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CpG Island Methylation of *TMS1/ASC* and *CASP8* Genes in Cervical Cancer

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Abstract
Background: Gene silencing associated with aberrant methylation of promoter region CpG islands is an acquired epigenetic alteration that serves as an alternative to genetic defects in the inactivation of tumor suppressor and other genes in human cancers.
Aims: This study describes the methylation status of *TMS1/ASC* and *CASP8* genes in cervical cancer. We also examined the prevalence of *TMS1/ASC* and *CASP8* genes methylation in cervical cancer tissue and none – neo plastic samples in an effort to correlate with smoking habit and clinicopathological features.
Method: Target DNA was modified by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequently amplified by Methylation Specific (MS) PCR with primers specific for methylated versus unmethylated DNA. The PCR product was detected by gel electrophoresis and combined with the clinical records of patients.
Results: The methylation pattern of the *TMS1/ASC* and *CASP8* genes in specimens of cervical cancer and adjacent normal tissues were detected [5/80 (6.2%), 3/80 (3.75%)-2/80 (2.5%), 1/80 (1.2%) respectively]. No statistical differences were seen in the extent of differentiation, invasion, pathological type and smoking habit between the methylated and unmethylated tissues ($P > 0.05$).
Conclusion: The present study conclude that the frequency of *TMS1/ASC* and *CASP8* genes methylation in cervical cancer are rare (<6%), and have no any critical role in development of cervical cancer.

Key words: Methylation, *TMS1/ASC*, *CASP8*, cervical cancer

Introduction

Cervical cancer is the second most common cancer and an important cause of death in women worldwide [1]. Therefore, it is likely that host genetic and epigenetic events play an important role in cervical carcinogenesis .The term "epigenetic"is used to describe mitotically and meiotically heritable states of gene expression that are not due to changes in DNA sequence [2]. DNA methylation is an epigenetic mechanism used for long-term silencing of gene expression. The methylation pattern is established during development and is normally maintained throughout the life of an individual. It has been shown that such epigenetic mechanisms can be important in initiating tumorogenesis and supporting the malignant state of cancer cells [3].

Apoptosis is mediated by a family of cystine proteases called caspases. *CASP8*, located at chromosome 2q33-34, encodes caspase 8, an initiator caspase that plays an important role in the Fas- ligand pathway [4]. Alterations of these genes have been described in several neoplasias, such as mutations in colon cancer and promoter hypermethylation in medulloblastomas and neuroblastomas [5]. *TMS1* gene located on 16p11.2-12 chromosome has function such as activates pro caspas-1-8, modulates NF-KappaB activation pathway [6]. *TMS1/ASC* is a bipartite protein comprising two protein-protein interaction domains, a pyrin domain (PYD) and a caspase recruitment domain (CARD). Proteins containing these domains play crucial roles in regulating apoptosis and immune response pathways, and mutations in a number of PYD- and CARDcontaining proteins have been linked to auto-inflammatory diseases and cancer [7]. This gene is also known as *ASC* (Apoptosis Speck like protein containing a CARD) [8]. So, the down regulation of *TMS1/ASC* in breast cancer cell lines correlates with dense methylation on the CpG islands [9]. Methylation of the promoter region of *TMS1/ASC* has also been identified in small cell lung cancer and non-small cell lung cancer [10], human glioblastoma [11], ovarian tumors [12], colorectal cancer [13], neuroblastoma [14], and melanoma [15]. It was appeared no correlation between methylation of the *TMS1/ASC* gene and acute lymphoblastic leukemia [16]. Tischoff et al. [17] reported that promoter methylation of the proapoptotic genes *CASP8* and *TMS1* are involved in the malignant epithelial liver tumor. The role of epigenetic (gene inactivation) in tumorigenesis in gynecologic malignancies have been poorly understood. So, we investigated the promoter methylation status in *CASP8* and *TMS1* genes and relationships between clinopathologic parameters and methylation status with risk of cervical cancer.

Materials and Methods

Study Subjects

The case -control study involved collection of tissue samples of 160 North Indian subjects. 80 cases were newly diagnosed, previously untreated and histologically confirmed as cervix cancer patients. The samples were collected from the Post graduate Institute of Medical Education and Research (PGIMER) Chandi-

garh and Government Medical College (GMC), Chandigarh. The control tissue samples (n = 80) were collected from the same institute with no history of cancer or pre cancer. Informed consent was obtained from all the cases and controls.

DNA Extraction

Genomic DNA was isolated from tissue samples by the procedure of Roe et al. [18]. For the DNA methylation studies, 1 μg of genomic DNA was processed and modified with sodium bisulfite using the Intergen CpGenome DNA modification kit (Intergen, Norcross, GA). Briefly, genomic DNA was modified by sodium bisulfite, desulfonated with sodium hydroxide, and then purified and resuspended in TE (10 mM Tris, 0.1 mM EDTA, pH 7.5). Negative control (no sample) and positive control (in vitro methylated and bisulfite treated human placenta DNA) were included in all reaction . The methylation-specific (MS) PCR conditions and the sequences of premiers are used for *TMS1/ASC* and *Caspes-8* previously described by Liu et al. [19] and Lázcoz et al . [20] respectively .

