CD34 and Wnt3 expression in potentially malignant oral disorders

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ABSTRACT:

Background: Potentially malignant oral disorders (PMOD) are common precursors of oral squamous cell carcinoma (OSCC). Neoangiogenesis and signalling are important intermediate biomarkers that may govern the progression of dysplastic mucosa into carcinoma.
Aims: Evaluate the importance of CD34 and Wnt3 expression in PMOD and OSCC in relation to their clinicopathological parameters.
Settings and Design: Prospective cross-sectional study.
Materials and Methods: Immunohistochemical staining for CD34 and Wnt3 was performed for 41 samples. These included 27 PMOD, six OSCC and eight normal gingival and alveolar mucosa. Analysis of variance (ANOVA) and post-hoc tests were applied. P<0.05 was considered statistically significant.
Results: CD34 expression showed a significant difference between groups (P<0.05). CD34 expression decreased in patients who had PMODs, and it was seen to correlate with clinical staging in OSCC patients. The alveolar epithelia had lower microvessel density (MVD) (9.3±.88) than the gingiva (17.47±5.09) (P<0.05), whereas the lichen planus without dysplasia had lower MVD (8.85±3.95) than both the gingiva and the dysplastic epithelia (14.46±3.89) (P<0.05). On the other hand, Wnt3 expression was not detected in the alveolar mucosa, but scattered perinuclear and nuclear expression in the gingival mucosa was observed. Cytoplasmic Wnt3 expression was seen in all oral lichen planus (OLP) and some leukoplakia cases with no nuclear staining, whereas its expression in proliferative verrucous leukoplakia was only nuclear. Furthermore, OSCC showed both cytoplasmic and nuclear expression.
Conclusion: MVD may represent a useful biomarker preceding oral cancer development. It increases from normal mucosa to dysplasia to carcinoma. Aberrant cytoplasmic expression of Wnt3 is detected in PMOD and OSCC. Thus, Wnt3 may be involved in disease progression.
Keywords: potentially malignant oral disorders, oral squamous cell carcinoma, microvessel density, CD34, Wnt3. **(J Bagh Coll Dentistry 2017; 29(3):59-67)**

INTRODUCTION:

A precancerous lesion is a benign, morphologically altered tissue that has a greater than normal risk of malignant transformation and is not necessarily showed clinical alteration. Malignant transformation potential is defined as the likelihood of cancer being present in a precancerous condition or lesion, either at initial diagnosis or in the future.(1) Dysplasia represents a spectrum of changes with no precise, distinct stages. The inconsistency in dysplasia assessments is attributed to the lack of guidelines for interpretation. Pathologists still need to improve consistency in interpreting transitions in the grades of severity of dysplasia.(2)

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This term conveys that not all lesions and conditions described under this term will transform into cancer; rather, this term refers to a family of morphologically altered lesions that may have increased the potential for malignant transformation.(3) Oral squamous cell carcinomas (OSCC) appear to arise in the apparently normal mucosa, in otherwise healthy people; however, some lesions are preceded by clinically obvious PMOD.(4) The main PMOD described in the literature are actinic cheilitis, erythroplasia, leukoplakia (homogeneous, speckled, nodular-verrucous, proliferative-verrucous, sublingual, candidal, syphilitic), erosive oral lichen planus (OLP), submucous fibrosis, palatal lesion in reverse smokers, and other systemic conditions associated with PMOD.(4) Erosive OLP has a 0.4–2.5% chance of carcinoma transformation; its behaviour differs from that of other OLP forms or lichenoid eruptions that are not reported to be PMODs.(1) The screening of PMODs, particularly those that appear normal but have tumorigenic potential, is crucial to treatment success. The early diagnosis

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of oral lesions is the key to preventing progression to advanced stages of the disease.[5] Currently, visual and cytology-based techniques are used by clinicians to detect dysplasias and early stage OSCCs. These approaches are limited in their ability to judge the severity of lesions and are primarily useful after the appearance of visual changes.[5]

