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Status of p16^{INK4a} and E-Cadherin Gene Promoter Methylation in Moroccan Patients With Cervical Carcinoma

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Aberrant methylation of tumor suppressor gene promoters has been extensively investigated in cervical cancer. Transcriptional silencing, as a main consequence of hypermethylation of CpG islands, is the predominant mechanism of *p16^{INK4a}* and *E-cadherin* gene inactivation in malignant epithelial tumors. This study was conducted to evaluate the promoter methylation status of *p16^{INK4a}* and *E-cadherin* genes in 22 specimens of cervical carcinomas, four cervical cancer cell lines (HeLa, SiHa, Caski, C33A), and 20 human papillomavirus negative specimens, obtained from normal cervical swabs, using the methylation-specific PCR approach. Hypermethylation of the 5′ CpG island of the *p16^{INK4a}* and *E-cadherin* genes were found in 13 (59.1%) and 10 (45.5%) of 22 cervical cancer samples, respectively. Furthermore, our findings did not show any correlation between promoter methylation of *p16^{INK4a}* and *E-cadherin* genes and clinicopathological parameters, including HPV infection, phenotypic distribution, and stage of the disease. However, hypermethylation of E-cadherin gene promoter appears to be age related in cervical cancer, whereas the frequency of aberrant methylation of p16^{INK4a} gene promoter is unchanged according to the age of patients. Thus, caution must be made to use these markers in the diagnosis of cervical cancer. However, dietary or pharmaceutical agents that can inhibit these epigenetic events may prevent or delay the development of cervical cancer.

Key words: Cervical cancer; E-cadherin; Hypermethylation; p16^{INK4a}

INTRODUCTION

Worldwide, cervical cancer is one of the most important female malignancies with about 500,000 news cases and 230,000 deaths (1). Among the new cases estimated each year, approximately 80% occur in developing areas. In Morocco, cervical cancer is the second most common cancer among women and represents a major public health problem (2).

Clinical and epidemiological studies have clearly established human papillomavirus (HPV) persistent infections as the central cause of cervical cancer development (3,4). Certain types of HPV are considered as high-risk (HR) types due to the great odds ratios of association with cervical cancer and their ability to integrate into the host genome (5). HR HPV infections are widespread in the world but the majority of HPV-associated lesions such as cervical intraepithelial neoplasia (CIN) will remain stable or spontaneously regress over time (4–7), suggesting that other genetic and epigenetic events are likely to be involved in cervical carcinogenesis.

Epigenetic alterations leading to activate or inactivate the expression of some genes are important keys in the development of various cancers. Thus, in many human cancers epigenetic hypermethylation in the promoter regions of a number of genes has been recognized as an important change in the carcinogenesis (8). Controlled by DNA methyltransferases, global DNA hypomethylation and site-specific hypermethylation have been reported as the hallmark of cancer (9). Methylation of cytosine is widely found in mammal genomes in the context of the palindromic sequence 5′-CpG-3′. Most CpG dinucleotide pairs are methylated except at some areas called "CpG island" where the methylation is developmentally controlled. CpG islands are CpG-rich areas of approximately 1 kb, usually located in the vicinity of genes and often found near the promoter of widely expressed genes (10,11). It has become increasingly apparent that DNA

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hypermethylation with subsequent epigenetic silencing of tumor suppressor genes (TSGs) through chromatin remodeling is associated with loss of function, which may constitute the second hit of the "two hit" hypothesis, providing a selective advantage during carcinogenesis (8,9,12). Moreover, genes that are frequently aberrantly methylated in specific tumors have been used as molecular targets for the detection of neoplastic cells in body fluids such as urine and plasma (13,14).

