



## Original Research Article

## Expression of Cyclin D1 in oral squamous cell carcinoma and its correlation with histopathological differentiation

Vigyan Mishra<sup>1</sup>, Subhransu Kumar Hota<sup>1</sup>, Ranjana Giri<sup>1,\*</sup>, Urmila Senapati<sup>1</sup>, Subrat Kumar Sahu<sup>2</sup>

<sup>1</sup>Dept. of Pathology, Kalinga Institute of Medical Sciences, Bhubaneswar, Odisha, India

<sup>2</sup>Dept. of Oncosurgery, Kalinga Institute of Medical Sciences, Bhubaneswar, Odisha, India



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## ABSTRACT

**Background:** Cyclin D1 is a vital protein that has a widespread role in cell cycle regulations, providing control over G1 to S Phase transition and governing cell proliferation rates. Cyclin D1 overexpression has been reported in variety of tumors. Present study was carried out to study the expression of cyclin D1 and its association with histopathological differentiation and stage of oral squamous cell carcinoma.

**Materials and Methods:** 48 formalin fixed paraffin embedded tissue blocks of biopsy specimens of oral squamous cell carcinoma were immunohistochemically evaluated for expression of cyclin D1.

**Result:** Cyclin D1 expression was seen in 98.5% (46) cases of OSCC. It did not correlate with site and staging. Cyclin D1 expression was seen in 94.6% of well differentiated tumor, 100% in moderately differentiated and 100% in poorly differentiated oral squamous cell carcinoma, but it was not statistically significant ( $p=0.065$ ).

**Conclusion:** Relatively higher frequency of Cyclin D1 immuno-reactivity observed in patients with less differentiated tumours suggest inverse correlation of Cyclin D1 expression with histological differentiation of tumour.

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### 1. Introduction

Oral cancers constitutes the sixth most common cancer worldwide<sup>1</sup> and its incidence is much higher in many developing countries.<sup>2</sup> In India, incidence of oral cancer is 10.4% of all cancers.<sup>3</sup> Five year survival rate is 82% in early stage and 27% in advanced stages.<sup>3-5</sup> About 90-95% of the oral cancers are squamous cell carcinoma.<sup>6</sup>

Despite improvements in both diagnostic and therapeutic strategies, the prognosis of oral squamous cell carcinoma (OSCC) has not changed significantly over the last decades.<sup>7</sup> Moreover, the biological behaviour of OSCC has been found to be varied. That is why it becomes important to find out molecular markers to predict and prognosticate the disease apart from refinement of treatment strategy. The

multistep process of carcinogenesis involves progressive acquisition of mutations and epigenetic abnormalities in the expression of multiple genes with an important group being those involved in cell cycle control.<sup>8</sup>

Escape of cancer cell from the cell cycle mechanism reflects the fundamental hallmark of cancer progression and even emerging as central role in oral carcinogenesis.<sup>8</sup> Indeed, the strongest connection between cyclins and oncogenesis comes from the study on Cyclin D1. Dysregulated or overexpressed Cyclin D1 may lead to shortening of the G1 phase, increased cell proliferation and reduced dependency on growth factors.<sup>9</sup> It causes disturbance in the normal cell cycle control and mitogenic signaling pathways enhancing the cell transformation and tumorigenicity.<sup>8</sup>

Amplification and overexpression of Cyclin D1 has been reported to be more frequent in head and neck, oral,

\* Corresponding author.

E-mail address: [drranjana.pth@gmail.com](mailto:drranjana.pth@gmail.com) (R. Giri).

laryngeal and nasopharyngeal carcinoma. However, studies regarding Cyclin D1 expression and their relation to stage, grade and nodal status are less. With this background, the present study has been undertaken to evaluate the immunohistochemical expression of Cyclin D1 in oral squamous cell carcinoma. And to correlate their expression with histopathological differentiation and stage of tumor.

## 2. Subjects and Methods

The present study includes 48 cases of oral squamous cell carcinomas. Oral squamous cell carcinoma cases which have undergone radical excision and diagnosed on histology were included, while recurrent cases, small biopsies or cases where nodal status or tumor stage is not known and patients taken neo-adjuvant therapy were excluded.

Specimens were fixed overnight in 10% buffered formalin and processed. Grossing of the specimens was done as per the AJCC guidelines.<sup>10</sup> Four to five micrometer thick formalin fixed, paraffin embedded tissue sections were subjected for haematoxylin and eosin staining.

Histologic examination was performed. Typing and grading of tumor was done according to the criteria outlined in the World Health Organization<sup>11</sup> (WHO) classification of tumours and classified into well, moderately and poorly differentiated cases.

