



## Original Research Article

## A study to detect numerical aberrations of cyclin D1 gene using Fluorescence in situ hybridization technique among patients of oral squamous cell carcinoma

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## ABSTRACT

**Introduction:** The Cyclin D1 gene (CCND1) located on chromosome 11q13 is a positive regulator of the cell cycle. It encodes a nuclear protein that plays an important role in the tumorigenesis of human cancers.

**Aim:** The aim of the study is to evaluate numerical aberration of Cyclin D1 gene by using Fluorescence in Situ Hybridization (FISH) in Oral Squamous Cell Carcinoma (OSCC).

**Materials and Methods:** Formalin-fixed paraffin embedded tumor section obtained from histological confirmed 50 OSCC patients from Department of Oncology, ENT and Pathology, S.M.S. Medical College, Jaipur. The FISH technique was used to detect the numerical aberrations of Cyclin D1 using the Vysis protocol.

**Results:** The CCND1 numerical aberration was found positive in 18(36.0%) of 50 patients of OSCCs. Low level amplification was found in 9(11.3%), Cluster amplification 6(7.5%), polysomy 2(2.5%) and deletion of Cyclin D1 1(1.3%) respectively. There was not statistically significant association of histopathological differentiation, site of carcinoma, lymph node metastasis with gender. A significant association (P=0.004) was present between stage of carcinoma and types of numerical aberration of Cyclin D1 gene. Cyclin D1 aberration showed a significant association with lymph node metastasis (P=0.038). We have found no significant association between risk factors (tobacco chewing, smoking and alcohol consumption) to aberration of Cyclin D1 gene.

**Conclusions:** Analysis of the CCND1 numerical aberration using FISH on paraffin embedded tumor section may be a useful and practical method for predicting aggressive tumors, recurrence and clinical outcome in patients with OSCCs.

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## 1. Introduction

Cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018. Globally, about 1 in 6 deaths is due to cancer. Tobacco use is the most important risk factor for cancer and is responsible for approximately 22% of cancer deaths.<sup>1</sup>

Head and neck squamous cell carcinomas (HNSCCs) is the most prevalent malignant neoplasm (90% approximately).<sup>2,3</sup> Approximately 6,50,000 new cases of HNSCC including OSCC have been reported annually worldwide, and are responsible for 350,000 deaths per year, with half of them occurring in developing countries like India.<sup>4,5</sup>

OSCC mainly affects the tongue and floor of mouth.<sup>6</sup> SCC of the lip, hard palate and maxillary gingival are not frequently metastasize to regional lymph nodes and have

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a relatively favorable prognosis rather than SCC of the tongue, floor of mouth and mandibular gingival.<sup>7</sup>

OSCC arises through a multistep process of genetic alterations usually as a result of individual predisposition and the exposure to environmental agents, thus cancer is a genetic disease of somatic cells.<sup>8</sup> The aggressiveness of a malignancy due to chromosomal and genetic alterations can affect tumor progression, treatment and prognosis.<sup>9</sup> The genetic changes occurring in OSCC have retained the focus attention in dentistry, mainly in oral and maxillofacial pathology.<sup>10</sup>

Carcinogens like tobacco products and alcohol in solution constantly accumulate in the floor of mouth and bathe the tissues of the floor of mouth and ventrum of tongue. So these carcinogens rapidly penetrate the epithelium to reach the progenitor cell.<sup>11</sup>

Oral cancers have a multifaceted etiology. Lifestyle and environmental factors has been identified as the risk factor for oral cancers. In spite of this human papilloma virus (HPV) infection especially HPV 16 and 18 can also affects of oral, tonsillar and oropharyngeal OSCC.<sup>12</sup>

Molecular Cytogenetic is the field in which cloned DNA probes are used to examine chromosomes. This is relatively specialized area now, but it may become part of routine clinical cytogenetic practice in future.<sup>13</sup>

Among the advanced molecular techniques, fluorescence in-situ hybridization (FISH) has a perfect balance of high specificity and sensitivity with advantage of rapidity, which is being used in routine clinical laboratory for genomic diagnosis.<sup>14</sup>

Deregulation of the cell cycle mechanism is a critical event in carcinogenesis and it is emerging as a central theme in oral carcinogenesis. The genes involved in cell cycle regulation represent targets of oncogenic abnormalities among which Cyclin D1 is most involved.<sup>15</sup>

