Expression of cyclin D1 correlates with p27^{KIP1} and regulates the degree of oral dysplasia and squamous cell carcinoma differentiation.

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STATEMENT OF CLINICAL RELEVANCE:

Our study demonstrates that the expression of both cyclin D1 and p27^{KIP1} regulates the degree of differentiation of Oral Squamous Cell Carcinoma and could be used to determine the degree of oral dysplasia.

ABSTRACT

Objectives. The aim of this study was to show an association or link between cyclin D1 and P27^{KIP1} protein expression and dysplastic changes or progression.

Study design. Oral mucosal biopsies with a diagnosis of non-neoplastic tissue (Gingivitis) (n=10), mild to moderate oral epithelial dysplasia (n=12) and oral squamous cell carcinoma (n=11) were evaluated by using immunohistochemistry. Scanning software was used to determine cyclin D1 and p27^{KIP1} intensity of expression, location and pattern.

Results. A significant increase in expression of cyclin D1 and a decrease in expression of p27^{KIP1} proteins were observed in oral epithelial dysplasia and less differentiated OSCC. There was a more diffuse distribution of cyclin D1 protein expression extending from the basal cell layer into the prickle cell layers in epithelial dysplasia, and extending within all epithelial layers in OSCC. Cases of oral epithelial dysplasia showed moderate infrequent expression of p27^{KIP1}. There were no p27^{KIP1} positive cells in OSCC. The percentage of cells with both nuclear and cytoplasmic cyclin D1 staining was higher in oral squamous cell carcinoma specimens than control groups and oral epithelial dysplasia.

Conclusions. The expression of both cyclin D1 and p27^{KIP1} correlate with the grade of oral epithelial dysplasia and degree of OSCC differentiation. The results obtained will be verified through a basic follow up of the cases to determine the prognosis/progression of oral dysplasia.

INTRODUCTION

Cyclins are a family of proteins synthesized during the cell cycle, and are capable of controlling the progression of cells by activating cyclin-dependent kinase (CDK) enzymes. At least 30 cyclins have been described ¹ that are active in different parts of the cell cycle and cause the CDK to phosphorylate different substrates. The Mitotic cyclins and the G1 cyclins ² are major classes recognized according to the phase of action. Mitotic cyclins are essential for the control of the cell cycle at the G2/M transition (mitosis). They usually accumulate steadily during G2 and are abruptly destroyed as cell exit from mitosis. While the G1 cyclins are essential for the control of the cell cycle at the G1/S transition ².

Cyclin D has three isoforms D1, D2 and D3 and is involved in regulating cell cycle progression in the G1/S phase transition and appears to be responsive to external growth stimuli rather than internal controls of the cell cycle ³. Cyclin D1 is the best described D-type cyclin and one most frequently associated with cell cycle control and oncogenesis. Cyclin D1 is localized at chromosome 11q13 and elevated levels induce apoptosis ². However, cyclin D1 is an important proto-oncogene, its overexpression leads to shortening of the G1 phase and to less dependency on exogenous mitogens, resulting in abnormal cell proliferation that in turn may favour the occurrence of additional genetic lesions ⁴.

Cyclin D1 is reported as being overexpressed or amplified in a number of primary human cancers supporting its role as an oncogene. In many tumours genetic alterations affecting the cyclin D1 gene frequently result in overexpression of cyclin D1 protein. Several studies have shown overexpression of cyclin D1 is associated with at least half of all invasive breast cancers ⁵. Many studies of mantle cell lymphoma have demonstrated increased activity in cyclin D1 ⁶. It has been shown that overexpression of cyclin D1 by lymphocytes in the mantle zone impairs the capacity of these cells to exit the cell cycle and to differentiate into mature plasma cells ⁷. Studies of oesophageal cancer also showed amplification and overexpression of cyclin D1 in 30% of the cases. Cyclin D1 have been observed in 10% of

hepatocellular carcinoma⁸. Overexpression of cyclin D1 has also been associated with decreased survival and worse prognosis⁹.

