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HISTOLOGICAL AND IMMUNOHISTOCHEMICAL INVESTIGATION OF SMOKING-INDUCED CHANGES IN HUMAN GINGIVAL TISSUE: A FOCUS ON P16 AND CD34 EXPRESSION

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ABSTRACT

Background: Tobacco smoking is frequently linked to the development of oral cancer and precancerous lesions. It causes alterations that can be seen histologically even in smokers' clinically normal oral mucosa. p16 is a critical protein involved in the activation of apoptotic pathways, which is frequently altered during carcinogenesis. The significance of angiogenesis in the growth and proliferation of cancerous lesions has received a lot of attention. Changes in blood vessel size are a consequence of smoking. Using the endothelial cell marker CD34, researchers examined alterations in angiogenesis. Furthermore, as CD34 overexpression progresses from normal mucosa to dysplasia to carcinoma, it is thought to be a good marker for oral cancer progression.

Objectives: This work aimed to investigate the smoking-induced histological changes in gingival tissue, aside from assessing the immunohistochemical expression of protein 16 and CD34.

Material and methods: Gingival biopsies were obtained from healthy twelve middle-aged male patients during badly decayed teeth extraction. The control group consisted of two nonsmokers, while the smokers' group consisted of ten patients. Histological and immunohistochemical examinations, as well as morphometric and statistical evaluations of p16 and CD34 expression, were performed on the samples.

Results: In the current study, Smokers' gingiva showed some dysplastic alterations. Furthermore, compared to the control group, the smokers' group exhibited significantly higher P16 immunoreactivity. However, there was a non-significant increase in CD34 immunoexpression.

Conclusion. Our results concluded that although smoking is a known environmental risk factor for malignancy, smoking-induced oral epithelial dysplasia does not predict malignant transformation, since apoptosis and angiogenesis were not significantly affected.

KEYWORDS: Smoking; dysplasia; gingiva; p16; CD34; immunohistochemistry

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INTRODUCTION

Smoking causes premature death, illness, and disability throughout the world.[1] According to studies, smoking causes respiratory distress, coronary heart diseases,[2] complicated pregnancies, male impotence, osteoporosis,[3] senile cataracts, and delayed recovery.[4] Moreover, smoking has undeniably established itself as one of the primary causes of cancer.[5]

Oral cancer is an extremely serious oral ailment provoked by smoking. In accordance with the National Cancer Institute, smoking causes many cancers, including oral neoplasms (lip, tongue, etc.).[6] Oral carcinogenesis is a multi-step process including genetic defects and gene alterations.[7] Accumulated genetic changes cause the shift from normal oral epithelium to oral dysplasia and cancer.[8] Deactivation of the tumour suppressor genes p53 and cyclin-dependent kinase 2 (CDKN2) is a common early occurrence linked with potentially precancerous changes in the oral mucosa.[9] CDKN2 gene produces the protein p16, which is a negative cell cycle regulator.[10] p16 expression has been suggested as a marker for oral mucosal dysplasia and malignant transformation.[11,12,13] In non-small cell lung carcinomas and squamous cell carcinomas of the head and neck, a relationship between pl6 and smoking has been reported.[14,15,16]

Smoking has a deleterious effect on oral health. It is indicted with unaesthetic tooth staining, bad breath, impaired healing of wounds, increased risk of dental implant failure, precancerous conditions and oral cancer.[17] Furthermore, cigarette smoking has been related to periodontal affection as a determinant for the disease's onset and progression.[18] Increased loss of attachment and loss of alveolar bone are also factors to consider.[19] On probing, smokers, on the other hand, were found to have inadequate symptoms of clinical inflammation and bleeding.[20] The reduced intensity of the gingival response could be linked to vascular changes caused by smoking.[19]

The consequence of angiogenesis in the proliferation and enlargement of tumors has received a lot of attention.[21] Endothelial cells express various surface markers; some are found inherently, while others are augmented or produced de novo as response to various stimuli.[22] CD34, a surface antigen expressed in hematopoietic progenitor cells throughout the embryonic phase and thereafter in vascular endothelial cells, is a well-known indicator. It has a lifelong expression within these cells.[23] CD34 is a useful biomarker for predicting oral cancer development since it rises from normal mucosa to dysplasia to carcinoma.[24]

The current work aimed to examine the smoking-induced histological changes and immunohistochemical expression alterations of protein 16 and CD34 in gingival tissue.

MATERIAL AND METHODS

Study population

Twelve male individuals ranging from thirty to forty years old were included in the study. The patients were visiting the outpatient department at the Faculty of Dentistry, Cairo University for extraction of badly decayed teeth. Upon oral examination, a clinically healthy gingiva has been revealed. The study population was free from systemic disease and receiving no medication. The patients were classified into two groups. The control group (C) consisted of two healthy nonsmoker patients with clinically healthy gingiva. The smoker group (S) consisted of ten patients who smoked ≥ 10 cigarettes per day for ≥ three years.[25]

Sample collection

Gingival tissue biopsies obtained from both groups were of 1mm average size excised from the extraction sites at the interdental papilla. Informed consent was obtained from all patients after explaining the purpose of the work.

