RESEARCH ARTICLE

Promoter Methylation and Relative mRNA Expression of the p16 Gene in Cervical Cancer in North Indians

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Abstract

Background: Cervical carcinoma is one of the main causes of mortality in women worldwide as well as in India. It occurs as a result of various molecular events that develop from the combined influences of an individual's genetic predisposition and external agents such as smoking and menstrual hygiene, for example. However, infection with human papillomavirus (HPV) is the established major risk factor. The aim of the current study was to investigate p16 CpG island methylation and establish any correlation with mRNA expression in north Indian population. Materials and Methods: We analyzed 196 woman volunteer out of which 98 were cases and 98 healthy controls. For the analysis of methylation pattern, DNA extracted from blood samples was modified with a bisulfate kit and used as template for methylation specific PCR (MSP). Quantitative real-time PCR (QRT-PCR) was performed to check mRNA expression. Results: Correlation between methylation status of p16 gene and poor menstrual hygiene was significant (p=0.006), high parity cases showed methylation of p16 gene (p=0.031) with increased risk up to 1.86 times for cervical cancer and smoking was a strong risk factor associated with cervical cancer. We analyzed methylation pattern and found 60.3% methylation in cases with low mRNA expression level (0.014) as compare to controls (1.24). It was also observed that promoter methylation of p16 gene was significantly greater in FIGO stage III. Conclusions: We conclude that p16 methylation plays an important role in cervical cancer in the north Indian population and its methylation decreases mRNA expression. It can be used as an important and consistent blood biomarker in cervical cancer patients.

Keywords: Cervical cancer - p16 methylation - p16 mRNA expression - North Indian population

Asian Pac J Cancer Prev, **17 (8)**, 4149-4154

Introduction

Among all gynaecological cancers, cervical cancer is the most prominent global health malignancy (Parkin et al., 2006). Despite advances in diagnosis and treatment, prevalence of this disease is still rising universally. Cervix is the most common primary site of cancer among Indian women and second most common cancer in women worldwide with 5,300,00 new cases every year amounting to 2,700,00 deaths approximately in 2013 (Vaccarella et al., 2006). It is mainly notable in the low economy developing countries with more than one fifth of all new cases being diagnosed in India (GLOBOCAN, 2013).

It occurs as a multistep process with HPV infection as major risk factor and various molecular events such as genetic alterations as well as epigenetic modification that drive the progressive transformation of normal human cells into highly malignant derivatives (Bosch et al., 1995: Hanahan et al., 2000). The major epigenetic modifications in eukaryotes are DNA methylation and histone acetylation (Sharma S et al., 2010). DNA methylation is a common covalent modification by addition of methyl group to cytosine and immediately followed by guanine (so called CpG Island). In normal tissues it was observed that large numbers of genes are unmethylated at promoter CpG islands while methylated to various types of human cancer in multiple degrees (Esteller, 2002). A methylated CpG island has been shown in transcriptional repression of numerous genes including tumour suppressor genes (as like p16 gene) that function to prevent tumour growth or development. The cellular p16, encoded by human CDKN2A (cyclin-dependent kinase inhibitor) gene, a tumour suppressor protein and acts as a negative regulator of the cell cycle. Genome is not methylated homogeneously and includes regions of unmethylated segments interspersed with methylated region (Weinberg, 1995; Luo et al., 2006; Cao et al., 2009).

It has been reported that p16 gene with promoter hypermethylation was associated with a loss of expression level in many cancerous cells. Based on these findings, methylation of the p16 gene is considered to be an important and early event in the evolution of squamous cell

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neoplasia (Nuovo GJ et al., 1999). The aim of the current study is to investigate promoter p16 hypermethylation and correlates its mRNA expression in north Indian population thereby contributing in better understanding of molecular mechanism as well as in the diagnosis and treatment of disease.

Materials and Methods

Subject recruitment and sample collection: This is a case-control study, conducted in the department of Obstetrics and Gynecology, King George's Medical University (KGMU), Lucknow, U.P., India for the period of April 2014-December 2015. Clinically diagnosed 196 women who volunteered were enrolled in this study after written informed consent. Histopathologically confirmed patients (n=98) were included in case group while those who had normal tissue morphology were grouped in control (n=98). Clinical details and demographic information like smoking habits, parity, abortion and use of contraception of both group subjects were collected through general questionnaire, case history sheet, histopathology and clinical diagnosis reports. All recruited subjects for both groups were aged between 38-70 years. Cases having double malignancy and co-morbid conditions such as diabetes, tuberculosis, HIV and not willing to participate were excluded.

