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$p16^{INK4a}$ and $p15^{INK4b}$ gene promoter methylation in cervical cancer patients

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Abstract. The aim of this study was to determine the methylation status of the $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$ genes and the subsequent effect of hypermethylation on the expression of these genes in cervical cancer patients. Methylation-specific PCR (MSP) was performed to analyse the methylation status of $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$ genes and was confirmed by sequencing. Reverse transcription PCR (RT-PCR) was carried out to determine changes in the expression of the genes due to hypermethylation. Hypermethylation of the $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$ gene promoters was observed in 36, 8.8 and 11.2%, respectively, of 125 cervical cancer samples from a north Indian population. Methylation of $p16^{INK4a}$ was significantly (P<0.001) associated with the cervical cancer cases. Significant association of $p16^{INK4a}$ hypermethylation with passive smoking and oral contraceptive use was also observed. Methylation of $p15^{INK4b}$ was also found to be significant (P<0.05). Our findings did not reveal any correlation between the promoter methylation of $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$ with factors, including age and human papillomavirus infection. mRNA expression was significantly reduced in patients with a methylated promoter (P<0.001) of $p16^{INK4a}$ compared to patients with an unmethylated promoter. In conclusion, this is the first study demonstrating significant hypermethylation of $p15^{INK4b}$ and $p16^{INK4a}$ genes among cervical cancer patients in a north Indian population, and a significant association of $p16^{INK4a}$ hypermethylation with passive smoking and oral contraceptive use.

Introduction

Cervical cancer is the third most common type of cancer in females, and the seventh most common type overall, with an estimated 529,000 new cases in 2008 (1,2). Epigenetic changes are as equally responsible as genetic changes in the development and progression of various types of cancer, including cervical cancer. These epigenetic changes include DNA methylation and histone deacetylation.

Aberrant promoter hypermethylation of tumor suppressor genes has been shown to be involved in human neoplasia (3). The genomic organization at 9p21 houses two members of the INK4 family of cyclin-dependent kinase inhibitors (CDKIs), *p15* and *p16*, and an unrelated gene $p14^{ARF}$. Notably, $p14^{ARF}$ utilizes two of the same exons as *p16*, but is translated in an alternative reading frame. *p16* is the most commonly altered gene in human malignancies (4).

p15 is located 25 kb from the *p16* gene and its protein shares significant areas of homologous amino acid sequences with *p16* (5). *p16* is a major target in carcinogenesis, rivaled in frequency only by the *p53* tumor suppressor gene (6). The major mechanism of *p15* gene inactivation in acute myelogenous leukemia (AML) is methylation of the 5' promoter region of the gene, which leads to transcription silencing (7). The *p15* gene is also aberrantly methylated in several human neoplasms, particularly among hematopoietic malignancies (8).

Hypermethylation of the *p16* promoter region has been detected in various types of human cancer (9,10). In their study, Feng *et al* reported similar promoter methylation patterns in genes from exfoliated cell samples and corresponding biopsy specimens. Furthermore, the frequency of hypermethylation increased statistically significantly with the increasing severity of neoplasia present in the cervical biopsy (11). *p16* hypermethylation is an early event in cervical carcinogenesis, but no study has examined its association with risk factors, including passive smoking, oral contraceptive (OC) use and age at first sexual intercourse (AFSI). Additionally, no study has examined the significant hypermethylation of *p14* and *p15* genes in cervical cancer.

Materials and methods

Sample collection. A total of 125 biopsy samples were collected with informed consent from patients diagnosed with cervical cancer. Ethics approval was provided by the Mohan Dai Oswal Cancer Treatment and Research Foundation, Ludhiana, India. One hundred blood samples from the healthy females (control) were collected in tubes with anticoagulant (heparin). Twenty-five biopsy samples, obtained from females in which hysterectomy had been carried out, but the cervix was normal, were used as controls.

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DNA extraction. Cells obtained from tissue biopsies and 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.

Human papillomavirus (HPV) infection and HPV-16 typing. The HPV consensus primers, MY09 and MY11, were used in the PCR assay for HPV infection (12). A total of100 ng of HPV-16 viral genome cloned into pBR322 was used as the positive control. The HPV-16 pBR322 plasmid DNA was a gift from E.M. DeVilliers of Deutsches Krebsforschungszentrum, Heidelberg, Germany. For typing of HPV-16, the primer sets and the methodology used was performed as previously described (13).