In human cells, cell division is controlled by the activity of Cyclin-dependent kinases (CDKs) and their essential activating coenzymes, the CDK inhibitors, which may be influenced by genetic variations in the corresponding genes.<sup>16–18</sup>

Cyclin-D1 is a protein that is encoded by the CCND1 (Cyclin D1) gene. CCND1 is a proto-oncogene is located on the long arm of chromosome 11 (band 11q13). It is 13,388 base pairs long, and translates into 295 amino acids. Initial studies indicated that cyclin D1 is localized predominantly in the nuclei of asynchronously growing cells.<sup>19</sup> A frequent target in carcinogenesis is the deregulation of G1 to S phase progression of the cell cycle. The transition through G1 to S phase is regulated by cyclins, cyclin-dependent kinases (CDK)-CDK4 and CDK6 and their inhibitors. Cyclin D1 is a key regulatory protein at G1/S checkpoint of the cell cycle. It forms complexes with CDK4 or CDK6 and is responsible for the phosphorylation of the retinoblastoma tumour suppressor protein, resulting in the release of E2F

transcription factors that allow cell to enter into S phase. The G1/S checkpoint is frequently altered in many epithelial tumours and may confer growth advantage and enhanced tumor genesis.<sup>20</sup>

Cyclin D1 (CCND1) have been detected in oral squamous cell carcinomas (OSCCs), suggesting that abnormalities of these genes may play an important role in the genesis or progression of OSCCs and serve as independent prognostic indicators.<sup>21</sup>

FISH analysis requires very little tumor tissue. This Method is rapid and does not involve radioactivity. Interphase FISH eliminates the necessity and time of cell culture and enables enumeration of gene copy number compared with a control probe on the same chromosome in large populations of cells even in the absence of metaphase chromosomes. The interphase FISH technique produces direct visualization of chromosomal aberrations in cell nuclei using fluorescently-labeled DNA probes.<sup>22</sup>

Alarming numbers of the population from North India including state of Rajasthan and adjoining region currently suffering from cancer and a substantial numbers of patients comprise of OSCCs. Understanding the epidemiology and the risk factors for oral cancers can help early identification and prompt treatment of patients with oral cancers.

The present study was design to detect Cyclin D1 gene numerical aberrations in OSCCs by using fluorescence in situ hybridization technique (FISH).

## 2. Subjects and Methods

The samples required were collected from the 50 patients of Oral Squamous Cell Carcinoma (OSCC) who were attending and enrolled in the Out Patient Department (OPD) of the institution. This is retrospective study.

### 2.1. Inclusion criteria

1. Histopathologically proved OSCC patients.
2. Age: 25 years and above it.

### 2.2. Exclusion criteria

1. Patients who have been exposed to chemo/radiotherapy.
2. Subjects with any other malignancy, any other systemic disease or medication using from long time were excluded.

### 2.3. Tissues and Patient characteristics

Formalin-fixed paraffin embedded tumor section obtained from histological confirmed 50 OSCC patients. Paraffin embedded tumor section were collected from the Out Patient Department (OPD) of the institution.

The institutional ethics committee cleared the protocol and the information pertaining to the patients. Informed

consent was obtained from all patients in accordance with our Institutional ethics committee guidelines. Clinical data of all patients with regard to of patients including age, gender, weight, height, tobacco chewing, smoking, alcohol consumption, duration of symptoms and the presence of lymph node metastasis factors helpful in study were record.

The mean age of patients was  $46.80 \pm 12.34$  years (range, 22-70 yeras). The mean of BMI was  $21.56 \pm 3.2$  kg/m<sup>2</sup> (range, 16.94-33.89 kg/m<sup>2</sup>). The 50 OSCC samples were derived from the buccal mucosa (23), soft palate (3), lateral surface of tongue (11), retro-mandibular region (3), root of tongue (4), lower alveolar mucosa (6) and the floor of the mouth (4). The clinical staging was defined on the basis of the American Joint Committee on Cancer (AJCC).<sup>23</sup> TNM classification: Stage I (12), stage II (12), stage III (8), stage IV (7), Stage IV (2) and stage IVC (9). The tumors were classified histopathologically in to well (17), moderately (26) and poorly differentiated (7) according to their cellular differentiation as defined in the World Health Organization classification.<sup>24</sup> Duration of delay in diagnosis was  $5.8 \pm 7$  months (range, 1-24 months).

