingival specimens of HS immunoexpression in correlation to those of light smoker exhibited a highly significant increase with p<0.001 (table A, graphs 1&2). In reference to the immunoexpression of CK5, LS gingival specimens revealed a highly significance increased immunoreactivity in correlation with those of control specimens. Similarly, the HS gingival specimens compared to those of LS revealed a highly significant increase with p<0.001 (table A, graphs 1 & 2).

2-Optical density: Analysis of the intensity of immuno-expression of p53 and CK5 in gingival specimens of LS revealed a highly significant increased reactivity when compared to the corresponding specimens of control groups with p<0.001 for both biomarkers (table B, graphs 1 & 2). However, when comparing the heavy smoker gingival specimens immunoreactivity for p53 with those of light smokers only a significant statistical increase appeared with p<0.05. On the other hand, the intensity of immunostain of CK5 in gingival specimens of HS when statistically correlated with LS group was not significant as p>0.05 (table B, graphs 1 & 2).

IV-Statistical analysis of area percentage using ANOVA test

The statistical analysis of area percentage of p53 in gingival specimens of all groups (control, LS & HS) revealed a highly significant difference correlation with p<0.001. Similarly a highly significant statistical correlation existed in the gingival specimens when comparing the different groups of CK5 with p<0.001 (table C).

Table A. Comparing The Area % Of P53 And CK5 Among The Different Groups And Its Statistical Significance Using t-Test

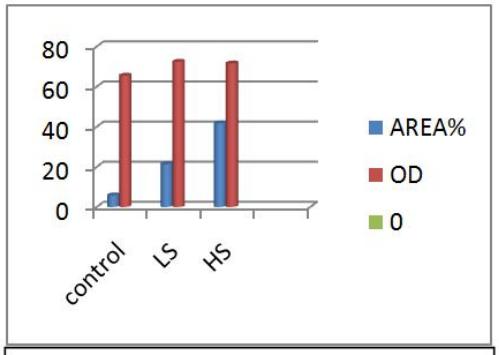
Groups	p53		CK5	
Control	12.1 ±1.42		5.83 ±1.37	
LS	18.0 ±1.76	P=0.001	21.4 ±4.28	P<0.0005
HS	31.1 ±1.76		41.8 ±1.61	
LS	18.0 ±1.76	P<0.0005	21.4 ±4.28	P<0.0005

Data are expressed as mean (M) ± standard deviation (SD)
□□P value ≥ 0.05 …….. not significant (ns).
□□P value≤ 0.05…… significant*.
□□P value≤ 0.001…….. highly significant**

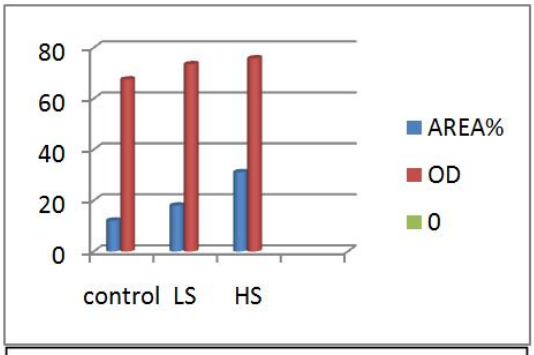
Table B. Comparing The Optical Density Of P53 And CK5 Among The Different Groups And Its Statistical Significance Using t-Test

Groups	p53		CK 5	
Control	67.5 ±1.64		65.5 ±1.52	
LS	73.5 ±1.05	P<0.0005	72.4 ±2.11	P<0.0005
HS	75.8 ±2.04		71.6 ±0.760	
LS	73.5 ±1.05	P=0.022	72.4 ±2.11	P=0.470

Data are expressed as mean (M) ± standard deviation (SD)
□□P value ≥ 0.05 …….. not significant (ns).
□□P value≤ 0.05…… significant*.
□□P value≤ 0.001…….. highly significant**



Graph 1. Bar chart illustrating the difference in area % and optical density of p53 expression among the different groups of the current study.



Graph 2. Bar chart illustrating the difference in area % and optical density of CK5 expression among the different groups of the current study.