Amplification of cyclin D1 gene has been demonstrated in 17%-55% of HNSCC in several studies¹⁰. Over expression of cyclin D1 protein has also been shown in 21%-64% of HNSCC and associated with a poor prognosis, more frequent recurrence, and a shorter time to recurrence^{11,12}. Moreover, overexpression has been shown to be associated with lymph node metastasis¹³.

P27^{KIP1} is a cyclin-dependent kinase inhibitor and a tumour suppressor that regulates G0 to S phase transitions¹⁴. P27^{KIP1} has two main functions in the regulation of cyclin D/CDK4 and cyclin D/CDK6 complexes by promoting assembly and nuclear import of cyclin D1/CDK complexes by tyrosine phosphorylation in the early G1 to mid-G1 stage¹⁵, and under adverse situations it inhibits cyclin D/CDK complexes¹⁵. P27^{KIP1} is not a classic tumour suppressor like P53, but loss of p27^{KIP1} is detected in both carcinoma in situ and invasive tumours and could result in resistance to growth inhibitory factors, deregulation of cell proliferation, and oncogenic changes.

Study of prostate carcinoma showed that variable degrees of reduction in p27^{KIP1} staining were frequently observed in the prostatic intraepithelial neoplasia adjacent to invasive carcinoma¹⁶. Studies of breast cancer also have similar results; p27^{KIP1} is reduced in premalignant and non-invasive malignant lesions, including ductal carcinoma in situ of the breast¹⁷. Furthermore, a comparison of p27^{KIP1} levels in primary colon carcinoma and metastatic tumour demonstrated a reduction of p27^{KIP1} staining in the metastatic tumour¹⁸. Therefore, reduction in p27^{KIP1} level may contribute to cancer progression in the transitions from carcinoma in situ to invasive tumour, and from localized primary tumour to metastatic tumour.

Moreover, decreased levels of p27^{KIP1} may be related to high tumour grade and stage in human colorectal¹⁹, gastric²⁰, breast²¹, prostate^{16,22} and other cancers. Reduction of p27^{KIP1} protein in tumours correlates significantly with decreased survival in colorectal²³, gastric²⁴, breast²⁵ and oesophageal²⁶ squamous cell carcinoma patients. These studies have suggested that p27^{KIP1} plays an important role in tumour suppression. In fact, identification of the p27^{KIP1} proteolysis pathways has opened new avenues for therapeutic interventions in cancer²⁷.

The expression of cyclin D1 and its correlation with p27^{KIP1} in normal oral mucosa, oral dysplasia, and OSCC has not been clearly described in literature. The aim of this study was to determine cyclin D1 and p27^{KIP1} intensity of expression, location and pattern in oral epithelial dysplasia and OSCC by standard immunohistochemistry, and to show an association or link between cyclin D1 and P27^{KIP1} protein expression and dysplastic changes or progression.

MATERIAL AND METHODS

The project received category – B ethical approval from the University of Otago Ethics Committee (07/210) and approval from the Ngai Tahu Research Consultation Committee.

Sample collection and selection criteria

Formalin-fixed, paraffin-embedded (FFPE) oral mucosal biopsies with a diagnosis of non-neoplastic and non-dysplastic tissue (gingivitis) (n=10), mild to moderate oral epithelial dysplasia (n=12) and OSCC (n=11) for the period from 2005 to 2013 were obtained from the archived records of the University of Otago, School of Dentistry, Oral Pathology Diagnostic

Laboratory. The specimens were selected for the experiment according to the following selection criteria:

1. All tissues were obtained by biopsies
2. All samples had the patient's signed approval to be stored in the histopathology archive and to be used in experimental studies
3. All samples had adequate tissue bulk to represent the lesion
4. Diagnoses of oral epithelial dysplasia were confirmed by review using established criteria²⁸
5. Diagnoses of OSCC were confirmed by review using established criteria²⁸

Positive control for cyclin D1 and negative control for p27^{KIP1} were breast carcinoma tissue. Normal thyroid tissue was negative control for cyclin D1 and positive control for p27^{KIP1}. The specimens were categorized into five groups; Group 1, control group, n=10; Group 2, mild oral epithelial dysplasia, n=9; Group 3, moderate epithelial dysplasia, n=3; Group 4, well-differentiated OSCC, n=8; Group 5, moderately differentiated OSCC, n=3.

