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ROLE OF THE WNT SIGNALING PATHWAY IN ORAL CANCER

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ABSTRACT

The sixth most dangerous cancer worldwide is oral cancer. Pre-cancer disorders are often present in many patients before oral cancer develops. Several types of human cancer have been linked to abnormal activation of Wnt/β-catenin signaling, which is characterized by β-catenin translocation and overexpression of Wnt ligands. β-catenin interacts with the transcription factor during canonical Wnt/β-catenin signaling to activate downstream target genes such as Cyclin D1, which is primarily associated with cellular proliferation. This study aims to evaluate the role of β- catenin and Cyclin D1 in epithelial dysplasia and various stages of oral squamous cell carcinoma (OSCC) via the Wnt signaling pathway. For the present study, 5 cases of normal oral epithelial tissues, 10 cases of severe epithelial dysplasia, and 30 cases of various grades of OSCC (10 cases for each grade) were collected as paraffin-embedded blocks. All specimens were immunohistochemically stained for β-catenin and Cyclin D1 antibodies. β-catenin immunostaining showed the highest mean area percentage found in normal epithelial tissue, whereas the lowest values were recorded in poorly-differentiated OSCC, while Cyclin D1 immunostaining showed the lowest mean area percent was recorded in normal epithelial tissue, whereas the highest values were noted in poorly-differentiated OSCC. Conclusion: Aberrant cytoplasmic expression of β-catenin act as a co-factor, activating Cyclin D1 lead to high proliferative activity and invasion potential. Thus associations of both molecules has greater role in transformation from normal to dysplastic cells and increase their invasiveness to OSCC.

KEYWORDS: Oral cancer, β-catenin, Wnt signaling pathway,Cyclin D1

INTRODUCTION

Oral cancer ranks the sixth most prevalent malignant neoplasm within countries with variations in survival rates. Squamous cell carcinoma accounts for more than 90% of oral cancer. Oral epithelial dysplasia frequently precedes OSCC and is characterized by many tissue and cellular alterations that are comparable to carcinoma but limited to the surface epithelial layer. It displays surface squamous epithelium morphological alterations, such as varied cellular atypia, proliferative activity, and loss

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of typical differentiation patterns. Diagnostic delay in oral cancer is associated with the advanced stage at the time of diagnosis; it affects patient survival, influenced by the tumor's biological properties.[1-3]

Oral carcinogenesis is a complex process that involves both genetic and molecular modifications that result in defective DNA repair systems, modulated signaling pathways, a deregulated cell cycle, and limited apoptosis. Unchangeable genetic and epigenetic changes in oral malignancies impair the expression and action of proteins such as pRB, cyclin, p53, and Wnt/β-catenin pathway factors. Wnt/β-catenin signaling is one of these alterations; it is required for cell survival and tumorigenesis. It is also involved in embryonic development, adult organ, tissue maintenance and the pathogenesis of many diseases, including various cancers.[4-7]

The Wnt signaling pathway includes a wide range of signaling factors. The dysregulation of these factors or their mutations affects gene expression by controlling the level of β-catenin, which activates Wnt signaling expression of the target gene. β-catenin is a protein with many functions that participates in a variety of cellular events and human diseases. In its physiological form, β-catenin is found in the cell membrane, where it forms an adhesion complex with E-cadherin. The establishment and maintenance of tissue architecture and function are regulated by the cadherin-catenin adhesion complex, which regulates cell-cell adhesion and recognition. Decreased cell adhesion, enhanced cell motility and invasiveness are brought on by the release of catenin from E-cadherin. The proteasome system degrades the unbound cytoplasmic β-catenin once it has been phosphorylated, ubiquitinated, and then removed from the cytosol, preventing the protein from moving to the nucleus.[8-11]

If β-catenin is not degraded physiologically, it accumulates in the cytoplasm, which is linked to the activation of Wnt canonical oncogenic pathway. When β-catenin build up in the cytoplasm and moves to the nucleus, it acts as a co-factor for the T cell factor (TCF) family of transcription factors, allowing them to activate downstream target genes required for cell proliferation, migration, invasion, cell cycle progression, and metastasis. One of these target genes is Cyclin D1, which controls cell division. Therefore, the key step in pathway activation is the nuclear localization of β-catenin.[12-13]

