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Hypermethylation of DAPK Gene in OSCC Patients among North Indian Population [Version 1, Awaiting Peer Review]

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Abstract

The promoter hypermethylation of *DAPK* gene was found in 53.33% of OSCC patients among north Indian population. The tumor suppressor gene, *DAPK*, is altered in many human tumors, particularly in those caused by environmental carcinogens, such as tobacco smoke. Alterations of DAPK occur very early during the multistep process of carcinogenesis.

According to the present study, promoter hypermethylation of *DAPK* gene was detected in 16 out of 30 cancer patient samples. This is the first report on the promoter hypermethylation of *DAPK* gene in OSCC patients among north Indian population. The present study needs to be carried out on a large scale to make a significant statistical conclusion.

Keywords

OSCC; DAPK gene; Promoter Hypermethylation

Introduction

Cancer is when abnormal cells divide in an uncontrolled way. Some cancers may eventually spread into other tissues. Cancer is a leading cause for morbidity and mortality worldwide, approximately 14 million new cases are arising yearly starting 8.2 million cancer related death in 2012. Statistical studies have shown that among head and neck cancer OSCC is one of the most commonly occurring cancer [1]. The fact that there are more than 100 different types of cancer, with most cancers having multiple possible causes cancer has become a war that needs to be fought over as it is a leading cause for morbidity and mortality worldwide. Oral cancer, a subtype of head and neck cancer, is any cancerous tissue growth located in the oral cavity. Statistical studies have shown that among head and neck cancer oral squamous cell carcinoma (OSCC) is one of the most commonly occurring cancer. It accounts for 3–5% of all human malignancies and it is ranked sixth most common cancer in the world [2]. Oral carcinogenesis is modulated by endogenous and environmental factors. OSCC arises as a result of multiple molecular events that develop from the combined influences of an individual's genetic predisposition, immune deficiency and external agents such as dietary factors and viruses and like human papillomavirus and Epstein barr virus [3]. Revised investigations about prevalence and/ or incidence of OSCC concludes that smoking is a major risk factor in ¼ of cases, between 7 to 19% of cases are attributable to alcohol consumption and 10 to 15% cases are caused due to micronutrient deficiency [4].

The highest incidence and prevalence of OSCC is found in the Indian subcontinent where the risk of developing OSCC is increased by the very prevalent habits of chewing tobacco, betel quid and areca-nut. The mutagenic effects of tobacco, alcohol, betel quid or areca-nut are dependent upon dose, upon frequency and upon duration of use, and are accelerated and exaggerated by the concurrent use of two or more of these agents.

The survival rate remains 50% despite in the improvements from last five decades. The outcome of the patients can be improved with OSCC, which is a pivotal to understand the molecular biology of distinctive tumors and find predictive biomarkers for targeted therapy [5]. In addition to mutation, there are several epigenetic changes which lead to the cancer. Two type of epigenetic changes are there: Histone modifications and DNA Methylation. Histone modification is a covalent post-translational modification (PTM) to histone proteins which includes methylation, phosphorylation, acetylation, ubiquitylation, and sumoylation and DNA methylation is an epigenetic mechanism used by cells to control gene expression.

Squamous cell carcinoma which may affect any anatomical site in the mouth, commonly affects the tongue and the floor of the mouth and accounts for 90% of all oral cancer.

Epigenetic changes are one of those changes which occurs due to environment and dietary factors. The word "epigenetics" was coined by the developmental biologist C.H. Waddington in 1942 [6]. Epigenetic refers to the change in the gene expression without the change in the sequence of the gene. The epigenetic changes refer to any reversible heritable modifications in gene expression without alterations of the DNA sequence. They occur more frequently than gene mutation.

Epigenetics is another major event in the development and progression of OSCC. The epigenetic changes refer to any reversible heritable modifications in gene expression without alterations of the DNA sequence. They occur more frequently than gene mutations.

DNA Methylation can physically inhibit the transcription of the gene or methylation can lead to the recruitment of transcription factors that repress transcription, leading to the same end result- down regulation of gene expression that leads to decreased levels of the tumor suppressor protein. In DNA methylation, a methyl group is covalently added to the fifth carbon of the cytosine ring to form 5-methyl cytosine. Cytosine is one of the five nucleotides in the nucleic acids of DNA and RNA. Along the linear DNA chain, there are sites of DNA where a cytosine is followed by and linked via a phosphate to guanine, another nucleotide. These sites are called CpG sites. Regions of DNA that have a high density of CpG sites are called CpG islands. DNA methylation occurs predominately on the CpG islands. DNA methylation is actively involved in regulating cell differentiation and function. When too much or too little methylation occurs, it can often negate a gene's function and thus causes unwanted alterations in the cell and even result in diseases.