#### 2.4. Fluorescence in Situ Hybridization (FISH)

FISH was carried on thin sections ( $4\mu$ ), cut from formalin-fixed, paraffin-embedded tissue blocks. The slides have been baked and aging have done at 70°C in oven for 3 hrs. Immerse slides in xylene solution A, B and C for 10 mints. Dehydrate slides in 100% ethanol solution A and solution B for 3 minutes. Immerse slides in 0.2N HCl for 20 minutes. Slides dip in sodium thiocyanate solution (pretreatment solution) at 85°C for 30 minutes in hot water bath. Immerse slides in wash buffer (2×SSC) for 3 minutes, then Immerse slides in pepsin solution at 37°C for 11 minutes. Immerse slides in wash buffer (2×SSC) for 3 minutes. Dehydration slides in 70% EtOH, 80% and 100% EtOH for 1 mint each and air dry on room temperature for 2-5 minutes. At room temperature, 10μl of respective probe mixer was prepared (7 μl hybridization buffer, 2 μl purified water and 1 μl probe) in dark room. Add 2.5 μl probe mixer on a one slide. Seal using rubber cement. Keep the slides in silver paper covered box and then put the box in oven (75°C for 7 mints). After that slides keep in moist box and put it in incubator at 37°C for overnight. Next day remove the slides from the incubator and using the forceps remove rubber cement seal. The slides place immediately in 2×SSC agitating the slides for 2-3 seconds. Transfer the slides in (Wash buffer I) 0.4×SSC kept at 71°C for 30 seconds in hot water bath. Wash slides in 2×SSC with 0.1% NP-40 at room temperature for 1 minute. Dehydration with help of ethanol series (70%, 80%, and 100%) (1 mints in each gradation). Thaw the DAPI and apply DAPI counter stain to the target area of the slides and put the cover slip. Incubate at 4°C for 5 mints. Transfer the slides to microscopy room in dark box.

#### 2.5. Fluorescence Microscopy

A Leica DM2500 Fluorescence microscope equipped with 10x, 20x, 40x dry, and 100x oil immersion objectives with triple-pass filter for spectrum Green/Spectrum Orange and DAPI (Vysis) was used to count the fluorescent signals. To capture images the fluorescent microscope is attached to a digital camera Leica DCF420C installed on the C-mount of the DM2500 and results were interpreted using Leica application suite (LAS) software for image acquisition (fixed images). Overlapping and damaged nuclei were ignored and only intact nuclei were evaluated.

#### 2.6. Evaluation of FISH analysis

Enumeration of the fluorescent signals was done in 200 nuclei per slide under objective 100x, using a Leica DM2500 fluorescent microscope equipped with single band sets for DAPI, Fluorescein isothiocyanate (FITC) green and Tetramethyl rhodamine spectrum orange to discriminate the color signals of green for chromosome 11 centromeric DNA and orange for Cyclin D1 during scoring.

##### Dual Probe Color setup

Green Signal: for chromosome 11 centromeric DNA

Orange Signal: for Cyclin D1 gene on chromosome 11

In a cell with normal copy number of the Cyclin D1 gene (11q13 region) and chromosome 11 (11p11.11-q11), two respective spectrum orange color signal for Cyclin D1 and two respective green color signal (chromosome 11 (11p11.11q11)) were observed. When  $\geq 20\%$  of the nuclei exhibited  $\geq 3$  signals for CCND1, the tumor was considered to have a "CCND1 numerical aberration." Simultaneously the copy number of chromosome 11(11p11.11q11) were quantified by enumeration of the respective centromeric probe (11p11.11-q11) green signal within the same cell.

#### 2.7. Types of chromosomal aberrations

- 1. Normal (No aberrations)-** In a nucleus, two respective spectrum orange color signal for Cyclin D1(CCND1) gene (11q13 region) and two respective green color signal for chromosome 11 (11p11.11q11 region). The signals ratio of the orange signals to the green signals is 1. Figure 1 (A)
- 2. Low Level amplification-** Abnormal copy number of Cyclin D1(CCND1) gene was indicated by 3 or more respective orange color signals with two respective green color signal (chromosome 11 (11p11.11q11)). When  $\geq 20\%$  of the nuclei exhibited  $\geq 3$  signals for CCND1, the tumor was considered to have Low Level amplification. Figure 1(B)
- 3. High (Cluster type) level amplification-** Clusters of CCND1 (orange) signals were present in nucleus with two respective green color signal (chromosome 11 (11p11.11q11)). When clusters of CCND1 signals (orange) were observed in more than 20% of 200

nuclei, this was considered as showing Cluster-type amplification of CCND1. Figure 1(C)

4. **Polysomy** - The copy number of chromosome 11 (11p11.11q11) were quantified by enumeration of the respective centromeric probe (11p11.11-q11) green signal within the cell. In a nucleus, green signals were >2 and orange signals were also quantified according green signals. Figure 1(D)
5. **Deletion of CCND1 gene (Missing)** - In one nucleus, there were one or no orange color signal for Cyclin D1 (CCND1) and two respective green color signal for chromosome11 (11p11.11q11).