Ethical statement

The study protocol was approved by the Ethics committee of the Faculty of Dentistry, Cairo University, Egypt (approval No. 14/5/21).

Experimental procedures

All the gingival specimens were washed in sterile saline solution 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:

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 then coverslipped.

The procedure was carried out to analyze the altered histological features of gingival tissues and the structural changes evoked by smoking.

Immunohistochemical investigation

The samples were subjected to immunohistochemical analysis using the Mouse monoclonal antibody against CD34 (mouse monoclonal primary antibody, AM353-5 M, Biogenex, USA). Also, p16 monoclonal antibody (dilution: 1/40) was used (Biogenex, USA) in the gingival specimens to detect any possible dysplastic change**.**

Immunohistochemical Analysis

The immunohistochemical sections were examined by the image analyzer computer system using the software Leica Qwin. The immunoreactivity for CD34 and p16 was measured by 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.

Statistical analysis

The Leica Qwin image analyzer obtained and recorded data were statistically analyzed using SPSS version 25. The information were checked to be normally distributed by normality tests obtaining means and standard deviations using the Student t-test as a Parametric Test. p-value ≤ 0.05 and p-value ≤ 0.001 were considered significant and highly significant respectively.

RESULTS

Histological changes induced by smoking

The gingiva of group C appeared to consist of keratinized stratified squamous epithelium with normal histological features overlying the lamina propria. The papillary layer (part of connective tissue interdigitating with the epithelial ridges) appeared as loose moderately vascular connective tissue with fine collagen fibers and small size blood vessels (Figs. 1a & 1b).

The gingival specimens of group S revealed alteration from the normal histological features. Most of the specimens showed hyperplastic changes or different levels of dysplasia (Figs. 2- 4). The cytological alterations accompanied with the dysplastic epithelium were mainly hyperchromatism, nucleus pleomorphism and increased nuclear cytoplasmic (N/C) ratio (Figs. 2a & 2b). There was some degree of loss of normal configuration of epithelial ridges; they became broad and flattened (Fig. 2a). Clubbing of the epithelial ridges, acanthosis and basilar hyperplasia along with atypical mitosis in upper prickle cells (Fig. 2b) was observed. The granular or superficial cells and part of the higher prickle cells appeared with intracellular vacuolization or edema forming a sharply demarcated perinuclear

halo surrounding deeply stained nuclei of variable size (Koilocytic atypia/ koilocyte) (Fig. 2a).

Atypical mitotic figures were identified in many specimens and were numerous throughout the whole epithelial thickness in case of severe dysplasia (Figs. 3a & 3b). Moreover, disorganization and loss of polarity of the basal cells were also evident and were highly obvious in many specimens (Fig. 3a). Furthermore, pleomorphic bizarre-shaped nuclei were detected all over the epithelial layers (Fig. 3b). Karyorrhexis and karyolysis were detected associated with severe dysplasia (Fig. 3b).

Concerning 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. 4) Abnormal keratinization was also identified. Some specimens appeared non keratinized (Figs 2 & 4) and may present irregular surfaces composed of swollen faint cells with pyknotic nuclei (Fig. 2a).

Unlike group C, the lamina propria of group S showed a variable degree of vascular changes in the form of vasodilatation and extravasations of RBCs (Fig. 5). However, a rich capillary network was identified as subepithelial 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. Moreover, it may encompass wide blood vessels, and sometimes aggregate in clusters pattern of hyperchromatic cells (Figs. 5c & 5d).

Immunohistochemical expression of p16 and CD34 p16 expression

In the gingival sections of group C, p16 protein was negatively expressed in both epithelium and lamina propria. Moreover, higher magnification of group C sections confirmed negative p16 protein immunohistochemical expression in basal and parabasal epithelial layers as well as in the lamina propria (Figs.6a & 6b).

In comparison to group C, the sections obtained from group S revealed mild to moderate expression of p16 protein in the basal and parabasal cell layers of the gingival epithelium. In addition, higher magnification of the same group specimens showed that the immunohistochemical expression of protein p16 was both nuclear and cytoplasmic in the basal and parabasal cell layers while the lamina propria showed negative staining (Figs. 6c & 6d).

