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Original Research Article

Salivary *Cyclin D1* and *Ki 67* for staging of oral cancer

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Abstract

Aims & Objectives: To study the usefulness of salivary abnormal expression of *Cyclin D1* and *Ki-67* proteins for staging of oral cancer.
Materials and Methods: The study was approved by the Institutional Ethics Committee. Saliva was collected from each of the patients taking appropriate precautions. The vials used were sealed tightly and stored at -40 degrees. ELISA test was collected with these samples to estimate Cyclin D1 and Ki 67 levels.
Results: Among the malignant oral lesions, 2 subjects had stage 1 disease, 6 subjects had stage 2 disease, 1 had stage 3 disease and 2 subjects had metastatic stage 4 disease. In the study there was no significant correlation for Stage of cancer with Cyclin D1 and Ki67 levels. Both correlations were negative, i.e. with increase in stage of cancer there was decrease in Cyclin d1 and Ki 67.
Conclusion: Cyclin D1 and Ki 67 levels in saliva as a screening strategy for oral malignant lesions appears to be a very promising prospect in a quest to develop simple, reliable noninvasive tool. Both the biomarkers seem to be useful for staging of oral cancer also.
Keywords: *Cyclin D1, Ki 67,* oral cancer.

1. Introduction

1.1 Aims & Objectives:

To study the usefulness of salivary abnormal expression of *Cyclin D1* and *Ki-67* proteins for staging of oral cancer.

1.2 Need of the Study

Oral malignancy is one of the most common types of cancer. worldwide. Majority of oral cancers are squamous cell carcinomas. About one-third of the burden of this malignancy occurs in Indian subcontinent.[1]

Being easily accessible, one would expect these lesions to be detected early. But unfortunately they are diagnosed often at very late stage, which results in poor outcome and survival rate. Because of its direct contact with oral malignancy, saliva would contain abnormal DNA, RNA and protein molecules released by the neoplastic cells. These can be easily accessed and utilised for diagnosis at early stage, for monitoring after therapy and for prognostication of the malignant lesions.

1.3 Review of Literature

Molecular signatures used for the diagnosis of oral squamous cell cancer are:

1.3.1 Changes in the Cellular DNA

Point mutations, deletions, translocations, amplifications and methylations and alteration in cyclin D1, epidermal growth factor receptor (EGFR), presence of HPV [2] are some important changes observed in the host DNA of the malignant cells.

Several prognostic markers have been identified for oral cancer. Cyclin D1, Ki 67 [3] being proliferation markers, have been found to indicate poor prognosis. 8-oxoguanine DNA glycosylase, phosphorylated-Src and mammary serine protease inhibitor (Maspin) have been found be decrease in saliva of subjects with OSCC. HPV (human papilloma and EBV (Epstein Barr virus) are showing promise as possible DNA markers for diagnosis of OSCC and tumor progression.

Ki-67:

Ki- 67 is a nuclear protein needed for cellular proliferation and ribosomal RNA transcription.[3] Inhibition of Ki-67 leads to reduced ribosomal RNA synthesis. [5] Gonzalez-Moles *et al* have found Ki-67 expression in non-tumour epithelium adjacent to oral cancer a risk marker for oral cancers. [6] The Ki-67 protein (also known as MKI67) is a marker for cellular proliferation It is being used for prognostication of oral cancers. Ki-67 is an important protein present during all phases of the cell cycle ($G_1$to mitosis). Ki-67 is an excellent marker to determine the growth fraction of a given cell population. Ki-67-positive tumor cells fraction (the Ki-67 labeling index) correlates with clinical staging of cancer.

Cyclin D1:

It is protein encoded by CCND1 gene, which is located at 11q13 locus belongs to cyclin family. Different Cyclins function as regulators of various Cyclin dependant kinases (CDK). Different cyclins have distinct expression patterns and contribute to coordination of each mitotic event. Cyclin D1 acts as a regulatory subunit of CDK4 or CDK6, which is critical for cell cycle G1/S transition. Cyclin D1 interacts with tumor suppressor protein Rb and the expression of Cyclin D1 is regulated by Rb. Various alterations of Cyclin D1, including Mutations, amplification and overexpression, which have important effect on cell cycle progression, are noted in a variety of neoplasias and may contribute to genesis of these tumors.[7]

Microinjected antibodies in cultured cells, which neutralize Cyclin D1, cause cessation of cell cycle progression. Uma Swaminathan, Elizabeth Joshua *et al* [8] studied expression of p53 and Cyclin D1 squamous carcinoma of oral cavity and normal mucosa. They found that mutant p53 expression increases as with the malignant transformation of normal mucosa. Cyclin D1 is amplification and overexpression was also found in oral squamous cell carcinoma. There was positive correlation noted between increased mutant p53 and cyclin D1 expression in subjects with squamous cell carcinoma of oral cavity.[8]

2. Materials & Methods

The study was conducted adhering to the ICMR and MCI guidelines with regard to ethical issues to be followed in research activity.