### 2.1. Immunohistochemical analysis

Immunohistochemical evaluation of Cyclin D1 was done on formalin fixed paraffin embedded tissue sections (4-5 micrometer thick) on poly-L lysine coated slides by using polymer two-step indirect method. The antibodies and chemicals used were from DAKO, USA. Monoclonal Rabbit antibody to Cyclin D1 (clone-EP12)<sup>12,13</sup> was used for immunohistochemical evaluation of Cyclin D1.

The manufacturer's instructions were followed after standardization in our laboratory. The following steps were followed: deparaffinization done by placing slides on hot plates at 60° for 30 minutes followed by Xylene de waxing – 3 changes 10 minutes each. Followed with rehydration by dipping the slides for 5 minutes each in decreasing concentration of alcohol (100%, 85%, and 75%) and then washing by keeping the slides in running tap water for 5-10 minutes followed by dipping in deionized water for 2 minutes. For antigen retrieval (Microwave heating method) slides were dipped in antigen retrieval solution (Tris buffer (1.2 gm) + Sodium EDTA (0.37 gm) +1 L distilled water (pH-9) ) kept in Coplin jars and are heated in the microwave oven for 6 cycles 3 minutes each. Then cooling at room temperature for 30 minutes and washing the slides in wash buffer (pH – 7.6 – Distilled water (1 L) + Sodium chloride (8.5 gm) + Tris buffer (6.05)) solution for 3 times, 2 minutes each was done. Power block solution (100% methanol + 0.5% H<sub>2</sub>O<sub>2</sub> + 2.5%

NaCl) was added on the slides and kept for 12 minutes at room temperature. It prevents background staining. Primary antibody was added (without any dilution) and incubated for 1 hour at room temperature in a covered jar. Then washing three times in wash buffer solution. Incubation in super enhancer solution for 30 minutes done for enhancement and amplification followed again by three times washing in wash buffer. Secondary antibody tagged with HRP was added and incubated for 30 minutes at room temperature. Three times washing in wash buffer solution followed by addition of chromogen DAB solution on slides and kept for 5-10 minutes. Then washing 5 minutes in distilled water and counterstaining with Haematoxylin 1-2 dips done. Lastly dehydration by dipping in increasing concentrations of alcohol (75%, 85% and 100%) and mounting with DPX.

External positive and negative control slides were used with each batch of staining. Cyclin D1 positive control slides were prepared from tonsil. Negative control slides were prepared from the same tumour block under study. The primary antibody step was omitted and slides were incubated with phosphate buffer saline instead.

### 2.2. Assessment of staining

The assessment of immunohistochemical staining was carried out by three independent pathologists. The corresponding H&E section were thoroughly examined before evaluating IHC. The sections were initially scanned at low power (4x and 10x objective magnifications) and the slides showing positive reactivity were further evaluated for Cyclin D1. To determine the Cyclin D1 expression we used a more objective and inclusive method as depicted by Guy et al. 2010.<sup>14,15</sup> Both nuclear and cytoplasmic staining was considered as positive. Cytoplasmic staining in absence of nuclear staining was considered negative.

In every slide ten hot spot areas were selected and observed under higher (400X) magnification. Percentage of IHC positive tumour cells per hot spot was calculated and the mean percentage per slide (labelling index) was determined. A labelling index score of 1, 2, 3, or 4 was assigned for labelling indices 1–25%, 26–50%, 51–75%, and >75%, respectively.

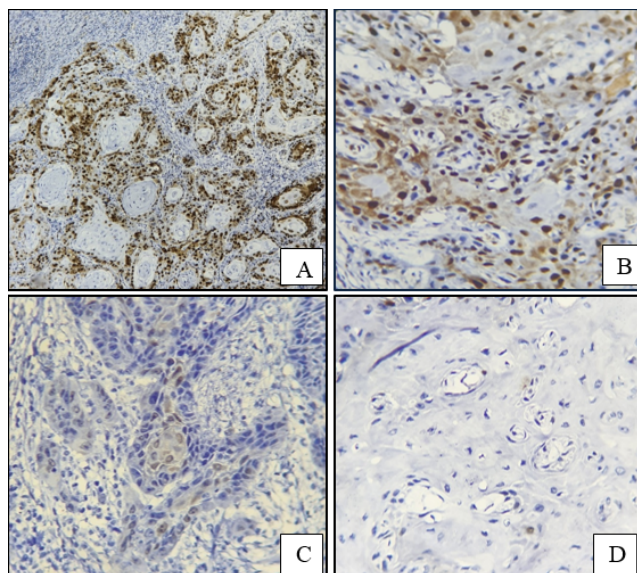
The intensity of Cyclin D1 immunostaining was evaluated<sup>15</sup> on the basis of microscopic appearance as weak, intermediate, or strong and an intensity score of 1, 2, or 3 was assigned to them, respectively. A final expression score was calculated by multiplying labelling index score with intensity score, based on which the Cyclin D1 expression was determined as weak (score 1–4), moderate (score 5–8), or strong (score 9–12). (Figure 1A,B,C,D)

