ORIGINAL RESEARCH

A Study of Cyclin D1 Gene Numerical Aberrations in Blood Samples of Oral Squamous Cell Carcinoma Patients of North India

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ABSTRACT

Introduction: The present study aims to detect Cyclin D1 gene numerical aberrations in blood samples of OSCCs patients (50) and controls (30) by using fluorescence in situ hybridization technique (FISH) along with risk factors.

Materials and Methods: Present study is an observational, retrospective Case control study comprising samples from North Indian population. Peripheral blood samples were collected randomly from histological confirmed 50 OSCC patients from outpatient department (OPD) of Oncology and ENT of regional tertiary care hospital, and also from 30 healthy controls of 25 years or above age. FISH interphase technique was used to detect the numerical aberrations of Cyclin D1 using the Vysis protocol (Vysis CCND1 CEP11 FISH Probe Kit, Abbott Molecular Inc. Des Plaines IL 60018 USA). Mean and SD were calculated. Pearson Chi-square and the 2-tailed Fisher's exact test (FET) were used for comparison of parameters association among themselves (*P* value <0.05).

Results: Cyclin D1 gene numerical aberrations were absent in controls; where as 5 (6.3%) cases were positive in OSCC group. Among positive cases low and high level amplification were found in 3 (3.8%) and 2 (2.5%) cases respectively. Other than all factors measured Cyclin D1 gene aberrations showed a significant association only with lymph node metastasis (*P*=0.004) and stage of Carcinoma (*P*=0.001) respectively.

Conclusions: The present study suggests the prognostic importance of blood as a medium for assessing Cyclin D1aberrations in advance stage OSCC patients.

Keywords: Cyclin D1, FISH Technique, Gene Amplification, HNSCC.

INTRODUCTION

The genetic changes occurring in OSCC have retained the focus attention in dentistry, mainly in oral and maxillofacial pathology.[1] Carcinogens like tobacco products and alcohol in solution constantly accumulate in the floor of mouth, so these carcinogens rapidly penetrate the epithelium to reach the progenitor cell.[2]

Fluorescence in-situ hybridization (FISH) technique is highly specific and sensitive with advantage of rapidity, now used in routine clinical laboratory for genomic diagnosis. The interphase FISH technique produces direct visualization of chromosomal aberrations in cell nuclei using fluorescently labeled DNA probes.[3,4]

The genes involved in cell cycle regulation represent targets of oncogenic abnormalities among which Cyclin D1 is most involved. In human cells, cell division is controlled by the activity of Cyclin-dependent kinases (CDKs) and activating coenzymes, the CDK inhibitors, which may be influenced by genetic variations in the corresponding genes.[5,6]

Cyclin D1 proto-oncogene is an important regulator of G1 to S-phase transition in numerous cell types from diverse tissue. CCND1 is a proto-oncogene is located on the long arm of chromosome 11 (band 11q13). 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 tumor 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 tumors and may confer growth advantage and enhanced tumor genesis. The detection of CCND1 using a simple and sensitive method would be valuable for the development of effective treatment modalities for oral cancer.[7,8]

Alarming numbers of the population from North India region currently suffering from cancer and 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 primary objective of this study was to detect Cyclin D1 gene numerical aberrations in blood samples of controls and OSCCs patients from North Indian population by using fluorescence in situ hybridization technique (FISH). Secondary objective was to analyse the association between Cyclin D1 gene numerical aberrations with other risk factors.

MATERIALS AND METHODS

Present study is an observational, retrospective case control study. The primary objective of this study was to detect Cyclin D1 gene numerical aberrations in blood samples of controls and OSCCs patients from North Indian population by using fluorescence in situ hybridization technique (FISH).

PATIENT CHARACTERISTICS

The random samples were collected from 50 OSCC patients (42 males, 8 females) of the Outpatient Department (OPD) of Oncology and ENT respectively. The samples were also obtained from 30 healthy controls age and gender mached. All the samples comprise of North indian population.

INCLUSION CRITERIA

For cases histopathologically proved OSCC patients. And for controls age 25 years or above, clinically normal and asymptomatic.

EXCLUSION CRITERIA

OSCC patients who have been exposed to radio/chemotherapy and subjects with any other malignancy, any systemic illness or long term medication were
excluded.