Sample preparation

Biopsies were fixed in 10% formalin, processed and embedded in paraffin. Ten 4 µm serial sections were cut using a microtome and mounted onto a positively charged slide and stored at 4°C.

Histological examination

The first slide of each specimen was stained with H & E and examined using a light microscopy (Leica CTR5000, Leica Microsystems, Wetzlar, Germany) with X10, X20, and X40 magnification objectives to confirm the diagnosis using WHO criteria.

Immunohistochemistry (IHC)

The second and third slides from each group and control tissues (n=2) were used to establish optimum dilution for each antibody and the time required for conditioning the tissue cells in the automated slide stainer (BenchMark XT, Ventana Medical System, Inc. AZ, USA).

Sections were deparaffinized in xylene, rehydrated through graded ethanols and endogenous peroxidase activity was blocked by using 3% hydrogen peroxide for five minutes. Immunoreactivity of the target antigens was enhanced by microwaving the sections for ten minutes in a 0.1M-citrate buffer. Slides were washed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with monoclonal rabbit anti-human cyclin D1 antibody (Ventana Medical Systems, Inc. 0.07µg/ml), or p27^{KIP1} monoclonal mouse anti-human p27^{KIP1} antibody (Dako Denmark A/S Company 1µg/ml). Normal rabbit IgG (Santa Cruz Biotechnology, Inc. sc-2027, 0.4 mg/ml) was used as a negative control for monoclonal rabbit anti-human cyclin D1 antibody. Normal mouse IgG (Santa Cruz Biotechnology, Inc. sc-2027, 0.4 mg/ml) was used as a negative control for monoclonal mouse anti-human p27^{KIP1} antibody. The Dako Envision™ detection system (Dako, Denmark A/S Company) provided the secondary detection system, and colour development was obtained using 3,3'-diaminobenzidine (DAB) in accordance with the manufacturer's instructions. Sections were then counterstained with Haematoxylin and mounted.

Quantification and Evaluation of immunohistochemistry for cyclin D1 and p27^{KIP1}

The slides were examined under a light microscope (Leica CTR5000) at 10x, 20x and 40x objective magnification and representative photomicrographs were taken with a digital camera (Leica DC500; Leica Microsystems, Wetzlar, Germany). The presence of brown-coloured end product at the site of target antigen was indicative of positive immunoreactivity.

Cyclin D1 and p27^{KIP1} immunohistochemistry was evaluated using image analysis software, by analyzing at least 25 representative fields from each section with the highest staining count at (20X) magnification. Positive cells were counted with square size dimensions of the graticule grid (Figure 1). To determine the number of cyclin D1 and p27^{KIP1} positive cells as a proportion of the total epithelial cell population (expression score), an image analysis system IMAGEJ v 1.47 (National Institutes of Health, Bethesda, MD, U.S.A.), and the IMAGEJ plugins Grid and Cell Counter were used. Images were calibrated to 2.741 pixels/ μm by using a standard 20x scale bar image. Analysis showed a value of 4900 μm^2 per square for images with a resolution of 1600 $\times$ 1200 to be suitable for the study. This resulted in each image consisting of thirty-five (4900 μm^2) squares, and six squares were selected randomly for cell counting. Incomplete squares or squares containing no epithelial cells were not selected for analysis. The percentage of IHC positive cells per representative area were calculated by dividing the total number of the positive epithelial cells by the total number of epithelial cells. Finally, the intensity, pattern and location of cyclin D1 and p27^{KIP1} staining were also described:

Intensity:

Visual characterization of the intensity of the immunohistochemical staining was performed using a scale of 0–10 (with 0 indicating a lack of brown immunoreactivity and 10 reflecting intense dark brown staining) by three observers. All observers evaluated all slides and observations outside of the 5th to 95th percentile of the remaining observations were considered outlying data and were excluded from the analysis. The mean was calculated and the results were converted into grades:

0 = negative staining

1–3 = mild staining

4–6 = moderate staining

> 7 strong or intense staining

Pattern:

- 1- No nuclear or cytoplasmic staining.
- 2- Nuclear staining.
- 3- Cytoplasmic staining.
- 4- Nuclear and cytoplasmic staining.

Location:

- 1- Cells in basal epithelial layers.
- 2- Cells in basal to prickly epithelial layers.
- 3- Cells in the granular cell layers only.
- 4- Diffuse cell distribution throughout the thickness of epithelium.

Statistical analysis

Data was entered into a Microsoft Excel 2011 spreadsheet (Microsoft Corporation, USA) and the relationship between tissue diagnosis to expression of cyclin D1 and p27^{KIP1} was tested by ANOVA using SPSS version 22 (IBM Company, NY, USA) with statistical significance at $P < 0.05$.

RESULTS

Histological examination (Haematoxylin and Eosin Staining)

Representative images of the histological structure of the various lesions and different degrees of epithelial dysplasia are presented in Figure 2 showing features consistent with their diagnosis.

Immunohistochemistry (IHC)

Features of cyclin D1 staining

It was found that cyclin D1 staining was mainly restricted to epithelial cells in the experimental and control tissue. In the control groups, scattered cells showing cyclin D1 protein expression were seen in the basal epithelial layers (Figures 3a, b). Either nuclear or nuclear and cytoplasmic staining was detected in the control and experiment groups. On average, 20% of the epithelial cells in the control group were positive expressing cells. The highest cyclin D1 protein expression score in the control group was 27% of cells, therefore it was determined that protein expression levels above this represented overexpression of cyclin D1. In contrast to the distribution in the control group, in mild epithelial dysplasia there was a

more diffuse distribution of cells showing cyclin D1 protein expression extending from the basal cell layer into the prickle cell layers (Figures 3c, d). Thirty-one percent of the epithelial cells in mild epithelial dysplasia were positive expressing cells. In the cases of moderate oral epithelial dysplasia, moderate to strong immunostaining was detected in basal, and prickle cell layers. The percentage of positive cells per total number of epithelial cell was 45%. While in cases with well-differentiated OSCC and moderately differentiated OSCC, an intense positive cyclin D1 expression for the cancer cells throughout the thickness of the epithelium, was a constant finding (Figures 3e, f). The protein expression score for well-differentiated OSCC and moderately differentiated OSCC were 52% and 69% respectively.

Quantitative analysis of cyclin D1 expression

In comparing the control group with oral epithelial dysplasia, the oral epithelial dysplasia specimens revealed significantly higher cyclin D1 expression than the control group ($P=0.003$). Similarly, the OSCC specimens showed significantly higher cyclin D1 expression than the control group ($P= 2 \times 10^{-7}$). In addition, higher cyclin D1 expression was observed in OSCC ($P= 4 \times 10^{-5}$) compared to oral epithelial dysplasia. Mild oral epithelial dysplasia specimens had significantly lower cyclin D1 expression ($P= 0.021$) than moderate epithelial dysplasia. A high expression of cyclin D1 was observed in moderately differentiated OSCC when compared with well-differentiated OSCC ($P= 0.007$) (Figure 5a).