The positions of the 191bp PCR product representing the methylated and unmethylated 196 bp alleles for *TMS1/ASC* were separated on 3% agarose gels (Fig.1). In case of *Caspes-8*, the PCR products for the methylated and unmethylated alleles which separated on 3% were 321 and 320 bp respectively (Fig.2).

Statistical Analysis

The results combined with the clinical records of Patients were analyzed with the the Epi-Info software (Epi-Info, version 3.2, Centers for Disease Control and prevention, Atlanta, GA, USA) and the software package SPSS, version 10.0 (SPSS, Chicago, IL, USA). Significance was set at $P \leq 0.05$.

Results

The methylation status of the *TMS1/ASC*and *Caspase-8* in primary cervical cancer and control non-neoplastic cervix tissue specimens derived from north India population was analyzed.

There were no methylation frequency association for *TMS1/ASC* gene with various clincopathological parameters including: age, smoking, histological type, and stage ($P > 0.05$) (Table 1). Similarly, same result was observed for *Caspase-8* gene with various clinicopathological parameters (Table.2).

The status of promoter methylation for *TMS1/ASC*and *Caspase-8* genes in 80 primary cervical cancer tissue and 80 control non-neoplastic cervix tissue specimens were examined. The promoter methylation frequency for *TMS1/ASC* gene in cervical cancer and control were 6.2% (5/80), 3.75% (3/80) respectively.

The methylation status of the *Caspase-8* gene was detected in cervical cancer tissue 2.5% (2/80) and normal tissue 1/80 (1.2%). There was no deference significant methylation frequency for the *TMS1/ASC* and *Caspase-8* genes as compared to controls non-neoplastic cervix.

Discussion

The effect of DNA hypermethylation in gene promoter regions is similar to genetic loss-offunction mutations [21]. Many cellular pathways are inactivated by this epigenetic event, including DNA repair, cell cycle, apoptosis, cell adherence, and detoxification [22]. The specific patterns of CpG island hypermethylation between tumor types may provide a useful signature for tumor diagnosis and prognosis [23]. *TMS1/ASC* gene was originally identified as a target of methylation-induced silencing using cell lines that over express DNA

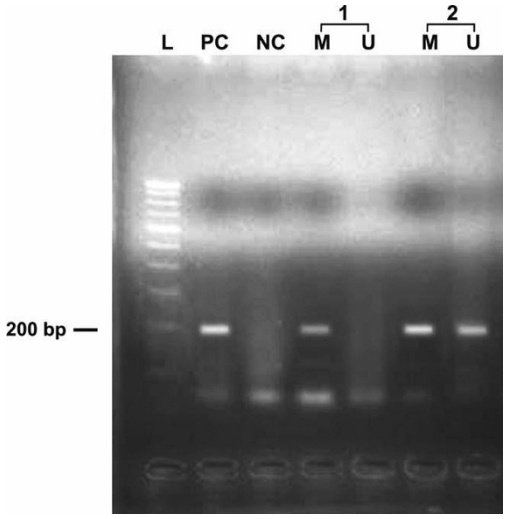

Fig. 1. Methylation analysis of *TMS1/ASC* in cervical cancer. Lane U: amplified product with primers recognizing unmethylated sequence; Lane M: amplified product with primers recognizing methylated sequence. NC, normal control; PC, positive control for methylation. L: ladder (100bp).

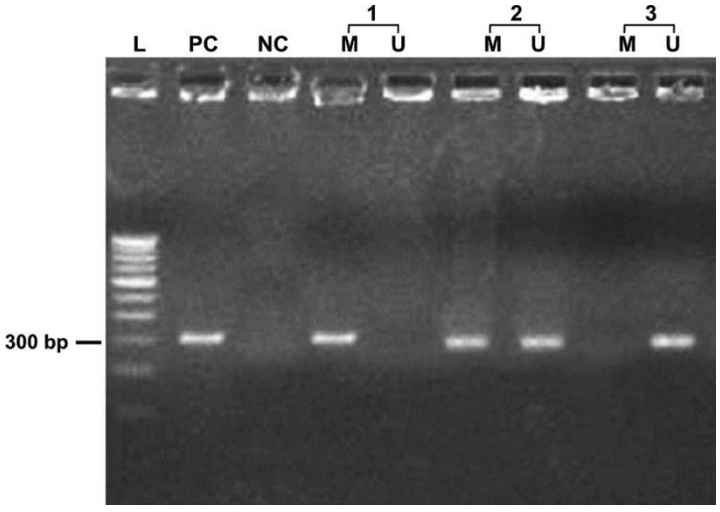

Fig. 2. Methylation analysis *CASP8* in cervical cancer. Lane U: amplified product with primers recognizing unmethylated sequence; Lane M: amplified product with primers recognizing methylated sequence. NC, normal control; PC, positive control for methylation. L: ladder (100bp).