The institutional ethics committee (No.2879/MC/EC/2016 Dated16/12/2016) 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 the patients including age, gender, weight, height, dietary habits,

tobacco chewing, smoking, alcohol consumption, duration of symptoms and the presence of lymph node metastasis factors helpful in study were record.
The clinical staging (TNM classification) was defined on the basis of the American Joint Committee on Cancer **(**AJCC).[9] The tumours were classified histopathologically in to well, moderately and poorly differentiated according to their cellular differentiation as defined by Pindborg JJ et al.[10]

SAMPLE COLLECTION AND PROCESSING

FISH technique was carried on blood samples by adopting Vysis protocol (Vysis CCND1 CEP11 FISH Probe Kit, Abbott Molecular inc. Des Plaines IL 60018 USA). Peripheral venous blood collected from the OSCC patients and controls, then immediately transferred into a sterile, heparinized tube and processed. 500 µl blood sample was taken in centrifuge tube and then 5ml pre-warmed KCl was added. Put it at 37°C in Incubator for 25 minutes. From the corner of the tube 3 ml freshly prepared chilled Carnoy's fixative was added. After that solution became black colour, then put it in refrigerator (2-4°C) for 10 minutes and after that centrifuged at 1000rpm for 10 minutes. Supernatant removed leaving behind 3ml solution with pellet. Slides were prepared by adding 10-30µl of the centrifuged cell pellet on to it, and hybridization areas marked with a diamond tipped scribe. Slides were put on the slide warmer (60°C) for 1hour. Slides then put through an alcohol gradient (freshly made each time) 70%, 80% and 100% for 2 min each and completely dried. Keep the slides in **Solution A** (49ml D/W, 500µl 0.01NHCl, 100µl Pepsin) for 5 mints at 37°C in incubator. Then Keep the slides in **Solution B** (Formaldehyde 1.34ml (1340µl), 49ml D/W) for 5 mints at 4°C. Freshly prepared probe locus specific cyclin D1 (CCND1) (11q13), and centromeric probe (11p11.11-q11) (control probe) were added to one target area immediately and the cover slip was laid. Hybridization procedure was done overnight and then post hybridization washes were given with 2X SSC. Five microliter counterstain (DAPI diamidino-2-phenylindole) had applied to the target area of the slide and coverslip was placed. The slides were viewed under single bandpass fluorescence filter on a fluorescence microscope.

EVALUATION OF FISH ANALYSIS

Evaluation of the preparation was observed by counting 200 interphase nuclei according to criteria described by Hopman et al.[11] 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) spectrum green and Tetramethyl rhodamine isothiocyanate (TRITC) spectrum orange to discriminate the colour signals of green for chromosome 11 centromeric DNA and orange for Cyclin D1 during scoring.

DUAL PROBE COLOUR SETUP

Green Signal: for chromosome 11 centromeric DNA
Orange Signal: for Cyclin D1 gene on chromosome 11
The hybridized signals appear as small spot since the region of a chromosome occupies only a small region of the nucleus. Results were interpreted using Leica application suite (LAS) software for image acquisition. At least 200 nuclei were scored using a 100X objective in each defined area, and each nucleus was assessed for the chromosome copy number.

TYPES OF CHROMOSOMAL ABERRATIONS

NORMAL (NO ABERRATIONS)

In a nucleus, two respective spectrum orange colour signals for Cyclin D1(CCND1) gene (11q13 region) and two respective green colour signals for chromosome11 (11p11.11q11 region). The signals ratio of the orange signals to the green signals is 1. (Figure 1A)

LOW LEVEL AMPLIFICATION

Abnormal copy number of Cyclin D1(CCND1) gene was indicated by 3 or more respective orange colour signals with two respective green colour signals (chromosome11 (11p11.11q11))**.** If the signals ratio of the orange signals to the green signals is more than 2, it was considered positive amplification. When ≥20% of the nuclei exhibited ≥3 signals for CCND1, the tumour was considered to have a low-Level amplification. (Figure 1B)

HIGH (CLUSTER TYPE) LEVEL AMPLIFICATION

Clusters of CCND1 (orange) signals were present in nucleus with two respective green colour signals (chromosome11 (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 1C)

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.