Cyclin D1 expression pattern

Cyclin D1 expression (nuclear and/or cytoplasmic) was detected in all specimens. Nuclear staining was defined as those cells showing nuclear staining alone, cytoplasmic staining was defined as those cells showing cytoplasmic staining alone, and nuclear and cytoplasmic

staining was defined as those cells showing both nuclear and cytoplasmic. In this study, cyclin D1 was predominantly localized to the nucleus in both control and experimental group, whereas nuclear and cytoplasmic cyclin D1 was less frequent in control group. The frequency of nuclear and cytoplasmic cyclin D1 overexpression was higher in oral epithelial dysplasia and squamous cell carcinoma specimens than in control groups. OSCC specimens showed the highest score of nuclear and cytoplasmic staining (Table 1). There was no statistically significant differences among these three groups.

Clinical features of p27^{KIP1} staining

It was found that p27^{KIP1} staining was mainly restricted to epithelial cells in the experimental and control tissue. In the control group, positive p27^{KIP1} stained cells were found in the superficial and intermediate thirds, associated with epithelial differentiation and granular cell layers (Figures 4a, b). Only nuclear staining was detected in the control and experiment groups. On average, 39% of the epithelial cells in the control group were positive expressing cells (Figure 5b). Since no cases showed a p27^{KIP1} expression score below 24%, we determined that protein expression levels below this represented under-expressed p27^{KIP1}.

Cases of mild oral epithelial dysplasia showed infrequent expression of p27^{KIP1} with 25% of the epithelial cells showing positive (Figure 5b). In comparing the control group with the mild and moderate epithelial dysplasia specimens, a reduction in p27^{KIP1} expression was detected ($P=0.031$ and $P=0.003$ respectively). Compared to mild oral epithelial dysplasia, only a few scattered positive cells were detected in the granular cell layer in moderate oral epithelial dysplasia (Figure 4c, d). In comparing the moderate oral epithelial dysplasia with the mild oral epithelial dysplasia, no statistically significant differences were detected in the

expression score ($P=0.08$). Only 10% of the epithelial cells in the moderate oral epithelial dysplasia were positive expressing cells. There were no positive cells in OSCC (Figure 4e, f).

DISCUSSION

It has been postulated that carcinogenesis of OSCC involves an accumulation of genetic alterations in the oral epithelium. It has been estimated that six to ten genetic mutations occur independently in the development of head and neck squamous cell carcinomas²⁹. Expression of cyclin D1 and P27^{KIP1} is important during cell proliferation and differentiation and alterations may be implicated in malignant transformation³⁰. This study was carried out to correlate immunohistochemically the expression of cyclin D1 and p27^{KIP1} in non-neoplastic, non-dysplastic epithelium, oral epithelial dysplasia, and OSCC.

Relatively few studies have examined cyclin D1 protein expression in oral epithelial dysplasia and the reported prevalence rates of cyclin D1 overexpression vary from low in low-grade oral epithelial dysplasia³¹ to high in all grades of oral epithelial dysplasia³². These discrepancies and variations reflect the general lack of objective quantification methods to examine protein expression since most of the studies used subjective assessment of staining intensities³², while others used semi-quantitative scoring³³. In the present study, computer image analysis software was used to quantify cyclin D1 and p27^{KIP1} nuclear/cytoplasmic staining and therefore provided an objective assessment of protein staining levels in oral epithelial dysplasia and OSCC.

The threshold for cyclin D1 expression in normal epithelium was established by showing that the expression score did not go above 27%. As such overexpression of cyclin D1 in OSCC was found compared with the control groups, which is in line with previous studies³⁴. Moderately differentiated OSCC demonstrated a significantly higher incidence of

cyclin D1 overexpression than well-differentiated OSCC specimens. These results are consistent with previous studies³⁵⁻³⁸. However, the exact reason for this relationship remains unclear. Studies on myoblasts and intestinal epithelial cells demonstrated cyclin D1 overexpression can inhibit the differentiation of these two cell types^{39,40}. This implies that cyclin D1 overexpression may be able to inhibit the differentiation in OSCC.

