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Research Article

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Elucidation of β-Catenin Cyclin D1 Pathway in Oral Squamous Cell Carcinoma

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Abstract

Background: *Oral cancer is sixth most common cancer in India with poor overall disease free survival. In last decade major changes in the cancer management has happened but no such advantage has been seen in the survival of oral cancer patients. One major reason for the poor survival of head and neck squamous cell carcinoma (HNSCC) is lack of good predictive and prognostic biomarkers. Different studies have shown that in cancer cells, cell-cycle regulatory protein expression is altered. Cyclin D1 is a key regulatory molecule in cell cycle regulation.*

Many of the molecular alterations that cause abnormal biologic behaviour of cancer cells are based on aberrations of cell cycle regulation. Studies have demonstrated that Cyclin D1, c-Myc and MMP7 were important target genes of WNT signaling pathway and overexpression of them was highly associated with accumulation of β-Catenin and mutational defects of the WNT signaling pathway in numerous tumor types.

Aim: *This study was planned to characterize the β-Catenin and Cyclin D1 transcript level expression pattern in oral squamous cell carcinoma (OSCC) samples.*

Materials and Methods: *Expression patterns of β-Catenin and Cyclin D1 were studied in OSCC at the transcript and protein levels by using qRT-PCR and immunohistochemistry (IHC) respectively. χ2, t-tests and ANOVA were used for the statistical analyses.*

Results: *β-Catenin and Cyclin D1 were significantly overexpressed in oral squamous cell carcinoma cases when compared to normal. Correlation regression analysis showed the expression of Cyclin D1 and β-Catenin at mRNA level were positively correlated. Further, in immunohistochemical analysis β-Catenin showed cytoplasmic staining rather than nuclear.*

Conclusion: *It is concluded that β-Catenin and Cyclin D1 mRNA level analysis using Real-time PCR could serve as biomarkers in oral squamous cell carcinoma since their expression is consistently altered in majority of the oral squamous cell carcinoma samples.*

Keywords: Oral squamous cell carcinoma, Cell-cycle, Cyclin D1, β-Catenin, Real-Time PCR

Abbreviations

HNSCC - Head and Neck Squamous Cell Carcinoma
OSCC - Oral Squamous Cell Carcinoma
IHC - Immunohistochemistry
CDK - Cyclin-Dependant Kinases
NSCLC - Non-Small-Cell Lung Cancers
WDSCCs - Well-differentiated SCCs
MDSCCs - Moderately-differentiated SCCs
PDSCCs - Poorly-differentiated SCCs

Introduction

Cancer is probably the deadliest human affliction. Cancer of the lip and oral cavity accounted for 300,000 cases in 2012 (2.1% of the world total), with two-thirds occurring in men. Worldwide, 145,000 deaths occurred (1.8% of the world total), of which 77% were in the less developed regions [1]. HNSCC represents a major worldwide health problem.

One reason for the poor survival of this tumor is lack of good predictive and prognostic biomarkers [2]. Moreover, molecular markers which are linked to malignant transformation may provide a non-surgical therapeutic approach by targeting these molecules through gene therapy or anti-sense molecules [3]. Many of the

molecular alterations that cause abnormal biologic behaviour of cancer cells are based on aberrations of cell cycle regulation [4].

The *Cyclin D1* gene is located on chromosome 11q13 and encodes a nuclear protein that is a positive regulator of cell cycle, facilitating G_1 to S phase transition [5]. Overexpression of *Cyclin D1* prevents cells from entering quiescence and therefore enhances the proliferative fraction of tumor cells [6]. However the underlying mechanism leading to this aberrant expression remains poorly understood in head and neck cancer.

Studies have demonstrated that *Cyclin D1*, *c-Mycand MMP7* were important target genes of WNT signaling pathway and overexpression of them was highly associated with accumulation of β-Catenin and mutational defects of the WNT signaling pathway in numerous tumor types [7-13]. β-Catenin is a subunit of the cadherin protein complex. Reduction and loss of β-Catenin or other molecules might disrupt stability and integrity of the E-cadherin-Catenin complex and disturb cellular adhesive junction, resulting in cell proliferation, invasion and metastasis of the tumor [14-17]. Though several immunohistochemical studies have been done on Cyclin D1, β-Catenin and their role in carcinogenesis, only very few mRNA level tests have been conducted especially in oral carcinoma. In this study we have addressed the issue of whether altered expression of *Cyclin D1* is associated with abnormal expression of *β-Catenin* in oral squamous cell carcinoma. We have studied the expression patterns of *β-Catenin* and *Cyclin D1* in OSCC at the mRNA and protein levels by using qRT-PCR and immunohistochemistry respectively. The ultimate goal being to use biomarkers for early diagnosis of cancer.