DELETION OF CCND1 GENE (MISSING)

In one nucleus, there were one or no orange colour signal for Cyclin D1 (CCND1) and two respective green colour signals for chromosome11 (11p11.11q11).

STATISTICAL ANALYSIS

The results of FISH were compared with the clinic pathologic information of patients included patient age, gender, tumour site, disease stage, histopathology differentiation and the presence of lymph node metastasis, using IBM SPSS version 19 (IBM Corp., Armonk, NY, USA) computer program for windows. Mean and SD were derived for the continue 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.

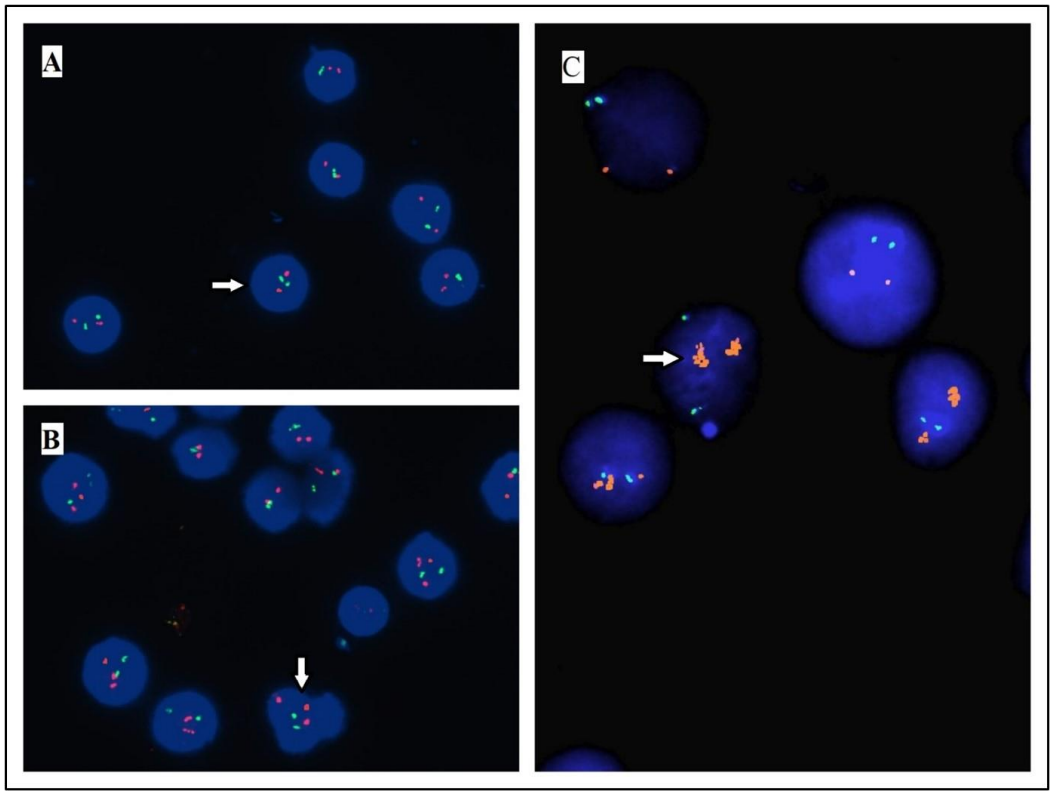

Figure 1: FISH for Cyclin D1 gene showing blood cells interphase nuclei (Arrow), green signals for chromosome 11 centromere and orange signals for Cyclin D1 gene with DAPI counterstaining at 100x. A- Normal, B- Low level Amplification, C- Cluster Amplification.

RESULTS

The mean age of controls and patients were 45.27±10.03 and 46.80±12.34 years respectively (range, 22-70 years). The mean Body mass index (BMI) of controls and patients were 22.67±3.15 kg/m^2 and 21.56±3.2 kg/m^2 respectively. The 50 OSCC tumour sites were recorded as 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). TNM classification shows cases as Stage I (12), stage II (12), stage III (8), stage IV-A (7), Stage IV-B (2) and stage IV-C (9) [Table 1].