2.1 Ethics:

The study was approved by the Institutional Ethics Committee, Karnataka Institute of Medical Sciences, Hubli. The study was carried out at Multidisciplinary Research Unit of Karnataka Institute of Medical Sciences, Hubli. It was a Prospective Observational study. Written and informed consent subjects were obtained from the subjects. Demographic details were noted including age, sex and personal history about alcohol consumption, tobacco chewing or smoking.

The subjects were the patients suffering from Oral cancer.

Saliva was collected from each of the patients. They were asked to follow the below mentioned instructions before including them into study.

- Should not have alcohol for 12 hours before sample collection.
- Do not eat a major meal within 60 minutes of sample collection.
- Dairy products not to be consumed for 20 minutes before sample collection.
- Avoid foods with high sugar or acidity, or high caffeine content, immediately before sample collection, since they may compromise the assay by lowering saliva pH and increasing bacterial growth.
- Mouth to be rinsed with water to remove food residue before sample collection. Participants should not have brushed teeth within 45 minutes prior to sample collection.
- Dental extraction should not have been performed within 48 hours prior to sample collection.

Saliva samples visibly contaminated with blood would be discarded and recollected.

Passive drool method was the method used for collection of saliva. Falcon tubes were used for collection of sample collection. Subjects were asked to pool saliva inside the mouth. With head tilted forwards, participants were asked to drool down saliva into the falcon tube. The procedure was repeated till the sufficient sample is collected. The vials used were sealed tightly and stored at -40 degrees.

2.2 Method:

2.2.1 Sample collection

Saliva samples (10 to 15mL) from all the study Subjects was collected in a falcon tube, sealed tightly and stored at -40˚C.

2.2.2 Reagent Preparation

2.2.2.1 Washing Buffer:

It was prepared by diluting the wash solution from 30X to 1X using deionised or distilled water.

2.2.2.2 Standard Preparation

Ki-67:

Standard solution of the kit has a concentration of 48ng/ml. Standard solution is prepared as per instructions. Standard diluent was used as control.

Cyclin D1:

Standard solution available in the kit has a concentration of 64ng/ml. Standard solution is prepared as per instructions. Standard diluent was used as control.

2.3 Procedure:

2.3.1 Ki-67:

- All the reagents were cooled to room temperature.
- Loading of Blank, Standards and sample in wells was carried out as per Manufacturer's instructions.
- Washing with washing solution for 30 seconds.
- Colour development: Chromogen reagent B was added to each well after Chromogen reagent A, incubated for 10 minutes at 37˚C for 10 min in dark conditions for the colour (Blue) to develop.
- Stop solution is added which results in a colour change from blue to yellow.
- The absorbance is measured at 450nm wavelength, within 10 minutes after adding stop solution.
- Standard curve is plotted. Then according to the OD values of the sample, concentration of Ki67 in the sample is calculated using standard curve.

2.3.2 Cyclin D1:

The concentration of Cyclin D1 in each sample was calculated using the procedure similar to Ki 67.

2.4 Statistical analysis

Data was entered into Microsoft excel data sheet and was analyzed using SPSS 22 version software. Categorical data was represented in the form of Frequencies and proportions. Continuous data was represented as mean and standard deviation.

Graphical representation of data:

MS Excel and MS word was used to obtain various types of graphs such as bar diagram.

p value

(Probability that the result is true) of <0.05 was considered as statistically significant after assuming all the rules of statistical tests.

Statistical software:

MS Excel**,** SPSS version 22 **(**IBM SPSS Statistics, Somers NY, USA) was used to analyze data.

3. Results and data analysis

A total of 13 patients with oral cancer were studied (Table 1).

Among the malignant oral lesions, 2 subjects had stage 1 disease, 6 subjects had stage 2 diseases, 1 had stage 3 disease and 2 subjects had metastatic stage 4 disease.

Table 1: Baseline characteristics of the patients

	Malignant (n=13)
AGE	56.23±13.42
SBP	131.23±20.29
DBP	79.5±10.7
Alcohol	38.1%
Family hist	15.4%
Gutkha chewing	46.1%
Smoking	61.5%

Table 2: Spearman's rho correlation between Stage of cancer and Cyclin D1 and Ki 67

Correlations					
			Stage cancer	CY d1	Ki 67
Spearman's rho	Stage cancer	Correlation Coefficient	1.000	-0.215	-0.192
		Sig. (2-tailed)	.	0.481	0.530
		N	13	13	13

In the study there was no significant correlation for Stage of cancer with Cyclin D1 and Ki67 levels. Both correlations were negative, i.e. with increase in stage of cancer there was decrease in CY d1 and Ki 67. Non significant correlation may be due to small sample size.