Angiogenesis is a complex process that encompasses the growth and migration of endothelial cells and capillary morphogenesis. CD34 is a sensitive marker for endothelial cells. It is a transmembranous glycoprotein that functions in early hematopoiesis.[6] Immunohistochemical staining for CD34 is used to evaluate microvessel density (MVD) in numerous tumours, and intratumoral MVD has been established to be useful in predicting relapse or metastasis.[7] Siar et al. indicate that formation of angiogenic squamous dysplasia-like complexes in oral precursor lesions may be a useful predictive marker of oral malignancy.[7]

On the other hand, Wnt paracrine signalling molecules consist of a family (19 members in humans) of well-conserved secreted glycoproteins.[8] Wnt3 expression is detectable in dysplastic lesions (oral leukoplakia), but not in normal oral epithelia, suggesting a potential involvement of this marker in dysplasia.[9]

The present study aimed to investigate the importance of CD34 and Wnt3 as markers for malignant transformation of PMODs in comparison to their importance in oral carcinomatous lesions and to correlate the findings with the clinicopathological parameters.

MATERIALS AND METHODS:

This prospective cross-sectional study was conducted in Sulaimani, during the period from April 1st, 2012 to the end of November 2014. The study was approved by the ethical and scientific committees of the Faculty of Medical Sciences. Signed informed consent, medical history, and clinical data were obtained from each participant before sample collection.

A total of 41 samples were collected. The negative control group included eight clinically normal-looking oral epithelia taken from non-smoking patients aged between 20 and 40 years. Four of these were keratinized oral mucosa obtained from the gingiva of patients undergoing impaction removal; these are thick mucosa with papillary hyperplasia. The other four samples were non-keratinizing lining alveolar mucosa that showed poorly developed papillae (thin mucosa) obtained during dental implantation. The test group included 33 cases; 27 PMODs, and six OSCCs. The PMODs subgroup included 21 cases of erosive lichen planus and six cases of leukoplakia diagnosed during the study period.

Formalin fixed paraffin embedded blocks were prepared, and three serial tissue sections were cut. One section was stained with haematoxylin and eosin for histopathological diagnosis and dysplastic staging. The other sections were subjected to immunohistochemical staining. The slides were kept in the oven (60ºC, six hours) and then tissue sections were deparaffinised, rehydrated and washed with phosphate buffer saline (PBS) (five minutes). The sections subjected to antigen retrieval (boiling citrate buffer pH 6, for 15 minutes), then they were allowed to cool and washed with PBS (three minutes); the excess buffer was tapped off gently. Hydrogen peroxidase was applied (37ºC for 10 minutes) followed by protein blocker (37ºC for 10 minutes). Next, primary monoclonal anti-CD34 antibody (1:150) and polyclonal anti-Wnt3 (1:100) (Abcam) were applied separately, one on each of the sections subjected to staining in a humid chamber (37ºC for 45 minutes). Complement was added (10 minutes). Goat anti-rabbit horseradish peroxidase conjugate was applied (15 minutes), followed by diaminobenzidine (DAB) chromogen (five minutes in a dark field), then counter-stained with hematoxylin for 20 seconds and washed in distilled water. Lastly, sections were dehydrated, cleared and mounted. The negative controls for the staining procedure were performed by omitting the primary antibody and applying the antibody diluents alone. The positive control for CD34 involved internal staining of arterioles in the tissue sections,[7] whereas OSCCs served as a positive control for Wnt3.[9]

Sections stained with CD34 were initially evaluated at low power (x40) to identify four ‘hot spot’ areas in the connective tissue papillae and lamina propria. The MVD counting was performed at a higher magnification (x400). The CD34 positive endothelial cells or clusters of endothelial cells that were separate from adjacent microvessels or other connective tissue elements were regarded as a single countable microvessel. The average MVD score was calculated.[7] In normal oral epithelia and PMODs, microvessels located just underneath the epithelium were considered, whereas in OSCCs those located in between the islands were considered.

Sections stained with Wnt3 were evaluated according to its distribution in the epithelial layers (basal, suprabasal and spinous layers), and intracellular localisation (nucleus

and cytoplasm). Four fields were randomly selected to identify positive cells at x400 [9].