P27^{KIP1} is a member of the universal CDK inhibitor, which is considered as a negative regulator of the cell cycle, and is found abundantly in quiescent cells and less in proliferative cells⁴¹. A number of studies have shown that decreased expression significantly correlates with cancer progression in a number of cancers including prostate cancer¹⁶, breast cancer⁴², primary colon carcinoma⁴³, gallbladder cancer⁴⁴, extrahepatic bile duct cancer⁴⁵ and parathyroid cancer⁴⁶ suggesting that the p27^{KIP1} cell cycle inhibition pathway plays an important role in human carcinogenesis and tumour progression. In this study, the control groups exhibited high levels of nuclear p27^{KIP1} immunoreactivity, whereas decreased p27^{KIP1} expression in oral epithelial dysplasia and lack of immunoreactivity in OSCC specimens were observed. This strongly suggests that dysfunction of p27^{KIP1} is involved in the pathogenesis of OSCC. This is supported by a previous study by Venkatesan et al., 1999 where variable levels of P27 intensity levels and frequency were demonstrated in cancer cases. Most of the P27 expression was nuclear with unclear cytoplasmic staining. In control samples, P27 were limited to the superficial epithelial layers with only less than 5% of the basal cells showing a positive reaction to P27 which is also in agreement with the present study⁴⁷. Furthermore, the above mentioned study correlated low levels of P27 expression with unfavourable treatment response, shorter overall survival, and shorter disease-free survival⁴⁷, while the present study did not include follow up cases to demonstrate the above correlations. However, it seems plausible that p27^{KIP1} is a negative regulator of the cell cycle in the oral cavity and that

reduced expression of p27^{KIP1} protein reflects the potential for malignant formation in oral potentially malignant lesion⁴⁸.

An interesting finding was that reduced p27^{KIP1} expression was closely associated with cyclin D1 overexpression. Given that both cyclin D1 and p27^{KIP1} affect the cell cycle by regulating the same targets, CDKs, their concomitant under and overexpression is understandable. CDKs interact with Rb protein by phosphorylation, as a result the whole complex (CDK-Rb) releases the transcription factor E2F, which stimulates the transition from G1 phase into the S phase. Both decreased p27^{KIP1} expression and cyclin D1 overexpression result in increased CDK activity, and subsequently lead to uncontrolled cell proliferation. This is supported by a study by Shintani et al., 2002 where Cyclin D1 was overexpressed in squamous cell carcinoma when compared to control cases in order to provide a new growth avenue for dysplastic cells, and P27^{KIP1} was either poorly expressed (7.1% of cases) or not observed at all in cases of epithelial dysplasia, this reduction in P27^{KIP1} was reported to be associated with early stages of squamous cell carcinoma development⁴⁹. P27^{KIP1} expression was reported to be reduced in a number of studies related to oral cancer^{50, 51}. Over expression of cyclin D1 has been confirmed in oral cancer with a high variability in levels of expression between studies due to subjectivity and difference in scoring systems used in individual studies^{52, 53}. In the present study we used a computer imaging software for localization of cyclin D1, P27^{KIP1} immunostaining, which was augmented by individual interpretation of staining intensity. Both methods resulted in a more objective assessment of areas, levels and patterns of immunostaining in the present study.

Studies suggest cyclin D1 is a nuclear protein⁵⁴. However, in this study it was observed that OSCC had strong cyclin D1 nuclear and cytoplasmic staining. In addition, the percentage of cells with both nuclear and cytoplasmic cyclin D1 staining was higher in OSCC than in the control group and oral epithelial dysplasia, which is in agreement to previous

studies⁵⁵. The exact mechanism for this is unknown, but it may be due to the presence of two different isoforms of cyclin D1 or that cyclin D1 normally accumulates in the nucleus throughout G1 phase, but relocates to the cytoplasm during the interphase⁵⁴.

