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IMMUNOHISTOCHEMICAL EXPRESSION OF CD44 AND DNA ANALYSIS IN ORAL PREMALIGNANT AND MALIGNANT LESIONS

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ABSTRACT

Objectives: The aim of the present study is to evaluate CD44 expression to detect cancer stem cell activity along with analyzing the DNA ploidy, to predict the activity of oral carcinogenesis.

Materials and method: Forty formalin fixed paraffin embedded tissues of oral lesions were used and divided equally into 4 groups; different premalignant white lesions, well, moderate, and poorly differentiated squamous cell carcinomas. All specimens were immunostained using CD44 antibodies. Moreover, the same paraffin blocks for each case used to prove the differentiation in DNA ploidy between groups using flow cytometer.

Results: The CD44 immunoexpression showed a marked statistical significant difference between premalignant white lesions and poorly differentiated squamous cell carcinomas. Flow cytometric analysis of DNA parameters between the tested groups revealed a powerful difference of DNA ploidy between the premalignant group and other malignant groups. The S-phase fraction showed significant difference between poorly differentiated OSCC and premalignant lesions with no effective differences between the malignant lesions.

Conclusions: The findings of the present study suggest that the CD44 immunoexpression collaborates with the DNA content analysis presented a real indicator in prediction the behavior of oral premalignant and malignant lesions.

KEY WORDS: Cancer stem cell, CD44, DNA Ploidy, Premalignant lesions, OSCC.

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INTRODUCTION

Oral carcinomas are among the most popular cancers worldwide. A bulk of oral cancers arise in pre-existing conditions called the oral potentially malignant disorders. They are defined as a band of oral mucosal lesions with a high hazard of cancer shift[1]. The five years' survival rate of oral cancer span from 15% to 50%, mainly because most oral tumors have proceeded to a progressive stage, at the diagnosis date[2]. The survival rate of oral cancer can reach 80% of patients, if it is recognized and controlled early[3]. Oral squamous cell carcinoma (OSCC) generated as various genetic mutations, which motive abnormal deviations in cell cycle. These alterations rise and devote the growth of lesions[4]. Therefore, studying of the biologic rules of these transformations allow proper diagnostic and prognostic evaluation. Up to now, a visual oral investigation is the routine first-line method of checking oral diseases. Although, this manner has certain limitations and as a result, patients are either diagnosed when their cancer reached an advanced stage or a high-risk patient with oral potentially malignant conditions is misdiagnosed and left untreated[5]. Certain diagnostic aids have been matured to allow the early detection of oral carcinomas and some of these have been reworded into regulatory approved in vitro diagnostic systems or medical fittings[6].

To overcome doubt in the prognosis of OSCC, studies on the recognition of prognostic markers and therapeutic objects are vital and apparently called for[7]. The cancer cell nature, more defiant to non-surgical treatment; as radiotherapy and chemotherapy, their identification remains challenging. Within this situation, cancer stem cells (CSCs); also known as tumor-initiating cells are studied relevant targets[8]. The CSC theory suggests that only a few cancer cells with a capacity for high tumorigenicity, self-renewal, and differentiation are responsible for the maintenance and growth of tumors. Similar to normal tissue stem cells, CSCs can also exist within a supportive niche. Though CSCs are adhered to activate OSCC development and recurrence[9]. CD44 is a cell surface glycoprotein biomarker that is involved in many cell regulation pathways, including cell proliferation and migration[10]. In normal epithelium, CD44 manifested primarily in the basal and suprabasal layers of lining epithelium. When dysplasia processes, CD44 expression migrates into the active proliferative layers, implicating its role in early stages of carcinogenesis[11]. Additionally, CD44 is overexpressed on the surface of metastatic tumors when compared to the primary tumor. CD44 is released in soluble set from the surface of cells by metalloproteinases that are high expressed in advanced cancers[12,13].