$p16^{INK4a}$ and E-cadherin are two important proteins used as immunohistochemical markers in various gynecological cancers. $p16^{INK4a}$, the product of *CDKN2A* gene, is a component of $p16^{INK4a}$-Cdk4-6/cyclin D-pRb signaling pathway and is perturbed in many cancers. In these tumors, the functions of $p16^{INK4a}$ may be lost due to the mutations or suppression of the transcription by promoter methylation (15). In HR HPV-positive cervical cancer, the oncogene E7 disrupts pRb/E2F interaction, releases active E2F, and induces the pRb degradation (16). The existence of the regulatory feedback in the pRb/p16 pathway leads to an overexpression of $p16^{INK4a}$ in cervical tumors (17). However, the overexpression of $p16^{INK4a}$ is accompanied with hypermethylation of the promoter region in 19% to 61% of cervical carcinomas as determined by methylation-specific PCR (MSP) (17, 18). Recently, several studies have shown high sensitivity and specificity of p16-based cytology to detect high grade CIN (19).

E-cadherin is a transmembrane glycoprotein coded by the *CDH1* gene. It is localized on the surface of epithelial cells and mediates adhesion through Ca^{2+}-dependent homotypic binding. Based on its biological functions, E-cadherin is regarded as an invasion and metastasis suppressor. Loss of E-cadherin expression or function correlates with increased invasiveness and metastasis in carcinomas of several anatomical sites (20,21). The E-cadherin-mediated cell adhesion system is inactivated by multiple mechanisms. It may be inactivated as a result of genetic alteration, reduced gene expression, or changes of other cadherin–catenin complexes (22). It has been reported that aberrant hypermethylation of CpG islands in the E-cadherin promoter region, together with alterations in chromatin structure and transcription factor activity, may conspire to suppress E-cadherin expression (22). In cervical cancer, previous data showed that the presence and localization of cytoplasmic E-cadherin were significantly correlated with cervical CIN grade. In invasive types, the expression of E-cadherin was significantly reduced (23) and this is mainly due to gene silencing by methylation processes (24).

The present study was designed to investigate the promoter methylation status of the $p16^{INK4a}$ and *E-cadherin* genes in cervical tumors from Moroccan patients and cervical carcinoma cell lines using the MSP approach. The aim of this study is to evaluate the potential use of $p16^{INK4a}$ and/or E-cadherin promoters' methylation as a marker for cervical cancer management in Morocco.

MATERIALS AND METHODS

Specimens

Twenty-two freshly frozen cervical samples were collected from women with cervical cancer visiting the Oncology Centre of Casablanca (Centre Hospitalier Universitaire Ibn Rochd) Morocco. The samples were immediately frozen in liquid nitrogen until use for DNA extraction. Tumor tissue was selected from an area with >75% malignant cells as determined on a hematoxylin and eosin-stained slide. Twenty cervical swabs diagnosed on Pap smear as normal squamous epithelium were used as controls.

Cell Lines

The human cervical cancer cell lines HeLa, SiHa, C33A, and CaSki were obtained from American Type Culture Collection (ATCC). SiHa and CaSKi cell lines harbor HPV 16, HeLa cell line harbors HPV 18, whereas C33A is HPV free. Theses cell lines were used as controls for MSP as their p16 methylation status has already been reported by Ivanova et al. (17) and Nehls at al. (18). Cells lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS).

DNA Extraction

Cells obtained from tissue biopsies, cervical swabs, or cell cultures were lysed in digestion buffer (Tris-HCl 10 mM, pH 8.0, EDTA 10 mM, NaCl 150 mM, and SDS 2%) containing proteinase K (0.2 mg/ml). DNA was then purified using the standard phenol-chloroform extraction and ethanol precipitation method. DNA precipitate was then resuspended in sterile distilled water and stored at −20°C until use (25).

HPV Detection

HPV DNA was amplified by PCR using consensus primers MY09 and MY11, and HPV genotyping was realized by Southern blot hybridization using specific probes as described by Amrani et al. (2).