Table C. Comparing the area % of p53 and CK5 among the different groups and its statistical significance using ANOVA test

Groups	p53	CK5
Control	12.1 ±1.42	5.83 ±1.37
LS	18.0 ±1.76	21.4 ±4.28
HS	31.1 ±1.76	41.8 ±1.61
LS	18.0 ±1.76	21.4 ±4.28
P value	<0.0005	<0.0005

Data are expressed as mean (M) ± standard deviation (SD)
□□P value ≥ 0.05 …….. not significant (ns).
□□P value ≤ 0.05…… significant*.
□□P value≤ 0.001…….. highly significant**

4. DISCUSSION

Tobacco smoking is a major risk factor for oral epithelial dysplasia (OED) and invasive or squamous cell carcinoma (OSCC). Individuals who smoke more than 20 cigarettes per day are highly susceptible to developing OED. Oral epithelial dysplasia is a histopathological diagnosis and helping tool in predicting subsequent development of invasive carcinoma.It was sometimes diagnosed without specific clinical appearance [9]. The

histopathological results of the present investigation showed a variable degree of hyperplastic and dysplastic changes. Different degree of keratinization ranging from hyperkeratosis to complete loss of keratinization was encountered. Hyperkeratosis was found in the gingiva of about 20% of cases and this finding was previously reported in several studies. *Hedin et al*[10]*; Rahman et al*[11] concluded that smoking induced hyperkeratosis and was suggested to be a mean of protection against noxious and thermal effect of smoking on gingival tissues. On the other hand, the majority of cases of the present investigation showed some degree of diminished keratinization to the extent of complete loss of keratinization that was found in 50% of cases in heavy smoker group. Loss of keratinization associated with smoking was previously demonstrated by *Katchburian and Arana*[12] who attributed this alteration to the presence of a material similar to glycogen in clinically inflamed areas. The inflammation can interfere with the epithelial maturation process and thus the original structural arrangement of tonofilaments compromising the complete keratinization.The authors verified the presence of glycogen-like material inside the epithelial cells indicating a disturbance in the cellular differentiation process. It is believed that this glycogen-like material dissolved during histological preparation give the cells vacuolization appearance or koilocyte picture.

In the current study, squamous cells with enlarged nuclei and sharply demarcated perinuclear clear zone, surrounded by a rim of cytoplasm, “koilocytes” (from Greek, a hollow cell) was found in most of the gingival specimens of smoker groups. They were observed in the granular and superficial prickle cell layers in LS groups while it extended to a deeper level in HS. Consequently, this might indicate a proportional rise with the increasing effect of tobacco consumption and noxious by-products. Similar finding was reported by *Raulin et al*[13] who added that such cells were found as a common finding in injured cells exposed to high concentration of nicotine. Consequently, koilocytes might be a result of affection of cellular metabolism caused by uptake and storage of nicotine by cell [14].

Acanthosis was a common finding in the gingival epithelium of both light and heavy smoker groups. *Luomanen et al*[15]*; Bajagic et al*[16] had previously reported an increased thickness of epithelium in specimens obtained from gingiva of smokers. *Pejcic et al*[17] had also found in the samples obtained from smokers that the spinous cell layer occupied more than 50% of total epithelial thickness. *Alonge et al*[18] contributed acanthosis to the increase in local temperatures and by-products produced from tobacco oxidation which induce an increase in oral mucosa and gingival epithelium thickness. Acanthosis might be a result of increased mitosis or alteration in normal mitosis as well as the atypical mitotic figures encountered in the gingival specimens along with the cellular edema demonstrated in the prickle cells.

In the current study, heavy infilammatory cells infiltrated the lamina propria of gingiva of LS and more intensively in the HS group. The acute effect of smoking on inflammation and oxidative stress was studied by many authors[11,19]. This chronic inflammatory cell infiltration might be explained by either contact irritation of smoking to gingival tissues or local penetration of cigarette by-products deeply into lamina propria. It could be also attributed to noxious by-products passing through blood circulation and gingival vasculature to evoke the inflammatory response. *Van der Vaart et al*[20] noted that acute cigarette smoking is chemotactic to neutrophils and macrophage and activate these cells. Furthermore, acute smoking results in tissue damage by the increased products of lipid peroxidation and matrix degradation products. Moreover, tobacco smoke was found by *Grocki*[21] to block important inflammatory mediators as Leukotriene A4 Hydrolase that the body needs to heal inflammation. The chemicals inhaled from tobacco release inflammatory-inducing chemicals that interfere with the enzymatic respective tasks. This enzymatic blockage may aggravate the inflammatory response and the damaging effect of noxious materials.

