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DETECTION OF P16^{INK4A} PROMOTER METHYLATION IN THE BLOOD SAMPLE COMPARED WITH CLINICO-PATHOLOGICAL FINDINGS OF CERVICAL CANCER PATIENTS: A NORTH INDIAN STUDY

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ABSTRACT

Epidemiologic and clinical data show that the development of cervical cancer is a multifactorial process in which infection with human papillomavirus (HPV) takes a central place along with other risk factors such as smoking, immunosuppression, immunodeficiency, diet, parity, age at first full-term pregnancy and family history. Detection of p16 Methylation in Blood Samples Compared with Clinicopathological findings analyzed in the north India population among cervical cancer patients. Methylation status of p16 promoter was analysed in cervical cancer and controls subjects by PCR using specific primer for p16 after methylation-insensitive restriction enzyme digestion of genomic DNA. Methylated status of p16 gene promoter was analysed in cervical cancer cases and controls using RE digestion followed by PCR. The methylation-insensitive restriction enzymes cut regions containing unmethylated CpG sequences at the promoter region. Methylation pattern analysis of cervical cancer out of 64 cases 51(79.7%) of them contained methylated CpG island at promoter region compare to controls containing only 13(40.6%) out of 32 subjects methylated CpG. It was observe that methylated p16 promoter subjects have higher risk of cervical cancer in comparison with unmethylated p16 promoter subjects. This result supports that p16 methylation in blood could be a novel marker for the malignancy of cervical cancer.

KEYWORDS: Cervical Cancer, Methylation, p16, north Indian population.

INTRODUCTION

Cervical cancer is the most common neoplastic disease affecting women, the highest incidence being in developing countries out of which 25% is from India. A mortality of 2,700,00 cases and 5-year prevalence of 1,547,161 was reported in 2013.[1] Epidemiologic and clinical data showed that the development of cervical cancer is a multifactorial process in which infection with human papillomavirus (HPV) takes a central place along with other risk factors such as smoking, immunosuppression, immunodeficiency, diet, parity, age at first full-term pregnancy and family history.[2, 3]

Several mechanisms including point mutations, homozygous deletions and methylation lead to alter the functions of many genes. DNA methylation, a common covalent modification by addition of methyl group to cytosine and immediately followed by guanine (so called CpG Island).[4] In normal tissues it was observed that large numbers of genes are unmethylated at the position of CpG islands while methylated to various types of human cancer in multiple degrees.[5]

A methylated CpG island has been shown in transcriptional repression of numerous genes that function to prevent tumour growth or development.[6] The cellular p16 encoded by human CDKN2A gene, a tumour suppressor protein, acts as a negative regulator of the cell cycle. It binds and inhibiting CDK-4 (cyclin-dependent kinase 4) and CDK-6 and also phosphorylates serine and threonine residues of RB (retinoblastoma) protein. It arrests the cell cycle by decelerating cell progression from G1 phase to S phase.[7] CpG methyalation in the exon1 of CDKN2A has been found to be associated with a wide variety of malignant tumours, such as NSCLC, colorectal, pancreatic cancer, oral squamous cell carcinoma and cervical cancer.[8-10]

Based on these findings, methylation of the p16 is considered to be an important and early event in the evolution of squamous cell neoplasias. The aim of study to investigate p16 CpG island hypermethylation and reflects the expression of p16 transcripts in north Indian population. There is lack of early sensitive and specific detection of cervical cancer at molecular level. Thus this study help us a better understanding of molecular mechanism that are responsible for cervical cancer development and progression will help improve the diagnosis and treatment of the disease.

MATERIALS AND METHODS

Patient selection and Sample Collection

Cervical cancer subjects (64) and normal control subjects (32) have been enrolled form north Indian origin from January 2013-September 2014 at outpatients and inpatients wards of Department of Obstetrics and Gynaecology of a tertiary care teaching hospital. Due approval of Institutional Ethics Committee and written consent form all subjects were obtained. 3 ml of blood specimens were collected in EDTA vials from the patients and stored at -20°C until further use. Subjects who were already in follow up and who refused to participate were excluded in this study.

