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Promoter Methylation Status Of P16 Gene In Biopsy Samples Of OSCC Patients Among North Indian Population.

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ABSTRACT

Worldwide, large number of deaths are associated with cancer. Cancer is the leading cause of death worldwide causing millions of death. Oral cancer is the 6th most commonly occurring malignancy leading to high mortality across the world. Smoking and chewing tobacco are the major causes of oral cancer. Cancer is genetic alteration of the gene in which mutation and epigenetic changes are responsible . These are caused due to environmental and dietary factors. p16 is a tumor suppressor gene which gets hypermethylated due to several riskfactors. It was observed that the p16 hypermethylation was found to be significant in the north Indian population (p-value= 0.0013). p16 hypermethylation was observed in 48% (24 out of 50) of the samples.
Keywords: Oral Cancer, Epigenetic changes, p16 gene, Hypermethylation,

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INTRODUCTION

Cancer, (malignant tumor/ neoplasm) is a disease which leads to abnormal cell growth (immortal, continuous dividing) with the capacity to spread towards other parts of the body from origin. Normal cells in the body maintains a regulated path of growth, division, and death. Cancer was the leading causes for morbidity and mortality globally, with across 14 million new cases and 8.2 million deaths due to cancer in 2012. OSCC is most prominent cancer and is sixth most common cancerworldwideand accounts for 3–5% of all malignancies [1]. There are more than 100 different variety of cancer, in which some of the cancer have multiple risk factors. 90% of all oral cancers are found to be squamous cell carcinoma. The floor of the mouth and tongueare anatomically most common site for 90% of oral cancer. Pre-existing oral lesions have the potential for causing the oral cancer [2]. Oral Squamous Cell Carcinoma (OSCC) consists of one third of total cancer malignancies.

Early detection of the oral cancer leads to better treatment of the cancer. The 5-year survival rate is <30% among patients with stage IV cancer. The death rate of the patients suffering with the oral cancer is increasing because the early detection of cancer is less frequent. Therefore, identifying reliable biomarkers for the early diagnosis of OSCC may help to improve the rate of survival and prognosis in OSCC patients.

Epigenetics refers to the alteration of the expression of gene without inducing any change in the sequence of the gene. Covalent modification of the DNA and histone modification are important part of epigenetic changes. Robin Holliday defined epigenetics as “the study of the mechanism of temporal and spatial control of the gene activity during the development of complex organism”. Epigenetic changes consists of DNA hyper methylation and histone modifications.

In India, oral cancer is leading cause for the deaths and it is ranked first in case of males and ranked third in case of females. Tobacco smoking and alcohol consumption are established as the most prominent cause for the OSCC [3].

EPIDEMIOLOGY

Occurrence of the cancer in oral cavity, pharynx, larynx, epithelium of the nasal and head and neck region which leads to tumors [4]. Estimated 400,000 fresh cases of the oral cavity, lip and pharynx cancer diagnosed globally in 2008 (3% of the total cases) [5]. Oral SCC more frequently affects males as compared to females (M:F = 1.5:1). With increase in the period of exposure of the risk factors the probability of developing oral SCC increases, and increasing age adds a further dimension of age-related mutagenic and epigenetic changes. Among some region higher prevalence of oral cancer was found [6]. Globally, the occurrence of oral cancer was depend over various risk factors in which more than 50% to betel quid chewing then 25% attributed to tobacco usage (chewing and/or smoking), 7–19% to alcohol drinking, 10–15% to micronutrient deficiency [7]. Smoking for long time period (nearly 20 or more) produced significantly elevated odds ratios (OR= 2.1; 95% CI= 1.1- 4.0) [8]. In India, a case-control study was performed in which 388 patients of oral cancer were taken (n=388). As control same number of people were taken having equal age and sex match for analysis of lifestyle factor which may leads to oral cancer (diet, tobacco chewing, alcohol drinking, smoking and dental care). Result shows that tobacco chewing and drinking alcohol found to be essential risk factor for oral cancer (odds ratio OR=11.34). Through increase in awareness among general public and by effective policy, control and prevention of oral squamous cell cancer may be achieved [9].

p16 G

p16 (also known as cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1) is a tumor suppressor protein, encoded by the *CDKN2A* gene [10]. p16 regulates cell cycles by decelerating cell progression from G1 phase to S phase. Hypermethylation was found in the promoter regions of p16 gene from tissue samples of primary oral squamous cell carcinoma (OSCC) showing a significant increase of methylation in CpG islands in the promoter region of p16. The function of tumor suppressor gene is suppressed due to this epigenetic change. The methylation can repress the transcription through the recruitment of transcription factors, down regulation of gene expression leading to decrease in p16 expression [11]. Therefore, p16 acts as a tumor suppressor gene, useful in the prevention of cancers.

