Research Paper

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Clinico-Pathological Correlation Of The Expression Of Cyclin D1 And Bcl-2 In Oral Squamous Cell Carcinomas -A Tissue Microarray Study

Medical Science

KEYWORDS : Oral Squamous cell carcinoma, tissue microarray, tumor grade, protein expression, prognosis

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ABSTRACT

Oral squamous cell carcinomas (OSCC)containa heterogenous tumor cell population that are at different stages of differentiation and with different levels of proliferative potential. Hence, the behavioural pattern of individual cases is poorly predictable. Several molecules play a vital role in the step-wise process of oral carcinogenesis. A better understanding of the interaction of these molecules will help in identifying the subgroup of patients with poor outcomes.

Aim and Objective

To analyse the immunohistochemical expression of the cyclin D1 and bcl-2 in oral squamous cell carcinomas. To evaluate the possible association between cyclin D1 and Bcl-2 during the progression of oral squamous cell carcinomas.

Settings and Design

A systematic approach of tissue microarray technology and immunohistochemistry analysis for a cohort of oral squamous cell carcinoma patients.

Materials and methods:

Formalin Fixed Paraffin Embedded (FFPE) tissue blocks of histological proven cases of OSCC (n=119) were selected. Tissue microarray (TMA)paraffin blocks were constructed using a "handy tissue microarrayer". 4 µm thick sections were cut from the TMA blocks and the sections were immunostained with cyclin D1 and Bcl2. A semiquantitative analysis of the immunohistochemical expression of these markers in the tumor cells was carried out.

Introduction

Squamous cell carcinoma of the head and neck is the sixth most common human malignancy, with a worldwide incidence of 500,000 new cases annually[1]. Oral Cancer is a major health problem in India, where it accounts for 40% of all malignancies[2,3].In India, annually 1,30,000 people succumb to oral cancer with approximately 14 deaths per hour. In developed countries 40% ofpatients present with disease at its advanced stage, but in India, as high as 80% of patients present with disease at its advanced stage[4]. In India, the age standardized incidence rate of oral cancer is reported to be 12.6 per 100,000 people[5]. The widespread habit of using tobacco, betel quid and tobacco based products play a major role in the aetiology of the disease in India[6,7]India accounts for 86% of the world's oral cancer cases as per a study conducted by National Institute of Public Health in February 2011. Oral cancer is the most common cancer in India; as 4 in 10 of all cancers are oral cancers[4].

Although there have been vivid improvements in the surgical techniques, radiotherapy and chemotherapy for management of cancers, the overall survival of patients with head and neck squamous cell carcinomas have remained unchanged.[8]

Oral carcinogenesis is a step wise process, wherein, there is a disruption in the regulatory mechanisms that control basic cellular functions including cell division, differentiation and cell death[9]. Cyclinsare the key regulatoryproteins of the cell cycle, that along with kinases result in a cascade of protein phosphorylations that are required for a cell to pass through a specific stage of cell cycle. Cyclin D1 is believed to control the transit from the G1 to the S phase[10,11],The role of cyclin D1 as a prognostic marker in HNSCC remains controversial. Overexpression had been associated with a more aggressive tumor phenotype and reduced patient survival[12].

Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. It is topographically restricted to cells in the proliferating zones and cells with long life spans. The protein is is down regulated in terminally differentiated cells[13].

Previous studies have investigated the prognostic role of the expression of Bcl-2,few studies have indicated that a high positivity for bcl-2 is related to a better clinical behavior, while others have suggested that a high level was associated with a poor prognosis[14,15]. Very few studies have investigated the interaction between the cyclin D1 and Bcl-2 [14].

The aim of thisstudy is to first, evaluate the immunohistochemicalexpression of cyclin D1 and Bcl-2 in a cohort of oral squamous cell carcinomas.Then, to correlate the expression of these biomarkers with clinicopathological parameters and patient outcome.And lastly, to observe the association between cyclin D1 and Bcl-2 during tumor progression.