Most healthy human cells express the protein Cyclin D1, which is encoded by the CCND1 gene on chromosome 11q13. It serves as a crucial regulator of the checkpoints of the cell cycle, initiated during G1 and drives the G1/S phase transition by acting as a regulator of the cyclin-dependent kinase4 (CDK). Retinoblastoma (RB) protein is phosphorylated by the active Cyclin D1/CDK4 complex after moving to the nucleus, which controls the transcription of particular genes necessary for cell proliferation. Entering S phase and starting DNA replication is a crucial decision that force cells to divide, and they must be controlled throughout G1 phase. Uncontrolled cell growth is caused by dysregulation of Cyclin D1 transcription, ubiquitination and accumulation, as well as the assembly and overactivation of its associated CDK. Thus, Cyclin D1 is considered an oncogenic driver in several cancer forms including melanoma, lung cancer, and breast cancer.[14-16]

In fact, the majority of signaling pathways that control cell proliferation exert their effects in the G1 phase. Deregulation that results in Cyclin D1 overexpression may minimize the demand for growth factors, shorten the G1 phase, and accelerate cell proliferation. This could lead to an accumulation of unrepaired DNA mutations that lead to losing control of the cell cycle, which could lead to tumor formation. In the division cell cycle, canonical WNT signaling was first explained as a trigger for G1 phase progression via c-Myc and Cyclin D1 transcription. Abnormal Cyclin D1 expression through transcriptional up-regulation participates in loss of normal cell cycle control, linked to a higher risk of tumorigenesis.[17-19]

Little research has been done on the correlation of expression of both β-catenin and Cyclin D1 proteins in oral dysplasia, and different grades of OSCC; therefore, the aim of this study is to evaluate the role of β-catenin and Cyclin D1 in epithelial dysplasia and various stages of oral squamous cell carcinoma (OSCC) via the Wnt signaling pathway.

MATERIALS AND METHODS

Case Selection

Blocks of paraffin-embedded, formalin-fixed specimens were used in this study. They are collected from the archives of the Oral and Dental Pathology Department, Faculty of Dental Medicine for Girls, Al-Azhar University and Pathology Department, National Cancer Institute, Cairo University. Specimens were categorized into 3 groups: normal oral epithelial tissues (10 cases of normal gingival tissues adjacent to hyperplastic gingival tissue excised from patients undergoing gingivectomy); severe epithelial dysplasia (10 cases); and 30 cases of various grades of OSCC (well-differentiated (WD), moderately-differentiated (MD), and poorly-differentiated (PD), 10 cases for each grade. Ethical code and approval of the current study was obtained from the Ethical Committee of Faculty of Dental Medicine, Al-Azhar University (Code. No. P-PD-23-02).

Histopathological analysis

Using H&E for reevaluation of the aforementioned cases was carried out to validate their diagnosis and determine the histopathological grade in accordance with WHO classification. [20]

Immunohistochemical analysis

4-μm tissue sections were cut from paraffin blocks, mounted on electrically positive charged glass slides. They are deparaffinized by xylene incubation overnight and then rehydrated in gradually decreasing concentrations of ethanol followed by phosphate buffered saline (PBS) wash. 3% hydrogen peroxide (H2O2) was used for 5min at room temperature to block the endogenous peroxidase activity. Tissue sections were put in a glass jar with 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval, and they were then microwaved twice for 5 min each to increase immunoreactivity. After allowing slides to cool, they were washed with PBS (pH 7.2). The immunohistochemical staining of β-catenin and Cyclin D1 antibodies was carried out in accordance with the manufacturer's instructions by use of β-catenin mouse monoclonal antibody (Product Code: NCL-B-CAT, NovocastraTM Lyophilized) and Cyclin D1 mouse monoclonal antibody (Cat. No. RM-9104-R7, Thermo Scientific), ready to use for immunohistochemical staining. The dilution used was 1:50 in phosphate buffered saline (PBS). The detection was done using a universal kit (DAKO, Denmark) by first cleaning slides in PBS for 5 min, followed by 30-min incubation with a secondary antibody of biotinylated goat serum conjugated with mouse and rabbit sera. Sections were then rinsed for 5 min in PBS before being exposed to diaminobenzidine [DAB] in PBS that contained 40% H2O2 to develop antigen antibody visualization. Sections were rinsed under running tap water for 10 min, then Mayer's hematoxylin was used as a counterstain and it was mounted.