To improvement limitations of histopathological evaluation of malignant changes, biomarkers act to improve the understanding of the molecular pathogenesis of oral cancer. DNA ploidy is act as a malignant biomarker. Atypical nuclear DNA is an aspect of malignant cells and their precursors. Detection of aneuploidy in oral neoplasms at a very early stage is considered a mark of genomic instability, which indicating for cancer development and progression. Aneuploidy as a measure of abnormal DNA content. It is a cancer-type-specific oncogenic matter that may have clinical importance as a prognostic marker and as a powerful curative target[14]. DNA ploidy position resolved by flow cytometry (FCM) using tissue samples is an objective and adjunctive diagnostic method to actually measure the allocation of nuclear DNA content. Although DNA aneuploidy has been accepted to be a marker of malignancy in certain organs in addition to oral cancer detection[15].

The study aimed to highlight the accuracy of CD44 expression with DNA analysis as an adjuvant tool in prediction carcinogenesis and aggressiveness in oral premalignant and malignant lesions.

MATERIALS AND METHOD

The research was conducted on 40 formalin fixed paraffin embedded tissue blocks of premalignant white lesions, well, moderate, and poor OSCC. The blocks were obtained from the Department of Oral Pathology, Faculty of Dentistry, Alexandria University and the Department of Oral and Maxillofacial Pathology, Faculty of Dentistry, Assiut University over the last four years (2018-2021). The study was conducted following the ethical guidelines by the Faculty of Dentistry, Alexandria University (IRB No. 00010556 - IORG 0008839). All blocks were obtained from incisional biopsies before the patients have received any treatments. The tissue blocks are classified into 4 equal groups. Group I, the premalignant lesions including white spotted, fungating, ulcerated or other suspicious oral lesions. They are showing moderate to high grade epithelium dysplasia, which associated with a high risk for malignant shift to cancer. Group II, 10 blocks diagnosed as early well differentiated OSCC. Group III, 10 blocks of moderate differentiated OSCC. The last Group IV of poorly differentiated OSCC. The number of samples are equal in each group for ideal statistical comparison to obtain significant results.

In this study's tissue samples, one section was cut from each block and stained with hematoxylin and eosin stained (H&E) for the verification of diagnosis. Importantly, histopathological grading reassures that the neoplasm tissue constitutes more than 70% of the section, with minimal hemorrhagic and necrotic foci. This step is essential in obtaining accurate results and avoiding errors produced by analysing normal, inflammatory or necrotic tissues[16].

Immunohistochemical staining

The tissue paraffin blocks were stained by monoclonal rabbit anti-human CD44 antibody marker (dilution 1:100; Abcam, Cambridge, UK). The staining steps were conducted while adhering to the universal immunostaining protocols. The strength of the CD44 immunoreaction was evaluated in terms of means area (%) using Image J software (version 1.52p)[17]. Unmarked sections were blindly examined by 2 pathologists, selected microscopic areas at x100 magnification.

DNA analysis results

Evaluation of DNA content was using the FACS Calibur Flow Cytometer (USA), Becton-Dickinson (B-D). The staining material is The Cycle TEST™ PLUS DNA Reagent Kit (BD Biosiences). Ploidy classification set according to the value of the DNA index (DI) as follows: diploid (DI = 0.9–1.10), aneuploid (DI = 1.15–1.93) [15]. In the case of double peaks, the DI of the most prominent peak was considered. The S-phase fraction (SPF) is the fraction of the full cell residents that are present in the S-phase of the cell cycle and is usually asserted as a ratio. The cut off for the SPF was done as the mean ±2 standard deviation (SD) and considered as either being has low or high S-phase activity. The ploidy histograms were investigated blind to pathological examination, histological grading and immunohistochemical outcome.