For histopathological investigations, tumour was graded according to degree of differentiation and keratinization of tumour cells; Grade I-well-differentiated, Grade II-moderately differentiated, Grade III-poorly differentiated and Grade IV-anaplastic tumour. Clinical diagnosis of cervical cancer patients was carried out according to the American Joint Committee and the International of Federation of Gynecology and Obstetrics (FIGO) system for cervical cancer staging (Pecorelli S et al., 2009; NCCN Guidelines, 2013). The study was ethically approved by Institutional Ethic Committee of KGMU, Lucknow [56 E.C.M.IIB/P8].

3 ml of blood sample of both groups collected in separate vials containing EDTA and trizol separately (Invitrogen, Carlsbad CA, USA) and stored at -80°C till further processing. Blood samples in EDTA vial were used for DNA isolation and methylation while trizol vials were used for RNA isolation and gene expression.

DNA extraction: Genomic DNA (gDNA) was extracted from peripheral blood mononuclear cells (PBMCs) using salting out method (Miller SA et al., 1988). Concentration of gDNA was determined by taking absorbance at 260nm in a double beam UV-vis spectrophotometer (Shimadzu, Japan). 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. The DNA samples were stored at -20°C until further use.

Table 1. Effect of Socio Demographic Characteristics on Cervical Cancer

Demographic profile	Controls N(%) (n=98)	Cases N(%) (n=98)	OR;95%CI	p-value
Age in years				
≤40	32(32.6)	29(29.6)	1.154;0.630-2.113	0.644
>40	66(67.4)	69(70.4)		
Place of Residence				
Urban	35(35.7)	19(19.4)	0.433;0.226-0.829	0.011*
Rural	63(64.3)	79(80.6)		
Educational Status				
Literate	21(25.3)	59(56.5)	5.547;2.9-10.411	0.001*
Illiterate	77(74.7)	39(43.5)		
Socio-economic Status				
Upper	9(9.2)	6(6.2)	0.352;0.206-0.601	0.001*
Middle	51(52.0)	34(34.7)		
Lower	38(38.8)	58(59.1)		
Parity				
None	05(5.1)	11(11.2)	0.112;0.043-0.291	0.001*
≤2	56(57.2)	29(29.6)		
>2	37(37.7)	58(59.2)		
Age at first full term pregnancy				
≥20	67(68.4)	59(60.2)	1.429;0.794-2.570	0.234
<20	31(31.6)	39(39.8)		
Menstrual Cycle				
Regular	63(64.3)	65(66.3)	1.094;0.608-1.971	0.764
Irregular	35(35.7)	33(33.7)		
Menstrual Hygiene				
Napkins	41(41.8)	45(46.0)	0.365;0.204-0.652	0.001*
Cloths	57(58.2)	53(54.0)		
Use of Contraception				
None	50(51.0)	61(62.3)	0.738;0.403-1.352	0.326
OCP	48(48.9)	37(37.7)		
Smoker				
Passive	75(76.6)	56(57.2)	0.409;0.221-0.756	0.004*
Active	23(23.4)	42(42.8)		

* Implies significant at 5% level, Bold denote significant p-values

Bisulfite Modification: DNA methylation patterns in the CpG islands of the p16 gene were determined by chemical modification of unmethylated regions. These cytosines to uracil conversion were taken through bisulfite conversion method. Bisulfite conversion of gDNA (300-800ng) was performed using commercially available kit as per manufacturer's protocol (EpiTech Bisulfite Kit, QIAGEN, UK). The bisulfite converted DNA was used as template for methylation-specific PCR (MSP).

Methylation specific PCR (MSP): Methylated status of p16 gene promoter was analyzed in both cases and controls by using MSP. The modified DNA samples were amplified by specific primers for both methylated (M) and unmethylated (UM) sequences: unmethylated reaction was p16 UM (Forward) 5'-TTATTAGAGGGTGGGGTGGAT-3'; (Reverse) 5'-CAACCCCAAACCACAACCATAA-3'. Methylated reaction were p16 M (Forward) 5'-TTATTAGAGGGTGGGGCGGATCGC-3'; p16 M (Reverse) 5'-GACCCCGAACCGCGACCGTAA-3' (Herman JG et al., 1996). PCR was performed in a 25μl reaction mixture containing 100 ng of template DNA, buffer (100 mMTris, pH 9.0; 500 mMKCl; 15 mM MgCl2; 0.1% gelatin), 200μM dNTP, 10pmol of each primer and 1.5 units Taq DNA polymerase. The PCR amplification of the modified DNA samples consisted of 1 cycle of 95^{o}C for 5 min; 35 cycles of 95^{o}C for 30s, 61^{o}C for 30s and 72^{o}C for 30s and 1 cycle of 72^{o}C for 5 min. The resulting PCR products for p16 were checked on 3% agarose gels (Figure 1).