Histomorphometric analysis

Computerized image analysis using Leica image analyzer (Germany) was used. The immunoreactivity of β-catenin and Cyclin D1 was determined by estimating the proportion of immunostained cells in relation to the area. The image analyzer was automatically adjusted to transform the measurement units (pixels) generated by the image analyzer program into real micrometer units. The area percentage of both β-catenin and Cyclin D1 reactive areas were calculated in relation to a reference measuring frame of area 11434.9 μm^2 using magnification (x200). A blue binary colour was used to mask the reactive areas of positive immunostaining using

colour detection. Ten fields were obtained sequentially from each slide for histomorphometric evaluation. Then, the mean values for each specimen were determined.

Statistical Analysis

A mean and standard deviation (SD) value was used to present the data. The different groups were compared using a one-way ANOVA. When the ANOVA test was significant, Tukey's post-hoc test was employed to compare the groups in pairs. Significant relationships between various markers were found using Pearson's correlation coefficient. $P \leq 0.05$ was used as the significant level. With IBM's, SPSS Statistics Version 20 for Windows, statistical analysis was carried out.

RESULTS

Histopathological findings

The normal gingival tissue specimen showed keratinized stratified squamous epithelium with normal thickness. Condensed collagen fibers, fibroblasts, and a few localized inflammatory cells were seen in the connective tissue. Each epithelial layer demonstrated dysplastic features, with the basement membrane remaining intact in cases of severe dysplasia. Cytological and architectural changes were clearly seen, such as loss of polarity, pleomorphism and hyperchromatism. In some parts of the connective tissue, there were chronic inflammatory cells present as well as engorged blood vessels. Well-differentiated OSCC cases revealed invasive nests within the underlying connective tissue. The existence of individual cell keratinization and multiple, different-sized keratin pearls were two of the most noticeable characteristics. In moderately-differentiated OSCC, neoplastic cells were less similar to squamous epithelial cells. There were variations in the size and shape of cells with minimal keratin formation. The malignant cells in poorly-differentiated OSCC displayed an abnormal histological pattern and bear no similarity to the parent tissue. Deep invasion of the underlying connective tissue by very anaplastic cells without keratinization [Fig., 1(A-E)].

Immunohistochemical findings

β-catenin immunostaining was found in the cell membrane of normal oral epithelium, as well as in the cell membrane and cytoplasm of cases of severe epithelial dysplasia and well-differentiated OSCC. While β-catenin immunostaining was present only as cytoplasmic staining in moderately and poorly-differentiated OSCC grades. Decreasing cytoplasmic immunostaining from well to moderate to poorly- differentiated OSCC was found [Fig., 1 (F-J)].

Cyclin D1 immunostaining was noted mainly in the cytoplasm and nucleus of both normal oral epithelial tissue, severe epithelial dysplasia, and all grades of OSCC. Increasing cytoplasmic and nuclear immunostaining from well to moderate to poorly-differentiated OSCC was noticed [Fig., 1 (K-O)].

Statistical analysis

Statistically, considering β-catenin immunostaining, normal oral epithelial group showed the statistically significant highest mean area percent (52.4%). There was statistically significant difference between oral epithelial dysplasia and OSCC group, both are lower than normal [(32.5%) and (22.3%)], respectively. Tukey post hoc test revealed no statistically significant differences of area percent between severe epithelial dysplasia (32.5%) and well-differentiated OSCC group (30.5%). Among all studied groups, poorly-differentiated OSCC showed the statistically significant lowest mean area percent (Table 1, 2& Fig 2, 3). Regarding Cyclin D1 immunostaining, OSCC showed the statistically significant highest mean area percent (60.5%), followed by oral epithelial dysplasia, which showed lower mean area percent (32.1%). Normal oral epithelial tissues showed the statistically significant lowest mean area percent (21.1%).