Material and Method

Patient Selection

Following approval from the institution ethical committee (UEC 13/2012) 119 patients with primary oral squamous cell carcinoma who were treated between July 2009 and December 2012were considered. None of the patients suffered from any other systemic disease and there was no evidence of distant metastasis. The clinical staging of the patients was according to the criteria of the International Union against cancer [16]. The tumors were classified as well, moderately and poorly differentiated as per the WHO classification[17].The survival rate of the patients was measured from the date of initial examination to the date of death or last follow- up. The follow-up data was available for cases.

Construction of tissue microarray

The formalin fixed paraffin embedded tumor blocks and the corresponding H and E stained sections of the 119 cases of OSCC was selected. The hematoxylin and eosin stained sections of these blocks were reviewed and areas where the tumor

cells were least differentiated were marked. Using the marked slide as a template the corresponding area on FFPE blocks was marked. Two cores of 2mm diameter each from these representative areas were obtained from each FFPE block using a "handy tissue microarrayer" (Patent filed). These cores were carefully arrayed on a recipient paraffin block (Fig 1). As the cores were arrayed, their identification detail was recorded in a formatted excel sheet.

Immunohistochemistry

Four micron thick sections were cut from the TMA blocks and mounted onto 3'aminopropylethoxysilane (APES, Sigma, Aldrich Chemical Co, St Louis, MO, USA) coated microslides. Sections were treated with 3% H2o2 in methanol for 20 minutes to block the endogenous peroxidase activity in the tissues. For unmasking the epitomes the micro slides were immersed in preheated 0.01mM sodium citrate buffer (pH 6) and boiled in a decloaking chamber (Pascal, Dakocytomation. Sections were treated with 10% goat serum for 30 minutes to block the non-specific antigens. The tissue sections were incubated with primary antibody cyclin D1 (Clone SP 4, Prediluted, Dakocytomation, Denmark and Bcl-2 (Bcl-2/100, PredilutedBiogenix) for 60 minutes in a humid chamber. The streptavidin peroxidase system (Sigma, Aldrich Chemical Co, St Louis, MO, USA) was used to build the immune reactions. The reaction was developed with 3 '3 diaminobenzidinetetrahydrochloride, (Dakocytomation Denmark). Finally the sections were counterstained with Mayer's hematoxylin for 1 min, dehydrated, cleaned and mounted with DPX. Negative control was carried out by replacing the primary antibody with normal goat serum.

Evaluation of cyclin D1 expression and Bcl-2 expression was performed using a standard (Leitz, Labor lux) light microscope. The brown stain in the nuclei of tumor cells was regarded as positive for cyclin D1 (Fig 2 and Fig 3) and brown stain of the cytoplasm was considered positive for Bcl-2 expression (Fig 4 and Fig 5). The level of cyclin D1 and Bcl-2 was graded semiquantitatively into four scores (0, +, ++ and +++). The scoring system for cyclin D1 was as follows: 0: No staining, + : <30% of tumor cells are positive, ++:30%-60% tumor cells are positive, +++ : > 60% cells are positive.[14]. The cytoplasmic expression of Bcl-2 was evaluated semi quantitatively with a scoring system that was described by Singh BB et al 13 with minor modifications, 0: < 5% cell stain positive, +: Staining in 5%-24% of tumor cells, ++: Staining in 25%-50% of tumor cells +++ when >50% of cells are positive

Statistical analysis

Statistical analysis was carried out using the SPSS package (version 15). Mean +/- SD, minimum and maximum was used to summarize the age. Frequency with percentage was used to summarize the categorical variables. Chi square was used to find the association between the staining pattern of the makers (Cyclin D1 and Bcl-2) and the clinico- pathological parameters. A p value of less than 0.05 was considered to be statistically significant.