In the current study, changes in vasculature particularly the extensive vasodilatation and extravasation were demonstrated in both LS and HS and might be considered sequalae of inflammation. This finding agrees with *Baab and Oberg*[22] who indicated that cigarette smoking contributed to a significant increase, rather than decrease in human gingival circulation. According to *Rahman et al*[11] the examination of gingival tissue of smokers revealed a relative increase in blood supply along with epithelial keratosis, inflammation and fibrosis of the connective tissue. *Kumar and Fairzuddin*[6] had reported that percentage of small blood vessels increased in smokers.

In the present study, the smoker groups showed variable degrees of dysplasia ranging from mild to moderate in LS and mild to severe (carcinoma in situ) in HS (table 1 & 2). Whereas, one case of heavy smoking individuals was reported with early invasive squamous cell carcinoma. However, gingival specimens appeared clinically normal. According to many authors[23,24] smoking tobacco is associated with an increased risk of oral cancer. They suggested that smoking tobacco is not only an important risk factor of developing oral cancer, but also may have its greatest impact during early stages of oral carcinogensis that precede malignant transformation.

It is possible that some oral cancers arise from normally appearing mucosa[25]. It is not known how many oral squamous cell carcinomas arise from precursor lesions and how many develop from apparently normal oral mucosa. However, studies have shown that 16-62% of oral carcinomas are associated with white lesions when diagnosed [26,27]. An Indian survey showed that about 80% of oral cancers were preceeded by oral precancerous lesions[27]. Others considered the vast majority of oral cancers to arise from otherwise clinically normal mucosa[28]. In the present investigation, pleomorphism, anisonucleosis, anisocytosis, hyperchromasia, increased N/C ratio, increased normal and atypical mitotic figures and increased number and size of nucleoli were obviously demonstrated. *Bouquot et al*[29] had considered pleomorphism to be unusual finding outside cancerous and precancerous lesions.

Mitotic activity is considered abnormal either by presence of abnormal mitotic figures or by presence of normal mitosis in a more superficial position than the stratum germativum[13]. Increase in mitotic activity with abnormal mitotic figures (star shaped, tripolar, Y-shaped and others)along with the high level mitosis were also

obvious in gingiva of smokers of the present study. They have been considered indicative for precancerous changes [29].

Increased size and number of nucleoli was a common finding presented herein associated with smoking and is considered by the World Health Organization (WHO) a cytological feature of dysplasia [30]. *Raulin et al*[13] reported that cells exposed to high concentration of nicotine exhibited an increased number of nucleoli suggesting possible affection of RNA synthesis.DNA damage induced by smoking[31] would play important role in hyperchromasia, affect size and shape of nuclei and alter mitosis that would be reflected on size and shape of daughter cells.

Cell nest formation or loss of cellular cohesiveness (acantholysis) was also shown in this work in gingival samples of both light and heavy smoker groups. It is considered a major sign that upon its presence epithelial dysplasia should be upgraded[29]. Loss of cellular cohesiveness may also contribute to abnormal basal cell orientation or loss of polarity.

In the present study, pyknotic nuclei, karyorrhexis and karyolysis were found in association with severe dysplasia. These features were considered hallmarks of necrosis that might be induced in some cells by the dysplastic dividing neighbors that overgrew their nutritional supply[32].

In the current study one of the most important changes in tissue architecture was the alteration of retepegs (bulbous or drop shaped retepegs). *Bouquot et al*[29] had considered them worrisome regardless their size. Alteration of retepegs was suggested to be a result of excessive basal cell proliferation and basal hyperplasia together with loss of polarity as assumed by *Speight*[30]. Another finding that had been shown in this study and considered a worrisome dysplastic feature is the presence of nodular budding. *Bouquot*[29] found no physiological explanation for these secondary nodules, but upon their presence the histopathological grading of the lesion should be upgraded to a higher level.