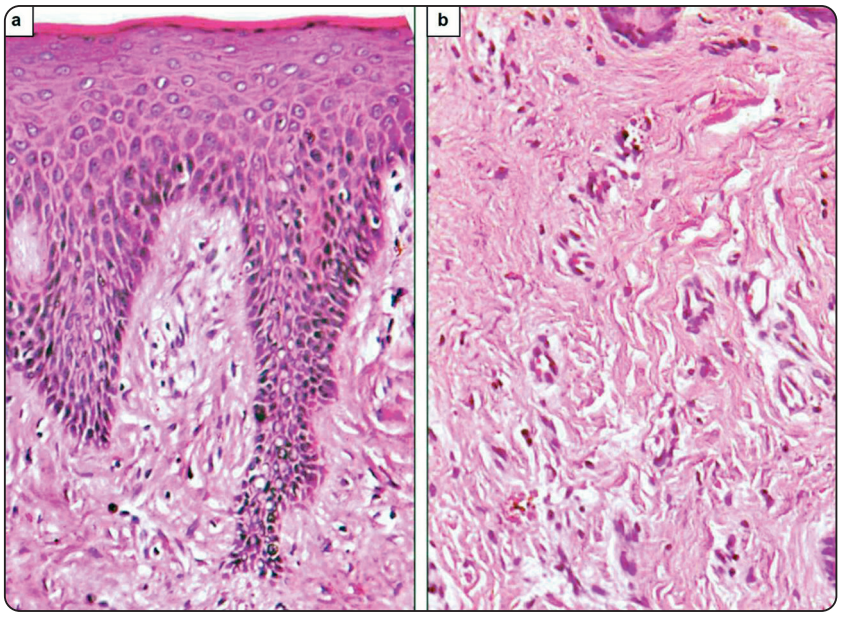

Fig. 1 (a) A photomicrograph of the gingiva of the control group, showing normal histological features of keratinized stratified squamous epithelium (H & E Stain, Orig. Mag. 100). (b) The lamina propria is composed of moderately vascular connective tissue, containing few mononuclear inflammatory cells (H & E Stain, Orig. Mag. 400)

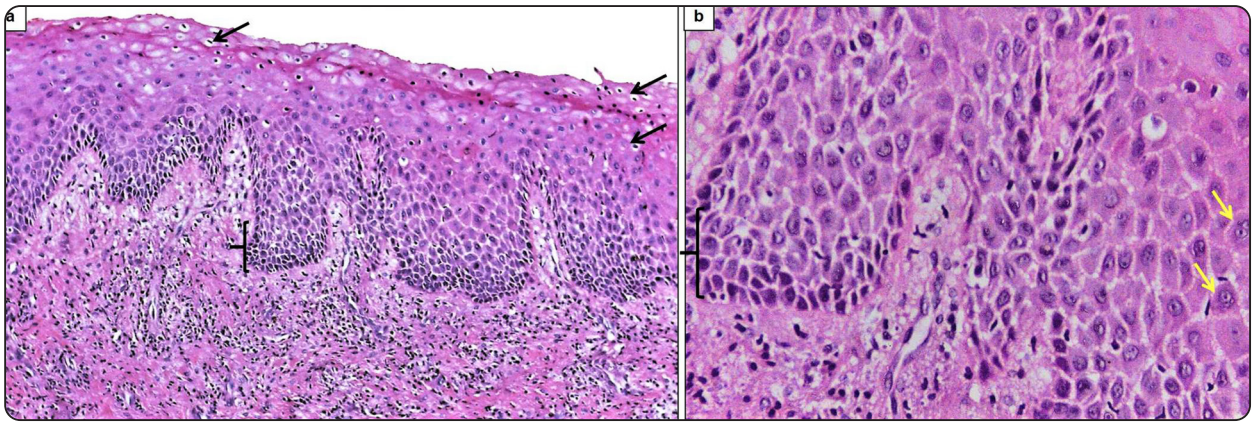

Fig. 2 (a) A photomicrograph of the gingiva of smoker group showing clubbing of the epithelial ridges, acanthosis and basilar hyperplasia occupy the basal third (black bracket), a picture of koilocytic atypia at the superficial squamous cells and some upper prickle cells (black arrows) irregular nonkeratinized epithelial surface. Lamina propria showing heavy inflammatory cell infiltrate (H & E Stain, Orig. Mag. 100). (b) A higher magnification showing remarkable basilar hyperplasia (black bracket) and atypical mitotic figures and mitosis in the upper prickle cell layer (yellow arrows) (H & E Stain, Orig. Mag. 400)