Results

In the present study the immunohistochemical expression of cyclin D1 and Bcl-2 was evaluated in 119 cases of histologically diagnosed cases of oral squamous cell carcinomas. The age of the patients in this study ranged from 26 years to 80 years (mean age of 54.49 years). In this study there were 95 male patients and 24 women patients. Among the 119 cases 78 (65.5%) of the cases were from the tongue, 24 (20%)cases were from the buccal mucosa. The remaining 17 cases were from the floor of the mouth, maxillary and madibular alveolus, soft palate, angle of the mouth and the lower lip. Among the 119 cases, 67 cases were well differentiated squamous cell carcinomas, 48 cases were moderately differentiated and 4 cases were poorly differentiated. The expression of cyclin D1 was evident in 90/119 (75%) of the cases and the expression of Bcl-2 was present in 96/119 (80%) of the cases.

Correlation of the expression with tumor size

Among the 7 cases of T1 tumors positive nuclear staining for cyclin D1 was observed in 5/7 (71%) casesand a postive expression for BCl-2 was evident in all the 7 (100%) cases. Among the 37 cases of T2 tumors 28 (75%)cases expressed cyclin D1 and BCl-2. Among the 31 cases of T3 tumors 25 (80%) cases expressed Cyclin D1 and 26 (83%) cases expressed Bcl-2. Among the 44 cases of T4 tumors ,cyclin D1 was expressed by 32 (72%) cases and Bcl-2 expression was evident in 35(75%) cases. (Table 1)

Correlation of the biomarker expression with the lymph node status

Among the 28 cases that did not have any clinically postive lymph nodes (NO), tumors positive nuclear staining for cyclin D1 was observed in 18 (64%) cases and a postive expression for BCl-2 was evident in 26 /28 (92%) cases. Among the 22 cases of N1 lymph node status 14 (63%) cases expressed cyclin D1 and 16 (72%) cases expressed BCl-2. Among the 40 cases with N2 Lymph node status 33 cases expressed Cyclin D1(82%) and 30 (75%) cases expressed Bcl-2. Among the 29 cases with N3 Lymph node status, 25 (86%) cases expressed cyclin D1 Bcl-2 expression was evident in 24 (82%) cases. (Table 2)

Correlation of the biomarker expression with tumor grade

Among the 67 cases of well differentiated squamous cell carcinomas a positive expression ofcyclin D1 was evident in 50 (74%)cases and the expression of Bcl-2 was present in 52 (77%)cases. Among the 48 cases of moderately differentiated squamous cell carcinomas a positive expression of cyclin D1 was evident in 37 (77%) cases and the expression of Bcl-2 was present in 41 (85%) cases. Among the 4 cases of poorly differentiated squamous cell carcinomas a positive expression of cyclin D1 was evident in 3(75%) cases and the expression of Bcl-2 was present in 3 (75%) cases. (Table 3)

Correlation between the co-expression of the marker

The expression of both cyclin D1 and Bcl-2 was seen in 74/119 (62%) cases. The expression of cyclin D1 alone was evident in 16/119 (13%)cases, whilst the expression of Bcl-2 alone was seen in 22/119 (18%)cases. There was no expression of either Cyclin D1 or BCl-2 in 7 cases (Table 4).

Clinico pathological correlation of expression of both Bcl-2 and Cyclin D1

Among these tumors a majority of the tumors 21/74 (28%) were T2 and T3 tumors, N2 Lymph node status was found in 24/74 (32%) tumors and most of the tumors 41/74 (55%) were well differentiated tumors (Table 5).

Discussion

Among the G1 cyclins, cyclin D1 appears to be most strongly implicated in human tumorigenesis (Morokura R, Arnold A. cyclins and oncogenesis. BiochimBiopyActa 1993; 1155:63-78). This protein is encoded by CCNDI gene (also known as bcl-I or PRADI) on chromosome 11q13 and is believed to control cell cycle transit from G1 to S phase.Cyclin D1 expression in squamous cell carcinomas of the head and neck and in oral mucosa in relation to proliferation and apoptosis.18

Overexpression of Cyclin D1 ranges from 34%- 68% in squamous cell carcinomas of the head and neck region.19.20,21In this study, overexpression of cyclin D1 was evident in 75% of the cases of oral squamous cell carcinomas. A strong expression (++,+++,++++) was seen in 55% (51/90) of the cases. Among them 28 cases were well differentiated tumors, 20 cases were moderately differentiated and 3/4 were poorly differentiated. In a study by Bova et al the moderately differentiated and poorly differentiated tumors demonstrated a significantly higher incidence of cyclin D122

Although the contribution of CCND1 gene amplification to overexpression has been well documented,23. other likely mechanisms include up regulation of receptor and signaling pathway that converge on cyclin D1 gene expression24.Cyclin D1 is a downstream mediator of ras. Activated ras oncogene induces cyclin D1 overexpression25.