Disruption of basement membrane integrity by inflammatory cells reported here in particularly in HS was previously demonstrated by many authors in lichen planus which is usually associated with smoking and has a high risk of malignant transformation[33]. *Zhou et al*[34] suggested that the destruction resulted from accumulation of mast cells and release of mast cell mediators specially proteases that create breaks for T-cells to migrate through the epithelium. Loss of basement membrane integrity was also found associated with massive subepithelial vascularity particularly in the gingival specimens of light smokers. Extensive subepithelial vascularity was considered an important feature in oral dysplastic lesions termed erythroplakia[35]. Moreover, dysplastic lesions in general were thought to activate an angiogenic switch that increase the subepithelial microvasculature and produces stromal inflammation[36,37,38]. On the other hand,actual invasion of dysplastic cells breaching the basement membrane barriers was encountered in the gingiva of one case of HS. The way by which disruption occur is not clearly elucidated. The invasive tumor cells were thought to degrade the basement membrane through specific matrix metalloproteinases or produce insufficient or defective basement membrane component and/or improperly assemble extracellular membrane constituents [39].

The changes in cell behavior in malignant transformation were thought to be due to organic toxic substances like tobacco-specific nitrosamine[40]. Moreover, long standing inflammation might be the leading factor of the early invasive SCC reported in this study, as the connective tissue in which the malignant cells settled was massively infiltrated with hyperchromatic pleomorphic inflammatory cells. Chronic persistent infection/ inflammation was suggested to play a role in the pathogenesis of cancer[41]. Furthermore, periodontitis has been found associated with poorly differentiated SCC[42].

Gene p53 mutations were assumed to be more common in smokers than non-smokers[43,44]. Moreover, Brennan *et al*[45] found that p53 mutation to be directly proportional to the extent of tobacco smoking. Tarapore and Fukasawa[46] found that mutation of p53 affects mitotic fidelity leading to defective mitosis including unequal distribution of chromosomes to daughter cells and failure to undergo cytokinesis. Binucleation demonstrated in gingival epithelium of smokers might be a sequale of this mechanism. Grizzle *et al* [35] had reported that mutations in p53 in dysplastic epithelium are represented by visible nuclear accumulation of p53. Muller *et al*[31] assumed that DNA damage arise from carcinogenic agents like tobacco smoke which results in aberrant mitosis and contribute to cancer. Wikinson[47] concluded that cigarette smoking interfere with normal cell division and causes mutation of the genes. Alteration of the cycle of cell regeneration and division was thought to last up to 20 years after a person quits smoking.

Over expression of p53 has been observed in dysplastic areas and cancer and confirm suspected diagnosis of dysplasia and assist with the distinction between low and high grade dysplasia. Moreover, positive expression of p53 in non-dysplastic lesions was suggested to be a first step in the progression toward neoplasia[48]. In the present study, all cases in the three groups showed some degree of p53 positivity. The control group showed p53 expression confined to the basal cell layer, and this coincides with previous results[49,50]. Expression of p53 in normal tissues was explained by several authors[51,52,53] through the role of p53 in regulation of cell proliferation, DNA damage repair or shifting to apoptosis. Hence p53 positive cells are expected in all normal tissues to be localized at the zone of cell proliferation (stratum germinativum of the gingiva).

Although p53 is a nuclear protein, some cases of the smoker groups in the present study showed cytoplasmic expression. Several authors[54,55,56] suggested that the mutant form of p53 protein binds to cytoplasmic heat shock proteins, which might be responsible for its cytoplasmic location. Another explanation was suggested by Bartek *et al*[57] that the cytoplasmic reactivity of the p53 protein might be due to mutation in the p53 gene. Li *et al*[58] concluded that the cytoplasmic expression of p53 protein might be correlated with tumourigensis. The regulation of p53 cellular localization depends on factors that influence its nuclear import and export and subnuclear localization. Also, a portion of p53 can be localized to mitochondria to induce apoptosis in an independent manner[59]. On the other hand, George[60] assumed that latent p53 may be cytoplasmic during part of

the cell cycle, while exposure to stress results in its accumulation in the nucleus where it exerts its biochemical activities. Maki[61] noted that p53 undergoes nuclear and cytoplasmic shuttling and is expressed in low levels in the nucleus and cytoplasm of unstressed cells. However, in response to stress p53 undergoes translational modification and accumulates in the nucleus. He added that recent studies have demonstrated the persistence of a significant fraction of p53 in cytoplasm and that indicates that both nuclear and cytoplasmic p53 participate in the tumor suppressive capacity.