Bisulfite Modification and Methylation-Specific PCR

DNA methylation patterns in the CpG islands of the $p16^{INK4a}$ and *E-cadherin* genes were determined by MSP (26). MSP distinguishes unmethylated from methylated alleles in a given gene based on sequence changes produced after bisulfite treatment of DNA, which converts

unmethylated (but not methylated) cytosines to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA. Sodium bisulfite treatment of genomic DNA was performed as described by Herman et al. (26). Briefly, 1 µg of DNA in a volume of 50 µl was denatured by NaOH (final concentration, 0.2 M) for 10 min at 37°C. Thirty microliters of 10 mM hydroquinone (Sigma) and 520 µl of 3 M sodium bisulfite (Sigma) at pH 5, freshly prepared, were added and mixed. Samples were then incubated under mineral oil at 50°C for 16 h. Modified DNA was purified using the Wizard DNA Cleanup System according to the manufacturer instructions (Promega, Madison, WI, USA) and eluted in 50 µl of water. Modification was completed by NaOH (final concentration, 0.3 M) treatment for 5 min at room temperature, followed by ethanol precipitation. DNA was then resuspended in water and used immediately or stored at −20°C.

After treatment, the unmethylated cytosine is converted to uracil, whereas the methylated cytosine remains unchanged. Uracil is recognized as thymine by Taq polymerase. The treated DNA was subjected to MSP using unmethylation-specific or methylation-specific primers. The two genes, *E-caderin* and $p16^{INK4a}$, were analyzed individually. Primer sequences, annealing temperatures, and the expected products sizes are listed in Table 1. Amplification reaction was performed in a total volume of 20 µl. The amplification mixture contained 2 µl of bisulfite-treated genomic DNA extracted from cervical carcinomas, cervical swabs, or cell lines, 0.25 µM of each primer, 250 µM of each dNTP, and 0.75 unit of hot *Taq* DNA polymerase (Amersham) in 1× *Taq* polymerase buffer. Thermal cycling was initiated at 95°C for 5 min, followed by 45 cycles at 95°C for 30 s, the specific annealing temperature for 30 s, and extension temperature at 72°C for 30 s; and a final extension at 72°C for 7 min. An untreated blood DNA from a normal individual was used as negative control. PCR products were run on 2% agarose gels and visualized after ethidium bromide staining. Samples with only the methylation-specific band were designated as M and the samples with only the unmethylation-specific band were designated as U.

Statistical Analysis

Statistical analyses were performed using Statistica 8 software that uses directly the Yates' chi-square test for small sample size. The associations between the discrete variables were assessed using chi-square test. Differences were considered statistically significant for $p \leq 0.05$. The power of the statistical analysis was calculated using Piface application from Java applets for power and sample size. Statistical analysis is considered conclusive if the power is more than 0.8.

RESULTS

Histopathology

Histopathological analysis showed that all cancerous specimens ($n = 22$) were invasive epidermoid carcinomas. According to the International Federation of Gynecology and Obstetrics (FIGO) classification, only stages II and III were found, with 41% (9/22) and 59% (13/22), respectively. Among them, 2 (9%) were poorly differentiated, 13 (59%) moderately differentiated, and 7 (31.8%) well-differentiated carcinoma.

HPV Analysis

Using the consensus PCR, HPV DNA was detected in 15 of the 22 specimens analyzed (68%). Molecular

Table 1. Primer Sequences, Annealing Temperatures, PCR Product Sizes, and MSP Primers Used for Amplification of *E-Cadherin* and $p16^{INK4a}$ Gene CpG Islands

Primers		Primer Sequences (5′–3′)	Size	Annealing Temperatures
p16 M	S	TTA TTA GAG GGT GGG GCG GAT CGC	150	65
	A	GAC CCC GAA CCG CGA CCG TAA		
p16 U	S	TTA TTA GAG GGT GGG GTG GAT TGT	151	60
	A	CAA CCC CAA ACC ACA ACC ATA A		
E-cad M	S	TTA GGT TAG AGG GTT ATC GCG T	116	57
	A	TAA CTA AAA ATT CAC CTA CCG AC		
E-cad U	S	TAA TTT TAG GTT AGA GGG TTA TTG T	97	53
	A	CAC AAC CAA TCA ACA ACA CA		

M: methylated-specific primers; U: unmethylated-specific primers; S and A represent sense and antisense primers, respectively.
Source: Herman et al. (26).

genotyping showed that HPV 16 and HPV 18, alone or in coinfections, were the most frequently identified types as they were each found in 46.6% of the cases (7/15), whereas HPV 31, HPV 33, and HPV 45 were found in 26.6% (4/15), 20% (3/15), and 40% (6/15), respectively. Among the 15 HPV-positive cases, no specimen harbored HPV 35, whereas two HPV-positive cases corresponded to other viral types that were not identified.