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In head and neck squamous cell carcinomas amplification of 11q13 has been reported in 30%-60% of tumors.26-29 .Amplification of 11q13 was found to correlate with increased tumor grade30.Overexpression of cyclin D1 was associated with reduced disease-free and overall survival with a higher incidence of local and regional recurrence.30,31,32.Michalides et al found that immunohistochemical overexpression of cyclin D1 is associated with a more frequent recurrence of HNSCC and a shortened patient survival rate.31 .Kuo et al found that patients with tumors containing more than 10% cyclin D1-positive cells had significantly shorter overall survival than those with tumors containing less than 10% cyclin D1-positive cells or with cyclin D1 negative tumors.33

Callenderet al reported that CCNDI amplification is observed more often in high grade and high stage HNSCCs and suggested that CCNDI amplification appears to be late event in the genesis of HNSCC27There was a progressive increase in the number of cases that showed a strong expression for cyclin D1 with the increase in the number of lymph nodes that were involved.

The cytogenetic abnormality places the bcl2 gene from chromosome 18 into juxtaposition with transcriptionally active immunoglobulin heavy chain locus on chromosome 14 resulting in inappropriately high levels of bcl2 gene expression.34Bcl-2 is localized to the outer the outer membrane of the mitochondria, endoplasmic reticulum and the nuclear membrane35. The expression of bcl-2 is found to range from 37% to 100%in oral squamous cell carcinomas36,37. In this study theimmunohistochemical expression of Bcl-2 was observed in 96/100 (80%) of cases. In this study of the 52/67 (76%) cases of well differentiated squamous cell carcinoma showed a positive expression for Bcl-2,(85%)of moderately differentiated cases showed a strong expression and (75%) of the poorly differentiated cases expressed Bcl-2. This observation of an increase in the bcl-2 positivity with the decrease in the differentiation probably reflects that bcl-2 is expressed in keratinocytes that have lost their ability to differentiate and have an increased capacity for survival. An increase bcl-2 expression in poorly differentiated Squamous cell carcinoma was also seen by Yu chen et al38and Muzio et al37. Jordan et al showed a higher percentage of immunoreactivity in poorly differentiated than in well differentiated carcinomas.39

A positive expression for BCL-2 was evident in 26/37 T3 tumors and 35/44 cases of T4 tumors. This overexpression possibly reflects the resistance of these tumor cells to apoptosis and increased cell survival.

A strong expression was seen in 30/40 N2 tumors and 24/29 of N3 tumors. Bcl-2 protein being an anti-apoptotic protein facilitates cell survival and allows further genetic mutations to take place34. These genetic changes result in more aggressive phenotype that are capable of invasion and metastasis

We could retrieve the follow-up details of 57 cases. Among the 57 cases 10 cases had recurrence and 4 patients died. In all the cases with a poor prognosis there was a strong expression (++, +++) of both Bcl-2 and Cyclin D1 except in one case the cyclin D1 was not expressed and in 2 cases Bcl-2 was not expressed. This variation in the expression pattern detectable by bcl-2 antibody could be because of elective caspase activation resulting in proteolytic degradation of bcl-2 in tumor cells.40

The activity of bcl-2 family is partly regulated by formation of homo and heterodimers and the relative concentration of the pro-apoptotic and anti-apoptotic members would decide the outcome of a cell challenged by an apopotic stimulus. However, as there is a functional interaction between different members of the bcl 2 as well as binding of this molecule to other cellular proteins their intrinsic activity is modulated during the a later stages of tumorigenesis15.Assessing the family of related genes of the BCl-2 family along with bcl-2 will help in a more accurate identification of patients with a poor prognosis.