Methods

The study was done in Government Dental College, in collaboration with the Division of Cancer Research, Regional Cancer Centre, Thiruvanathapuram. All patients who reported to the Department of Oral Medicine with lesions suggestive of oral cancer were ascertained histopathologically. The patients with previous history of any type of treatment for the cancer were excluded from the study. Accordingly we could obtain samples from 36 oral cancer patients and 10 normal tissues for further analysis.

Informed consent was obtained from the respondents at the start of the study. Consent was also obtained by presenting the study to the college ethical committee. Incisional biopsy was done under local anaesthesia and the tissue divided into two parts. One part was put in formalin and sent for histopathological examination. The other part was preserved in liquid nitrogen for future studies using qRT-PCR. Immunohistochemistry was done on the slides obtained by retrieving the paraffin blocks of the specimens.

RNA Isolation and cDNA Construction

RNA was isolated using the TRizol (Sigma) as per the manufacture protocol, the isolated RNA was treated with DNase to remove any DNA contamination. The cDNA construction was done using reverse transcription kit (Aplide biosystem) as per the manufacture protocol.

Real Time PCR

Cyclin D1 and *β-Catenin* transcript level expression was analysed using specific TaqMan probes. 2-ΔΔCt method was used to calculate relative fold changes of gene expression in tumor samples compared to normal tissue sample.

Immunohistochemistry

Protein level expression and sub-cellular localization of Cyclin D1 and β-Catenin was studied using IHC. The intensity of staining was evaluated using a three-point semi-quantitative scale. For assessing the positivity, the cells seen at the invading tumour front or the deep malignant islands were considered.

Statistical Analysis

The association between expression of *β-Catenin* and *Cyclin D1* was analysed by chi-square tests.

Results

Clinico-pathological evaluation of the collected samples showed the age range of 30-75 years. 23 out of 36 cases had the habit of using tobacco as pan chewing or smoking and majority had the habit of alcohol consumption (Table 1). 11(31%) OSCC cases belonged to stage I, there were 7(19%) cases belonging to stage II and 9(25%) cases each belonging to stage III and stage IV according to TNM classification (Table 2). t test expression analysis showed that in majority of cancer cases Cyclin D1 and *β-Catenin* mRNA were overexpressed with respect to normal samples (Figures 1 and 2). However, the *Cyclin D1* and *β–Catenin* expression difference between the histopathological group was not statistically significant (p=0.34 for *β-Catenin* and for *Cyclin D1* p=0.4) (Table 3). Correlation regression analysis showed that *Cyclin D1* and *β-Catenin* had a positive correlation (p=0.001). Protein level analysis showed that majority of samples (9/10) had membrane localization of β–Catenin, which indicate the non-activation of the *β–Catenin* pathway in OSCC (Figure 3). However, the Cyclin D1 was over-expressed in 6 out of 10 cases (Figure 4).

Table 1: Showing distribution of cases based on habits

	Alcohol	Tobacco + Alcohol	Tobacco	No Habits	Total
Normal	0	0	2	8	10
Cancer	1	9	23	3	36
					46

Table 2: Showing distribution of OSCC cases according to tumour stage

	β-catenin	Cyclin D1
MDSCC	1.1652	1.0237
WDSCC	0.8161	0.4377
PDSCC	1.3661	1.2702

Table 3: Showing the expression variation of Cyclin D1 and β-Catenin with respect to histopathology

	Frequency	Percent
Stage I	11	30.6
Stage II	7	19.4
Stage III	9	25
Stage IV	9	25

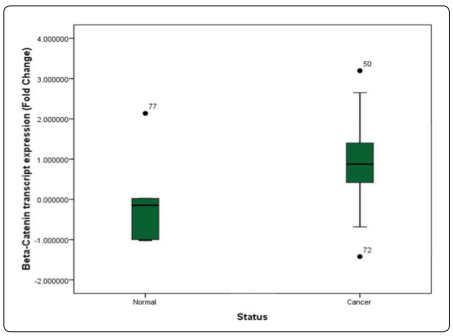

Figure 1: Box-plot showing the expression in fold change of β-Catenin between OSCC and normal cases

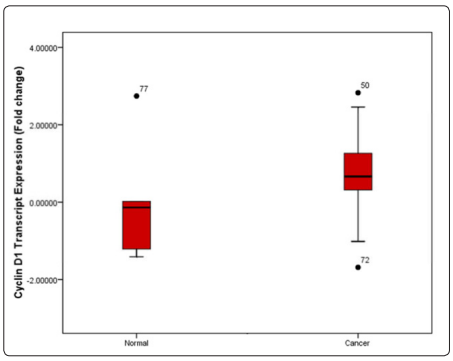

Figure 2: Box-plot showing the expression in fold change of Cyclin D1 between OSCC and normal cases