The tumours were classified histopathologically in to well (17), moderately (26) and poorly differentiated (7) according to their cellular differentiation. Duration of diagnosis after first sign and symptoms of OSCC was 5.8±0.7 months (range, 1-24 months).

Cyclin D1 gene numerical aberrations were found positive in 5 (6.3%) out of 50 patients with primary OSCCs. Controls (30) were negative for numerical aberrations. Among OSCC patients, low level amplification was found in 3 (3.8%) and high level or cluster amplification was found in 2 (2.5%) cases, whereas Polysomy and Deletion of Cyclin D1 (CCND1 gene) were not observed. There was not significant association of gender, primary site of carcinoma, histopathological differentiation of OSCC with numerical aberrations of Cyclin D1 gene (Table 1); However, a significant association was present between stage of OSCC and lymph node metastasis with numerical aberrations of Cyclin D1 gene with a P-value of 0.001 and 0.004 respectively. The association of risk factors (alcohol consumption, tobacco chewing, smoking and socio-economic status) with numerical aberrations. No risk factor shows significant association with numerical aberrations of CCND1 gene (Table 2).

Table 1: Association between Numerical aberrations of Cyclin D1 gene and Various parameters

Various parameters		Numerical aberrations of Cyclin D1 gene				Total		P-value
		Negative		Positive				
		n	%	n	%	n	%	
Gender	**Male**	37	74.0	5	10.0	42	84.0	**0.557***
	Female	8	16.0	0	0.0	8	16.0	
Primary Site of Carcinoma	**Buccal mucosa**	20	40.0	3	6.0	23	46.0	**0.741***
	Soft palate	2	4.0	1	2.0	3	6.0	
	Lateral surface of tongue	10	20.0	1	2.0	11	22.0	
	Lower alveolar mucosa	6	12.0	0	0.0	6	12.0	
	Retro-mandibular region	3	6.0	0	0.0	3	6.0	
	Root of tongue	4	8.0	0	0.0	4	8.0	
Histopathological grade of OSCC	**Moderately differentiated**	22	44.0	4	8.0	26	52.0	**0.204***
	Poorly differentiated	6	12.0	1	2.0	7	14.0	
	Well differentiated	17	34.0	0	0.0	17	34.0	
Lymph node metastasis	**No**	32	64.0	0	0.0	32	64.0	**0.004**†
	Yes	13	26.0	5	10.0	18	36.0	
Stage of OSCC	**I**	12	24.0	0	0.0	12	24.0	**0.001***
	II	12	24.0	0	0.0	12	24.0	
	III	8	16.0	0	0.0	8	16.0	
	IVA	7	14.0	0	0.0	7	14.0	
	IVB	1	2.0	1	2.0	2	4.0	
	IVC	5	10.0	4	8.0	9	18.0	
***Fisher's Exact test** **† Pearson Chi-Square, p- value <0.05**								

Table 2: Association between Numerical aberrations of Cyclin D1 gene and Risk Factors

Risk Factors		Numerical aberrations of Cyclin D1 gene				Total		P-value
		Negative		Positive				
		n	%	n	%	n	%	
Alcohol Consumption	**No**	28	56.0	1	2.0	29	58.0	**0.047**†
	Occasionally	3	6.0	2	4.0	5	10.0	
	Regular	14	28.0	2	4.0	16	32.0	
Smoking	**No**	22	44.0	2	4.0	24	48.0	**0.818**†
	Mild	2	4.0	0	0.0	2	4.0	
	Moderate	4	8.0	0	0.0	4	8.0	**0.818***
	Severe	17	34.0	3	6.0	20	40.0	
Tobacco Chewing	**No**	11	22.0	0	0.0	11	22.0	**0.271***
	Mild	2	4.0	1	2.0	3	6.0	
	Moderate	5	10.0	1	2.0	6	12.0	
	Severe	27	54.0	3	6.0	30	60.0	
Socio-economic Status	**Upper Class(I)**	15	30.0	3	6.0	18	36.0	**0.889***
	Upper Middle Class (II)	20	40.0	2	4.0	22	44.0	
	Middle Class (III)	5	10.0	0	0.0	5	10.0	
	Lower Middle	5	10.0	0	0.0	5	10.0	