MSP Analysis

The status of promoter hypermethylation of $p16^{INK4a}$ and E-cadherin genes was evaluated in four cervical cancer cell lines (HeLa, SiHa, C33A, and Caski), 22 cervical cancer samples, and 20 DNA specimens of cervical swabs diagnosed on Pap smear as normal squamous epithelium. Figure 1 shows the methylated and unmethylated alleles produced by MSP in four representative samples.

The four cervical cell lines, as well as the 20 DNA specimens from normal cervical swabs, did not show hypermethylation of $p16^{INK4a}$ promoter region. However, aberrant hypermethylation of CpG islands in the promoter region of the *p16*^{INK4a} gene was found in 13 of 22 cervical cancer cases (59.1%). Among them, eight showed only the methylation-specific bands (M) and five showed methylation-specific and weak unmethylation-specific bands (M/U).

MSP analysis showed that E-cadherin promoter region was hypermethylated in three cell lines (HeLa, SiHa, and C33A) without unmethylated bands, while the Caski cell line harbored both methylated and unmethylated alleles. In this cell line, MSP analysis showed DNA amplification both with the unmethylation-specific and methylation-specific primers. There were methylated alleles in none of the 20 normal cervical specimens. Aberrant methylation islands in the promoter region of the *E-cadherin* gene was found in 10 of 22 cervical cancer cases (45.5%). Among them, only one sample showed a methylation-specific band and a weak unmethylation-specific band (M/U).

Furthermore, methylation of at least one gene was detected in 86.3% (19/22) of cervical cancers. Among them, 18.2% (4/22) of cases were positive for methylation of both *p16*^{INK4a} and *E-cadherin* genes, 40.9% (9/22) were positive for methylation only in *p16*^{INK4a} gene and 27.3% (6/22) only in *E-cadherin* gene.

The evaluation of the relationship between $p16^{INK4a}$ and E-cadherin promoter's methylation status using chi-square test did not show any significant association (Table 2). The methylation for these two loci in cervical cancer and their association with clinicopathological parameters were also determined (Table 3). No significant association was found between promoter methylation of *p16*^{INK4a} and *E-cadherin* genes and HPV infection, differentiation status, and stage of disease. However, the frequency of E-cadherin promoter methylation shows a significant difference with age ($p = 0.0359$), as patients with promoter methylation were older than patients without promoter methylation. For each parameter, we have found that the power of the statistical analysis is more than 0.85.

DISCUSSION

Changes in the status of DNA methylation are among the most common molecular alterations in human neoplasia and specific pattern of hypermethylation was reported in each form of human cancer (27). Hypermethylation of CpG islands of gene promoter is an important epigenetic mechanism for gene silencing, which confers tumor cell growth advantage. Hypermethylation of CpG islands of promoter genes has been identified and associated with loss of expression of many potential key genes in cancer cells (8). Several studies have investigated the methylation status of genes in cervical cancer

