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Original Research Article

Copy Number Alteration of Cyclin D1 (CCND 1) Gene as a Prognostic Factor in Oral Squamous Cancers and its Correlation with Immunohistochemistry (IHC)

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Abstract

Ranganath Ratnagiri

Background Oral squamous cancers remain the most common cancers among males in India and the third most common cancer among men and women combined, with an age standardized ratio of 9.1 per 100,000 population. Surgery and chemoradiation have not improved survival rates significantly and hence, newer therapeutic targets are needed. Cyclin D1 (*CCND1*) is a proto-oncogene which is located on chromosome 11q13 and has been found to be amplified in many cancers including oral cancers. It is said to correlate with aggressive tumor growth and a poorer prognosis.

Aim This article correlates cyclin D1 (*CCND1*) gene copy number alteration with clinicopathologic prognostic factors in oral squamous cancers.

Materials and Methods Sixty-three patients who underwent surgery for oral cancer between January and June 2022 were included in the study after obtaining informed consent and ethics approval. Copy number alteration of CCND1 was assessed using quantitative polymerase chain reaction (Qf PCR) and correlated with clinical and histopathological prognostic factors, including short-term recurrence. Statistical analysis was performed using the SPSS software.

Results A statistically significant correlation was determined between the Qf PCR values of CCND1 gene and locoregional recurrence. Gene copy number alteration also correlated strongly with a higher grade of the primary tumor. There was also a significant correlation between the Qf PCR values and the immunohistochemistry for cyclin D1.

Conclusion Cyclin D1 offers a new therapeutic target in oral cancers and may improve survival without significant treatment-related morbidity.

Keywords

- oral squamous cancers
- cyclin D1 gene
- copy number alteration
- prognosis
- quantitative PCR
- immunohisto–chemistry

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Introduction

Oral squamous cancers are the most common cancers among males in India and the third most common cancer among men and women combined, with an age standardized ratio of 9.1 per 100,000 population, which is one of the highest in the world.[1] Most patients (~60%) present with locoregionally advanced disease.[1] Treatment involves extensive surgery and radiation (with or without chemotherapy) with their attendant morbidity and long-term sequelae. Prognosis, however, has not improved significantly over the past few decades.[2]

The poor prognosis is not only due to the delayed presentation of these cancers but also due to the inherent weaknesses in the currently used prognostic variables and accepted treatment models. Several molecular markers have been studied for their utility as prognostic factors in oral squamous cancers, in addition to the conventionally accepted histopathological variables like differentiation, lymphovascular invasion, perineural spread, nodal involvement, etc. Cyclin D1 (*CCND1*) is a proto-oncogene which is located on chromosome 11q13 in humans. This gene has been found to be amplified in many cancers including oral squamous cancers.[3] CCND1 activates cyclin dependent kinases (CDK) 4 and 6, causing phosphorylation of pRb. This drives the cell from the G1 phase to the S-phase in the cell cycle and has been postulated to lead to the propagation of unrepaired deoxyribonucleic acid (DNA) damage, accumulation of genetic errors, and consequent uncontrolled cell growth.[4]

Detecting a copy number alteration in the CCND1 gene may therefore help in identifying the subset of oral cancers which are biologically more aggressive and prone for locoregional recurrence. CDK inhibitors are already in clinical use in breast cancer, and may hence play a role in the management protocols of oral cancers in the future.

Materials and Methods

The study was designed as a prospective observational study to be conducted in our institute between January 2022 and September 2022, as part of a research grant by the Department of Health Research, Government of India. A total of 63 patients of oral squamous cell carcinoma who underwent surgery were included in the study after obtaining their informed consent. The clinical evaluation, investigative workup, surgical plan, and adjuvant therapy were as per the institute protocol. Samples were taken from the resected specimen on table, and sent in normal saline for genetic analysis. The patients were followed up according to international guidelines.

Copy Number Variation Analysis by Quantitative Polymerase Chain Reaction

DNA was isolated from blood or oral squamous tissue samples using QIAmp DNA mini kit (Qiagen GmbH, Hilden, Germany) or HiPuRA Mammalian Genomic purification kit (Himedia, Thane, Maharashtra, India), respectively, per the manufacturer's instructions.

Note that 25 mg of fresh frozen tumor tissue was taken to isolate tissue DNA, and 180 µL of lysis solution and 20 µL Proteinase K were added. Samples were then incubated at 55°C for 20 minutes. Note that 200 µL of another lysis solution was added, and samples were incubated for 10 minutes at 55°C. Column-based centrifugation was done at 6,500 × *g* for 1 minute, and flow-through was discarded. Columns are washed at 6,500 × *g* for 1 minute with wash solution and then dried. DNA was then eluted by adding 90 µL water and centrifugation at 6,500 × *g* for 1 minute.