Table1. The relationship between *TMS1* promoter methylation and clinicopathology parameter of cervical cancer.

Characteristic	overall	present	Absent	*P* Value
<50	44	2(4.5%)	42(95.4%)	0.65
>50	36	3(8.3%)	33(91.6%)	
Smoker	5	1(20%)	4(80%)	0.28
Nonsmoker	75	4(5.3%)	71(94.6%)	
Histological type				
SCC	68	4(5.8%)	64(94.1%)	0.56
AC	12	1(8.3%)	11(91.6%)	
Stage				
Ib	46	1(2.2%)	45(97.8%)	
IIa	12	2(16.6%)IIb	10(83.3%)	0.10
IIb	19	2(10.5%)	17(89.4%)	
IIIb	3	-	3(100%)	

Table 2. The relationship between *Caspase-8* promoter methylation and clinicopathology parameter of cervical cancer.

Characteristic	overall	present	Absent	*P* Value
<50	44	0	44(100%)	-
>50	36	2(5.6%)	34(94.4%)	
Smoker	5	1(20%)	4(80%)	0.12
Nonsmoker	75	1(1.3%)	74(98.6%)	
Histological type				
SCC	68	2(2.9%)	66(97.05%)	
AC	12	-		
Stage				
Ib	46	1(2.17%)	45(97.8%)	0.37
IIa	12	1(8.3%)	11(91.6%)	
IIb	19	-	-	
IIIb	3	-	-	

methyltransferase 1 (DNMT1). In another critical pathway mediating cell death via death receptors, *CASP8* acts as a key apoptotic enzyme by serving as an "initiator CASP"; moreover, *CASP8* was recently shown to be silenced by aberrant methylation [24]. However, because the 5' region of *CASP8* does not contain a typical CpG island, the relevance of methylation to its silencing remains unclear [25]. *TMS1/ASC* is a novel proapoptotic gene previously identified as a target of DNA methylation in breast cancer [26]. Terasawa et al. [27] showed that aberrant methylation of the 5' region of *TMS1/ASC* is well correlated with loss of expression in ovarian cancer. Notably, decreasing *TMS1/ASC* expression reduces sensitivity to chemotherapeutic drugs [28]. The methylationmediated silencing of *TMS1/ASC* would be expected to contribute to a survival advantage for tumor cells, by enabling them to escape apoptosis, which supports a role for aberrant methylation in human ovarian tumorigenesis [29]. Approximately 40% (10/17) of primary breast tumors also exhibited aberrant methylation of the *TMS1/ASC* gene. Different studies have confirmed the frequency of aberrant methylation of *TMS1/ASC* in primary breast cancers, ranging from 10% to 40%. Methylation-associated silencing of the *TMS1/ASC* gene was observed in 11% of gastric carcinomas, 40%-41% of small cell and non small cell lung carcinomas, 50% of malignant melanomas, and 44% of primary glioblastomas [30, 31]. Jens et al. [32] reported frequency of methylation for *Caspase-8* (1.2%) and *TMS1* (5.1%) in ovarian cancer, and concluded that, methylation of these genes in regulation of apoptosis was no significant ($P = 0.74$). In line of our study Feng et al. [33] demonstrated the rate of hypermethylation for *TMS1* to be 3.1% in controls and 6.7% in patient with cervical cancer. Liu et al. [34] found that aberrant methylation of the *TMS1* gene was detected in tumor tissues %36.1 with Choangio carcinoma, and in normal tissue 8.3%. So, there were no statistical differences in age, gender, pathologic type between the methylated and unmethylated tissues. Aberrant methylation of *TMS1* gene was detected in 15 of 80 ovarian cancer tissue (19%) but in none of the normal ovary specimens [35]. Michalowski et al.

[36] observed, hypermethylation of *Caspase-8* in 38% in neuroblastoma. Matinez et al. [37] strongly suggested that hypermethylation of the pro-apoptotic *Caspase-8* in glioblastoma ($P = 0.0035$). The present study's results were consonance with those of Yang et al. [38] who carried out a similar study in Chinas women. However, our study has several potential limitations. First, we were not able to incorporate into the model information concerning the relative level of hypermethylation of each specific gene. It is possible that other gene combinations, which may have increased sensitivity and specificity, will be identified through the use of real-time PCRbased assays such as MethyLight [39], which provided information on the relative level of hypermethylation of each specific gene examined. Second, we limited our search for useful hypermethylated genes to an assessment of 2 genes that had been previously reported to be associated with cancers at other sites. Identification of additional novel CpG islands that are specifically associated with cervical cancer will be needed to construct a panel with higher sensitivity that maintains high specificity, and studies examining detection of such a panel of genes using recently developed quantitative assays should be undertaken. More study using a much larger samples size are needed to further define the potential role of methylated DNA marker in cervical cancer management.

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