DNA Isolation and Methylation Analysis

Genomic DNA (gDNA) was extracted from peripheral blood mononuclear cells (PBMCs) using salting out method.[11] Concentration of gDNA was determined by taking absorbance at 260nm in a double beam UV-vis spectrophotometer. Purity of gDNA was determined by calculating the ratio at 260/280. Only gDNA that had absorbance ratio between 1.7-1.9 was used for subsequent analysis. Methylated status of p16 gene promoter was analysed in cervical cancer cases and controls using restriction endonucleases (RE) digestion followed by Polymease chain reaction (PCR). The methylation-insensitive restriction enzymes cut regions containing unmethylated CpG sequences at the promoter region. In PCR, methylated sequences were amplified and unmethylated sequences were not amplified. The methylation status of p16 gene promoter was analysed in 64 cervical cancer patient and 32 controls subjects. One site was examined in the p16 promoter, recognized by restriction enzyme *MspI* whose activity is blocked by CpG methylation. 100 ng of genomic DNA was completely digested with 5U of *MspI* in 20µl of sample containing water and buffer for 16 hours. A total of 100 ng digested DNA was then used in PCR to amplify a 240 bp target fragment which contain recognition site of *MspI* (methylated-insensitive) enzyme. After digestion 10µl (50ng) of digested DNA was used as template DNA in to 15µl reaction mixture contained buffer (100 mMTris, pH 9.0; 500 mMKCl; 15 mM $MgCl_2$; 0.1 % gelatin), 200 µM dNTP, 10 pmol of each primer and 1.0 unit Taq DNA polymerase. The primers was designed by Primer 3.0 online software were F-5'- GGAGTCACACACTCCACCT-3' and R-5'-CTGATTGGAAACCTTATTAAG-3'. The DNA sample without *MspI* enzyme was amplified as a positive control. The methylation results were cross checked randomly and were found to be in 98 % concordance.

RESULTS

Patient details showed that number of women in the age group of >40 years have a higher incidence of cervical cancer as compared to those in the lower age group (≤40 year). Risk factors such as higher parity, lower age at first full term pregnancy, irregular menstrual cycle, menstrual hygiene showed higher incidence of cervical cancer. A significant correlation with smoking (p=0.005) as well as high risk human papillomavirus (HR-HPV) incidence was observed in cases (p=0.003) (Table 1).

Number of methylatedand unmethylated subject has been shown in table 2. Subjects with methylation have no digestion in genomic DNA and after PCR found 240bp band product. In contrast, unmethylated genomic DNA digested then there is no PCR product obtained. Methylation status of p16 promoter was analysed in cervical cancer and controls subjects by PCR using specific primer for p16 after methylation-insensitive restriction enzyme digestion of genomic DNA (Figure 1). Methylation pattern analysis of cervical cancer out of 64 cases 51(79.7%) of them contained methylated CpG island at promoter region compare to controls containing only 13(40.6%) out of 32 subjects methylated CpG. These results indicated that the p16 methylation of controls was lesser in respect to cervical cancer subjects.

Table 2 presents the relation between the methylation status of p16 and clinical and pathological features. The methylation frequencies were higher in risk factors of cervical cancer such as HR-HPV positive, active smoker, lower age at first full term pregnancy, menstrual cycle with irregular menstrual hygiene and higher age of illiterate women lived in rural areas. It was observed that methylated p16 promoter subjects have higher risk of cervical cancer in comparison with unmethylated p16 promoter subjects. This result supports that p16 methylation in blood could be a novel marker for the malignancy of cervical cancer.

Table 1: Correlation of p16 methylation with clinicopathological parameters.

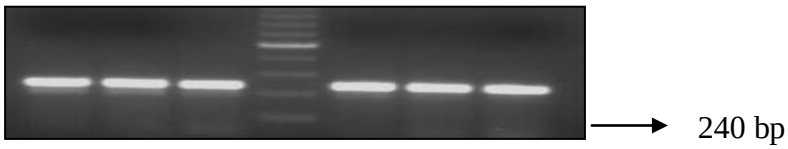
Demographic Profile	**Controls (n=32) (%)**	**Cases (n=64) (%)**	**P-value**	**Methylated cases (n=51) (79.7%)**
Age (in Years)				
≤40	7(22.0)	12(18.8)	0.717	6(50)
>40	25(78.0)	52(81.2)		45(86.5)
Place of residence				
Rural	18(56.2)	51(79.6)	0.018	41(80.3)
Urban	14(43.8)	13(20.4)		10(76.9)
Educational Status				
Illiterate	14(43.8)	50(78.0)	**0.001**	44(88.0)

Literate	18(56.2)	14(22.0)		7(50.0)
Socio-economic Status				
Lower	10(31.2)	42(65.6)	0.003	39(92.9)
Middle	15(46.8)	18(28.1)		12(66.6)
Upper	7(21.8)	04(6.2)		0.0(0.0)
Personal History				
(A) Parity				
None	02(6.2)	01(1.5)	0.067	0.0(0.0)
≤2	18(56.2)	24(37.5)		15(62.5)
>2	12(37.5)	39(60.9)		36(92.3)
(B) Age at first full term pregnancy				
≥20	24(75.0)	50(78.1)	0.731	39(78.0)
<20	8(25.0)	14(21.9)		12(85.7)
(C) Menstrual Cycle				
Irregular	02(6.25)	23(35.9)	**0.006**	17(73.9)
Regular	30(93.75)	41(64.1)		34(82.9)
(D) Menstrual hygiene				
Cloths	18(56.25)	39(60.98)	0.660	36(61.0)
Napkins	14(43.75)	25(39.01)		15(36.6)
Smoker				
Active Smoker	11(34.3)	12(18.75)	0.005	6(50.0)
Passive Smoker	21(65.7)	52(81.25)		45(86.5)
HR-HPV				
Positive	14(43.75)	48(75.0)	**0.003**	46(95.8)
Negative	18(56.25)	16(25.0)		5(31.2)