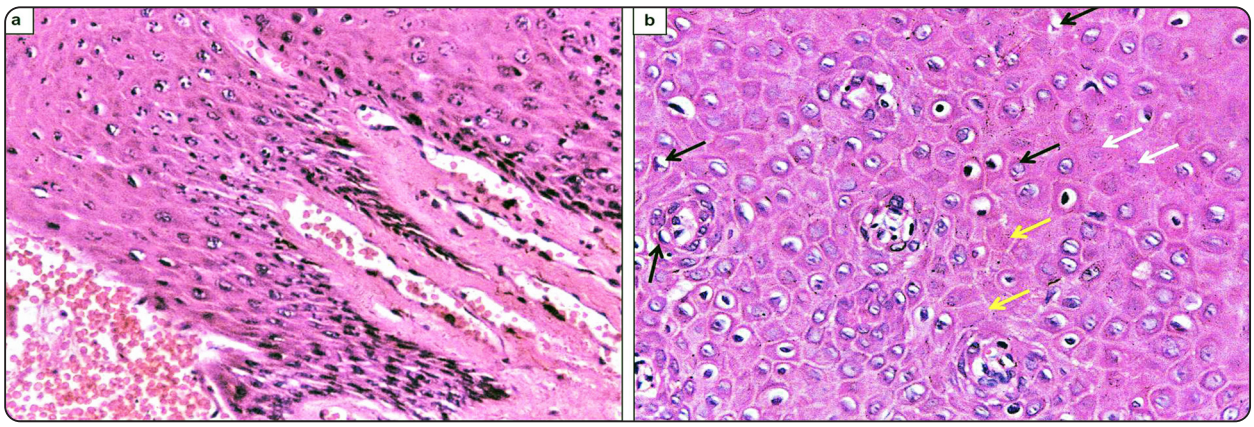

Fig. 3 (a) A photomicrograph of the gingiva of the smokers group showing loss of basal polarity and nuclear pleomorphism (H & E Stain, Orig. Mag. 200). (b) a higher magnification showing increased mitosis with excessive number of atypical mitotic figures (black arrows), karyorrhexis (white arrows) and karyolysis (yellow arrow) in addition to loss of normal arrangement of prickle cells (H & E Stain, Orig. Mag. 400).

CD34 expression

Specimens from group C showed mild immunohistochemical reactions to CD34 in the blood vessels of the gingival connective tissue (Fig. 7a & 7b). However, immunohistochemical expression of CD34 in the gingival specimens of group S showed a mostly moderate reaction in the blood vessels (Fig. 7c & 7d).

Statistical analysis of immunohistochemical results using Student t-test

The immunoexpression of p16 protein in the gingiva of group S showed a significantly increased immunoreactivity compared to those of group C with p= 0.002 using the t-test. However, the gingival specimens of the smokers' group CD34 immunoexpression in correlation to those of group C exhibited a non-significant increase with p-value = 0.23 (Table 1) (Figs. 8 & 9).

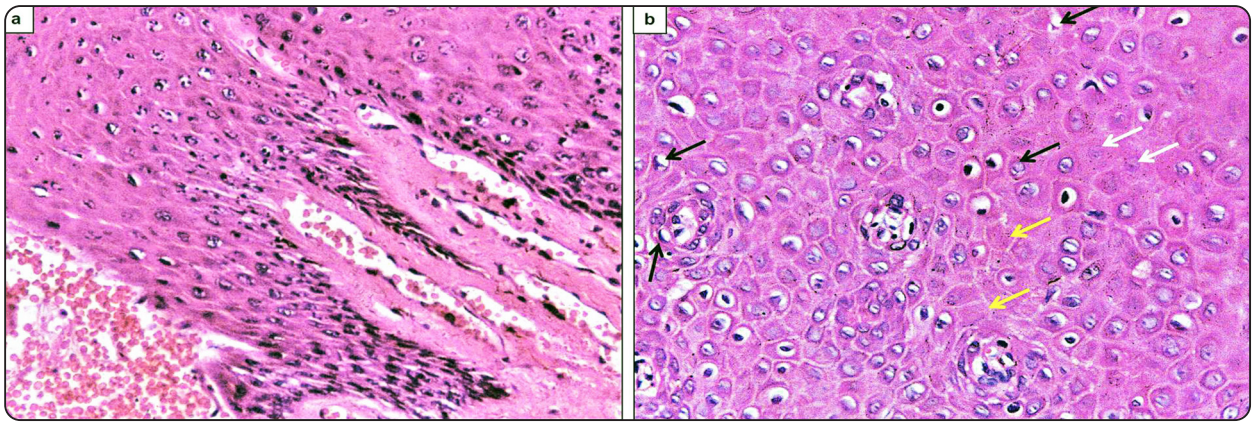

Fig. 4 (a,b) A photomicrograph of the gingiva of smoker group showing loosely oriented cells losing cohesiveness and begin a cell nest formation at the center of rete peg (yellow arrows) (H & E Stain, Orig. Mag. 400)