### 2.3. Statistical analysis

Statistical analysis was done to find out the expression of Cyclin D1 in oral squamous cell carcinoma and correlation

**Table 1:** Immunohistochemical expression of cyclin D1<sup>15</sup>

Score	Cyclin d1 expression
0	Negative
1 – 4	Weak staining
5 – 8	Moderate staining
9 – 12	Strong staining



**Fig. 1: A,B,C,D:** Cyclin D1 score on IHC. **A:** Cyclin D1 positive strong (score - 12). **B:** Cyclin D1 positive strong (score - 9). **C:** Cyclin D1 positive intermediate (score - 6) **D:** Cyclin D1 negative (score - 0)

of Cyclin D1 with histopathological differentiation. The relationship between qualitative parameters was determined using the chi-square statistics. Statistical significance was defined as  $p < 0.05$ . All the analysis was done by standard statistical software (Stata Version 15.1).<sup>16</sup>

### 3. Results

Cyclin D1 positivity was seen in 46 cases (95.8%) of OSCC cases. Further distribution of cyclin D1 reactivity in accordance with site, stage, and histopathological differentiation are explained in Table 2. The labelling index scores, intensity of staining, expressions are graded, and their correlations with clinical and histological parameters are elaborated in Tables 3 and 4.

Both cyclin D1 reactivity and expression did not show any correlation with site and staging of the OSCC (Tables 2 and 4). The histopathological differentiation revealed increase in Cyclin D1 expression with grade of tumor, however, there was no statistically significant correlation. (Tables 3 and 4). The labelling index score and intensity did not correlate with OSCC differentiation (Table 2).

### 4. Discussion

In present study we identified the immunohistochemical reactivity and expression of cyclin D1 and its association with site, staging, and histopathological differentiation of oral squamous cell carcinoma.

Out of 48 OSCC cases in the present study, Expression of Cyclin D1 was found in 95.80% (46/48) cases. Out of which, 54.35 % (25/46) cases had strong expression while 26.09% (12/46) had moderate expression and 19.56% (9/46) had weak expression of Cyclin D1. Our findings of Cyclin D1 positivity were in accordance with the study done by Uma Swaminathan et al. 2012, with 95% cases showing Cyclin D1 positive expression.<sup>17</sup> However, lower expression of Cyclin D1 was found in study done by Saawarn et al. 2012 and Huang et al. 2012, with 45.00% and 36.70% of cases respectively.<sup>15,18</sup> Ramos-García et al. in 2019, in their study of 54 cases Cyclin D1 expression was found in 28.70% cases only.<sup>19</sup> The over expression of Cyclin D1 suggests as expected, that there is increased proliferation in OSCC. These reported variations in reactivity may be due to diverse reasons like asymmetric labelling expression seen in different parts of same specimen owing to the fact that in a specimen at a given time, only about 20% of the neoplastic cells are under mitosis.<sup>20</sup>

In our study, the labelling index did not have any correlation with histopathological differentiation and the results could not be compared directly with other reported literatures, because of different criteria used for determining scores by different authors.<sup>21–32</sup> We have used an objective criteria for assessment of cyclin D1 expression as described by Guy et al. 2010.<sup>15,33</sup>

There was nonuniformity in staining intensity, showing maximum cases of strong staining followed by intermediate and weak and there was no statistically significant correlation with histological differentiation. Angadi and Krishnapillai<sup>34</sup> and Mishra and Das<sup>35</sup> noted a uniformly increasing intensity in relation to the histopathological differentiation, whereas Castle et al.<sup>36</sup> found no correlation.