Death Associated Protein kinase (DAPK) a novel calmodulin-dependent serine/threonine kinase which was first identified as a positive mediator of programmed cell death. Loss of DAP kinase expression was first demonstrated in highly metastatic cells, whilst re-expression of the protein resulted in delayed local tumour growth and a decreased incidence of metastasis.

Death-associated protein (DAP) kinase is a serine/threonine kinase that is important regulation of cell cycle. Hypermethylation of *DAP-kinase* is a strong indicator of the superficial bladder cancer associated with a high recurrence rate ($P < 0.001$; hazards ratio, 7.01). Detection of the aberrant hypermethylation of DAPK genes in blood DNA from non-invasive bladder cancer patients might offer an effective means for earlier auxillary diagnosis of the malignancy.

The treatment of oral squamous cell carcinoma (OSCC) following early detection is associated with good outcomes. Therefore, the survival and prognosis of OSCC patients could be hugely improved by identifying reliable biomarkers for the early diagnosis of the disease. A retrospective study of the epidemiologic profile of patients with oral squamous cell carcinoma (OSCC) conclude that strategies to overcome the present situation must be taken under consideration by oral health pro-

grams for the early diagnosis and prevention and management and follow up of oral cancer [7]. Death Associated Protein kinase (DAPK) a novel calmodulin-dependent serine/threonine kinase which was first identified as a positive mediator of programmed cell death. Loss of DAP kinase expression was first demonstrated in highly metastatic cells, whilst re-expression of the protein resulted in delayed local tumour growth and a decreased incidence of metastasis.

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Various case-control studies have been conducted in India to study the higher prevalence of OSCC in Indian subcontinent.

In one such study patient diagnosed with oral cancer (n=388), statistical parameters were studied including risk factors and an equal number of age and sex-matched controls to assess the effect of lifestyle factors (tobacco chewing, smoking, alcohol drinking, diet and dental care) on the risk of oral cancer. The results showed that use of tobacco (chewing) alone and alcohol drinking emerged as significant risk factors for oral cancer (odds ratio - OR=11.34).

The global prevalence of oral potentially malignant disorders ranges approximately from 1 to 5% [8].The first step in the prevention and control of OSCC is to increase awareness among the general public and policy makers to emphasize that these risk factors are modifiable [9].

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The motive was to study the activation of proto-oncogenes and inactivation of tumour suppressor genes and to see that they are the major genetic alterations involved in carcinogenesis. The increase in methylation at the promoter region of a tumour suppressor gene can lead to gene inactivation, selecting cells with proliferative advantage. Thus, promoter hypermethylation is considered a marker in a variety of malignant tumours, including oral cavity.

Materials and Methods

Sample Collection

Blood samples (30) were collected with informed consent of patients diagnosed with OSCC after obtaining the necessary ethical clearance from Dharamshila Cancer Hospital & Research Centre, New Delhi. The blood samples (15) from healthy individuals (as controls) were also obtained. The samples were further used for DNA extraction.

DNA Extraction

Cells obtained from blood samples were lysed in digestion buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl and 2% SDS) containing proteinase K (0.2 mg/ml). DNA was then purified using the standard phenol-chloroform extraction and ethanol precipitation.

Methylation-Specific PCR (MS-PCR)

DNA isolated from the blood samples was modified with sodium bisulphite and MS-PCR was carried out using specific primers for methylation and unmethylation for the *DAPK* genes (Table 1). The amplified products were run on a 2% agarose gel.

Table 1: Sequence of primers used.

Primers	Sequence	Volume 100 µM	Tm (°C)
DAPK Methylated Forward	GGATAGTCGGATCGAGTTAACGT	261.4	57.0
DAPK Methylated Reverse	CCCTCCAAACGCCGA	309.0	51.0
DAPK Unmethylated Forward	GGAGGATAGTTGGATTGAGTTA-ATGTT	162.8	55.0
DAPK Unmethylated Reverse	CAAATCCCTCCCAAACACCAA	217.2	52.0

Statistical Analysis

The association between hypermethylation of the genes and risk of OSCC was estimated by computing odds ratios (ORs) and 95% confidence intervals (CI) using the Chi-square test, Fisher's exact test and multivariate logistic regression analysis, which included several potential confounding variables. The reported OR may be interpreted as age-adjusted estimates of the relative risk of developing OSCC with the methylation of studied genes. Statistical analysis was performed using SPSS version 11.5 and Epi Info version 7.0. $P<0.05$ was considered to indicate a statistically significant difference.

Results

DNA Isolation

The DNA isolated from the blood samples of OSCC patients was run on 0.8 % agarose gel and visualized under gel documentation unit.