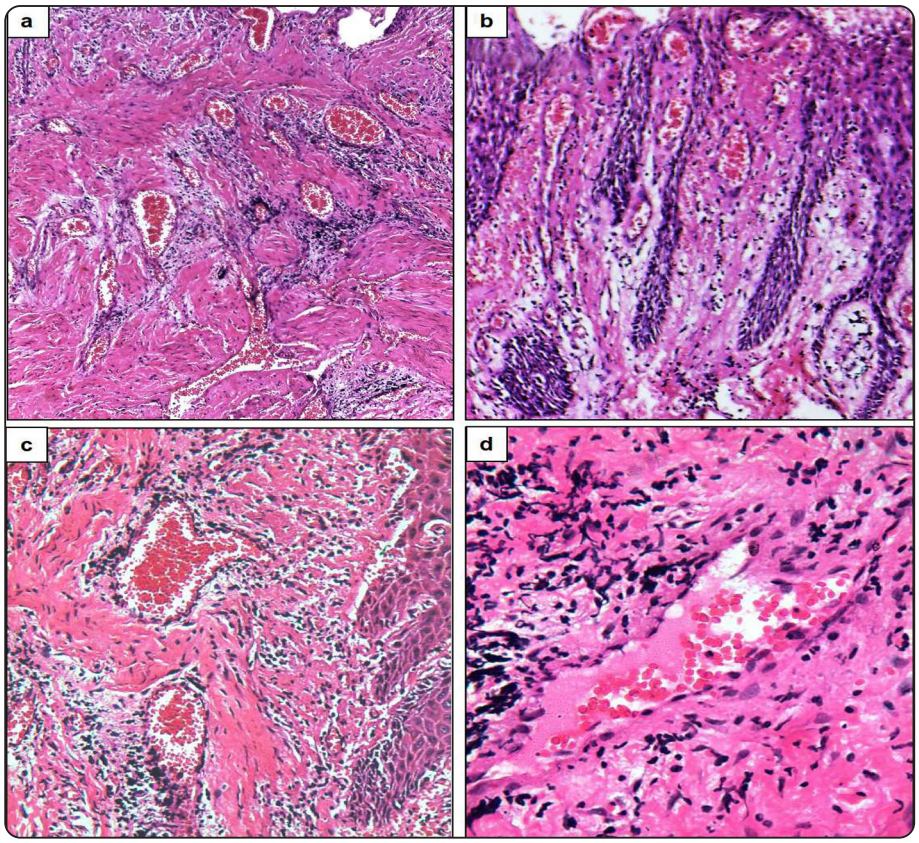

Fig. 5 (a, b) photomicrographs of the gingiva of smoker group showing multiple dilated blood vessels subepithelialy, increase in the number of hyperchromatic mononuclear inflammatory cells (H & E Stain, Orig. Mag. 100). (c, d) higher magnification showing heavy inflammatory cell infiltrate, dilated blood vessels engorged with RBCs and surrounded by bands of hyperchromatic inflammatory cells. Increase in the thickness & density of collagen bundles (H & E Stain, Orig. Mag. 400).

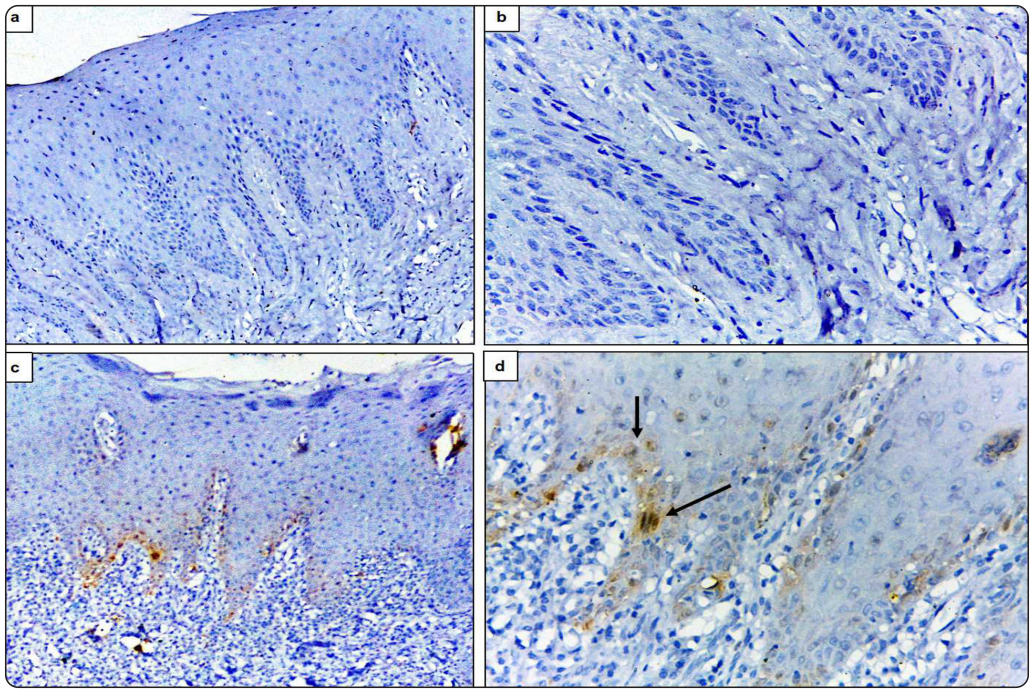

Fig.6 (a) Immunohistochemical photomicrograph of the control group showing negative staining of the P16 protein in both epithelium and lamina propria of the gingiva (P16 X 100). (b) Higher magnification of the gingiva of control group showing negative immunohistochemical staining in basal and parabasal epithelial layers (arrows) as well as in the lamina propria (P16 X 400). (c) Immunohistochemical photomicrograph of the smoker group showing mild to moderate staining of P16 protein in the basal and parabasal cell layers of the gingival epithelium (arrows) (P16X 100). (d) Higher magnification of the gingiva of smoker group showing both nuclear and cytoplasmic staining of P16 in the basal and parabasal cell layers (arrows) and negative staining of the lamina propria (P16 X 400)