p16 gene found at chromosome 9p21has vital role in cell cycle regulation. The inhibition of the cyclin-dependent kinases (4 and 6) is done by the p16 protein along with this these protein are also associated with phosphorylation of the serine and threonine residues to render the activity of retinoblastoma (Rb) protein [12]. The promoter hypermethylation of the p16 gene was found to be ranging between 17 to 43% for oral cancer, for primary tumor it was ranging 23 to 67% and for normal oral mucosa cells it was found to be 0% [13]. In premalignant oral lesions, there is a frequent hypermethylation of p16 gene. These lesions become the root cause for the generation of oral squamous cell carcinoma. The p16 gene (CDKN2 and MTS1) encoded protein are negative regulator of the cell cycle.

METHODOLOGY

SAMPLE COLLECTION

50 biopsy samples were collected in normal saline solution, from OSCC patients from Dharamshila Cancer Hospital and Research Center, Vasundhara Enclave, New Delhi after obtaining the necessary ethical clearance, along with 15 samples of normal oral mucosa.

DNA ISOLATION

The biopsy samples were lysed through application of digestion buffer [27 µl of proteinase K, 120 µl of 10% SDS, at 45^0 C] and further purification was done using phenol-chloroform extraction method [14].

BISULPHITE MODIFICATION

This method allows precise analysis of methylation in a certain region by converting all non-methylated cytosine into uracil, while methylated cytosines remain unchanged. The isolated DNA was treated with sodium bisulphite using agarose bead method.

MS-PCR

The amplification of the bisulphite treated DNA was done through methylation specific – PCR, and specific primers were used to identify the methylation in the promoter region (Table1).

Following steps and temperature conditions were used in the process: the initial denaturation was carried out at 95° C for 10 min s. The PCR cycles consisting of denaturation at 95° C for 45 sec, annealing at 60° C for 1 min and elongation step was done at 72° C for 45 sec for 30 cycles. The final extension was carried out at 72° C for 10 min.

STATISTICAL ANALYSIS.

The hypermethylation of the promoter region of p16 gene of OSCC patients was statistically analyzed through statcalc of Epiinfo tool version 7.2 which was used for computing Odds ratio (OR) and 95% confidence interval (CI) through Chi square test, Fisher exact test and regression analysis. Using this, the status of hypermethylation was analyzed.

RESULTS

ISOLATION OF DNA

The DNA isolated from the biopsy samples was run on 1% agarose gel and bands were visualized for qualitative analysis (Fig. 1)

STATUS OF PROMOTER HYPERMETHYLATION

24 out 50 samples were found to be promoter hypermethylated through MSP (Methylation Specific-PCR) in OSCC patients among north Indian population (Fig.2). The statistical analysis of promoter

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hypermethylation of p16 gene was found to be significant (p-value= 0.00133) with odds ratio of 0.065 (Table 2).

Table 1: Sequence of the primers for p16 gene

S.No.	PRIMER	SEQUENCE (5'- 3')
1	p-16 Methylated F	3'-TTATTAGAGGGTGGGGCGGATCGC-5'(24)
2	p-16 Methylated R	3'-GACCCCGAACCGCGACCGTAA-5' (21)
3	p-16 Unmethylated F	3'-TTATTAGAGGGTGGGGTGGATTGT-'5 (24)
4	p-16 Unmethylated R	3'-CAACCCCAAACCACAACCATAA-5' (22)

Table 2: The methylation status of the p16 gene in biopsy and normal mucosa samples.

Methylation	Population n=50 (%)	Control n= 15 (%)	OR (95 % CI)	p- value
p16	24 (48)	1 (0.067)	0.065 (0.008- 0.54)	0.00133