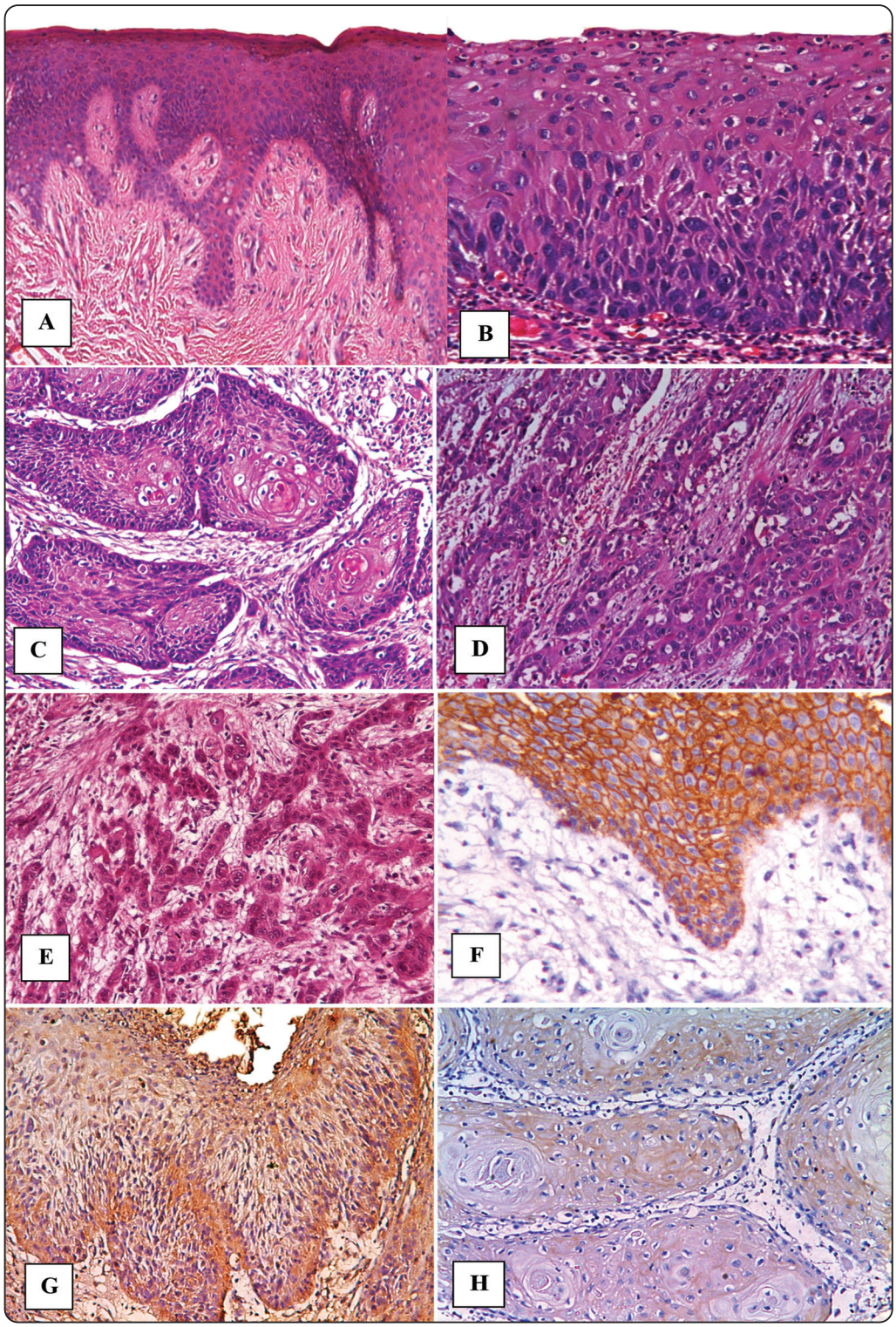

Fig. (1) (A) Normal gingival tissue showing layers of parakeratin, granular cells, spinous cells and basal cells, (B) severe dysplastic tissue displaying loss of polarity, pleomorphism, and hyperchromatism, (C) well-differentiated oral squamous cell carcinoma showing cell nests and numerous keratin pearls, (D) moderately- differentiated oral squamous cell carcinoma showing tumor cell nests less similar to squamous epithelial cells, (E) poorly-differentiated oral squamous cell carcinoma showing highly anaplastic cells (H and E, ×200).