	Class (IV)							
***Fisher's Exact test** **†Pearson Chi-Square, p- value <0.05**								

DISCUSSION

In the present study, we explored the feasibility using Cyclin D1 as a prognostic marker in OSCC by the FISH method on blood samples. We found 5(10%) cases positive for Cyclin D1 aberration. Similar to the results of Sunil PM et al,[3] in their study 7(35%) cases of OSCC had chromosomal alterations using blood samples and they did not observe any association with risk factors. In the study of Miyamoto R et al,[12] CCND1 numerical aberration was identified in 42.0% (21 of 50 patients) of the tumours. Out of 21 tumours which showed the CCND1 numerical aberration, 5 (24%) had multiple single copies of CCND1 associated with chromosome 11 polysomy. In current study, the presence of the CCND1 numerical aberrations did not correlate significantly with age, gender and the tumour site.

Myo et al,[13] 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. Rodrigo et al,[14] in their study correlated CCND1 amplification with clinicopathological parameters and found CCND1amplification was more frequent in T4 tumours and was associated with increased regional lymph node metastasis. In present study, a significant association was observed in between CCND1 amplification and TNM staging or lymph node metastasis.

Nimeus et al,[15] have reported positive Cyclin D1 amplification as low as 16% in OSCC cases while 56.5% in OSCCs of tongue has been reported by Fuji et al.[16] In the current study, the most common affected site of OSCC was buccal mucosa 20 (40%).

Huang X et al,[17] reported that clinicopathologic features of the cases failed to show any significant correlation with 11q13 amplification. Monteiro LS et al,[18] found that 43.3% (26) of the cases showed the presence of numerical aberrations. 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. This could be due to the small size of our series, differences in tumour sites or possible differences in geographic populations.

Uzawa N et al,[8] found that CCND1 numerical aberration was identified in 28 of 57 primary oral Squamous cell carcinoma (SCCs) (49.1%) and reported 8 tumours of cluster-type amplification for CCND1 out of 28 tumours. CCND1 numerical aberration was associated significantly with reduced disease-free survival (P=0.0004) and overall survival (P =0.0179). Mahdey HM et al,[19] observed positive amplification of Cyclin D1 was in 72% (36) of OSCCs. 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).

Ramadan O R et al,[4] detected that 8(26.7%) of the 30 formalin-fixed paraffin blocks cases were scored positive for CCND1 amplification and the relation between FISH and the demographic data (age, sex, the site of the tumour) of the patients, lymph node involvement, clinical stage and histological grade were not statistically significant. (*P*= 0.47, 0.67, 0.33, 0.15, 0.58 and 0.67 respectively).

Most of the studies related to Cyclin D1 numerical aberrations have used tumour cell sections whereas the present study used peripheral blood samples, which shows positive results. Study of Sunil PM et al,[3] have Supported the present study. Barrios L et al,[20] observed chromosomal aberrations in peripheral lymphocytes of patients with breast cancer. Allard WJ et al,[21] concluded that circulating tumour cells were present in the peripheral blood of various cancers but were not present in benign tumours. The usage of FISH technique on blood

samples makes the diagnosis much easier and very helpful in diagnosis of cancer avoiding unnecessary surgery, chemotherapy and radiotherapy.[22]

The present study is able to support the use of peripheral blood to detect Cyclin D1 aberrations by applying FISH interphase technique. The usage of blood sample for detection of gene aberrations supported by significant association with progression of OSCC. In the study, we use FISH technique on interphase cells which making it a better technique of molecular cytogenetics, because it eliminates the necessity required time for cell culture. The interphase FISH technique produces direct visualization of chromosomal aberrations in cell nuclei using fluorescently labelled DNA probes.

CONCLUSION

The study is able to support the use of peripheral blood to detect Cyclin D1 aberrations by applying FISH interphase technique. The usage of blood sample for detection of gene aberrations supported by significant association with progression of OSCC. Many studies have been done on Cyclin D1 in OSCC, and even though the controversy exists in the scientific literature, further discussion and research needed in different cancers with additional different criteria like lymph node involvement and metastasis as indicated in present study.

ACKNOWLEDGEMENTS

We like to show our gratitude to the Department of Oncology and ENT, for their support in this research. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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