Statistical analysis

The data were interpreted by Graphpad Prism software (Prism 8, version 8). All values were resulted as mean ± standard deviation (SD). Immunohistochemical expression analysis for the mean area percent of CD44 was analysed using one-way ANOVA test and further using Dunnett's multiple comparison posthoc tests. Comparison of FCM variables between the research groups was done using Mann Whitney U test for comparing categorical data, Chi square (±2) test was performed. The results were quantified using hazard ratio with 95% confidence interval (95% CI). The level of

statistical significance ($p<0.05$) was indicated on plots with asterisks (*).

RESULTS

Histologic evaluation

Histologic diagnosis of the tissue sections done to verify and assurance the lesions included in the study. Ten cases extracted from premalignant lesions revealed hyperplastic keratinized epithelium (the epithelial cells showed loss of polarity, pleomorphism, hyperchromatism, and an increase in the nuclear cytoplasmic ratio). Furthermore, the remaining 30 cases showed equal samples number of different grades of OSCC starting from well to moderate and finally poorly differentiated OSCC as shown in (Fig. 1a-d).

Immunohistochemical analysis of CD44

The immunoreaction to CD44 in the different tested groups showed variations in mean area (%) as shown in (Fig. 2a-e). The differences in mean CD44 area (%) were highly statistically significant ($p < 0.0001$), as shown in the comparison between premalignant Group I (11.16±3.11) %, and malignant groups as Group II (37.78 ± 5.23) %, Group III (64.1 ± 8.5) %, and Group IV (77.84 ± 11.65) %. The study results showed a marked statistically significant ($p < 0.0026$) in CD44 immunoexpression between well differentiated Group II and poorly differentiated OSCC Group IV. On the other hand, there is no important difference in the mean area (%) between moderate differentiation OSCC Group III and poorly differentiated type Group IV ($p= 0.528$). As well as, there is no real difference ($p= 0.046$) between Group II and Group III.

DNA ploidy analysis

A total of 13 cases were found to have a diploid cell population, 7 out of the 13 were in the premalignant conditions, 4 biopsies in Group II, and 2 lesions in Group III. The examination did not observe any diploid tissues in poorly differentiated OSCC. Aneuploidy was observed in 3 out of cases in Group I, 6 out of 10 tissues of Group II, 8 out of 10 specimens in Group III and all blocks of Group IV. The aneuploid tumors subdivided to: 5 hypodiploid lesions with DI ranged from 0.65 to 0.86 with a mean of 0.73 (1 in Group II, 2 in Group III, and 2 in Group IV), and 22 hyperdiploid tumors with DI ranged from 1.15 to 1.93 with a mean of 1.36 (3 in Group I, 5 in Group II, 6 in Group III, and 8 in Group IV). The significant differences in ploidy DNA content between Group I and Groups II, III, and IV were statistically significant ($p= 0.0001$). The differences in diploid and aneuploid state were statically significant between Group IV in relations