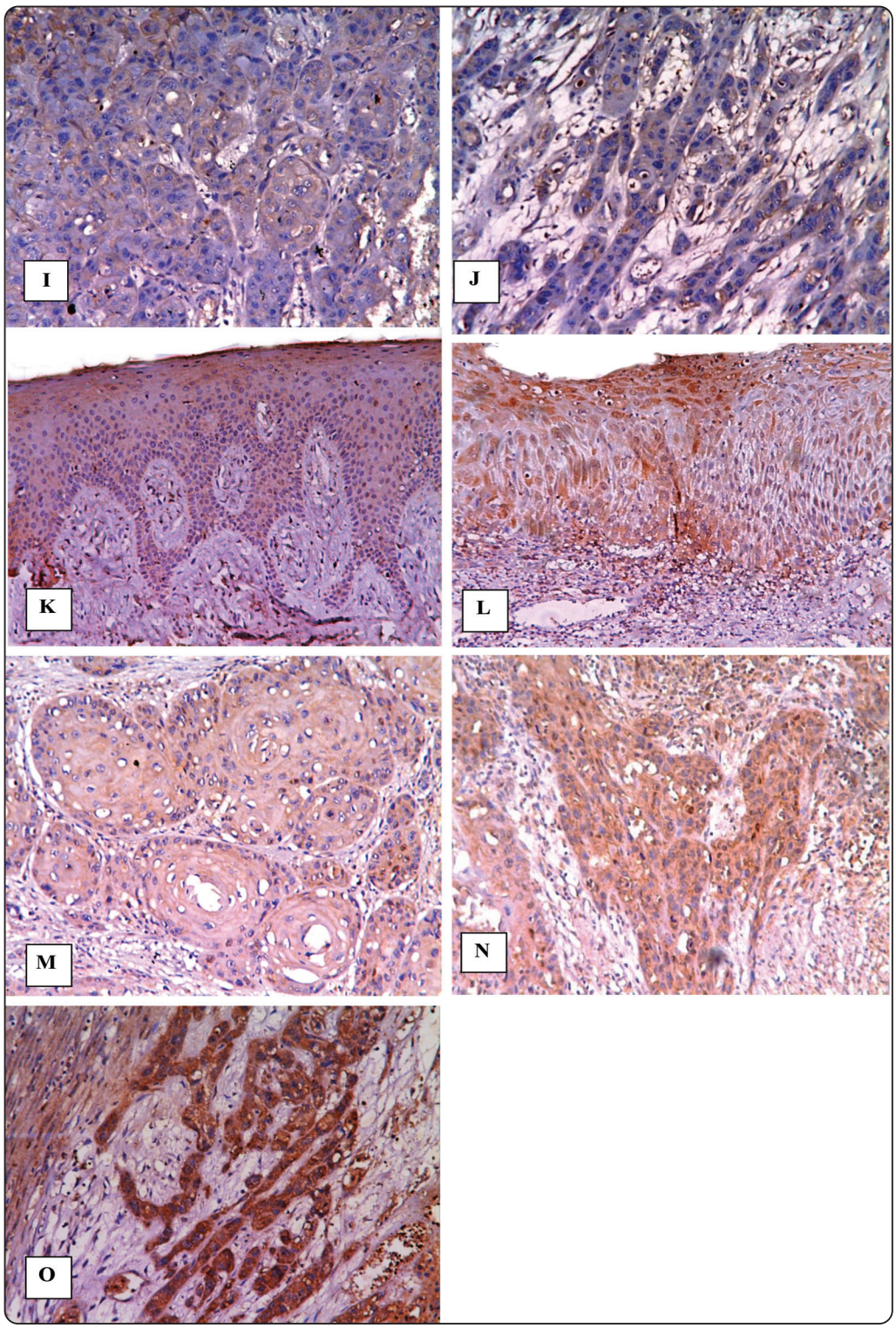

Fig. (1) β-catenin immunostaining was noted in the cell membrane of normal oral epithelium, as well as in the cytoplasm and cell membrane in severe epithelial dysplasia and well-differentiated OSCC. Only cytoplasmic immunostaining in moderately and poorly- differentiated OSCC (F-J, X 200). Cyclin D1 immunostaining was observed mainly in the cytoplasm and nucleus of both normal oral epithelial tissue, severe epithelial dysplasia, and all the grades of OSCC (K-O, X 200)

Tukey post hoc test revealed no statistically significant difference between moderately (60.2%) and poorly-differentiated OSCC (66.1%) groups, both showed the statistically significant highest mean area percent values, followed by well-differentiated OSCC (Table 3, 4 & Fig. 4, 5).

Pearson's correlation coefficient revealed a statistically significant positive correlation between β-catenin and Cyclin D1 in the dysplasia group (Fig. 6). In the OSCC group, there was a statistically significant negative correlation between β-catenin and Cyclin D1 (Fig. 7). The correlation coefficient between β-catenin and Cyclin D1 "r" (0.550, -0.498) for the dysplasia group and OSCC group, respectively.