In the current study the prevalence of p53 expression was found to be increased with increasing frequency and duration of smoking from light smokers group to heavy smoker group. A highly significant statistical difference was reported between the HS and LS and also between the LS and control with $p \leq 0.001$ in term of area percentage. However, in term of optical density a highly significant increase was detected between the light smokers and control with $p<0.001$. However, the statistical analysis revealed only a significant increase to exist between heavy and light smokers with $p<0.05$.This finding is in accordance with Wood *et al*[62] who reported a significant correlation between p53 expression and grades of dysplasia. Bansal *et al*[49] also found that number of p53 positive cells were significantly higher in specimens showing moderate or severe dysplasia than in specimens showing mild dysplasia. On the other hand, p53 expression in epithelial cell nests in gingivae of both light and heavy smokers appeared to be diminished in the center of the nests. Shi *et al*.[63], 1998 reported p53 positive cells only at the periphery of well differentiated cancer nest and suggested p53 positivity to be an early event in carcinogenesis.

In the current study, the IHC investigation showed that CK5 expression in the normal gingiva (control group) was confined mainly to the basal cell layer and associated with the cell membrane (membranous expression) with milder degree of expression suprabasally. The membranous expression is correlated to the CKs structure, being IFPs associated with plasma membranes and involving different components as desmosomes and hemidesmosomes[64].

Moreover, Barrett *et al*[65] had also mentioned that CK5 is strongly expressed in the basal cell layer of both keratinized and non-keratinized mucosae and its expression decreases superficially towards the spinous cell layer. Moreover, Matos *et al* upon[66] studying breast carcinomas reported that CK5 was expressed in basal epithelial cells of normal breast. Hence, they considered CK5 as important molecular marker used to distinguish basal phenotype in breast carcinomas.

In the present study, there was a noticeable increase in CK5 expression that appeared in all the examined gingival specimens of LS as compared to the control group in term of area percentage and optical density. A highly significant statistical difference was detected with $p<0.001$. This finding concurred with the results of *Vaidya et al*[67] who reported that the expression of CK5 is an early event of tobacco associated pathological changes. *Chatterjee*[5] also added that CK5 is expressed in suprabasal and spinous cell layers in case of dysplasia. According to *Kiyosue et al*[68] expression of CK5 was detected in the basal and parabasal layers of oral epithelium in the mild dysplastic specimens, and extended to almost all layers of the moderate dysplasia and severe dysplasia.

On the other hand, the HS in the present work showed a more intensive expression of CK5 in basal and suprabasal cell layers and extend suprabasally to include several layers of the spinous cells. However, in severe dysplastic features particularly in the epithelial cell nests, a striking decrease in expression was noted. This finding supports the assumption of *Vaidya et al*[67] that CK5 is not expressed with increasing grades of dysplasia. The wide variation in staining intensity among the specimens of heavy smoker group might be the leading cause to a non significant statistical correlation between heavy smoker and light smoker groups in term of optical density with $p> 0.05$.

Moreover, expression of CK5 in cell nests found in both smoker groups of the present study was very mild or almost missing particularly in their center and this support the histological finding where cells lose contact cohesiveness with their neighbors and start to move and arrange in whorled pattern forming cell nests. Also, it should be mentioned that the strong expression of CK5 in basal and spinous cell layers in HS was not as sharp demarcating lines delineating cell boundaries and cell-cell junctions as had been found in control or light smoker groups. Instead it became thicker and hazy which suggest a change in the cell-cell adherence, alteration in desmosomal junction and in the process of keratinization.

Thus, it could be speculated that tobacco smoking induced hyperplastic and dysplastic changes in gingival mucosa and the degree and severity of dysplasia seemed to be correlated with the frequency and duration of smoking. Finally it is recommended to give substantial attention to smoking as a health behavior to prevent and manage this hazardous habit. Moreover, schools should provide tobacco prevention programs to educate students about the dangers of smoking. Even with light tobacco consumption, epithelial cell nests (worrisome) were detected which would indicate how damaging even light smoking is for health, besides the different host susceptibility.

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