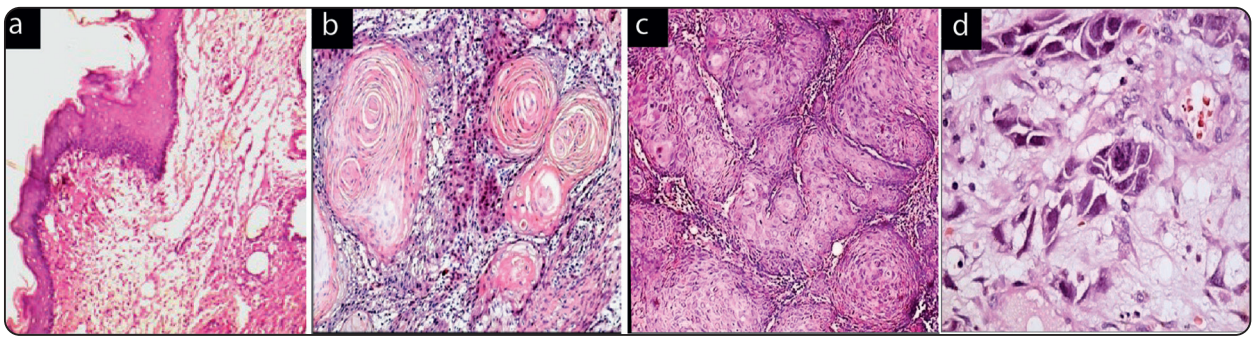

Fig. (1): Histologic evaluation. (a) Premalignant lesion showing mild hyperkeratosis and acanthosis (H&E x100). (b) Well differentiated OSCC showing a large keratin and epithelial pearls (H&E x100). (c) Moderately differentiated OSCC revealing malignant cell nests with no keratin formation (H&E x100). (d) Poorly differentiated OSCC exhibiting loss of adhesion between the malignant epithelial cells. Notice the abnormal mitotic figure (H&E x400).

to Group II and Group III (p= 0.0027). There is no symbolic difference (p= 0.057) in the DNA ploidy between Group II and Group III. The significant differences between hyperdiploid and hypodiploid cases with no ideal differences in all study groups (p= 0.672). (Fig. 3a-d)

The SPF values calculated for Group I ranged between 4.38% and 48.62% with a mean of 18.65%, whereas the SPF of the tumors in Group II ranged between 8.57% and 63.17% with a mean of 24.78%. Additionally, the values of SPF of Group III lesions demonstrated between 6.73% and 71.43% with a mean of 28.24%, Finally, the calculate SPF in Group IV neoplasms have a mean of 40.72% with range between 12.48% and 88.92%. The S-phase values were furthered classified into: high and low SPF. About 57.5% (23/40) of all specimens had high SPF (6 in Group I, 3 in Group II, 6 in Group III, and 8 in Group IV); numbers of cells in SPF were equal or more than 26.76%. On the other hand, the remaining 17/40 lesions had low SPF (4 in Group I, 7 in Group II, 4 in Group III, and 2 in Group IV). The SPF value is highly statically significant between lesions in Group I and Group IV (p= 0.0001). The differences in the vales of SPF with no significance difference between Group I in relation with Group II (p= 0.725) or Group III (p= 0.073). In addition, there is no difference in the relation of SPF values between the tumors in Group IV with Group II (p= 0.054) or Group III (p= 0.358). In the same hand, no difference between Group II and III in the SPF value (p= 0.548). Finally, it is no significant difference in the high and low SPF values between the study groups. (Table 1)

Correlation between CD44 staining along with DNA ploidy

Highly CD44 expression with aneuploidy DNA contents was powerfully associated with advanced tumor grade. There is a high significant difference (p <0.0001) between the specimens with high CD44 expression, aneuploid content and high SPF 7/10 (70%) in Group IV and the others lesions in remaining groups. This result detected an ideal relation between the increase of CSCs activity and the abnormality of DNA ploidy.