Methylation-specific PCR (MS-PCR). DNA isolated from the biopsy and blood samples was modified with sodium bisulphite and MS-PCR was carried out using specific primers for methylation and unmethylation for the $p16^{INK4a}$, $p14^{ARF}$ and $p15^{INK4b}$ genes (14-16). The amplified products were run on a 2% agarose gel.

Bisulfite sequencing. For sequencing, the MS-PCR product was purified using a gel purification kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. The sequencing was carried out by a 3100 ABI sequencer and the DNA sequence was then collected using a chromatogram.

Reverse transcription PCR (RT-PCR). RNA was isolated from the biopsy and blood samples using TRIzol reagent. An equal amount of RNA was used to synthesize cDNA using the RevertAid first-strand cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). RT-PCR was carried out to determine the alteration in the level of mRNA expression due to promoter hypermethylation using specific primers (15-17). β-actin was used as the internal control.

Statistical analysis. The association between hypermethylation of the genes and risk of cervical cancer 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 cervical cancer with the methylation of studied genes. The amount of mRNA expression was obtained by grading a ratio between the densitometry results of the gene and β-actin. Statistical analysis was performed using SPSS version 11.5 and Epi Info version 3.2. The γ-coefficient was calculated to correlate the hypermethylation of tumor suppressor genes with an increase in the stage of cervical cancer. P<0.05 was considered to indicate a statistically significant difference.

Table I. HPV infection and HPV-16 typing in cervical cancer patients and controls.

	Patients (%)	Control (%)
HPV-positive		
(+) ve	116 (92.8)	5 (5)
(-) ve	9 (7.2)	95 (95)
HPV-16 typing		
(+) ve	81 (69.8)	3 (60)
(-) ve	35 (30.1)	2 (40)

HPV, human papillomavirus.

Results

Epidemiological characteristics. The cases and the controls were well-matched with respect to age, gender and residence. The mean age ± SD of the cases and controls was 48.33±10.23 and 46.71±11.85 years, respectively. A total of 50.4% of patients were in the ≤45 years age-group, while 49.6% of patients were in the >45 years age-group (data not shown).

HPV infection and HPV-16 typing. It was observed that 116 out of 125 (92.8%) cervical cancer patients were HPV-positive. Typing was carried out for HPV-16, which demonstrated that out of the HPV-infected patients, 69.8% were HPV-16 (Table I).

Status of the promoter hypermethylation of $p14^{ARF}$ and INK family. No significant difference in the methylation of $p14^{ARF}$ (P>0.05) between patients and controls was observed, but a statistically significant difference in the methylation of $p15^{INK4b}$ (P<0.05) between patients and controls was observed. Methylation of $p15^{INK4b}$ was found to marginally increase the risk of cervical cancer (OR=1.54; 95% CI=1.20-1.99). Hypermethylation of $p16^{INK4a}$ was observed in 36% of cervical cancer patients (Fig. 1). A statistically significant difference in the methylation of $p16^{INK4a}$ (P<0.001) between patients and controls was observed. The hypermethylation of $p16^{INK4a}$ in cervical cancer patients was associated with an approximately 2.19-fold increase in the risk of cervical cancer (OR=2.19; 95% CI=1.85-2.59) (Table II). No statistically significant correlation was observed between $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ methylation and HPV infection in cervical cancer patients (P>0.05) (data not shown).

Table II. Frequency of methylation of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ with relative risk in cervical cancer patients and controls.

Methylation	Patient n=125 (%)	Control n=100 (%)	OR (95% CI)	P-value
$p14^{ARF}$	11 (8.8)	2 (2)	1.57 (1.21-2.05)	0.059
$p15^{INK4b}$	14 (11.2)	3 (3)	1.54 (1.20-1.99)	0.03
$p16^{INK4a}$	45 (36)	1 (1)	2.19 (1.85-2.59)	0.000

Table III. Frequency of methylation of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ compared to passive smoking in cancer patients and controls.

Methylation	Patient n=26 (%)	Control n=12 (%)	OR (95% CI)	P-value
$p14^{ARF}$	7 (26.9)	2 (16.6)	1.19 (0.77-1.84)	0.68
$p15^{INK4b}$	8 (30.7)	1 (8.3)	1.43 (0.99-2.07)	0.22
$p16^{INK4a}$	16 (61.5)	0 (0)	2.20 (1.39-3.48)	0.001

Table IV. Frequency of methylation of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ compared to use of oral contraceptives in cancer patients and controls.