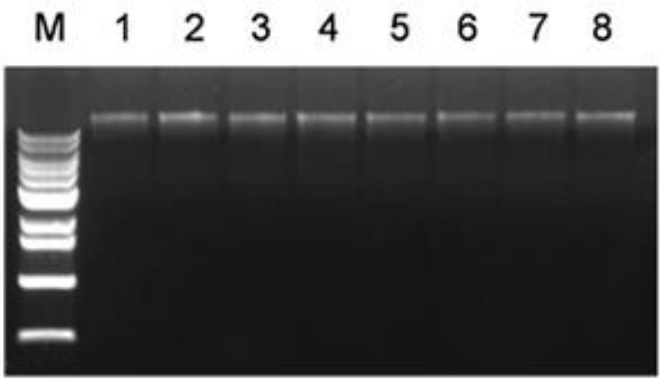

Figure 1: DNA isolated from blood samples of OSCC patients among north Indian population.

MS PCR

MS PCR was performed to check the promoter hypermethylation of DAPK gene in OSCC patients among north Indian population.

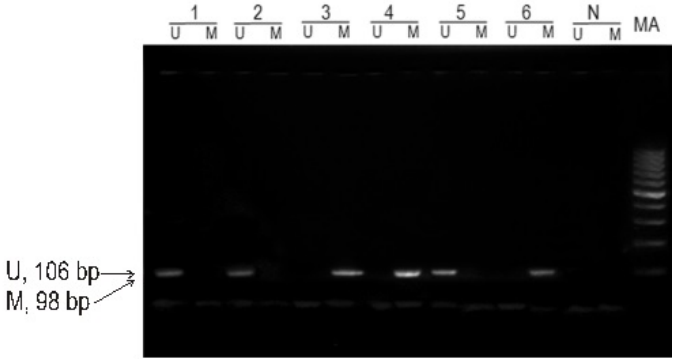

Figure 2: The MSB (methylation –specific band) and UMSB (unmethylation- specific band) of DAPK gene in blood samples of OSCC patients, along with ladder.

Table 2a: Number of samples found to be hypermethylated amongst the total 30 samples of OSCC patients.

Sample type	Samples found to be promoter hypermethylated	Total samples
Blood	16	30

Table 2b : Frequency of methylation of DAPK with relative risk of OSCC between patients and healthy controls.

	Patient n=30(%)	Control n=15(%)	OR (95% CI)	p value
Methylation of DAPK	16(53.33)	0(0)	2.071 (1.42-3.01)	0.0004

Discussion

Epigenetic role of promoter hypermethylation in early oral cancer is important to gain new insight into oral carcinogenesis, and to identify diagnostic and prognostic biomarkers, as well as potential therapeutic targets [10]. Epigenetic changes lead to the promoter hypermethylation which changes the expression of the gene without mutating the sequence of the gene. Covalent modification of the DNA or its packaging histones are responsible for transmitting epigenetic information. Epigenetic modification, such as acetylation, phosphorylation, methylation, ubiquitination and ADP ribosylation are also responsible for changes in the gene expression.

In epigenetic changes, the promoter region of the gene is hypermethylated which causeS global hypomethylation to the gene. Epigenetic changes vary from population to population and place to place as it mainly depends upon the environmental and dietary factors which varies greatly.

There are several reports on the promoter hypermethylation of the DAPK gene and it has been observed that in various populations DAPK gene was found to be hypermethylated in OSCC [11]. Till now no report exists on the DAPK gene hypermethylation in north Indian population.

Death Associated Protein kinase (DAP kinase) a novel calmodulin-dependent serine/threonine kinase which was first identified as a positive mediator of programmed cell death. Loss of DAP kinase expression was first demonstrated in highly metastatic cells, whilst re-expression of the protein resulted in delayed local tumour growth and a decreased incidence of metastasis.

Death-associated protein kinase (DAPK) is an important serine/threonine kinase involved in various cellular processes, including apoptosis, autophagy, and inflammation. DAPK expression and activity are deregulated in a variety of diseases including cancer. Methylation of the *DAPK* gene is common in many types of cancer and can lead to loss of DAPK expression. The prevalence of p16, death-associated protein kinase (DAPK) and O 6 -methylguanine-DNA-methyltransferase (MGMT) promoter hypermethylation in OSCC has been evaluated for several years while the results remain controversial.

The DNA was isolated from the collected blood samples of cancer patient (OSCC) and control samples (patients not suffering from cancer). Further isolation of DNA was followed by sodium bisulfite modification through agarose bead method, after which MSP (methylation specific PCR) was carried out using methylation specific primers.

In the present study on 30 samples, 16 samples showed promoter hypermethylation in blood samples respectively. After Gel electrophoresis, the PCR product was observed. The bands formed for methylated DNA was of 98 base pair (bp) whereas that for unmethylated DNA was 106 bp. The bands were observed in case of both methylation specific PCR as well

as unmethylation specific PCR because of the mixed population of cells (normal as well as cancerous cells) present in the blood cells. The percentage of promoter hypermethylation observed in blood samples was 53.33 %.

It was concluded that several environmental and dietary factors lead to promoter hypermethylation of DAPK gene among north Indian population.

The study needs to be carried out on large sample size to draw a complete statistical conclusion.

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