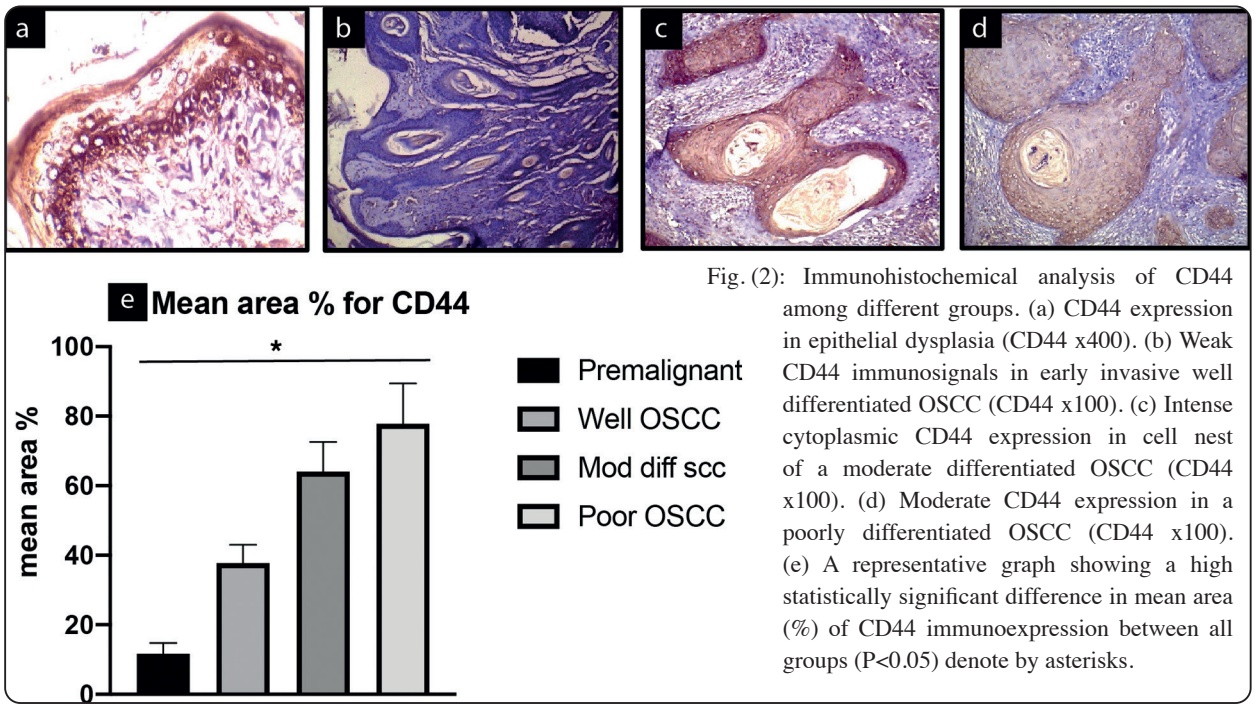

Fig. (2): Immunohistochemical analysis of CD44 among different groups. (a) CD44 expression in epithelial dysplasia (CD44 x400). (b) Weak CD44 immunosignals in early invasive well differentiated OSCC (CD44 x100). (c) Intense cytoplasmic CD44 expression in cell nest of a moderate differentiated OSCC (CD44 x100). (d) Moderate CD44 expression in a poorly differentiated OSCC (CD44 x100). (e) A representative graph showing a high statistically significant difference in mean area (%) of CD44 immunoexpression between all groups (P<0.05) denote by asterisks.

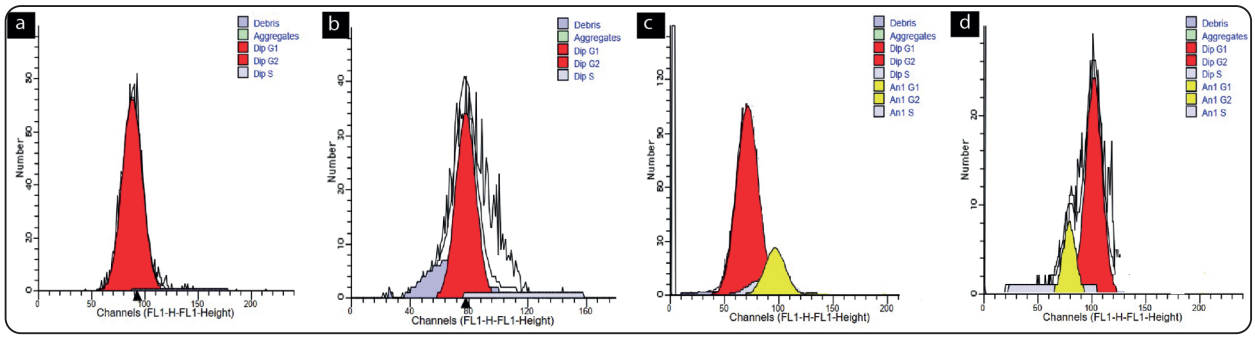

Fig. (3): DNA content analysis using the FACS caliber. (a) DNA frequency histogram of diploid peak obtained from a premalignant lesion, showing single G0/G1 peak and no SPF cells. (b) DNA frequency histogram of well differentiated OSCC, showing high SPF and diploid peak. (c) DNA frequency histogram of aneuploid moderate differentiated OSCC showing hyperdiploid, and low SPF cells. (d) DNA frequency histogram of aneuploid poorly differentiated OSCC showing hypodiploid and high SPF cells.