Methylation	Patient n=62 (%)	Control n=43 (%)	OR (95% CI)	P-value
$p14^{ARF}$	4 (6.4)	1 (2.3)	1.38 (0.86-2.20)	0.64
$p15^{INK4b}$	9 (14.5)	3 (6.9)	1.32 (0.91-1.91)	0.35
$p16^{INK4a}$	28 (45.1)	1 (2.3)	2.16 (1.67-2.80)	0.000

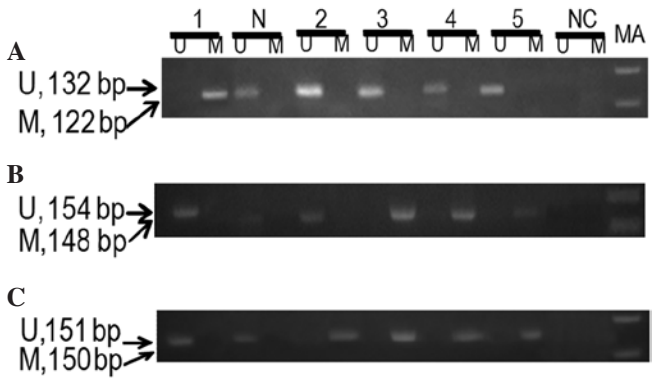

Figure 1. A representative agarose gel demonstrating the MS-PCR products of (A) $p14^{ARF}$, (B) $p15^{INK4b}$ and (C) $p16^{INK4a}$. N, sample from normal female (control); Lanes 1-5, samples from cervical cancer patients; MA, marker 100 bp; M, methylated primer; U, unmethylated primer; NC, negative control; MS-PCR, methylation-specific PCR.

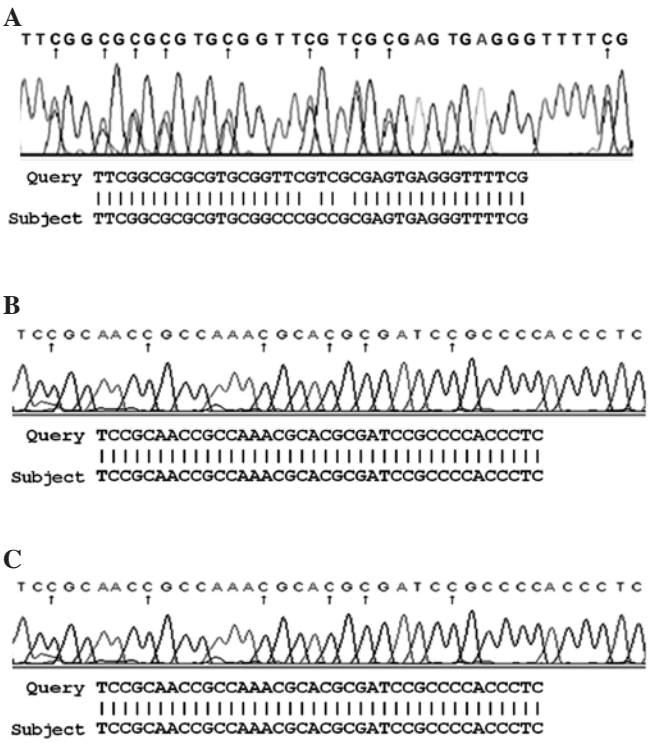

Figure 2. Bisulfite sequence of (A) $p14^{ARF}$, (B) $p15^{INK4b}$ and (C) $p16^{INK4a}$ genes.

Methylation of $p16^{INK4a}$ was found to be statistically significant in patients of the ≤45 and >45 years age groups (P<0.05). The risk of cervical cancer was increased 2.15-fold in the ≤45-year age group (OR=2.15; 95% CI=1.69-2.74), and 2.22-fold in the >45-year age group (OR=2.22; 95% CI=1.75-2.81). $p16^{INK4a}$ hypermethylation was found to be statistically significant in the passive smokers (P<0.05). The risk of cervical cancer increased 2.20-fold (OR=2.20; 95% CI=1.39-3.48) and 2.16-fold (OR=2.16; 95% CI=1.67-2.80) in association with $p16^{INK4a}$ methylation in the passive smokers and OC users, respectively (Tables III and IV).