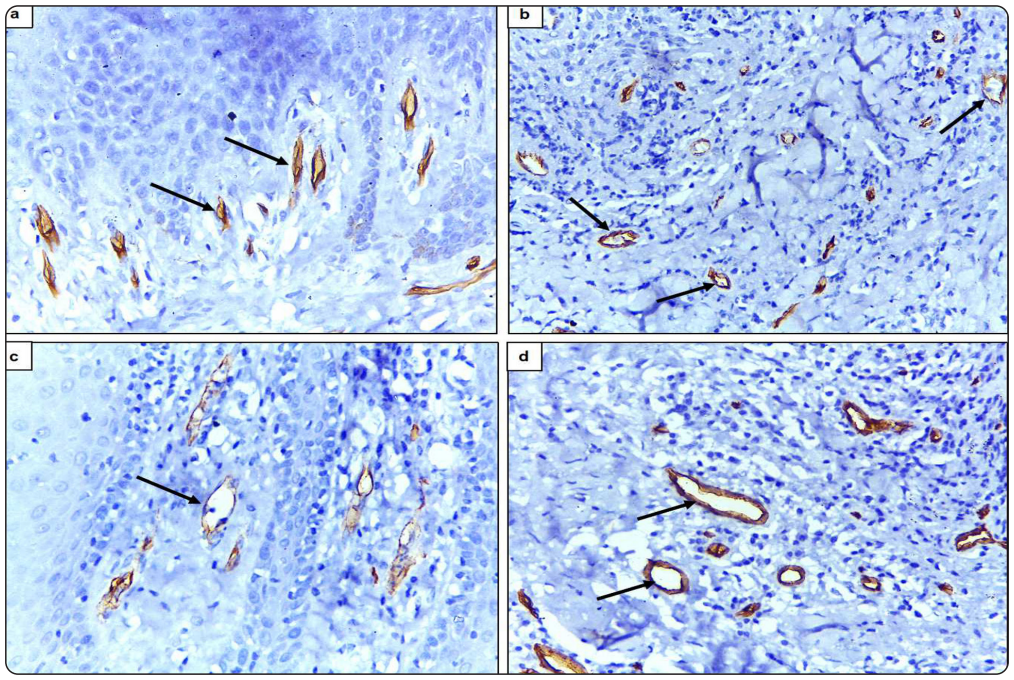

Fig. 7 (a) Immunohistochemical photomicrograph of the control group showing mild reaction to CD34 in the blood vessels of the lamina propria (arrows) (CD34 X100). (b): Higher magnification showing mild immunohistochemical reaction to CD34 in the blood vessels of the lamina propria of the control group (CD34 X 400). (c): Immunohistochemical photomicrograph of the smoker group showing mild to moderate staining of CD34 in the blood vessels of the lamina propria (arrows) (CD34 X 100). (d): Higher magnification showing moderate staining of CD34 in the blood vessels (arrows).

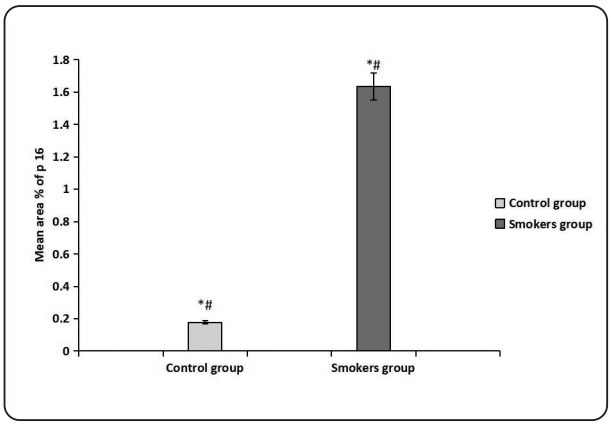

Fig. 8 A graph showing the immunoexpression of P16 protein in the gingiva of the control group compared to those of the smokers' group. The results are expressed as mean ± SD and # indicates significant difference between each two groups using T-test at *p value < 0.05

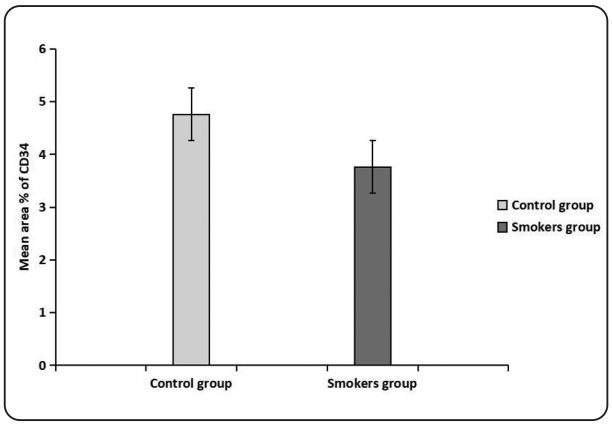

Fig. 9 A graph showing the immunoexpression of CD34 in the gingiva of the control group compared to those of the smokers' group. The results indicates non-significant difference between the two groups using T-test at p value > 0.05