RNA Extraction and cDNA synthesis: RNA was extracted from frozen blood samples with Trizol method (Chomczynski P et al., 1987) and purification done by phase separation method, treating with chloroform and isopropanol. RNA was quantified on a NanoDrop (Thermo Fisher, USA) at 260/280nm wavelength. 250 ng of the

Table 2. Relation of p16 Promoter Methylation with Demographic and Clinicopathological Risk Factors in Cervical Cancer Cases

Demographic profile	Cases (%) (N=98)	Methylated cases (N=59) (60.3%)	Unmethylation cases (N=39) (39.7%)	OR;95%CI	p-value
Age in years					
≤40	29(29.6)	9(15.2)	20(51.2)	2.33;1.01-5.36	0.046*
>40	69(70.4)	50(84.8)	19(48.8)		
Place of Residence					
Urban	19(19.3)	08(13.6)	11(28.2)	0.65;0.26-1.60	0.351
Rural	79(80.7)	51(86.4)	28(71.8)		
Educational Status					
Literate	43(55.1)	18(30.5)	25(64.1)	0.56;0.28-1.12	0.098
Illiterate	55(39.8)	41(69.5)	14(35.9)		
Socio-economic Status					
Upper	6(06.1)	2(3.3)	4(10.2)	0.83;0.47-1.45	0.521
Middle	34(34.8)	20(33.9)	14(35.9)		
Lower	58(59.1)	37(62.8)	21(53.9)		
Parity					
None	11(11.2)	2(03.3)	9(23.1)	1.863;1.05-3.28	0.031*
≤2	29(29.6)	13(22.0)	16(41.0)		
>2	58(59.2)	44(74.7)	14(35.9)		
Age at first full term pregnancy					
≥20	39(39.8)	11(18.7)	28(71.8)	0.347;0.16-0.74	0.007*
<20	59(60.2)	48(81.3)	11(28.2)		
Menstrual Cycle					
Regular	65(66.3)	39(81.3)	17(43.6)	0.990;0.50-1.95	0.977
Irregular	33(33.7)	20(18.7)	22(56.4)		
Menstrual Hygiene					
Napkins	45(45.9)	14(23.8)	31(79.4)	0.366;0.17-0.75	0.006*
Cloths	53(54.1)	45(76.2)	8(20.6)		
Use of Contraception					
None	61(62.2)	38(64.4)	23(58.9)	1.098;0.56-2.14	0.786
OCP	37(37.8)	21(35.6)	16(41.1)		
Smoker					
Passive smoker		24(40.7)	32(82.1)	0.514;0.26-0.99	0.047*
Active smoker		35(59.3)	7(17.9)		
FIGO Staging					
I		6(10.4)	12(30.7)	0.580;0.35-0.95	0.032*
II		17(28.9)	10(25.6)		
III		33(55.9)	15(38.5)		
IV		3(5.8)	2(2.2)		
Histological grading					
GI		5(8.5)	7(17.9)	0.787;0.46-1.34	0.337
GII		41(69.5)	11(28.2)		
GIII		13 (22.0)	21 (53.9)		

* Implies significant at 5% level, Bold denote significant p-values

total RNA was subjected to reverse transcription PCR using random hexamer primers with high capacity cDNA reverse transcription kit (Applied Biosystems, CA) per manufacturer's instructions.

Quantitative Real-Time PCR (QRT-PCR): For QRT-PCR with SYBR Green 1 master mix (Thermo Fisher, USA) was performed in a Light Cycler 480 real-time PCR machine (Roche, Germany) using specific primer for p16 and β-Actin (10 μM of forward and reverse primer) incorporated as internal control. Relative gene expression was determined by the 2–ΔΔCt method (Livak KJ et al., 2001). A negative control without template was run in parallel to assess the overall specificity of their action. All reactions were run in triplicates. Primer for QRT-PCR of p16 and β-actin as follows (Marjani HA et al., 2010):

Genes	Primers	Tm (°C)
P16 Forward	5'-GAAGAAAGAGGAGGGGCTG-3'	58
P16 Reverse	5'-GCGCTACCTGATTCCAATTC-3'	
β-actin Forward	5'-GTGGGGCGCCCCAGGCACCA-3'	58
β-actin Reverse	5'-CTCCTTAATGTCACGCACGATTTC-3'	

Data Analysis: Methylation frequencies between patients and controls were analyzed using Fisher's exact probability test and χ^2 test. All statistical analyses were performed with SPSS 21.0 version statistical software (IBM, India). Difference between variables was considered significant at $p<0.05$.