Analysis of the expression of Bcl-2 and Cyclin D1 gives an insight to the molecular status of the tumor. The interaction between the two biomarkers will reflect the biological behavior of the tumor. A better understanding of the biology of tumors will help in identifying patients who fairpoorly. These patients can be managed by applying more intense treatment protocols. Most patients suffer from poor out comes, succumbing to their primary tumor, a recurrent tumor, or a second primary tumor. For this cause, a molecular analysis will aid in better prognostication and help in planning better treatment protocols and will result in a better quality of life for the patient.

Table 1- Correlation of the expression of Bcl-2 and Cyclin D1 with the tumor size

Tumor Stage	T1 (n=7)	T2 (n=37)	T3 (n=31)	T4 (n=44)	Chi Square test
BCL2 expression					
0	0	9	5	9	
+	4	14	13	20	
++	3	11	12	12	.845
+++	0	3	1	3	
Cyclin D1 expression					
0	2	9	6	12	
+	3	14	9	13	
++	2	9	9	15	
+++	0	3	7	4	.530
++++	0	2	0	0	

Table 2- Correlation of expression of Bcl-2 and cyclin D1 with lymph node status

Lymph node status	No (n=28)	N1 (n=20)	N2 (n=40)	N3 (n=29)	Chi square test
BCL2 expression					
0	2	6	10	5	
+	14	8	18	11	
++	11	8	9	10	.494
+++	1	0	3	3	
Cyclin D1 expression					
0	10	8	7	4	
+	8	5	14	12	
++	6	8	12	9	
+++	4	1	5	4	.537
++++	0	0	2	0	

Table 3 Correlation between the expression of BCl-2 and cyclin D1 with the tumor grade

Histopathological grade	Well Differentiated (n=67)	Moderately differentiated (n=48)	Poorly differentiated (n=4)	Chi square test
BCL2 expression				
0	15	7	1	
+	24	25	2	
++	24	13	1	.717
+++	4	3	0	
Cyclin D1 expression				
0	17	11	1	
+	22	17	0	
++	19	14	2	.824
+++	7	6	1	
++++	2	0	0	

Table 4- Correlation between the expression of cyclin D1 and Bcl-2

Cyclin D 1	0	+	++	+++	++++	P value
BCL2 expression						.230
0	7	7	8	1	0	
+	16	17	11	5	2	
++	6	12	12	8	0	
+++	0	3	4	0	0	

Table 5: Association of the form of biomarker expression and the clinico-pathological parameters

Tumor Size						Chi square test
Biomarker expression	T1	T2	T3	T4		.883
Only BCl-2 (n=22)	2	7	5	8		
Only Cyclin D1 (n=16)	0	7	4	5		
Both cyclin D1 and Bcl-2 (n=74)	5	21	21	7		
No expression of either (n=7)	0	2	1	4		
Lymph node status						
Lymph Node	N0	N1	N2	N3		.126
Only BCl-2 (n=22)	9	5	6	2		
Only Cyclin D1 (n=16)	9	3	9	3		
Both cyclin D1 and Bcl-2 (n=74)	17	11	24	22		
No expression of either (n= 7)	1	3	1	2		
Histopath ological grade						
Tumor grade	Well differe ntiated	Moderately differ entiated		Poorly differe ntiated		.204
Only BCl-2 (n=22)	11	11		0		
Only Cyclin D1 (n=16)	9	7		0		
Both cyclin D1 and Bcl-2 (n=74)	41	30		3		
No expression of either (n=7)	6	0		1		

Fig 1- Tissue microarray blocks and slides

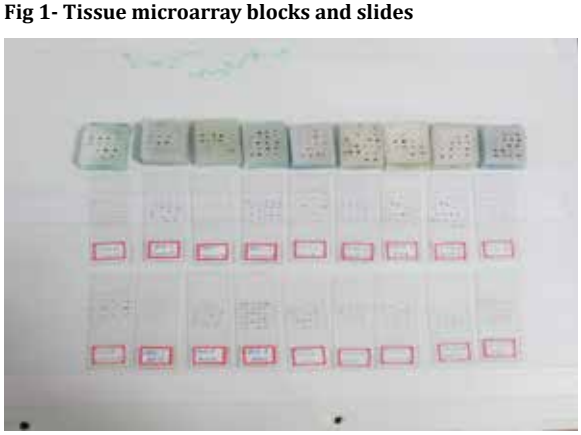


Fig 2.Moderate nuclear staining for cyclin D1 (IHC 40 X)