One-way ANOVA test and post-hoc tests (Tukey's test) were applied to estimate the differences among and between groups by using SPSS 20.0 software. P<0.05 was considered statistically significant.

RESULTS:

The clinical features of patients are illustrated in Table 1. The PMODs involved a wide age range (27–75 years) with nearly equal age group distribution. The cheek was the predominantly affected site. Most of the patients were non-smokers. PMODs had an equal frequency of presentation as homogeneously white (48.15%) and red and white lesions (48.15%) (Figure 1). On the other hand, oral carcinomas were seen more frequently in older age groups; 66.67% presented with an ulcer, and 33.3% involved the cheek. However, none of the patients reported being smokers (Table 1). Nevertheless, 50% of OSCC patients were at an advanced clinical stage (Table 2).

Histologically, four erosive OLPs (19.05%) displayed dysplastic changes, whereas all cases of leukoplakia showed dysplastic changes. Thus, the total number of PMODs with dysplastic changes was ten (37.04%). Five of these (50%) were in grade two and four cases (40%) were in grade three. Lastly, four cases (66.67%) of OSCCs were categorized as well-differentiated carcinoma (Table 2).

After immunohistochemical staining, six samples of PMODs were excluded (either they contained not enough surface epithelium to be evaluated or the connective tissue tore). Therefore, the remaining 21 samples of PMODs included 18 OLPs (16 without dysplasia and 2 with dysplasia) and three cases of leukoplakia were examined for CD34 and Wnt3 expression.

Immunohistochemical expression of CD34 was assessed in terms of MVD. In both OSCCs and PMODs the MVD did not differ in relevance to clinical parameters except for the significant Pearson correlation with clinical staging (r = 0.94, p =.006), (Table 3). Histologically, the mean of MVD revealed higher expression in gingival mucosa than in the alveolar mucosa (17.47 vs. 9.3, P<0.05). In addition, there was a significant difference between lichen planus without dysplasia (8.85, P<0.05) on the one hand and keratinized gingival epithelium and dysplastic epithelium on the other (Figure 2 and Table 4).

The immunohistochemical expression of Wnt3, was negative in alveolar oral epithelia, whereas the gingiva showed scattered perinuclear and positive nuclear expression. In OLPs and leukoplakia, the expression of Wnt3 was mostly cytoplasmic, whereas its expression in proliferative verrucous leukoplakia was nuclear. Wnt3 was positive in the OSCCs, both cytoplasmic and nuclear (Figure 3 and Table 5).

DISCUSSION:

The incidence and prevalence of PMOD in selected populations vary considerably.[10] In Sulaimani City, Sabri [11] reported the prevalence of these lesions as 1.5%. The reported prevalence of leukoplakia is 2%, but worldwide prevalence for other types of PMOD is unknown.[12] The rate of progression of PMOD to OSCC is estimated to be 36% when moderate epithelial dysplasia is present, but the rate increases up to 50% in lesions with severe dysplasia.[13]

There is a growing body of evidence that the angiogenic process commences in the pre-malignant stages of most cancers.[14, 15] The presence of capillary blood vessels closely juxtaposed to and projecting into metaplastic or dysplastic squamous bronchial epithelia is a significant morphology to identify the pre-invasive lesion.[16] Previous authors have shown that a statistically significant increase in vascularity occurs during the transition from normal oral mucosa to different grades of dysplasia to invasive carcinoma,[7, 17] whereas other authors have failed to demonstrate such an increase.[18] This study showed that MVD in patients had PMODs did not relate to any of the reported clinical parameters (sex, age, site, smoking, and presentation). It is worth to mention that Lindeboom et al.[19] also did not find significant differences between the gingival capillary density of smokers and non-smoking, healthy individuals. Furthermore, MVD showed no significant correlation with the dysplastic histological grade. The latter finding may be due to the few reported dysplastic lesions. Concerning OSCC, MVD is exclusive in its relation to clinical staging. Tae et al.[18] also mentioned that there were no relationships among various clinicopathological factors and MVDs in head and neck SCC.