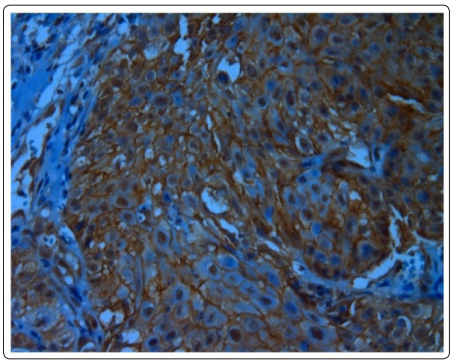

Figure 3: Expression of β-catenin with membrane localization in OSCC (40X)

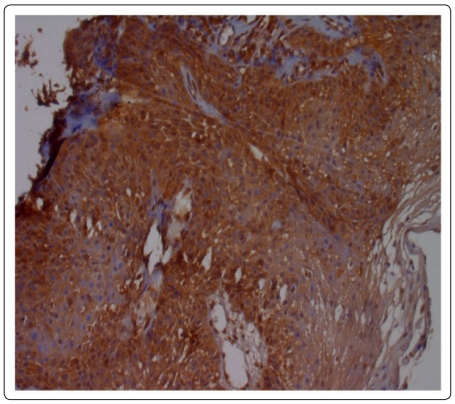

Figure 4: Expression of Cyclin D1 with cytoplasmic and nuclear localization in OSCC (40X)

Discussion

Cancer is a disease characterised by abnormal growth and development of normal cells beyond their natural boundaries. The management of cancer requires diagnosis at an early stage, which specifies the need for specific and sensitive biomarkers. Squamous cell carcinoma is the most frequent malignant tumor of the oral cavity [18,19].

Proliferation and progression of the cell cycle in mammalian cells is regulated in an orderly manner by a subset of cell-cycle genes that encode cyclin-dependant kinases (CDK) and their regulatory proteins the Cyclins and CDK inhibitors [20,21]. D-type Cyclins are synthesized as cells enter G_1 phase from quiescence (G_0) and are responsible for initiating cell cycle progression. *Cyclin D1* is expressed in most epithelial and fibroblast cells and *Cyclin D3* in lymphoid cells [22]. In conjunction with their partner kinases CDK4 and CDK6, these Cyclins link mitogenic signals to cell-cycle progression [23]. Dysregulation of *Cyclin D1* is a frequently observed feature of human cancers of diverse histological origin. Overexpression of Cyclin D1 protein has been found to occur in several malignancies and has been linked to a more aggressive tumour phenotype and a poorer prognosis [24-26].

Cancer occurs through multiple steps, each characterized by the sequential stimulation of additional genetic defects by clonal expansion [27]. Amplification leads to the increased expression of genes, which in turn can confer a selective advantage for cell growth [28]. Metastatic spread strongly reduces the possibility of cure; so it is obvious that, short of prevention of cancer, no achievement would confer greater benefit on patients than methods to prevent metastasis [29]. The first step in the metastatic cascade is a loosening of tumor cells. E-cadherins act as intercellular glues, and their cytoplasmic portions bind to β-Catenin. Free β - Catenin can activate transcription of growth-promoting genes. E-cadherin function is lost in almost all epithelial cancers, either by mutational inactivation of E-cadherin genes or by activation of β-Catenin genes [30].

The present study was designed to determine whether there is any correlation between expression patterns of *Cyclin D1* and *β-Catenin* at the mRNA level as potential biomarkers via expression analysis using qRT-PCR. Cyclin D1 and β-Catenin protein level expression have already been documented as potential biomarkers in different tumors [11,31]. However protein level analyses are subjective and very tedious compared to mRNA level analysis using Real-Time PCR which is quantitative and highly reproducible.

Uncontrolled cell proliferation is the hallmark of cancer and tumor cells have typically acquired damage to genes that directly regulate their cell cycles. Overexpression of *Cyclin D1* was involved in tumor genesis of non-small-cell lung cancers (NSCLC) from early stage and could be a molecular marker for a poorer outcome and progression in NSCLCs with resectability according to Keum JS, et al. (1999) [32]. Similar observations including correlation of CCND1 (*Cyclin D1gene*) amplification in HNSCC with an aggressive histologic appearance have been concluded from investigations carried out by Williams, Michael E, et al. (1993) and Sathyan KM, et al. (2008) [2,33].