Results

Association of cervical cancer cases with demographic risk factors: Number of cases was higher (70.4%) in age group >40 years. Lower socio-economic condition showed significant association (p=0.001) with cervical cancer cases. Place of residence, illiteracy rate and higher parity showed significant association (<0.001) with cervical cancer cases. Menstrual hygiene (p=0.001) and smoking (p=0.004) also were significantly associated with cervical cancer cases (Table 1).

P16 promoter methylation status by MSP and protein expression: In methylation pattern analysis, out of 98 cases 59 (60.3%) of them contained methylated CpG island at promoter region compared to controls containing only 20 (20.5%). Fold change expression was higher in methylated controls (1.240 *vs* 0.014). Unmethylated cases were found in 39 (39.7%) with fold change expression 0.020 as well as 78 (79.5%) in controls with fold change 1.460 (Figure 2a and 2b). It is clear that overall expression of p16 decreased in cervical cancer cases.

Relation of p16 methylation with demographic and clinicopathological risk factors: We calculated the association of promoter methylation of p16 gene with demographic and clinicopathological characteristics in cervical cancer cases (Table 2). We found that higher age grouped women in cases group (>40) had much methylation (84.8%) as compared to lower age group (15.2%; p=0.046) with increased risk upto 2.33 times. The frequency of methylation in illiterate, rural and lower socio-economic population cases was higher, but was insignificant Higher parity (>2; p=0.03) had increased risk upto 1.86 times in our population. Our

Figure 1. Cases and Controls Methylation and Unmethylation Status of p16 Gene in Agarose Gel (3%). Sample 1, 2 and 3 showed methylation in cases while 4 and 5 showed unmethylation in controls and 6 showed methyation in controls

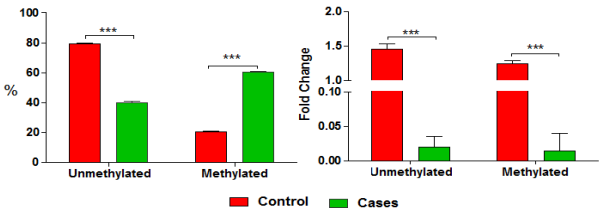

Figure 2. Methylation and p16 Gene Expression. (a) Frequencies of promoter p16 in both cases and controls. (b) Fold change expression of p16 gene in methylated and unmethylated cases and controls

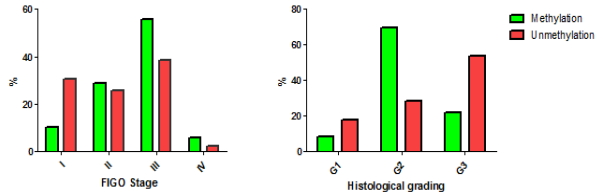

Figure 3. Methylation and Stage/Grade. (a) FIGO staging in methylation and unmethylation cases. (b) Histological grading in methylation and unmethylation cases

result showed that lower age at first full term pregnancy (<20) was significantly associated (p=0.007) with highly methylated (81.3%) cases. Women who used cloths in menstrual hygiene had highly methylated region (76.2%) significant showing association (p=0.006) with cervical cancer. Methylation was higher and significant in active smoker (59.3%; p=0.047) among our population. In clinical diagnosis of cervical cancer, out of 98 cases, 48 (48.9%) were in stage III and 5 (5.3%) in stage IV. 33 (55.9%) were methylated among stage III and 3 (5.8%) in stage IV cases. 27 (27.5%) cases were found to be stage II FIGO with 17 (28.9%) methylated and showed significantly difference (p=0.032) (Table 2; Figure 3a). Histopathological grading analysis showed 12.2% well differentiated, 53.1% moderately differentiated and 34.7% poorly differentiated carcinoma out of which 8.5%, 69.5% and 22.0% were methylated respectively (Figure 3b).

Discussion

In the present study, we correlate risk factors with the methylation of p16 gene and found higher age of women, lower age at full term pregnancy with high parity and smoking being significantly correlated with cervical cancer. This study show that women with >40 years age

have higher incidence of cervical cancer as compared to the lower age group (<40 years). This might be due to the fact that during adolescence (15–19 years) cervical epithelium is more susceptible to HPV; therefore, early age (<40 years) of sexual intercourse and subsequent pregnancy become risk factors for cervical cancer (Mitra S, 2009). It has been reported that the disease progression takes 10–15 years to manifest, therefore cases with HPV infection when subjected to other risk factors can causes disease beyond 40 years (Au et al., 2007). Similarly, our study also presented a significant association (p=0.046) between higher age group of cases and p16 gene methylation with increase risk upto 2.33 times. Akintola et al., 2008 reported that DNA methylation is an important mechanism for regulation of gene expression and affected by aging. This indicates that methylation of p16 gene rises with higher age group (>40 years) but its expression decreases increasing the risk of cervical cancer.