### 3. Statistical Analysis

The results of FISH were compared with the clinical pathological information of patients included patient age, gender, tumor site, stage, histopathology differentiation and presence of lymph node metastasis, using Microsoft excel computer program. Mean and SD were derived for the continue parameters. Frequency and percentage was assessed for cross tabulation of various parameters. Pearson Chi-square and the 2-tailed Fisher's exact test (FET) were used for comparison of parameters association among themselves. The significant P-value in these tests is <0.05.

### 4. Results

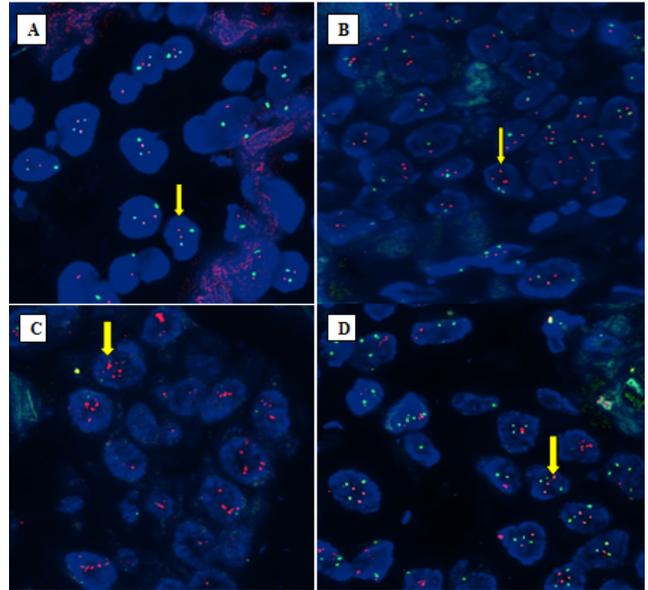
The CCND1 numerical aberrations found positive in 18(36.0%) of 50 patients of OSCCs. Table 1 shows the distribution of study population according to types of numerical aberration of Cyclin D1 gene. Out of 50 OSCC patients, low level amplification were found in 9(11.3%), high level or cluster amplification, polysomy and deletion of Cyclin D1 was found in 6(7.5%), 2(2.5%), 1(1.3) respectively. 32(40%) patients have no aberration. There was no significant association between primary site of carcinoma, histopathological differentiation and lymph node metastasis to gender (P=0.359, P=0.438, P=0.609).

Table 2 Shows the distribution of OSCC patients according to stage of carcinoma and types of numerical aberration of Cyclin D1 gene. Stage I and stage II were present in 12(24%) patients. 8(16%), 7(14%), 2(4%) and 9(18%) OSCC patients of different primary site of carcinoma were related to stage III, IVA, IVB and IVC respectively. A significant association was present between stage of Carcinoma and types of numerical aberration of Cyclin D1 gene in tissue (P=0.004).

Table 3 shows that 20 % positive numerical aberrations of Cyclin D1 gene found in buccal mucosa of OSCC. Moderately differentiated (grade II) of OSCC were showed positive numerical aberration of Cyclin D1 gene. The relation of Cyclin D1 gene numerical aberrations and primary site of carcinoma, histopathological differentiation were statistically not significant, but showed a significant

association with lymph node metastasis (P=0.038).

Distribution of OSCC patients according Risk factors and types of numerical aberrations of Cyclin D1 gene in Tissue is tabulated in Table 4. Risk factors like tobacco chewing, smoking and alcohol consumption were not associated with numerical aberrations of Cyclin D1 gene. There is zero patient without risk factor habits in the study.



**Fig. 1:** Eprezentative results of fluorescence in situ hybridization (FISH) forCyclin D1 (CCND1) gene in Formalin fixed paraffin section of Tumor (OSCC) in interphase nuclei. (A). **Normal**, normal cells were hybridized with probes for the chromosome 11 centromere (green) and the Cyclin D1 gene (orange), with 4,6-diamidino-2-phenylindole, dihydrochloride counterstaining. (B). **Low level amplification**, There were significantly more cells with more orange than green spots, (C)**High level or cluster amplification**, (D) **Polysomy** , FISH showed three copies of both signals. This case shows multiple copies of CCND1 with chromosome 11 polysomy.