The previously reported mean values of MVD in normal oral mucosa were either higher[18, 20–21] or lower [22– 24] than that reported in this study. These variations may be related to variations in sample location (gingiva, alveolar and buccal mucosa), sample size and CD34 interpretation. Some authors examined a larger field area at a lower magnification and did not justify the depth, whereas others depended on

digital images. Nevertheless, Tahir et al.[25] estimated the mean MVD obtained from five samples of normal oral mucosa to be 9.2, which is approximately equal to our result found in the alveolar mucosa. We reported a difference in MVD in relation to sample site variations in clinically normal-looking mucosa. The gingival mucosa showed higher MVD than the alveolar mucosa as well as to the dysplastic lesions; that may be related to physiological need rather than pathological changes.[17]. This finding was in line with the results of Tae et al.[18] However, in this study, the normal alveolar mucosa was found to have lower MVD than dysplastic PMODs; nevertheless, this difference did not reach a significant level, as Tae et al. reported. The present findings of MVD regarding dysplastic PMOD lesions are in agreement with those of the previous authors.[7, 17] Thus, increasing vascularity as the epithelium progresses from normal non-keratinized epithelium to dysplasia indicates that CD34 may be an intermediary biomarker in PMODs.

OLP is a chronic autoimmune disease with an inflammatory origin. It satisfies all the prerequisites of hypoxia that are essential for angiogenesis.[22] Previous authors clarified that angiogenesis is significantly increased in OLP compared to normal oral mucosa.[21, 22] The result of the present study did not support this concept. We observed that connective tissue papillae with dense lymphocyte aggregation underneath severely degenerated epithelia showed few blood vessels.

Wnt proteins consist of two groups based on their ability to activate Wnt/β-catenin signalling. Wnt3 is an efficient activator of the canonical pathway.[26] In the absence of Wnt, cytoplasmic β-catenin immediately phosphorylated and degraded, thus disappearing.[27, 28] In this study, normal alveolar mucosa did not show Wnt3 expression; this indicates that no signalling induction is required in the non-keratinized thin mucosa. While in the gingival mucosa, expression of Wnt3 is limited to the nuclei or perinuclear area in dispersed cells. This finding contradicts the results of a previous study on Wnt3 expression in the gingiva that showed negative expression [29] and supported that for negative expression of Wnt5a in the skin.[30] There are no previously published articles on the role of Wnt in the normal mucosa of other organs. The function of Wnt3 in the nucleus or the cytoplasm has not yet been discussed. However, a study comparing the distribution of the nucleus and cytoplasmic compartment to gene expression indicates that various membranous proteins (like Wnt3 in our study) can be stored in the nucleus or cytoplasm in relation to the ubiquitin cycle.[31] Such localisation reflects the movement and segregation of molecules that do not relate to pathological changes.

Different molecular studies were used in OLP to explore the possibility of malignant transformation, but this is the first study evaluating the expression of the Wnt family in OLP. Wnt5a expression in cutaneous lichen planus has shown over-expression in all dermis and epidermis layers when compared with negative normal healthy skin.[30] In our study, Wnt3 in OLP was characterised by positive cytoplasmic expression (lack of nuclear staining). Thus, positive aberrant cytoplasmic Wnt3 expression suggests its role in the disease process. Similarly dysplastic changes within OLP did not alter this expression. These findings can be attributed to the lack of basal cells, which are responsible for cell renewal and signal induction. Furthermore, the existence of positive and negative cytoplasmic with negative nuclear expression in oral leukoplakia in the present study is consistent with the results reported by Ishida et al.[9] they found a difference in Wnt3 expression between early stage and advanced stage dysplasia that could be related to nuclear β-catenin expression.

The subjective evaluation of Wnt3 expression provides quick, easy estimation of cellular expression and distribution and overcomes the use of the tedious counting method for a percentage of expression.

CONCLUSION

The study indicates that CD34 and Wnt expression may be useful biomarkers in malignant transformation of PMODs. MVD was revealed to markedly increase through the progression from normal oral epithelia to premalignant lesions, thus indicating the role of the CD34 marker in predicting the ability of these lesions to progress toward malignancy. Furthermore, the aberrant cytoplasmic localisation of Wnt3 may be involved in the progression of PMODs to OSCC.