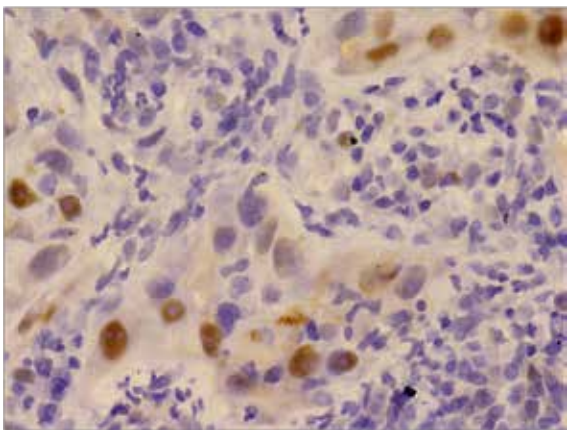


Fig 3.Strong nuclear staining for CyclinD1 (IHC 10X)

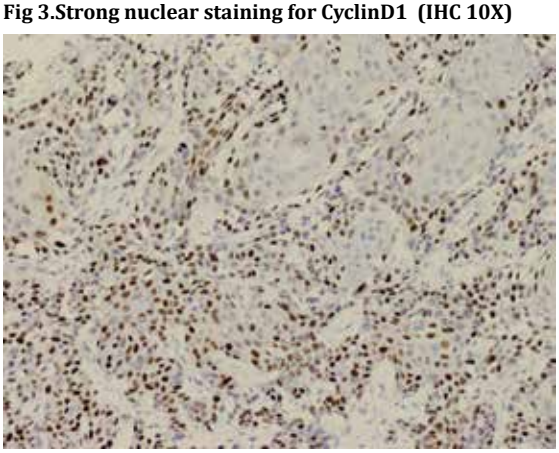


Fig 4. Cytoplasmic expression of Bcl-2 (IHC 10X)

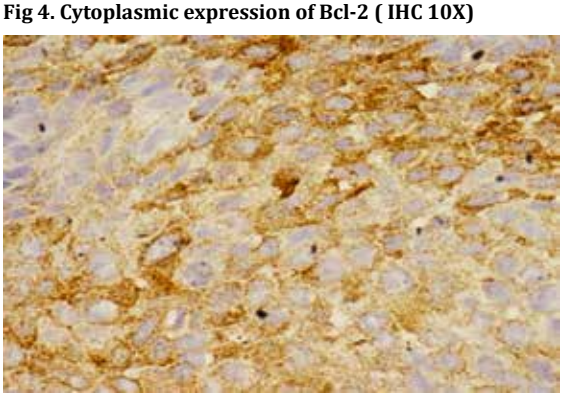
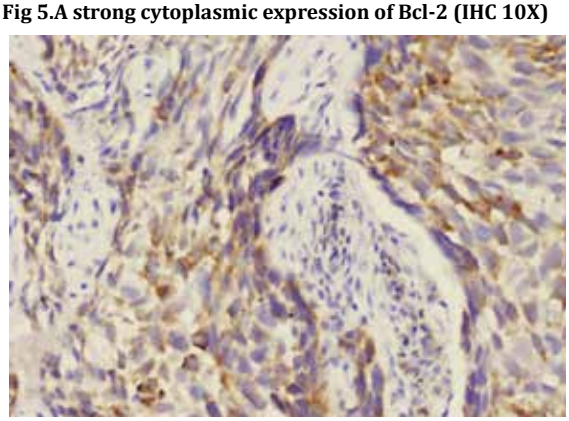


Fig 5.A strong cytoplasmic expression of Bcl-2 (IHC 10X)



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