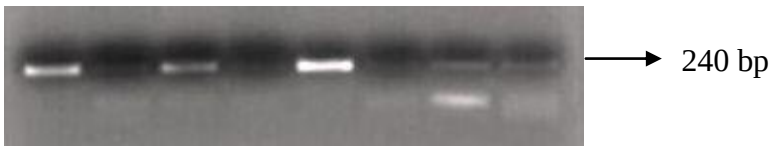
Table 2: Methylation frequencies of study subjects.

Genes	Controls (%) (n=32)	Cases (%) (n=64)
P16 (U)	19 (59.4)	13 (20.3)
P16 (M)	13 (40.6)	51 (79.7)

*U=Unmethylated; M=Methylated



(A)



(B)

Figure 1: Agarose gel (2.0%) showing methylated and unmethylated bands. (A): Lane 1-3, 5-7: Methylated; Lane 4: 100 bp ladder (B): Sample 1, 2, 3; Lane 1, 3, 5: positive control, Lane 2, 4, 6: unmethylated, Sample 4; Lane 7: positive control and Lane 8: methylated.

DISCUSSION

Cervical carcinoma is one of the major causes of death in women worldwide as well as in India. Cervical carcinoma is caused by multistep process with accumulation of genetic and epigenetic abnormalities. Human Papilloma Virus (HPV) infection plays important role in progression from precursor lesions to invasive cervical cancer along with some other factors such as smoking, age of full term pregnancy, parities etc. HPV infection with other cofactors induces changes in the host genome and epigenome. This genetic and epigenetic alteration in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes (TSGs). A genetic alteration, epigenetic inactivation of TSGs by promoter hypermethylation has been recognized as an important and alternative mechanism in tumorigenesis.[12, 13] DNA methylation is epigenetic alteration of DNA by addition of a methyl group to fifth base of cytosine. This epigenetic change does not alter the primary DNA sequence but it facilitates the organization of the genome into active and inactive regions with respect to gene transcription. Generally promoter regions of gene in normal tissues unmethylated CpG islands and transcriptionally active while genes with hypermethylated CpG, transcription of the affected genes may be blocked, resulting in gene silencing. In neoplasia, hypermethylation is now considered as one of the important mechanisms resulting in silencing expression of tumour suppressor genes, i.e. genes responsible for control of normal cell differentiation and/or inhibition of cell growth.[14] *p16*, tumor suppressor gene, is the most commonly altered gene in human malignancies. Hypermethylation is a major mechanism of inactivation of gene in various types of cancer, including cervical carcinoma. $p16^{INK4A}$ (p16) methylation was found to be significant in cervical cancer patients in the north Indian population and its methylation was found to increase the risk of cervical cancer ($P<0.001$). Methylation status of p16 in esophageal and colorectal found 82% and 47% methylation in Japanese population and 68% of abnormal promoter methylation in colorectal cancer patients.[15] In this study, we tested for the methylation status of p16 controls, only 13(40.6%) out of 32 subjects methylated CpG. This result indicated that p16 methylation play an important role in the tumorigenic pathway of these cancers. The p16 gene plays a vital role in controlling the cell cycle. Progressive methylation of the p16 gene has been reported in early lesions of squamous carcinomas of the lung and in 31% of cervical cancers. We found a 42% incidence of methylation for p16 in cervical cancers and 24% of high-grade dysplasias. Methylation was rare (3%) in nondysplasia/low-grade CIN specimens. Hypermethylation of p16 was localized only to the neoplastic cells in both in situ lesions and invasive cancers, and was associated with loss of

p16 protein expression. MSP-ISH allowed us to dissect the surprising finding that p16 hypermethylation occurs in cervical carcinoma.[16-19] In conclusion, our results indicate that it is not always true for all of the tumour suppressor gene or DNA repair genes to show a positive association between promoter hypermethylation and loss of expression in cancers. P16 methylation may be helpful in monitoring of multiple tumor types because inactivation of p16 by methylation is a common feature in human neoplasia in our study have potential clinical applications.

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Author Disclosure Statement

No competing financial interests exist.

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