The concentration of DNA was estimated using NanoDrop 2000 (Thermofisher Scientific, Massachusetts, United States). DNA was stored at –20°C till further use. Quantitative (Qf) reverse transcription-polymerase chain reaction (PCR) was set up using 5 µL of TB Green II Master Mix (Takara, Tokyo, Japan) with 100 ng of genomic DNA. Primers were added to a final concentration of 1 nM primer in a 10-µL reaction mixture. Initial denaturation was performed using PCR at 95°C for 10 minutes. Afterwards, 40 cycles were performed at 95°C, 60°C, and 72°C for 20 seconds each. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control. $2^{-\Delta\Delta Ct}$ was used to find fold change between CCND1 levels of blood and tissue. A fold change value of 1.8 was used as a cutoff for positive samples.

All the slides were assessed for tumor cell percentage, and only those slides with a tumor cell percentage of more than 70% were taken up for immunohistochemical (IHC) analysis. IHC analysis was done for cyclin D1 (BioGenex, EP12, RTU) on all the resected specimens and correlated with the Qf PCR values. We scored the IHC in terms of intensity of staining and percentage of cells stained (0–5 for each) and the product of both was used to report cyclin D1 positivity. A threshold of 6 was used as the cutoff value. ►**Fig. 1** shows the photomicrographs depicting IHC positivity of cyclin D1.

Statistical analysis was done with the help of the SPSS software using the chi-square test, logistic regression analysis, and analysis of variance.

Results

Most patients in our cohort were males above the age of 45 years (76% males and 65% more than 45 years of age). Almost all the patients gave history of tobacco use (95.2%), either in the form of chewable tobacco or by smoking. The mean duration of tobacco use in our cohort was 12 years. Concurrent with tobacco usage, more than half of the patients also gave history of alcohol intake for a mean duration of 10 years. Though most patients were in a poor nutritional condition, this was of recent onset after the appearance of cancer. Note that 39.6% of the cohort had associated medical illnesses, the most common ones being diabetes and hypertension. A fact of concern which was incidentally revealed during the course of our study was that the time from the first symptom (usually a nonhealing ulcer) to establishing the diagnosis of cancer and planning treatment was approximately 5 months (median). This was almost the same irrespective of the urban/rural background of the patient (►**Table 1**).

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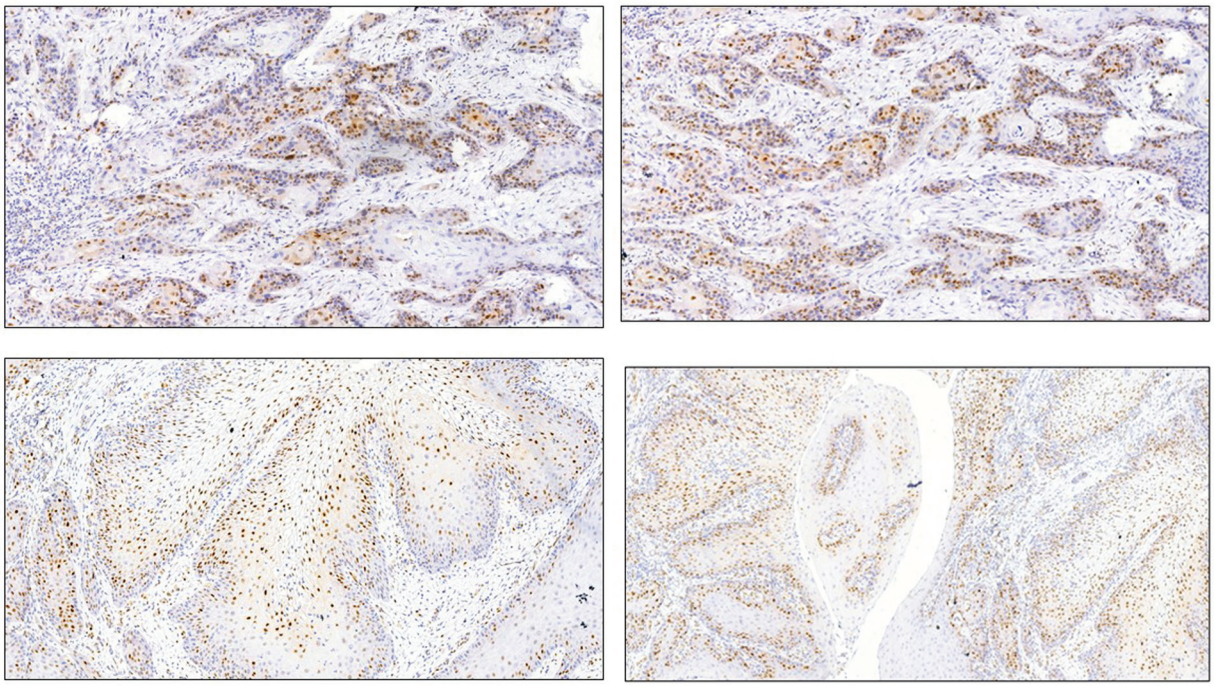