DISCUSSION

Smoking is a significant hazard for epithelial dysplastic alterations and eventual oral squamous cell carcinoma.[26] The gingival specimens taken from the smokers' group showed varying degrees of dysplastic changes in the current investigation. This is consistent with earlier research suggesting that smoking is not only a significant risk factor for oral cancer, but that it may have the greatest influence during the earliest stages of oral carcinogenesis, before the malignant transformation.[27,28] Furthermore, some oral malignancies can develop from what appears to be healthy mucosa.[29,30]

Pleomorphism, hyperchromatism, a rise in the nuclear-cytoplasmic (N/C) ratio, accelerated mitosis, and increasing size and number of nucleoli were all seen in this research. According to Bouquot et al.[30] pleomorphism is a rare occurrence outside of malignant and precancerous tumours. Aberrant mitotic activity was defined as atypical mitotic figures or normal mitosis in a position other than the stratum germinativum. Some authors have speculated that it is an indication of precancerous changes.[30] In accordance with the World Health Organization (WHO), the increasing size and number of nucleoli are cytological hallmarks of dysplasia. Raulin et al.[31] discovered that cells subjected to high nicotine levels had more nucleoli, signaling that RNA synthesis was altered. Moreover, changes in mitosis and hyperchromatism could cite to the DNA damage induced by smoking.[32]

In this study, severe dysplasia exhibited pyknotic nuclei, karyorrhexis, and karyolysis. These features were considered indicators of necrosis that might be produced in some cells by the dysplastic dividing neighbours that overgrew their nutritional supply. The formation of cell nests was as well observed in some smokers' gingival samples in this work. It is regarded as a sign that, upon its presence, epithelial dysplasia should be upgraded.[30] Acantholysis or loss of cellular cohesiveness may likewise contribute to abnormal basal cell orientation or loss of polarity. The current investigation encountered changes in tissue architecture and rete peg alteration. This was a worrying finding for some authors, who assumed it was caused by basal hyperplasia and polarity loss.[30,33]

Different degrees of keratinization, ranging from hyperkeratosis to complete loss of keratinization, have been seen in the ongoing investigation.[34,35]

Smoking-induced hyperkeratosis was found to be a protective measure against the smoke noxious and thermal effects on gingival tissues. On the contrary, some authors revealed that smoking causes keratinization loss, which they attributed to inflammation presence, which can interfere with epithelial maturation, affecting tonofilament structural arrangement and keratinization.[35,36] The presence of a glycogen-like substance inside epithelial cells was mentioned by some researchers, indicating a disruption in the process of cellular differentiation. This glycogen-like substance dissolved during histological preparation is thought to give the cells a vacuolization, or koilocyte, look.

Acanthosis was a common finding in group S gingival epithelium. Several studies had previously reported an increased thickness of epithelium in specimens obtained from the gingiva of smokers.[35,37,38] Some others contributed acanthosis to the rise in local temperatures and by-products produced from tobacco oxidation. Others suggested that acanthosis might be a result of accelerated mitotic rate and cellular oedema demonstrated in the prickle cells.[39]

In the current study, heavy inflammatory cells infiltrated the lamina propria of the gingiva of the smokers' group. Contact irritation from smoking or local penetration of cigarette by-products deep into the lamina propria or even passing through gingival vasculature could evoke inflammatory reaction. Some authors noted that acute cigarette smoking is chemotactic to neutrophils and macrophages. Moreover, smoking results in tissue damage by increasing lipid peroxidation and matrix degradation products. Furthermore, tobacco smoke was found to block Leukotriene A4 Hydrolase, which the body needs to heal inflammation.[40,41]

In the current work, changes in vasculature, particularly vasodilatation and extravasation, were demonstrated in the smokers' group and might be postulated as a sequela of inflammation. This finding agrees with Baab and Oberg[42] who indicated that smoking contributed to an increase rather than a decrease in gingival circulation in human. According to Jalayer Naderi et al.[35] investigating 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[25] reported that the percentage of small-sized blood vessels had increased in smokers. Extensive subepithelial vascularity is considered a feature in oral dysplastic lesions.[43] Furthermore, dysplastic lesions activate an angiogenic switch, increasing subepithelial microvasculature and causing stromal inflammation.[44,45,46]

The CDKN2 gene encodes the protein p16, which is a cell cycle negative regulator that controls the cellular passage from phase G1 to phase S. p16 is a critical protein implicated in apoptotic pathways activation, which is often altered during carcinogenesis. Environmental exposure, such as tobacco smoke, is frequently linked to carcinogenesis.[47] The value of p16 expression in dysplastic and nondysplastic lesions has been the subject of numerous investigations. Anti-p16 antibody immunohistochemistry of oral premalignant and malignant lesions has yielded mixed results, with some studies showing decreased expression[11,12,13] and others revealing overexpression.[48,49]

In the current investigation, gingival sections from group C showed negative expression of the p16 protein in both the epithelium and lamina propria. This is consistent with a study by Bradley et al.[50] that found negative p16 expression in normal non-dysplastic mucosa. Moreover, Agarwal et al.[51] also reported that p16 immunoreactive cells are not observed in normal oral mucosa.