TABLE (1) Values for the mean, standard deviation (SD) and the comparison's findings between β-catenin area percent in the three groups

Normal		Dysplasia		Squamous cell carcinoma		*P*-value
Mean	SD	Mean	SD	Mean	SD	
52.4 [a]	12.2	32.5 [b]	7.9	22.3 [b]	9.2	<0.001*

: Significant at P ≤ 0.05, Different letters are statistically significantly different

TABLE (2) Values for the mean, standard deviation (SD) and the comparison's findings between β-catenin area percent in normal epithelium, severe dysplasia and grades of OSCC.

Group	Mean	SD	*P*-value
Normal	52.4 [a]	12.2	
Severe dysplasia	32.5 [b]	5.5	
Well differentiated SCC	30.5 [b]	9	<0.001*
Moderately differentiated SCC	22.9 [c]	3.1	
Poorly differentiated SCC	13.7 [d]	4.8	

: Significant at P ≤ 0.05, Different letters are statistically significantly different

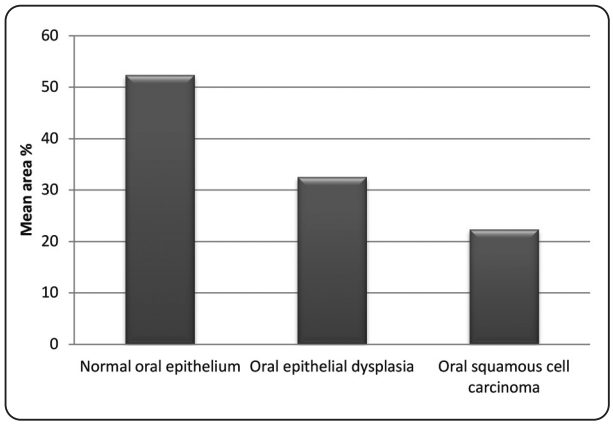

Fig. (2) Bar chart representing mean β-catenin area percent in the three groups.

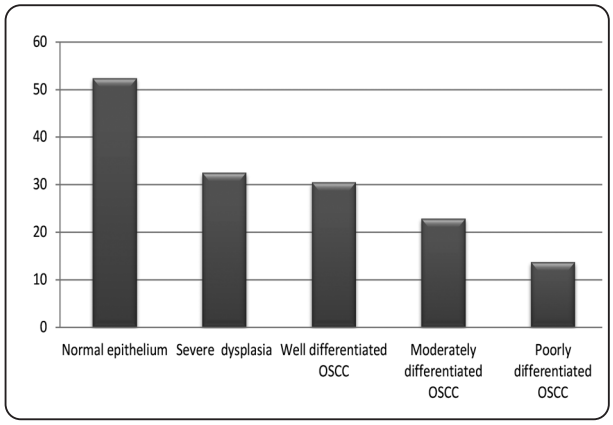

Fig. (3) Bar chart representing mean β-catenin area percent in normal epithelium, severe dysplasia and grades of OSCC.

TABLE (3) Values for the mean, standard deviation (SD) and the comparison's findings between Cyclin D1 area percent in the three groups.

Normal		Dysplasia		Squamous cell carcinoma		*P*-value
Mean	SD	Mean	SD	Mean	SD	
21.1 [c]	6.1	32.1 [b]	5.9	60.5 [a]	9.7	<0.001*

: Significant at P ≤ 0.05, Different letters are statistically significantly different

TABLE (4) Values for the mean, standard deviation (SD) and the comparison's findings between Cyclin D1 area percent in normal epithelium, severe dysplasia and grades of OSCC.