CONCLUSION

Cyclin D1 protein was frequently overexpressed in OSCC than in non-neoplastic and non-dysplastic epithelium and correlated with the grade of oral epithelial dysplasia and the degree of OSCC differentiation. Normal expression of p27^{KIP1} could be observed in non-neoplastic and non-dysplastic epithelium. A decrease in p27^{KIP1} expression was found in oral epithelial dysplasia and a lack of immunoreactivity was observed in OSCC. Reduced p27^{KIP1} expression was associated with cyclin D1 overexpression. It was found and concluded that cyclin D1 accumulates in different compartments and this may be through transit across the nuclear pores. Both nuclear and cytoplasmic cyclin D1 staining were observed more in OSCC than non-neoplastic and non-dysplastic epithelium. The exact mechanism remains unknown, but it may be due to the presence of two different isoforms of cyclin D1. A follow up of the cases was deemed necessary to confirm the correlation between cyclin D1, p27^{KIP1} expression, the progression of oral dysplasia and the degree of OSCC differentiation.

CONFLICTS OF INTEREST

None declared

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Figure 1: Representative section of cyclin D1 positive epithelial cells demonstrating graticule grid used for counting.

Figure 2: Representative image of hyperplastic gingivitis (Control Group) (a & b), mild oral epithelial dysplasia (c & d), moderate oral epithelial dysplasia (e & f), well-differentiated oral squamous cell carcinoma (g & h), moderately differentiated oral squamous cell carcinoma (i & j).

Figure 3: (a): Representative image of hyperplastic gingivitis (Cyclin D1 IHC X 10). (b): Showing scattered positive nuclear staining in cells of the basal epithelium layer (Red arrows) and positive nuclear and cytoplasmic staining in the prickle cell layer (Black circles) (Cyclin D1 IHC X 20). (c): Representative image of mild to moderate oral epithelial dysplasia (Cyclin D1 IHC X 10). (d): Showing a more diffuse distribution of moderate to strong nuclear staining within mild to moderate oral dysplasia extending from the basal layer into prickle cell layer (Red arrows) (Cyclin D1 IHC X 20). (e): Representative image of a well differentiated oral squamous cell carcinoma (Cyclin D1 IHC X 10). (f): Showing the diffuse strong nuclear and cytoplasmic staining of Cyclin D1 positive cells within the well differentiated OSCC (Red arrows) (Cyclin D1 IHC X 20).

Figure 4: (a): Representative image of Hyperplastic gingivitis (P27^{KIP1} IHC X10). (b): Showing positive nuclear staining in p27^{KIP1} cells in the granular cell layer (Red arrows) (P27^{KIP1} IHC X 20). (c): Representative image of moderate oral epithelial dysplasia (P27^{KIP1} IHC X10). (d): Showing scattered positive nuclear staining in p27^{KIP1} cells in the granular cell layer of moderate oral epithelial dysplasia (Red arrows) (P27^{KIP1} IHC X 20). (e): Representative image of moderately differentiated oral squamous cell carcinoma (P27^{KIP1} IHC X10). (f): Showing no positive p27^{KIP1} cells in the moderately differentiated oral squamous cell carcinoma (P27^{KIP1} IHC X 20).

Figure 5: Bar graph with standard deviation illustrates the percentage of the cells with (a): cyclin D1 and (b): P27^{KIP1} protein staining among control group, mild oral epithelial dysplasia, moderate oral epithelial dysplasia, well-differentiated OSCC and moderately differentiated OSCC.

Table 1. Showing Cyclin D1 expression pattern.

	Nuclear staining (Mean $\pm$ SD)	Cytoplasmic staining (Mean $\pm$ SD)	Nuclear and cytoplasmic staining (Mean $\pm$ SD)
Control group (Gingivitis)	68 $\pm$ 11%	0	33 $\pm$ 11%
Oral epithelial dysplasia	54 $\pm$ 12%	0	46 $\pm$ 11%
OSCC	48 $\pm$ 14	0	55 $\pm$ 13

Figure 1.tif

Figure 2.tif

Figure 3.tif

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Figure 5.tif

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