Kudo Y, et al. (2004) concluded that, invasion and metastasis of OSCC cells require methylation of E-cadherin and /or degradation of membranous *β-Catenin* [34]. According to Tetsu O and McCormick

F, (1999) cells expressing mutant *β-Catenin* showed high levels of *Cyclin D1* messenger RNA and protein constitutively. They concluded that abnormal levels of β-Catenin may therefore contribute to neoplastic transformation by causing accumulation of Cyclin D1 [35].

In the present study out of the total 36 cases of OSCC majority were males. This may be due to the increased association of habits in the male population.

The results showed that the *β-Catenin* and *Cyclin D1* mRNA were significantly over-expressed in OSCC with respect to normal. These results substantiate other in-vitro and in-vivo reports showing the involvement of Cyclin D1 and β -Catenin proteins in tumorigenesis of different cancers [36-40].

Hui P, et al. (2003) have analysed *Cyclin D1*mRNA in mantle cell lymphoma [41]. Real-Time PCR analysis of *β-Catenin* mRNA in sporadic colorectal cancers was done by Qin YJ, et al. (2006) [42]. Different researchers have associated overexpression of *Cyclin D1* with recurrence, metastasis and poor survival rate in a variety of human malignancies [38,43-46]. Reduced β-Catenin protein expression has been linked with lymph node metastasis and an unfavourable prognosis [47]. The correlation between expression pattern of *Cyclin D1* and *β-Catenin* at mRNA level was analysed using correlation analysis and it was found that they were positively correlated ($p = 0.001$). This proves that *β-Catenin* may have a role in the transcript level overexpression of *Cyclin D1* in OSCC through activation of the WNT signaling pathway.

There have been earlier studies which have correlated the expression pattern of Cyclin D1 and β-Catenin immunohistochemically [11,31,48]. However, another immunohistochemical study by Goto H, et al (2002), has reported no correlation between pattern of *β-Catenin* expression and *Cyclin D1* overexpression in lingual squamous cell carcinomas [49].

There are some reports showing that Cyclin D1 and β-Catenin protein are overexpressed in less differentiated tumors [50,51]. Moderately-differentiated SCCs (MDSCCs) and poorly-differentiated SCCs (PDSCCs) are more aggressive as compared to well-differentiated SCCs (WDSCCs) and this can be correlated with our results in which *Cyclin D1* and *β-Catenin* mRNA level expression was higher in MDSCCs and PDSCCs. Cyclin D1 is a cell cycle regulatory protein and increase in its level can lead to increased cellular proliferation.

The main prognostic determinators in OSCC are stage, location and microscopic grading of the disease [52-56]. In the present study 50% of the cases were stage III and IV. Kouraklis, et al (2006) described poor survival rate in patients with advanced stages of cancer [57].

Mutated *β-Catenin* can act as an oncogene and the mutation results in accumulation of the protein in the cytoplasm and /or nucleus of the cancer cell [58,59]. Studies have correlated the expression of Cyclin D1 and β-Catenin immunohistochemically in different cancer cases [11,31]. From the immunohistochemical analysis of protein level expression pattern of Cyclin D1 and β-Catenin, only one sample had nucleolar localization for β-Catenin, hence from the present study we can infer that *β-Catenin* has no direct influence in transcript level overexpression of *Cyclin D1* in OSCC.

The results from our study, demonstrate that the overexpression of *Cyclin D1* and *β -Catenin* mRNA was a common event in OSCC and could serve as potential biomarkers. Any step of gene expression may be modulated, from the DNA-RNA transcription step to post-translational modification of a protein. The stability of the final gene product, whether it is RNA or protein, also contributes to the expression level of the gene - an unstable product results in a low expression level. In general gene expression is regulated through changes in the number and type of interactions between molecules that collectively influence transcription of DNA and translation of RNA [60-62]. Hence any of the above reasons could be attributed to overexpression of *Cyclin D1* in OSCC leading to uncontrolled cell proliferation via activation of the cell cycle.

Conclusion

The conclusion that can be arrived at from the present study is that the *β-Catenin* and *Cyclin D1* mRNA level analysis using qRT-PCR could serve as biomarkers in OSCC since their expression is consistently altered in majority of the cancer samples and technically it is convenient, easy to quantitate and highly reproducible. However, further studies using a larger cohort with larger number of early stage and late stage samples of OSCC and correlation with immunohistochemistry are necessary to have a better understanding of their role in carcinogenesis.

It is extremely likely that, in the not too distant future, molecular profiling will become an adjunct in the diagnosis, classification and management of cancer. This type of analysis also may reveal novel gene targets for tumors and development of new drugs. Therapy may be tailored to the specific genes dysregulated in a given patient. Who knows, advertisements for "designer genes" may appear side by side with ads for "designer jeans"! [30].

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