Group	Mean	SD	*P*-value
Normal	21.1 [d]	6.1	
Severe dysplasia	32.1 [c]	8	
Well differentiated SCC	55.3 [b]	9.9	<0.001*
Moderately differentiated SCC	60.2 [a]	8.6	
Poorly differentiated SCC	66.1 [a]	8.3	

: Significant at P ≤ 0.05, Different letters are statistically significantly different

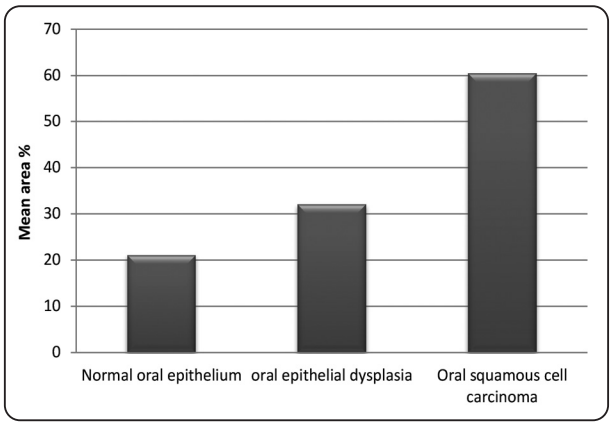

Fig. (4) Bar chart representing mean Cyclin D1 area percent in the three groups. .

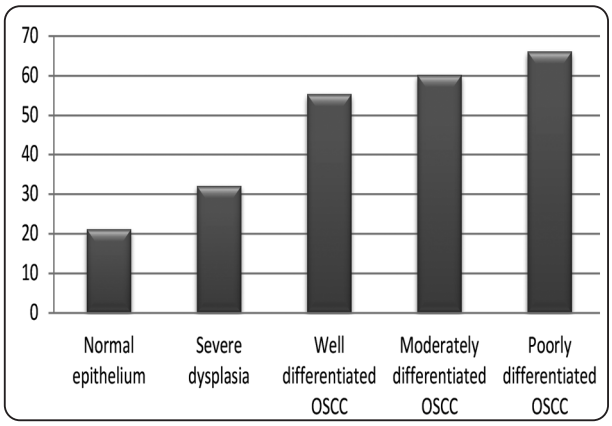

Fig. (5) Bar chart representing mean Cyclin D1 area % in normal epithelium, sever dysplasia and grades of OSCC.

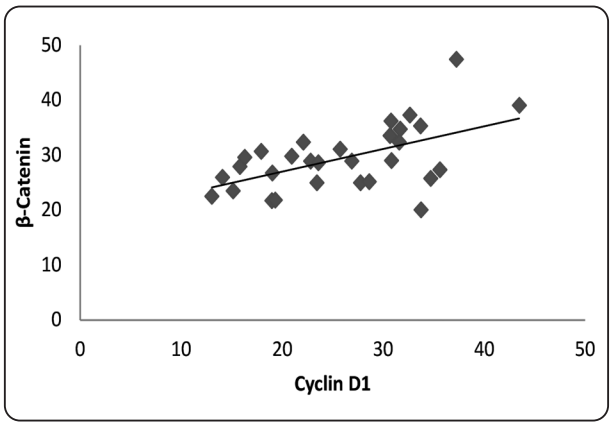

Fig. (6) Scatter diagram showing positive correlation between β-catenin and Cyclin D1 in dysplasia group.

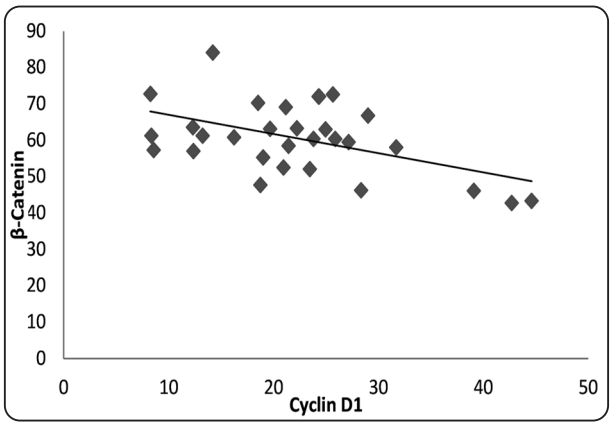

Fig. (7) Scatter diagram showing negative correlation between β-catenin and Cyclin D1 in OSCC group.