TABLE (1): DNA ploidy differences between oral premalignant lesions and different histologic grades of OSCC.

Groups	No.	DNA Analysis				
		Ploidy State			SPF	
		Diploid (n %)	Aneuploid		High n %	Low n %
			Hyper. (n %)	Hyper. (n %)		
Group I, n	10	7 (53.8)	3 (13.6)	0 (0)	6 (26.1)	4 (23.5)
Group II, n	10	4 (30.8)	5 (22.7)	1 (20)	3 (13)	7 (41.2)
Group III, n	10	2 (15.4)	6 (27.3)	2 (40)	6 (26.1)	4 (23.5)
Group IV, n	10	0 (0)	8 (36.4)	2 (40)	8 (34.8)	2 (11.8)
Total, n	40	13	22	5	23	17
%		32.5%	67.5%		57.5%	42.5%
			81.5%	18.5%		

DISCUSSION

Oral cancer represented more than 80% of head and neck malignancies. Identifying novel biomarkers to predict survival and prognosis is urgently needed for the management of malignant patients[2]. Cell motility is considered as the dominant element in the progression and metastasis of cancers. One of the theories regarding carcinogenesis is that the cancer growth is processes on CSCs; that have the abilities of self-renewal and promote tumor initiation, progression as well as metastasis[8]. CD44 is a single chain transmembrane glycoprotein that was proposed as the ideal CSCs marker[18]. The expression of CD44 was determined in 40 oral cases. In normal epithelium the CD44 expression were almost in basal layer and were abundantly expressed in most abnormal cells[9]. A study by Prince et al. showed that expression of CD44 was detected in 41.7% head and neck SCC cases[19]. Sato et al. found that high CD44 expression was significantly associated with poorer clinical outcome in oral carcinomas[20]. In addition, Rodrigues et al. suggested that the CD44 overexpression was associated with disease-

related death and worst prognosis[21]. Furthermore, Dhumal et al. demonstrated that CD44 play a performative role in the tumorigenicity of CSCs in oral potentially malignant disorders and OSCC[22]. Moreover, Hussein et al. noted a link between higher CD44 expression levels and a prediction of poor survival in patients with oral cancer[23]. The present study results substantiate these findings as a high significant differentiation in the CD44 expression between the premalignant group and the malignant groups. In contrast, a study of Mack et al. investigating CD44 in head and neck mucosa, leukoplakia and carcinoma, the authors concluded expression levels of these molecules were not a suitable marker for malignant differentiation, as well as premalignant tissue from normal tissue[9]. Clay et al. suggested that it was unlikely that CD44 cells were an accurate population of CSCs and highlighted the need for its evaluation in combination with another marker[24]. This may be due difference in the number and proportion of studied subjects may produce different results. However, Tamatani et al. showed that CD44 could not be an important biomarker between well and moderate differentiated OSCC[25]. This observation goes in line with the results of the present work which demonstrated that CD44 could not be an accurate biomarker to differentiated between grades I and II oral cancer.