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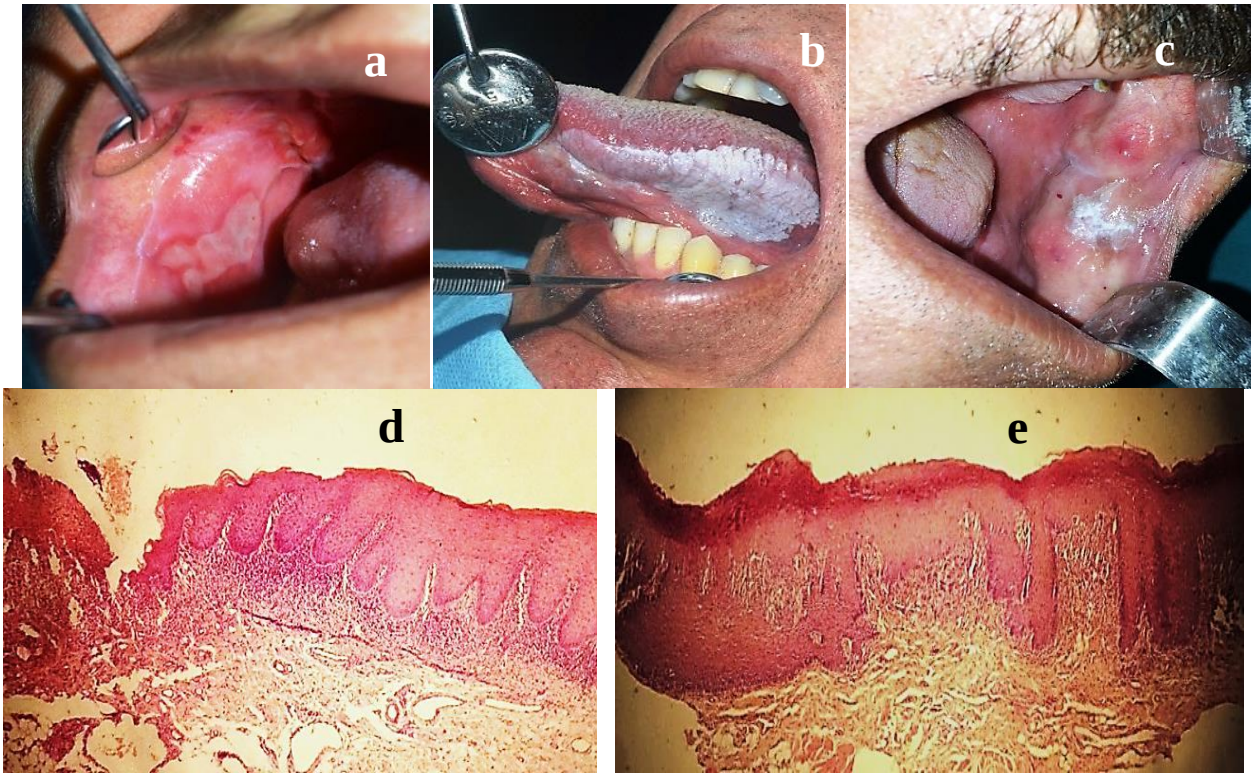

Figure 1: Clinical presentations of: a) erosive lichen planus. b &c) leukoplakia with surface keratosis. Histological appearance of d) lichen planus without dysplasia, e) lichen planus with mild dysplasia. (X100):