A significant association between the methylation of $p16^{INK4a}$ and risk of cervical cancer was observed in patients with AFSI ≤20 and >20 years (P<0.001). In addition, the hypermethylation of $p16^{INK4a}$ was associated with a 2.25-fold increased risk of cervical cancer in patients with AFSI ≤20 years and 2.11-fold in patients with AFSI >20 years.

Bisulfite sequencing. The bisulfite sequencing of samples hypermethylated in the promoter region of the three studied genes revealed a conversion of unmethylated cytosine, but not methylated cytosine (Fig. 2).

Correlation of methylation status with stage of cervical cancer. There was no increase in the percentage of methylation of $p14^{ARF}$ and $p15^{INK4b}$ with stage (data not shown). By contrast, an increase in the percentage of methylation of $p16^{INK4a}$ was observed with the increase in stage of cervical cancer. The methylation of $p16^{INK4a}$ was found to be higher in the advanced stages. However, to calculate the significance of methylation with the stage of disease more samples should be studied. The γ-coefficient was also not found to be significant for the three genes; *p16* (γ=0.200, P=0.115), *p14* (γ=0.314, P=0.166) and *p15* (0.348, P=0.079).

Semi-quantitative RT-PCR. The mRNA expression of $p14^{ARF}$ and $p15^{INK4b}$ was significantly reduced in patients with a

Table V. mRNA expression in methylated and unmethylated $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ in cervical cancer patients.

Gene	Mean (±SE)		Downregulated expression of genes (%)	P-value
	Unmethylated	Methylated		
$p14^{ARF}$	0.858 (±0.02)	0.594 (±0.03)	31.0	0.00
$p15^{INK4b}$	0.795 (±0.02)	0.634 (±0.01)	20.2	0.03
$p16^{INK4a}$	0.940 (±0.03)	0.118 (±0.03)	85.3	0.000

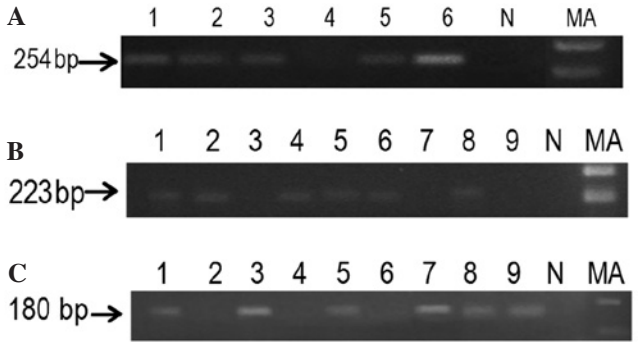

Figure 3. A representative agarose gel demonstrating the RT-PCR products of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$. (A) Lanes 1-6 and (B and C) Lanes 1-9, RT-PCR products of methylated and unmethylated samples. MA, marker 100 bp. NC, negative control; RT-PCR, reverse-transcription PCR.

hypermethylated promoter compared to the patients with an unmethylated promoter (Fig. 3). The mRNA expression was significantly reduced in patients with a methylated promoter (P<0.001) of $p16^{INK4a}$ compared to patients with an unmethylated promoter. Downregulation at the transcriptional level was approximately 85.3% due to the hypermethylation of $p16^{INK4a}$ (Table V).

Discussion

Cervical cancer is one of the most common types of cancer that affects female reproductive organs. It is the seventh most common type of cancer overall and the third most common among females (1). In developing countries, cervical cancer is often the most common cancer in females and constitutes up to 25% of all female cancers (2). The risk factors of cervical cancer include HPV infection and cofactors including age, smoking, oral contraceptives, low age at first sexual intercourse, deficient diet and a family history of cervical cancer.

HPV infection is the cause of almost all cases of cervical cancer (18). HPV 16 is the most commonly occurring subtype in cervical neoplasia, however, HPV 18 is associated with more advanced cervical neoplasia.

The incidence of cervical cancer has been reported to increase with age. The results of several case-control and cross-sectional studies indicate that females married to smokers experience a higher risk of cervical neoplasia than females married to non-smokers (19).

Several studies have reported that prolonged use of oral contraceptives increases the risk of cervical cancer. Early AFSI has been associated with an increased risk of high-risk HPV infection, a sexually transmitted infection, that in susceptible females is responsible for virtually all cases of invasive cervical cancer (ICC) (20).