DISCUSSION

Wnt/ β-catenin signaling pathway dysregulation is the tipping point in tumor progression, invasion, and metastasis via its effect on β-catenin and Cyclin D1.[21]

Concerning β-catenin results in the present study, immunopositivity was found in normal oral epithelium with membranous expression in all epithelial cell layers. These findings were consistent with previous study that found β-catenin expression was detected only in the cytoplasmic membrane of the normal oral mucosa. It is explained by the fact that β-catenin is essential component of E-cadherin and other cadherin-mediated cell-to-cell adhesion complexes necessary for the structural integrity and functional polarization of epithelia and other tissue structures.[22-24]

Regarding β-catenin expression in epithelial dysplasia, immunopositivity was found in the cytoplasm and cell membrane of dysplastic cells. This is consistent with another study which revealed that β-catenin showed weak membranous expression in parabasal and basal layers of severe dysplasia and a significantly increased expression in the cytoplasm.[25] Considering β-catenin expression in different grades of OSCC in the current study, the mean area percent of immunopositive carcinoma cells for β -catenin decreased from a well-differentiated grade to a moderately-differentiated grade, followed by a poorly-differentiated grade. The staining pattern varied from membranous and cytoplasmic in well-differentiated grades to only cytoplasmic immunostaining in moderately and poorly differentiated grades. These results are in agreement with another study that found significantly less membranous immunostaining in poorly-differentiated OSCC compared to well-differentiated OSCC, which could mean that the cells in this group of tumors are becoming even more dissociated from the basic tumor bulk and that invasion and possibly metastasis are occurring more frequently. It appears that loss of β-catenin membrane expression results in failure of cell adhesion and necessitates the loss of E-cadherin at the same time, it also has oncogenic actions. Thus, altered β-catenin expression has been linked to loss of cell differentiation and the development of an invasive phenotype. It seems that more occurrences of abnormal β-catenin changes, higher rate of aberrant cytoplasmic expression, alongside with less membranous expression can significantly lead to lower degree of cellular differentiation in OSCC.[26]

Concerning β-catenin expression, normal oral epithelial cells had the highest statistically significant mean area percent, followed by epithelial dysplasia; poorly-differentiated OSCC had the lowest mean area percent values. No statistically significant difference existed between oral epithelial dysplasia and OSCC groups, both are lower than normal. These findings were consistent with another study showed that membrane β-catenin expression was markedly reduced in cases of severe dysplasia compared to normal oral mucosa, followed by a change in the localization of β-catenin expression in the cytoplasm and increased staining intensity.[27]Also, these findings are in line with those of **Balasundaram P et al**., (2014), who found that OSCC tumor cells show lower levels of β-catenin expression compared to normal oral mucosa.[28]

Regarding Cyclin D1 in the present study, normal oral epithelial tissues showed the statistically significant lowest mean area percent. This was followed by oral epithelial dysplasia, which showed lower mean area percent compared to OSCC. These results align with those of **Siril YJ et al**., (2022), who reported that OSCC had higher Cyclin D1 levels than oral epithelial dysplasia. Additionally, Cyclin D1 expression varied statistically significantly depending on the grade of OSCC lesions.[16]

In the current study, it was found that both oral epithelial dysplasia and normal oral epithelium showed nuclear and cytoplasmic localization of Cyclin D1 immunopositivity in the basal and

suprabasal cell layers. These findings are in line with another study that discovered that Cyclin D1 immunopositivity was primarily found in the basal cell layer of the normal oral mucosa, whereas in most cases of dysplasia, it was found to extend up to the prickle cell from the basal cell layer and occasionally across the epithelium's thickness. [17]

In the present study, the mean area percent of immunopositive carcinoma cells for Cyclin D1 was found to increase from well-differentiated to poorly-differentiated grades. This result is consistent with another study that showed that the percentage of Cyclin D1 expression increased proportionally as the histopathological grade of the OSCC increased from being highly differentiated to being poorly differentiated. High-grade tumors have higher Cyclin D1 expression levels, which could be explained by the association between changes in Cyclin D1 and the lesions' high proliferative activity and invasion potential. [17]

Another study found that Cyclin D1 overexpression in tumor cells was indicative of an aggressive tumor and that it was associated with a bad prognosis. [29] Correlating between β-catenin and Cyclin D1 in the current study indicating that in normal epithelial tissue group there was no statistically significant correlation between different antibodies, while epithelial dysplasia group demonstrated a statistically significant positive correlation between β-catenin and Cyclin D1. Concerning oral squamous cell carcinoma group, β-catenin and Cyclin D1 had a statistically significant inverse correlation.

CONCLUSION

From the previous results, we can conclude that aberrant cytoplasmic expression of β-catenin act as a co-factor, activating Cyclin D1 lead to high proliferative activity and invasion potential. Thus associations of both molecules has greater role in transformation from normal to dysplastic cells and increase their invasiveness to OSCC.

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