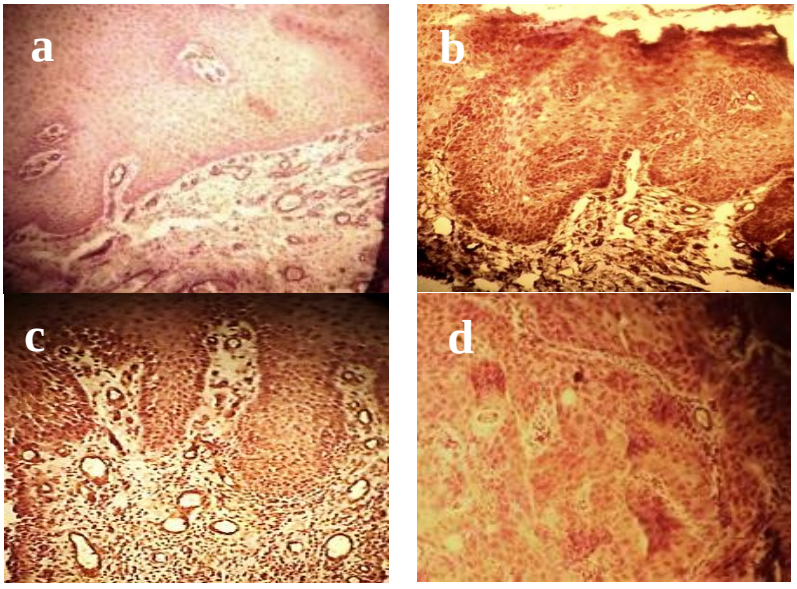

Figure 2 :Immunohistochemical expression of CD34 in (a) gingival mucosa. (b) proliferative verrucous leukoplakia. (c) oral lichen planus with mild dysplasia. (d) oral squamous cell carcinoma. (X400)