### 5. Discussion

In the present study, we explored the feasibility using Cyclin D1 as a prognostic marker in OSCC by the FISH method. HNSCC, the amplification of 11q13 may be an important biologic marker for poor prognosis.<sup>25</sup> The correlation between FISH results and the different studied parameters was statistically analyzed and revealed no statistically significant correlation between Cyclin D1 numerical aberration and clinicopathological features of the studied cases. A significant association was present between stage of Carcinoma and types of numerical aberration of Cyclin D1 gene (P=0.004). The lymph node metastasis of OSCC was significantly associated with numerical aberration of Cyclin D1 gene (P=0.038). However, Miyamoto et al states that the Cyclin D1

**Table 1:** Distribution and association of Cases between Gender and various parameters

Various parameters	Gender		Total	P-value	
	Male	Female			
Types of Numerical aberration of Cyclin D1 gene	Low level amplification	8	1	9	1.000*
	High level or Cluster amplification	5	1	6	
	Polysomy	2	0	2	
	Deletion	1	0	1	
	No aberration	26	6	32	
Primary Site of Carcinoma	Buccal mucosa	20	3	23	0.359# 0.359*
	Soft palate	3	0	3	
	Lateral surface of tongue	10	1	11	
	Lower alveolar mucosa	5	1	6	
	Retro-mandibular region	2	1	3	
Histopathological differentiation of OSCC	Root of tongue	2	2	4	0.438# 0.438*
	Moderately differentiated	22	4	26	
	Poorly differentiated	7	0	7	
Lymph node metastasis	Well differentiated	13	4	17	0.609# 0.609*
	No	27	5	32	
	Yes	15	3	18	

Pearson Chi-Square, \*Fisher's Exact test

**Table 2:** Distribution of OSCC patients according Stage of Carcinoma and Types of Numerical aberration of Cyclin D1 gene in Tissue with association

	Types of Numerical aberration of Cyclin D1 gene in Tissue					Total		
	Low level amplification	High level or Cluster amplification	Polysomy	Deletion	No aberration	N	%	
	N	N	N	N	N	N	%	
Stage of Carcinoma	I	0	0	1	0	11	12	24.0
	II	1	0	0	1	10	12	24.0
	III	1	2	0	0	5	8	16.0
	IV A	2	1	1	0	3	7	14.0
	IV B	2	0	0	0	0	2	4.0
	IV C	3	3	0	0	3	9	18.0
	Total	9	6	2	1	32	50	100.0

\*Fisher's Exact test

**Table 3:** Association between Numerical aberration of Cyclin D1 gene and various parameters

Various parameters	Numerical aberration of Cyclin D1 gene				Total		P-value	
	Negative		Positive		N	%		
	N	%	N	%	N	%		
Primary Site of Carcinoma	Buccal mucosa	13	26.0	10	20.0	23	46.0	0.118*
	Soft palate	0	0.0	3	6.0	3	6.0	
	Lateral surface of tongue	9	18.0	2	4.0	11	22.0	
	Lower alveolar mucosa	4	8.0	2	4.0	6	12.0	
	Retro-mandibular region	3	6.0	0	0.0	3	6.0	
Histopathological differentiation of OSCC	Root of tongue	3	6.0	1	2.0	4	8.0	0.140# 0.129*
	Moderately differentiated	15	30.0	11	22.0	26	52.0	
	Poorly differentiated	3	6.0	4	8.0	7	14.0	
Lymph node metastasis	Well differentiated	14	28.0	3	6.0	17	34.0	0.038#
	No	24	48.0	8	16.0	32	64.0	
	Yes	8	16.0	10	20.0	18	36.0	

#Pearson Chi-Square, \*Fisher's Exact test

**Table 4:** Distribution of OSCC patients according Risk factors and types of Numerical aberration of Cyclin D1 gene in Tissue