Table 3: Mean Cyclin D1 and Ki67 with respect to Stage of cancer

		Cyclin D1		**Ki 67**	
		Mean	**SD**	**Mean**	**SD**
Stage cancer	Stage 1	1.555	0.643	0.890	0.765
	Stage 2	1.085	0.673	1.131	0.486
	Stage 3	1.232	0.808	0.888	0.520
	Stage 4	0.871	0.836	0.788	0.763
P value		0.780		0.865	

In the study there was no significant difference in mean Cyclin D1 and Ki67 with respect to stage of Cancer.

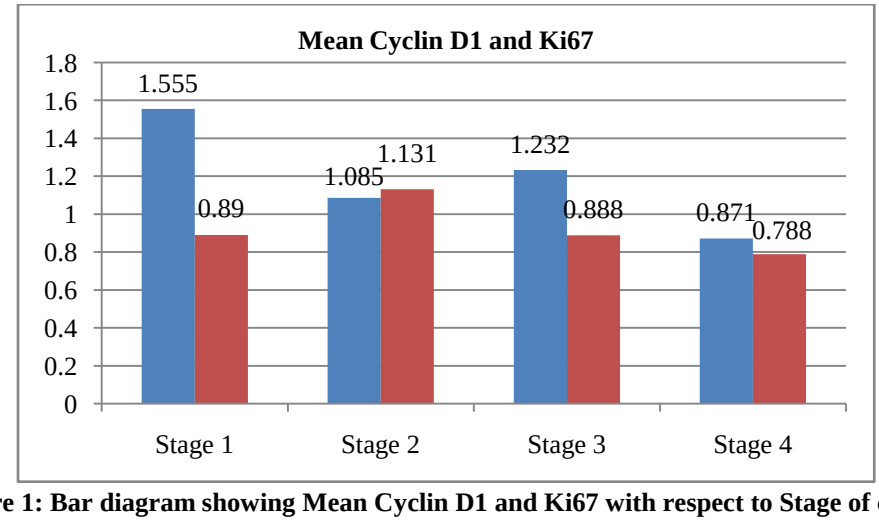

Figure 1: Bar diagram showing Mean Cyclin D1 and Ki67 with respect to Stage of cancer

4. Discussion

Oral malignancies are seen commonly in middle aged and elderly individuals. This was noted in our study also. Gutkha chewing was the commonest predisposing factor found in subjects with oral cancer. Smoking cigarettes and beedies was higher in individuals with these lesions. About 15% of the study subjects had family history of oral malignancy in first degree relatives. Majority of our study subjects belonged to lower and middle socioeconomic class. We have attempted to identify newer biomarkers for diagnosis as well as for prognosis of oral malignancies. Compared to the earlier reports, we have found both Cyclin D1 and Ki 67 having negative correlation with the stage of oral cancer, though the negative correlation found was statistically insignificant (Table 3, Figure 1). Due to the sample size, the generalization of the results is difficult. The results are pointing towards need for more research regarding utility of Cyclin D1 and Ki 67 in arriving at management decisions. Study by Shpitzer T *et al.*[9] showed both Cyclin D1 and KI 67 increased in saliva in subjects with oral malignacies. Other studies have found Cyclin D1 and Ki 67 to be good predictors for local and regional regional spread and also for recurrence of Oral and Head and neck Squamous cell malignancies. [10, 11]

Another study reported that Ki 67 levels in areas distant from oral Squamous cell cancers serves as prognostic factors for these malignancies.[12] Unlike others studies which have been reported about these molecules, our study is pointing towards decline in the level of these biomarkers as neoplasia develops. The explanation for negative correlation is not clear. But several factors may be contributing to this; lack of standard protocols for conducting these tests may be one important factor. Whether different storage time of different samples alters the values is also not clear. And technical aspects like extent of dilution of the samples needed also needs clarity. Cyclin D1 and Ki67 are known regulators of cell-cycle, and shown to be correlated with tumor cell proliferation, progression, metastasis and poor prognosis. But our study is showing opposite results compared to earlier reported studies with regard to alteration of these markers in neoplasias. Larger statistically powered study is indicated to clarify the role of these promising biomarkers for taking clinically important management decisions. Devising such simple, noninvasive methods for early detection, staging and for prognostication of malignant lesions of oral cavity can bring tremendous change in Primary and secondary prevention strategies in country like ours. Such a strategy has the potential to drastically reduce morbidity and mortality in economically productive population.

5. Conclusion

- Cyclin D1 and Ki 67 levels in saliva as a screening strategy for oral malignant lesions appears to be a very promising prospect in a quest to develop simple, reliable noninvasive tool.
- Both the biomarkers seem to be useful for staging of oral cancer also.

Limitations

- Generalization of the results not possible because of small sample size.
- Lack of standard protocols for processing of the saliva samples could have played a role in the result values.

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