Females getting pregnant at <20 years of age having high parity (>2) are more susceptible to cervical cancer (p< 0.001). Age at full term pregnancy with high parity can put women at higher risk for cervical cancer (Appleby P et al., 2007). Our results were consistent with previous studies (Weber et al., 2007; Gupta et al., 2016).

High parity cases showed methylation of p16 gene (p=0.031) with increased risk upto 1.86 times for cervical cancer. Our findings were similar to what was reported from other parts of world (Hildesheim A et al., 2001; Kruger-Kjaer S et al., 1998). There was increased risk of cervical cancer with high parity and HPV positive women who reported full term pregnancies at lower age (Dillner et al., 1997).

Most of the women (58.2%) in our study used homemade sanitary cotton napkins which were reused after washing indicating poor menstrual hygiene. This was independent risk factor for cervical cancer showing significant association (p=0.001). Our results were corroborated by other studies where reuse of home-made sanitary napkins was a risk factor for cervical cancer (Raychaudhuri et al., 2012; Bayo et al., 2002). Correlation between methylation status of p16 gene and poor menstrual hygiene was significant (p=0.006), it has already been shown that poor menstrual hygiene increases the cervical cancer with high risk HPV infection (Franceschi et al., 2003). In our study, majority of the women belonged to rural areas and low socio-economic status, which prompts them to use homemade napkins thereby, putting them at higher risk of cervical cancer.

Smoking was also found to be a strong risk factor associated with increased cervical cancer cases and the effect is dose-dependent (Brinton et al., 1986). Its effect was enhanced by interaction with HPV infection. Cigarette smoke contains carcinogens and their exposure induces gene mutation, chromosome aberration as well as promoter methylation initially in targeted tissues. Thus, these types of tissues are hallmark of cancer (Kjellberg et al., 2000). According to Global Adults Tobacco Survey (GATS), India (2009-10) passive smoking is more prevalent than active smoking in women of the reproductive age group. Passive smoking has been identified as risk factor for cervical cancer (Louie et al., 2011). Our study showed results on similar lines (Table 2). However, methylation of p16 gene was more in cases of active smoking (59.3%; p=0.004). Liu et al., 2015 have concluded that smoking increases incidence of promoter methylation of p16 gene in lung cancer. Thus, we can assume that high frequency of methylation in active smoking cases may also contribute in causation of cervical cancer.

According to FIGO staging of cases, higher percentage of cervical cancer as well as methylation status were found in stage III as compared to other stages and showed significant association (0.032). A previous study reported results along similar line (Virmani AK et al., 2001). In our study out of total cases, 52 (53.1%) were moderately differentiated in which 41(78.8%) were methylated.

Some other studies reported methylation of p16 gene being 82% in esophageal, 47% in colorectal and 30% in gastrointestinal cancer supporting that p16 methylation may have played a vital role in the tumourigenic pathway of these cancers (Hibi K et al., 2001). We too found high frequency of promoter methylation of p16 gene (60.3%) in cases than controls (39.7%). We also found that increased methylation was consistent with low mRNA expression of p16 gene in cases. This observation founds support in findings by Shim et al., 2003 who reported methyl¬ation of p16 gene and lack of p16 expression in hepatoblastoma. DNA methylation is an early event during the process of carcinogenesis, its progression and development and can be used as potential alternative molecular biomarkers for timely detection of cancer.

In conclusion, In this study, we conclude that p16 methylation plays an important role in cervical cancer patients in the north Indian population and its methylation was found to enhance the risk of cervical cancer. To the best of our knowledge, this is the first study to investigate methylation status of p16 in the normal blood sample of cervical cancer cases. It was also observed that promoter methylation of p16 gene was significantly more in FIGO stage III. mRNA expression of p16 gene was low in methylated cases than unmethylated controls. Future studies may focus on the promoter methylation of p16 gene in blood sample of pre-cancerous patients which can help in early diagnosis and further management of cervical cancer patients.

Acknowledgements

The authors wish to thank all those who have cooperated in the study. The authors also would like to thank Uttar Pradesh Council of Science & technology, Lucknow (UP-CST), India and for Junior Research Fellowships. Authors also acknowledge the institutional funding from King George's Medical University, Lucknow, INDIA.

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