The study profile of DNA aneuploidy is an indicator of numerical chromosomal changes, and its emergence is usually an early crucial step in carcinogenesis, lately its reputation as a marker of oral cancer progression was questioned. For formalin fixed paraffin-embedded biopsy tissues, DNA aneuploidy is reported to be a useful marker in predicting malignant transformation of oral potentially malignant disorders[26]. Giaretti et al. suggested that premalignant lesions and even normal appearing mucosa in the context of field cancerization already contain aneuploid cells[27]. This in agreement with the results of the present study, as three premalignant lesions showed aneuploid DNA content. Moreover, Remmerbach et al. concluded that DNA-aneuploidy is a very sensitive, highly specific, and objective adjuvant tool for the early identification of neoplastic epithelial cells in oral smears[28]. Similarly, Sudbo et al. reported that from the study patients with histologically diagnosed oral epithelial dysplasias more than 24% patients developed a squamous cell carcinoma during the follow up, 70% were diploid, 30% were aneuploid at initial diagnosis[29]. A study done by Pentenero et al. indicted that DNA aneuploidy an early onset of field effect in oral carcinogenesis[30]. Additionally, Li et al. reflected that the malignant progression of oral potentially malignant disorders is a typical multistep carcinogenesis processes with stepwise accumulations of DNA and genetic alterations[31]. Of particular interest, the presence of DNA aneuploidy in inflammatory bowel disease determined by flow cytometry was also associated with development of dysplasia or colorectal cancer[32]. In agreement with the results of the present work Li et al. demonstrated that DNA number alteration may be effective for prognosis prediction in oral leukoplakia patients with hyperplasia[33]. Also, Guimaraes et al. resulted that DNA ploidy have been shown to be good predictive markers of malignant transformation[34]. In contrast, Zargoun et al. recommended that the DNA ploidy alone may not be a good diagnostic tool to evaluate OSCC progression in the oral cavity. Although abnormal DNA content may be a necessary feature of OSCC, it is not specific to progressive lesions[35]. Notably, the various proportions of OSCC, dysplasia, and benign lesions enrolled in the study could produce different results, since detection of OSCC or dysplasia is the exposed factor.

In the current study, the SPF value is highly statically significant between premalignant lesion and the poorly OSCC with no significant difference between the malignant lesions. This observation goes with the result of the study done by Das et al. dem-

onstrate a significant relation between high SPF and poor histological grade[36]. Meanwhile, El-Deftar et al. analyzed that high SPF of primary OSCC tumors was significantly associated with decreased survival rates[37]. Additionally, Missaoui et al. showed that SPF appear helpful in making the distinction between benign and malignant neoplasms[38]. The study finding showed that there was no important relationship between the SPF of the malignant neoplasms. This result agrees with Zahran et al. study, which expressed that no significant SPF value in evaluating tumor activity[39]. Similarly, Pinto et al. report a borderline significance of SPF with high grade thyroid carcinoma[40].

The limitations awarded in this study that the biopsy tissues might not reflect the whole part of tumors. The present findings of this study might not be the results of CSC marker expression and DNA ploidy state of the whole tumors. Further studies in larger cohorts are needed to confirm the study findings, assess CSC marker expression and DNA aneuploidy in other relevant tissues, and investigate the differential in the context of OSCC prognostication. Also, realization that longitudinal studies with adequate follow-up and clinical endpoints are required to evaluate the efficacy of the CD44 expression and DNA aneuploidy as a surveillance tool for oral cancer development.

CONCLUSION

The findings of research suggest that the CD44 expression collaborates with the DNA content analysis presented a real indicator in prediction of the activity of oral carcinogenesis. Both CD44 expression with DNA diploid status had an equivalent positive and negative predictive value. This may serve a role in predicting the carcinogenesis process and aggressiveness of oral lesions.

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Conflict of Interests

The authors have no conflicts of interest to declare.

Author Contribution

Ahmed M. Hussein: conceptualization; data curation; formal analysis; investigation; methodology; project administration; resources; visualization; writing-original draft; writing review and editing. **Mohamed Badawy**: formal analysis; investigation; methodology; resources and writing-review. **Hany G. Gobran**: data curation; investigation; resources; writing review and editing. **Hesham A. Dameer**: formal analysis; methodology; project administration; supervision and writing-review. **Hazem L. Abdel-Aleem**: data curation; investigation; resources; writing review and editing. **Hend Abdel Hamid**: data curation; formal analysis; investigation; methodology; project administration; resources; supervision; writing-review and editing.

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