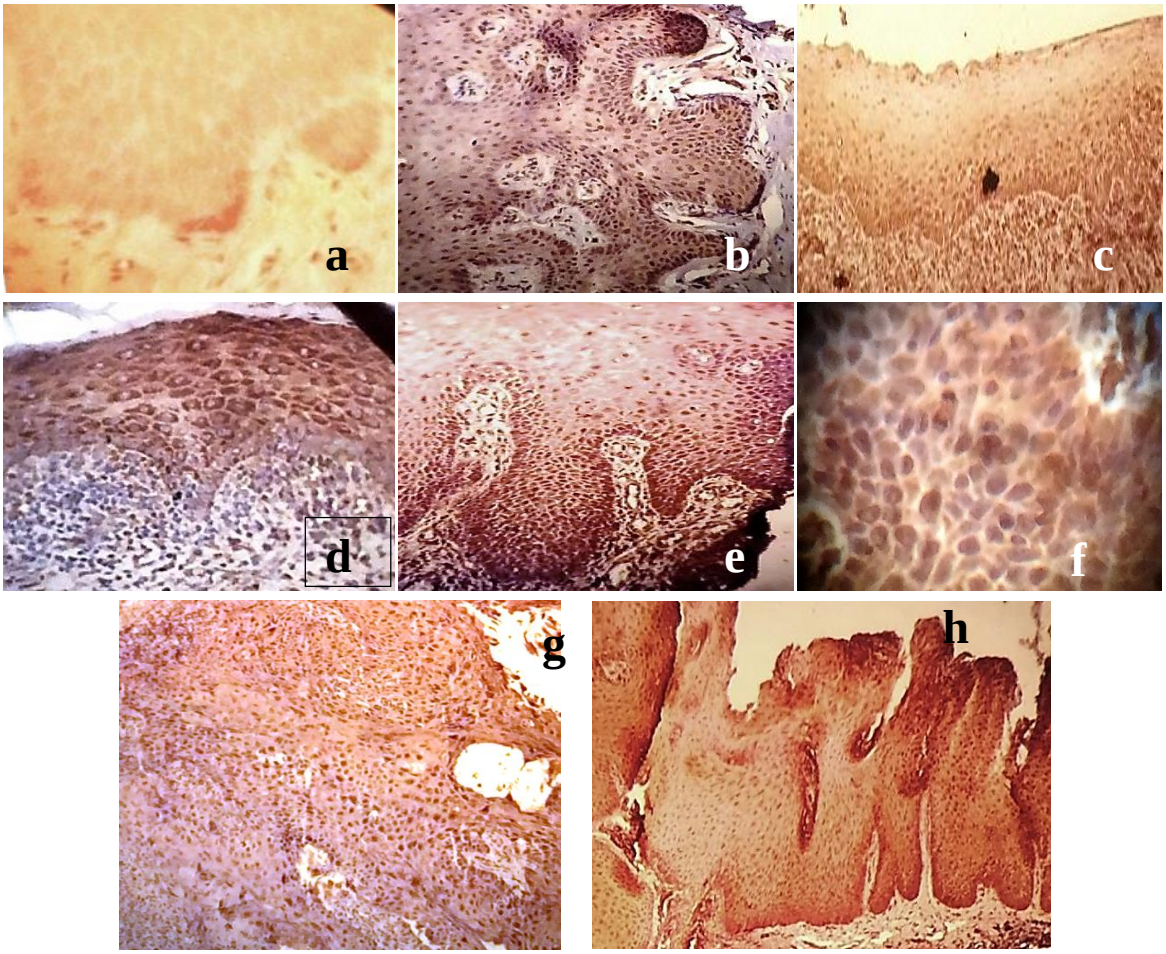

Figure 3: Immunohistochemical expression of Wnt3. (a) alveolar mucosa. (b) gingival mucosa .(c) oral lichen planus without dysplasia. (d) oral lichen planus with mild dysplasia. (e&f) leukoplakia with dysplasia. (g) OSCC.(h) proliferative verrucous leukoplakia. (X100 for all pictures except F, X 400).

Table (1): Clinical characteristics of patients

Groups		PMODs (27)		Carcinoma (6)	
		No.	%	No.	%
Sex	Male	13	48.1	2	33.33
	Female	14	51.9	4	66.67
Age	27-49	10	37	1	
	50-59	8	30	1	
	60-75	9	33	4	66.67
Site	Cheek	16	59.2	2	33.33
	Tongue	7	26	1	16.66
	Floor	1	3.7	1	16.66
	Alveolar	1	3.7	1	16.66
	Upper lip	1	3.7	0	
	Hard palate	1	3.7	1	16.66
Smoking	No	21	77.8	4	66.67
	Ex-smoker	3	11.1	2	33.33
	Yes	3	11.1	0	
Clinical Presentation	White (13)		48.15	Mass (2)	33.33
	White and red (13)		48.15	Ulcer (4)	66.67
	White and granular (1)		3.7		

Table (2): histopathological characteristics of PMODs and TNM staging of OSCC

Groups			Dysplasia	No.	%	Grading for dysplasia		
PMODs (27)	Lichen planus (21)		Without	17	62.96	I	II	III
			With (10) 37.04%	4	14.81		3	1
	Leukoplakia (6)	Idiopathic leukoplakia		4	14.81	1	2	1
		Chronic hyperplastic candidiasis		1	3.70			1
		Proliferative verrucous leukoplakia		1	3.70			1
OSCC (6)			Histopathologic grading			Clinical TNM staging		
			WD	MD	PD	I	II	III	IV
			4	1	1	1	2	0	3

Table (3): Mean value and standard deviation of MVD in relation to clinical parameters.

Clinical parameters		PMODs (n=21)				OSCC*			
		No.	Mean	SD	p	No.	Mean	SD	p
sex	Male	10	10.05	4.73	0.9	2	14	2.8	0.32
	Female	11	10.30	4.6		4	11.37	1.3	
Age	27-49	9	10.13	5.39	0.208	1	16		
	50-59	8	8.56	3.57		1	10.6		
	60-75	4	13.55	2.97		4	11.7	2.57	
Site	Tongue	6	11.08	4.21	0.58	2	9.3	0.42	
	Cheek	15	9.82	4.77		1	13.6		
Smoking	No	15	10	4.44	0.853	0			0.83
	Ex-smoker	2	12	7.07		2	12.53	2.3	
	yes	4	9.97	5.19		4	11.96	0.77	
Clinical presentations	White	13	8.96	4.27	0.119	Mass 4	13.7	1.6	0.54
	Red & white	8	12.17	4.53		Ulcer 2	10.3	0.4	

* Pearson correlation between MVDs and clinical SCC TNM staging ; r= -0.94, P=0.006.

Table (4): The relationship of the MVD to the histopathologic findings in all groups.