Fig. 1 Photomicrographs depicting immunohistochemistry (IHC) positivity for cyclin D1.

Note that 92.1% of our patients presented with a painful, nonhealing ulcer as the first symptom. About two-thirds (65%) complained of difficulty in speech and in mastication with 26.9% also complaining of bleeding from the ulcer. Note that 71.4% of patients experienced a delay of 5 months or more (median) from the onset of the ulcer to the diagnosis. This was partly due to lack of awareness and partly due to difficulty in access to health care facilities. This consequently led to the relatively high number of locoregionally advanced tumors (80.9%) in the study cohort and also was possibly the reason for the 20% recurrent tumors in the group (►**Table 2**).

The most common subsite in our patients was the oral tongue (42.8%) followed by buccal mucosa and the upper and lower alveolar regions (31.7 and 10%), respectively (►**Fig. 2**).

Upon correlating the Qf PCR for cyclin D1 values with various clinical and pathological parameters, we found that there was no correlation with the demographic characters like age, gender, and tobacco or alcohol use. The statistical correlation with grade of the tumor was tending toward significance (with a *p*-value of 0.09), and would have reached significance, if the cohort had more number of patients. There was a statistically significant correlation with recurrence, thus indicating that copy number alteration of the CCND1 gene had an effect in the pathophysiology of recurrent oral cancers. A significant relation was also demonstrated with IHC for cyclin D1 (*p* 0.009), thus suggesting that IHC can be a surrogate wherever PCR is not available (►**Table 3**).

When we attempted to correlate the locoregional recurrence with the known clinical and pathological prognostic factors (as described in literature), we found that margin positivity (*p* 0.075) and Qf PCR positivity for cyclin D1 (*p* of 0.05) were the only two variables reaching significance (►**Table 4**).

Discussion

The cyclin D1 gene (CCND1) is a proto-oncogene located on 11q13 and binds and activates CDK 4 and 6, thus promoting cell-cycle progression from the G1 to the S-phase.[5] The increased expression of cyclin D1 gene produces an increase in growth potential and causes tumorigenesis.[6] Some studies demonstrate

Table 1 Demographic pattern of the study cohort ($N = 63$)

Sl. no.	Parameter	Number	Percentage
1.	Age > 45 y	41	65
2.	Male:female	48:15	76.1:23.9
3.	Tobacco usage (smoking and smokeless)	60	95.2
4.	Alcohol usage	37	58.7
5.	Associated medical comorbidities	25	39.6
6.	Time from first symptom to diagnosis	5 mo (median)	

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Table 2 Clinicopathological descriptors of the study cohort ($N = 63$)

Sl. no.	Descriptor	Number	Percentage
1.	Painful ulcer as the first symptom	58	92.1
2.	Difficulty in chewing/talking	41	65
3.	Bleeding	16	26.9
4.	Duration of symptoms > 5 mo	45	71.4
5.	Trismus at presentation	32	50.7
6.	Palpable neck nodes at presentation	41	65.7
7.	Recurrent tumors	12	19
8.	Stage III/IV at presentation	51	80.9

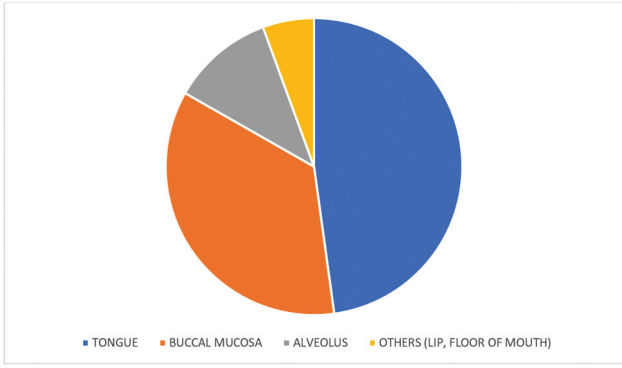

Fig. 2 Distribution of the tumors by primary site.