Increased Cyclin D1 expression was seen in tumors belonging to higher stage(III & IV) i.e. 100% whereas in lower stage(stage I)expressed Cyclin D1 in 100% cases while only 88.2% in stage II tumors. However, no statistically significant correlation was found between Cyclin D1 positivity and AJCC stage ( $p = 0.283$ ). Outcome of our study was similar to that of Huang et al. 2012,<sup>18</sup> which revealed increase in Cyclin D1 expression with higher stage and there was a statistically significant correlation of Cyclin D1 immunoexpression with higher tumor stage with a  $p$  value of 0.051. However, Ramos-García et al. 2019,<sup>19</sup> in their study also documented maximum number of cases are in stage IV (34.50%) with Cyclin D1 expression but it was not statistically significant. While, Saawarn et al. 2012,<sup>15</sup> also reported maximum number of cases in stage IV but immunoexpression of cyclin

**Table 2:** Distribution of site, staging and histopathological distribution of OSCC and their IHC reactivity

Distribution Category	Total	IHC reactivity		% of positive reactivity	P
		Positive (n)	Negative (n)		
Site					
Buccal mucosa	21	20	1	95.2%	0.992
Lip	1	1	0	100%	
Lower alveolar ridge	4	4	0	100%	
Palate	2	2	0	100%	
Retromolar trigone	2	2	0	100%	
Tongue	18	17	1	94.4%	
Stage					
Stage I	5	5	0	100%	0.283
Stage II	17	15	2	88.2%	
Stage III	9	9	0	100%	
Stage IV	17	17	0	100%	
Grade					
WDSCC	34	32	2	94.1%	0.651
MDSCC	11	11	0	100%	
PDSCC	3	3	0	100%	
Total	48	46	2	95.8%	

**Table 3:** Labelling index score and intensity in relation to histopathological differentiation

OSCC differentiation	Weak (n)	Intensity		P	Labelling index score				P
		Intermediate (n)	Strong (n)		Score 1	Score 2	Score 3	Score 4	
WDSCC	1	9	22	0.682	4	9	7	12	0.781
MDSCC	1	2	8		1	3	1	6	
PDSCC	0	0	3		0	0	1	2	
Total	2	11	33		5	12	9	20	

**Table 4:** Cyclin D1 expression in relation to site, clinical staging and histopathological differentiation

Distribution Category	Cyclin D1 expression			P
	Weak (n)	Moderate (n)	Strong (n)	
Site				
Buccal mucosa	3	5	12	0.087
Lip	1	0	0	
Lower alveolar ridge	0	2	2	
Palate	1	0	1	
Retromolar trigone	2	0	0	
Tongue	2	5	10	
Stage				
Stage I	0	0	5	0.130
Stage II	5	6	4	
Stage III	2	2	5	
Stage IV	2	4	11	
Grade				
WDSCC	7	16	9	0.594
MDSCC	2	6	3	
PDSCC	0	3	0	
Total	9	12	25	

D1 was seen highest in stage II patients, but not statistically significant. So, in current study it shows that Cyclin D1 expression was increased in higher stage compared to lower stage tumor, though, it was not statistically significant. This could be attributed to the low number of cases in the present study.

Cyclin D1 expression was found in 94.6% of Grade I tumor cases and in 100% Grade II and 100% in Grade III cases. Although with increase in tumor grade Cyclin D1 expression was found to increase but this was not statistically significant ( $p = 0.651$ ). Our results were similar to study done by Huang et al. 2012 and Ramos-García et al. 2019, who found increase in cyclin D1 expression with decrease in differentiation, but they found statistically significant correlation between Cyclin D1 expression and higher grade (moderately and poorly differentiated) of tumor with a  $p$  value of 0.031 and 0.030 respectively.<sup>18,19</sup> However, in a study by Saawarn et al. 2012, highest expression of Cyclin D1 was observed in 69.23% of lower grade of tumor i.e. well differentiated cases and there was statistical correlation with a  $p$  value of 0.011.<sup>15</sup>

These differences between studies could be due to differences in sample size, distribution of tumor site. Relatively higher frequency of Cyclin D1 immunoreactivity observed in patients with less differentiated tumours. However more sample size and study duration might reveal significant correlation.

To conclude, Cyclin D1 was expressed in significant number of cases in our study. Expression of Cyclin D1 also increases with increasing tumor grade and higher stage. Statistically significant correlation could not be made, may be attributed to small sample size. Further studies with large number of cases and using other ancillary molecular diagnostic techniques would throw further light on the exact alteration of Cyclin D1 in our population. The findings of this study will be an important adjunct along with staging and grading to determine the prognosis of the disease and also to design the treatment. It may lead to decrease in morbidity and improved survival of patients with OSCC

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## 7. Conflict of Interest

The authors declare they have no conflict of interest.

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### Author biography

**Vigyan Mishra**, Post Graduate Student

**Subhransu Kumar Hota**, Assistant Professor

**Ranjana Giri**, Associate Professor

**Urmila Senapati**, Professor and HOD

**Subrat Kumar Sahu**, Associate Professor

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