In comparison to group C, the smokers' sections showed low to moderate nuclear and cytoplasmic expression of p16 protein in the gingival epithelium's basal and parabasal cell layers but negative staining in the lamina propria. Tarakji,

Kujan and Nassani[10] showed that basal and suprabasal cells in the clinically normal oral epithelium of the smokers had significant p16 nuclear staining, which is similar to the current study findings. Some previous investigations only reported nuclear staining,[52,53] whereas others claimed both nuclear and cytoplasmic staining.[54] Cytoplasmic staining was found to be negative due to p16 inactivation.[51]

Smoking has reportedly been linked to the inactivation of the p16 gene.[55] Because of a mutation with enhanced expression or a deletion with a absence or reduced expression, oral premalignant and malignant lesions may have increased or decreased p16 expression.[51]

Although p16 gene inactivation is frequently observed during early carcinogenesis, Bradley et al.[50] also revealed a significant correlation between the lack of p16 expression and the severity of dysplasia. As a result, their findings advocated that p16 expression is ineffective in distinguishing dysplastic from non-dysplastic oral lesions. In contrast, squamous dysplastic lesions in the uterine cervix typically overexpress p16 as a result of infection with high-risk HPV strains. However, Bouland et al.[56] found that p16 overexpression was significantly related with low tobacco consumption and both longer recurrence-free and overall survival. Therefore, overexpression of the p16 protein could be viewed as a good prognosis factor for recurrence-free survival and overall survival for OSCC patients.

In previous work, the expression pattern of p16 was investigated in various grades of dysplastic epithelium. They reported that p16 expression in mild epithelial dysplasia was mostly identified in the basal and suprabasal cell layers, whereas in moderate dysplasia, it was found in the basal, suprabasal, and prickle cell layers.[51] Other investigations came up with similar findings.[57]

Since p16 is implicated in cell cycle regulation, its expression may differ depending on cell turnover periods, which vary among oral mucosa types.[58] Some authors[59] discovered the existence of p16 protein in normal oral mucosa using Western blotting. Moreover, others[60] described the presence of p16 protein in a wide range of normal human tissues, including the salivary gland, albeit it was not revealed in the epidermis. That study excluded normal oral mucosa, and many researchers who have utilized immunohistochemistry to assess p16 status in oral squamous cell carcinomas and premalignant lesions have commonly used stromal cells rather than normal oral epithelium as a control tissue.

Even in smokers' healthy-appearing mucosa, smoking has been shown to affect p16 immunoexpression.[10] Additionally, several writers claimed that the inflammatory and hyperplastic oral epithelium overexpressed the gene p16. An earlier investigation found that in non-neoplastic oral epithelium, smokers had considerably higher p16 immunoexpression than non-smokers. Smokers with oral epithelial hyperplasia displayed considerably higher p16 immunoexpression among the non-neoplastic oral epithelium than non-hyperplastic patients. According to the authors, p16 overexpression may work as a defense against malignant transformation.[61]

These findings highlight the importance of p16 in protecting against DNA damage and cell death. The significant increase in p16 immunoreactivity in the smoker group of the current work was closely connected to the histological finding of increased cell growth, especially in the basal and parabasal cell layers.

In the ongoing study, the control group's specimens had weak immunohistochemistry reactivity to CD34 in the gingival tissue's lamina propria blood vessels. Those in the smokers' group, on contrary, reacted in a mainly moderate manner. Anti-CD34 antibodies detect CD34, a transmembranous sialoprotein, in both endothelial cell progenitors and mature endothelial cells. As CD34 overexpression progresses from normal

mucosa to dysplasia to carcinoma, it is expected to be a good marker for oral cancer progression. Smoking has little effect on vascular density according to Mirbod, Ahing and Pruthi.[19] However, cigarette smokers, had a higher percentage of smaller blood vessels and a lower percentage of larger vessels. These findings are consistent with the current study, which found a non-significant increase in CD34 immunoexpression in the gingival specimens of smokers compared to group C.

On contrary, a significant increase in CD34 immunoreactivity was previously detected in smoking oral lichen planus patients compared to non-smoker patients. They related their results to the influence of smoking on enhancing releasing of pro-inflammatory cytokines.[62]

CONCLUSION

Smoking causes hyperplastic and dysplastic alterations in a smoker's gingiva. However, in dysplastic specimens, p16 expression was significantly higher, indicating acanthosis and basilar hyperplasia as signs of dysplasia. Although smoking-induced gingival epithelial dysplasia is a risk factor, it does not predict malignant transformation since apoptosis and angiogenesis were insignificantly affected.

RECOMMENDATIONS

Further studies using different tumor markers are recommended to differentiate between low risk and high risk gingival dysplasia caused by smoking.

Funding

Authors confirm that they didn't receive any fund to do this work.

Data availability

The authors declare that the data supporting the findings of this study are available within the manuscript.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval This study was carried out at the Faculty of Dentistry, Cairo university, Egypt, in accordance with the regulations and approval of the Ethics committee, Cairo university (Approval No. 14/5/21).

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