a strong association between cyclin D1 overexpression and poor survival,[7,8] whereas a few others have demonstrated no prognostic impact in oral squamous cancers.[9,10]

In our study, we have first demonstrated a copy number alteration in the cyclin D1 number by using the Qf PCR technique, correlated the overexpression by performing IHC analysis on the tumor samples from the same patient, and then tried to correlate this with histopathologic prognostic factors and local recurrence. Most studies in literature have used only IHC on paraffin blocks and correlated the results with nodal metastases, lymphovascular invasion, and other pathological variables.[11–13]

Studies have reported variable overexpression of cyclin D1 in oral squamous cell cancers ranging from 18 to 56%,[14] and the same was seen in our results with the IHC being positive in 68.3% of the patients, whereas the Qf PCR was positive for cyclin D1 in only 36.6%. This brings to view an important fact that even if the copy number is not altered, in approximately 32% of these patients there is functional overexpression of cyclin D1.

Table 3 Correlation of cyclin D1 Qf PCR scores with clinicopathological variables ($N = 60$)

Sl. no.	Variable	Number (percentage)	Qf PCR positive - number (percentage	*p*-Value
1.	Tumor stages III and IV	51 (85)	11 (18.3)	0.357
2.	Grade (high)	11 (18.3)	4(6.6)	**0.09**
3.	Lymphovascular invasion (LVI)	13 (21.6)	6 (10)	0.729
4.	Perineural invasion (PNI)	29 (48.3)	12 (20)	0.934
5.	Depth of invasion > 10 mm	41 (68.3)	15 (25)	0.233
6.	Positive margins	23 (38.3)	10 (16.6)	0.85
7.	Recurrence	23 (38.3)	14 (23.3)	**0.05**
8.	IHC for cyclin D1	41 (68.3)	22 (36.6)	**0.009**

Abbreviations: IHC, immunohistochemistry; Qf PCR, quantitative polymerase chain reaction.

Table 4 Relation of known prognostic factors with locoregional recurrence ($N = 60$)

Sl. no.	Variable	Number (percentage)	*p*-Value
1.	Tumor stage III and IV	51 (85)	0.816
2.	High grade	11 (18.3)	0.990
3.	Positive margins	23 (38.3)	0.075
4.	Qf PCR positive for cyclin D1	14 (23.3)	**0.05**

Abbreviation: Qf PCR, quantitative polymerase chain reaction.

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One plausible explanation for this apparent paradox is the hypothesis that there is always an accompanying mutation affecting the p16 protein, which leads to permanent activation of the kinase activity of cyclin D1, leading to downstream activation of pRb and consequent tumorigenesis.[15]

Moderately and poorly differentiated tumors had a significantly higher copy number alteration with a *p*-value of 0.09. This tendency toward statistical significance between the grade of the tumor and the cyclin D1 expression has been reported by others also.[16,17] The possible explanation being that cyclin D1 inhibits the expression of myoblasts and epithelial cells, thus preventing the differentiation of certain cell types.[18]

Of the 23 patients who developed a locoregional recurrence in our cohort, 14 (60.8%) were cyclin D1 positive. Mahdey et al have reported similar figures in their study and also have correlated the same with poorer overall survival.[19]

Limitations

Statistical significance could not be reached for some of the variables due to the number of patients being on the lower side. The same cohort is being followed up to assess the 5-year survival rates at present. A more holistic picture may form at the end of the 5-year follow-up.

Conclusion

Cyclin D1 copy number alteration and overexpression are independent prognostic factors causing increased local recurrences and decreasing the disease-free survival in oral squamous cell cancers. This is conclusively shown in our data even after controlling for grade and margin status. IHC is a good surrogate in institutions where facilities for Qf PCR are not available.

Ethical Approval

Ethical approval was obtained from the Institute Ethics Committee.

Authors' Contributions

R.R. was involved in conceptualizing the study, performing the surgeries, collating and interpreting the data, and writing the manuscript while S.J. was involved in performing the surgeries and in collection of data.

P.R. was involved in performing the genetic analysis, interpreting the results, and in reviewing the manuscript, M.S.U. was involved in performing the histopathological analysis of the specimens, performing and interpreting the IHC results, and in reviewing the manuscript and R.S. was involved in performing the surgeries and reviewing the manuscript before submission.

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Conflict of Interest

None declared.

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