Type of numerical aberration	Risk Factors							No habits	Total No. of cases
	Tobacco chewing only	Smoking only	Alcohol consumption only	Tobacco chewing and Smoking	Smoking and alcohol consumption	Tobacco chewing and alcohol consumption	Tobacco chewing, smoking and alcohol consumption		
Low level amplification	4	1	0	0	0	3	1	0	9
High level or cluster amplification	1	1	0	2	0	0	2	0	6
Polysomy	0	0	0	0	1	0	1	0	2
Deletion	0	1	0	0	0	0	0	0	1
No aberration	14	3	0	3	3	4	5	0	32
Total	19	6	0	5	4	7	9	0	50

numerical aberrations were significantly associated with an invasive tumor phenotype and pathologic lymph node status.<sup>26</sup> Myo et al has concluded that the aberration in Cyclin D1 numbers to be valuable in identification of patients at high risk of late lymph node metastasis in stage I and II OSCCs.<sup>27</sup>

On the other hand, Rodrigo et al correlated CCND1 amplification with clinicopathological parameters. CCND1 amplification was more frequent in T4 tumours and was associated with increased regional lymph node metastasis.<sup>28</sup> Interestingly, Kaminagakura et al found significant correlation between young age (<40 years) and CCND1 amplification, but failed to find any influence on prognosis.<sup>29</sup>

Miyamoto R et al found CCND1 numerical aberration was in 21(42%) of tumors. The presence of the CCND1 numerical aberration did not correlate significantly with age, gender, tumor site and stage. Tumors with a poorly differentiated and/or a more diffuse invasive pattern (the mode of invasion; Grade 4C, 4D) were associated significantly with the CCND1 numerical aberration (P=0.032 and P=0.047, respectively).<sup>30</sup>

Pathare et al found a significant correlation of +11q13 with high-grade OSCC.<sup>31</sup> Nimeus et al have reported positive Cyclin D1 amplification as low as 16% in SCC of oral cavity.<sup>32</sup> 56.5% positive Cyclin D1 amplification in SCC of tongue was reported by Fuji et al.<sup>33</sup> Huang X et al in their study where clinicopathologic features of the studied cases failed to show any significant correlation with 11q13 amplification.<sup>34</sup>

Monteiro L S et al found that 43.3% (26) of the cases showed the presence of numerical aberrations. In 19 cases (31.7%), there were more than six signals or cluster formations present per nuclei. They could not find an association of numerical aberrations in the 11q13 region such as CCND1 amplification with any clinical and pathological variables such as nodal metastasis and also with survival.<sup>35</sup>

Uazawa N et al found that CCND1 numerical aberration was identified in 28 of 57 primary oral SCCs (49.1%). CCND1 amplification was detected in 19 of 28 tumors that had CCND1 numerical aberrations. Only 9 tumors had multiple single copies of CCND1 associated with chromosome 11 polysomy. Of the 28 tumors that exhibited CCND1 numerical aberrations, 8 tumors demonstrated cluster-type amplification of CCND1. CCND1 numerical aberration was associated significantly with reduced disease-free survival (P=0.004) and overall survival (P=0.0179).<sup>21</sup>

Mahdey H M et al observed positive amplification of Cyclin D1 was in 72% (36) of OSCCs. Detection of positive amplification for Cyclin D1 was observed in 88% (22) and 56% (14) of the tongue and cheek tumors, respectively, where the difference was statistically significant (P=0.012). Lymph node metastasis of cheek SCCs showed a trend towards a significant association (P=0.098) with Cyclin D1 amplification whereas the lymph node metastasis of tongue SCC was clearly not significant (P= 0.593).<sup>36</sup>

Omenaya R R et al detected that 8(26.7%) cases were scored positive for CCND1 amplification, whereas 22 cases (73.3%) were scored negative. The relation between FISH and the demographic data of the patients as regards to the age, the sex, the site of the tumor, the lymph node involvement, the clinical stage, and the histological grade were not statistically significant. (P=0.47, 0.67, 0.33, 0.15, 0.58 and 0.67 respectively). Many studies have been done on cyclin D1 in OSCC, and even though the controversy exists in the scientific literature, it opens a window of opportunity for further discussion and research in different tumours with additional different criteria like lymph node involvement and metastasis.<sup>22</sup>

## 6. Conclusion

Evaluating the CCND1 numerical aberration by FISH on paraffin embedded specimens before treatment also helps in

the selection of more appropriate treatment for patients with OSCCs. Therefore, we conclude that the CCND1 numerical aberration is a useful tool, not only as a prognostic factor independently of the TNM classification, but also as an indicator to determine the most appropriate treatment for patients with OSCCs. To improve the overall survival rate of OSCC patients, more intensive treatment should be given to the patients with CCND1 numerical aberration-positive tumors.

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## 8. Conflicts of Interest

None.

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