The majority of recent studies have focused on the study of epigenetic changes resulting in many types of neoplasia (21). DNA methylation was the first epigenetic alteration to be observed in cancer cells (22). Recent studies have found that certain genes are hypermethylated in preinvasive lesions, raising the possibility that testing for methylation of these genes may prove to be a useful screening tool (23), particularly in cervical cancer as it evolves through a series of well-defined stages.

Methylation status of the $p14^{ARF}$ gene promoter has been found to be a useful biomarker for pathological and clinical outcome and prognosis of patients with colon, oral squamous cell carcinoma (24) and non-small cell lung cancer. However, of a panel of 16 genes selected for a study in cervical cancer, $p14^{ARF}$ did not demonstrate promoter methylation (23).

Hypermethylation of $p14^{ARF}$ was present in 8.8% of patients, and in the present population it was not found to be significant. Additionally, the risk factors did not have any significant impact on the methylation pattern of $p14^{ARF}$.

In this study, due to a low frequency of $p14^{ARF}$ and no significant correlation with risk factors, $p14^{ARF}$ does not appear to be a significant biomarker for the diagnosis of cervical cancer.

p15 promoter methylation was approximately 11.1% (5/45) in non-small cell lung cancer (25). However, hypermethylation of $p15^{INK4b}$ has not yet been reported in cervical cancer.

In this study, $p15^{INK4b}$ hypermethylation was observed in 11.2% of patients. $p15^{INK4b}$ methylation was found to be significant in cervical cancer patients in the north Indian population, and its methylation was found to marginally increase the risk of cervical cancer (P<0.05). This is the first study demonstrating significant hypermethylation of this gene in cervical cancer.

A significant trend towards an increase in the risk of cervical cancer was not observed with methylation of the $p15^{INK4b}$ gene in association with risk factors in the patients compared to the healthy controls.

The methylation profile and alteration in transcription suggests that the downregulated expression of $p15^{INK4b}$ by aberrant promoter methylation is an inactivating event in cervical cancer. However, this study requires extension to a larger population size in order to verify whether *p15* can be used as a significant or reliable marker in cervical cancer in the north Indian population.

p16 is the most commonly altered gene in human malignancies (4). Hypermethylation of the *p16* tumor suppressor gene and its effect on transcriptional downregulation or silencing is one of the major mechanisms of $p16^{INK4a}$ gene inactivation in various types of cancer, including cervical carcinoma (26).

$p16^{INK4a}$ methylation was found to be significant in cervical cancer patients in the north Indian population and its methyla-

tion was found to increase the risk of cervical cancer (P<0.001). The present result is consistent with other reports on cervical cancer (23,26) on other population groups.

Previous studies have not identified an association between *p16* alterations, including mutation or promoter hypermethylation, and HPV infection (27). In this study, a significant trend towards an increased risk of cervical cancer in association with HPV infection was not observed with methylation of the $p16^{INK4a}$ gene in patient samples compared to healthy controls.

Findings of a recent study showed hypermethylation of the $p16^{INK4a}$ gene promoter to be unchanged according to the patient age (28). Similar observations were also obtained in the present study, suggesting that age is not associated with the increase in the risk of cervical cancer with respect to *p16* hypermethylation.

Aberrant *p16* methylation has been reported to be associated with active tobacco use in patients with squamous cell carcinoma and high-grade dysplasia (29). Methylation of $p16^{INK4a}$ was found to be statistically significant even in the case of passive smokers, which increased the risk of cervical cancer. It also significantly increased the risk of cervical cancer among OC users (P<0.001). AFSI did not have a significant impact on the methylation of $p16^{INK4a}$ in the present study.

Results of a previous study showed that high-stage cancers exhibited an increased promoter methylation frequency for *p16* (22). In the present study, an increasing trend of methylation of *p16* was observed with increasing pathological change, confirming *p16* as a biomarker.

The results from the present study have shown that downregulation of the mRNA expression of $p16^{INK4a}$ by its aberrant promoter methylation is a significant inactivating event in cervical cancer. This gene may be used as a significant and reliable biomarker in cervical cancer in the north Indian population. Hypermethylation of *p16* was also found to be significantly associated with passive smoking and OC use. Hypermethylation of *p15* was also observed to be significant although at a low frequency.

Although the present findings require extension to a larger series, this study suggests that the pattern of aberrant methylation in females with or without dysplasia may help identify subgroups at an increased risk of histological progression or cancer development.

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