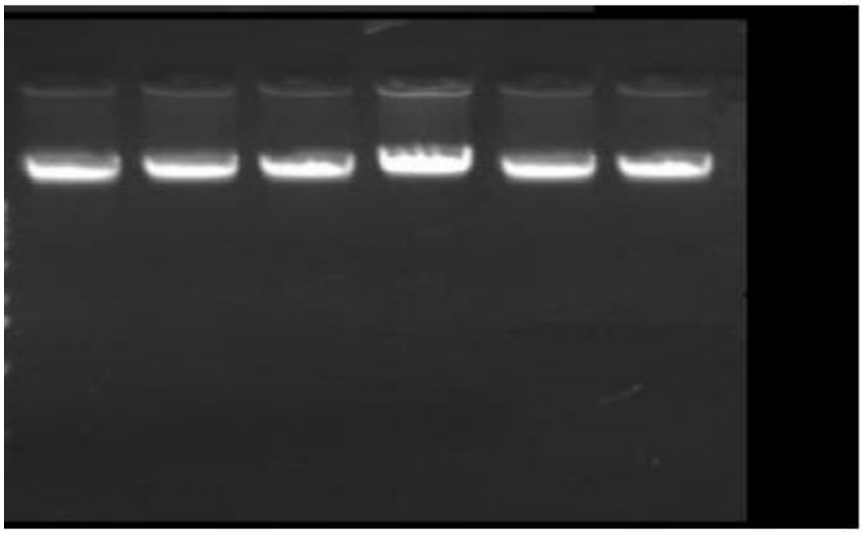

Figure 1: Qualitative Analysis of DNA

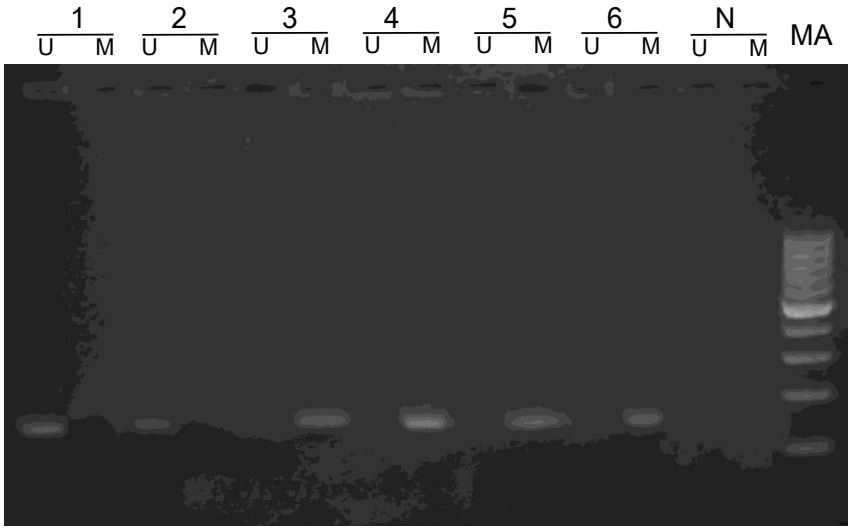

Figure 2: UMSB (Unmethylation Specific Band) and MSB (Methylation Specific Band) in biopsy samples of OSCC patient.

DISCUSSION

Epigenetics refers to the alteration in the pattern of expression of the gene without any change in the gene sequence. Epigenetics was coined by C.H Waddington in 1942 [15]. Promoter hypermethylation is part of epigenetic event. Modifications of the DNA or its histones packaging are responsible for epigenetic changes associated to the gene.

Epigenetic changes when deregulated leads to unnecessary silencing or regulation of the gene which cause cancer. In this changes there is no mutation or alteration in the sequence of the gene. This epigenetic silencing occurs due to biding of methyl group to 5' carbon of the cytosine through covalent bond. The methylation is done through DNMT (DNA methyl transferase).The excess methyl group is transferred to the promoter region leading to promoter hypermethylation along with global hypomethylation. In different regions people have different lifestyle and dietary habits thus these promoter hypermethylation of the gene varies greatly over the population of different areas [16].

Studies had been carried out among various populations on promoter hypermethylation of various genes associated with OSCC [17-19]. Till now, in north Indian population promoter hypermethylation has not been reported in case of p16 gene for OSCC, Since epigenetic changes vary with population, this study focused on studying the promoter hypermethylation of p16 gene in OSCC patients in North Indian population.

p16 gene is a cyclin – dependent kinase located on chromosome9p21. The p16 protein inactivates activity of cyclin-dependent kinases (4 or 6) and inactivate it and also renders the activity of retinoblastoma (Rb) protein [10]. This effect blocks the transcription of important cell-cycle regulatory proteins and results in cell-cycle arrest.

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

After Gel electrophoresis, the PCR product was observed. The bands formed for both methylated and unmethylated DNA was of 151 base pair (bp). The bands were observed in case of both methylation specific PCR as well as unmethylation specific PCR, in case of mixed population of cells.

The promoter hypermethylation of the p 16 gene was found to be 48% (24 out of 50 samples) along with p-value of 0.0133 and OR of 0.0659 (CI 95%). This statistical calculation was done using the Epiinfo 7.2.

Hence, it was observed that promoter hypermethylation of p16 gene is often found in the patients of OSCC among North Indian population. But this study needs to be carried out on a large scale as the sample size in present study was very small to draw any significant statistical conclusion.

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