Groups	Subgroups	No.	Mean	SD	ANOVA	**Tukey HSD**	
Control (8)	Alveolar mucosa	4	9.30	0.88	0.001	Groups	P value
	Gingiva	4	17.47	5.09		Alveolar vs gingiva	0.030
PMODs (21)	OLP without dysplasia	16	8.85	3.95		Gingiva vs OLP without dysplasia	0.002
	Dysplastic lesions*	5	14.46	3.89		Dysplasia vs OLP without dysplasia	0.045
OSCC		6	12.25	2.75			

* Three cases of leukoplakia and two cases of OLP

Table (5): Wnt3 expression and distribution in all groups.

Groups	No.	Subgroups	Cytoplasmic	Nuclear
Control	4	Alveolar mucosa	-ve	-ve
	4	Gingiva	Perinuclear +ve or cytoplasmic -ve	+ve scattered
PMODs	16	L.P. without dysplasia	+ve	-ve
	2	L.P. with dysplasia	+ve	-ve
	2	Leukoplakia(dysplasia)	+/-ve	-ve
	1	Proliferative verrucous leukoplakia	-ve	+ve
Carcinomas	6	OSCC	+ve	+ve

الخلاصة

خلفية الدراسة : الأصابات القابلة للتحول الى سرطان الفم هى اصابات شائعة. وتولد الاوعية الدموية يعتبر من العلامات المهمة لتحول هذه الاصابات الى مراحل متقدمة.

اهداف الدراسة : تكمن أهمية هذه الدراسة فى تقييم الفحص المناعي الكيميائي بأستخدام CD34 و Wnt3 كعوامل في تحديد التطور في الآفات القابلة للتحول الى أمراض خبيثة وسرطان الفم بالأضافة الى تحديد العلاقة بين الصفات السريرية مع الفحص المناعي الكيميائي لهذه الأمراض.

المواد و الطرائق: استخدم في هذه الدراسة واحد و أربعون عينة اشتملت على ثمانية عينات من نسيج اللثة الطبيعي و سبع و عشرون من اصابات الأمراض القابلة للتحول الى أورام خبيثة وستة عينات من سرطان الفم الحرشفي. تم فحص جميع العينات بأستخدام CD34 و Wnt3 بطريقة الفحص المناعي الكيميائي لجميع الحالات والتحليل الحصائي باستخدام ANOVA , post -hoc , واعتبرت القيمة الاحصائية $p<0.05$ ذات تاثير احصائي مهم .

النتائج: كان هناك ظهور احصائي واضح ل (CD34) بالمقارنة للمجاميع المستخدمة بالدراسة. واظهرCD34 انخفاض واضح في الأمراض القابلة للتحول الى أورام خبيثة وارتبط ب التقدم السريري لمرضى سرطان الفم . واظهرت ظهارة الغشاء المخاطي كثافة قليلة ل الاوعية الدموية (9.3±.88) بمقارنة مع اللثة (17.47±5.09) (P<0.05) , بينما اظهر الحزاز المسطح بدون الخلل النسيجي كثافه وعائية اقل (8.85±3.95) مقارنة باللثة والظهارة ذات الخلل النسيجي(P<0.05) (14.46±3.89).
لم يتم تحديد Wnt3 في الغشاء المخاطي للفم .بيمنما اظهر نسيج اللثة ظهور للعامل في النواة وحولها .جميع انسيجة الحزاز السطحي اظهرت تواجد في السايتوبلازم وبعض الطلوان الفموي .بينما ظهر تواجد نووي في الطلوان المفرط التقرن . سرطان الفم الحرشفي اظهر تواجد في كل من النواة والسايتوبلازم.
الأستنتاجات: تعتبر كثافة الأوعية الدموية المجهرية أحد العوامل الحيوية للتطور الى سرطان الفم . يتميز بالزيادة باتجاه التحول من النسيج الطبيعي الى سرطان الفم. تم تحديد ظهور السايتوبلازمي الشاذ ل Wnt3 في كل الأفات القابلة للتحول الى أمراض خبيثة و سرطان الفم الحرشفي, لذلك يمكن اعتبار Wnt3 عامل مهم في تحول هذه الأفات الى سرطان الفم.