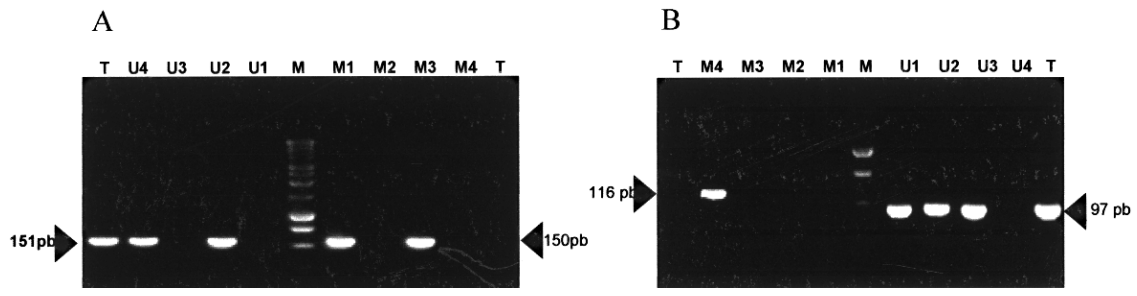

Figure 1. MSP analysis of the promoter regions of $p16^{INK4a}$ (A) and E-cadherin (B). The presence of a visible PCR product in lane U indicates the presence of unmethylated genes; the presence of a PCR product in lane M indicates the presence of methylated genes. Normal lymphocytes DNA (T) was used as a negative control for methylation. Case 1 and 3 were methylated at $p16^{INK4a}$ (A) and case 4 was methylated at E-cadherin (B). M: 100 bp ladder.

Table 2. Correlation Between $p16^{INK4a}$ and E-Cadherin Gene Promoter Methylation in Cervical Cancer

	$p16^{INK4a}$ M	$p16^{INK4a}$ U	*p*-Value
E-cadherin M	4	6	0.996
E-cadherin U	9	3	

M: methylated promoter; U: unmethylated promoter. *p*-Value was calculated by chi-square test.

and evaluated their implication in carcinogenesis (17, 18,28–35). In the present study we have investigated the methylation status of $p16^{INK4a}$ and E cadherin gene promoters in four cervical cell lines, 20 normal HPV negative cervical swabs, and 22 cervical cancer specimens from Moroccan women. These two genes are involved in many cellular pathways and have critical biological functions.

The cellular tumor suppressor protein p16 has a central function in the regulation of cell cycle activation. The *p16*^{INK4a} gene encodes a negative regulatory protein that regulates the progression of eukaryotic cells through the G_1 phase of the cell cycle (36). Loss of $p16^{INK4a}$ protein expression may be one of the steps required to overcome cell cycle arrest at senescence, leading to immortalization (36). In this study, the hypermethylation of CpG islands of $p16^{INK4a}$ gene promoter was found in 13 of 22 cervical cancer samples (59.1%). Our results of MSP analysis are in close agreement with other findings on different populations showing that hypermethylation of the same region of *p16*^{INK4a} gene using the same set of primers is observed in 19% to 61% of invasive cervical carcinoma (17,18). Moreover, the increased risk for disease progression was independent from clinical and pathological factors, suggesting that $p16^{INK4a}$ gene promoter methylation is an early event in cervical cancer development. Indeed, hypermethylation of the *p16* gene has been suggested to be a shared epigenetic alteration in multiple human cancers (27). Moreover, in HPV-induced cervical cancer, the cell cycle activation is not mediated by Cdks but by E7-related Rb disruption. The p16 inactivation would not confer any further growth promoting effect, because in this cancer the HR-HPV oncogene E7 induces a permanent release of E2F from its binding to pRb, leading to continuous cell cycle activation (18). Thus, methylation of $p16^{INK4a}$ promoter gene may be a result of genetic and epigenetic events produced during the carcinogenesis steps of cervical cancer development.

Table 3. Promoter Methylation of $p16^{INK4a}$ and E-Cadherin in Tissues From Patients With Cervical Cancer According to Clinical Data

	$p16^{INK4a}$		E-Cadherin	
	+	−	+	−
Cases	13	9	10	12
Age				
<50 years	7	6	3	10
>50 years	6	3	7	2
	$p = 0.6897$		$p = 0.0359$*	
HPV infection				
HPV positive	9	6	7	8
HPV negative	4	3	3	4
	$p = 0.7364$		$p = 0.7696$	
Stage				
I	0	0	0	0
II	4	5	5	4
III	9	4	5	8
IV	0	0	0	0
	$p = 0.4702$		$p = 0.7215$	
Phenotypic distribution				
Poorly differentiated	1	1	2	0
Moderately differentiated	8	5	6	7
Well differentiated	4	3	2	5
	$p = 0.7742$		$p = 0.7735$	

*Significant difference.

Cadherins are a family of cell–cell adhesion molecules that can modulate epithelial phenotype and morphogenesis in a variety of tissues. E-cadherin is the major cadherin expressed on the surface of normal epithelial cells (37). E-cadherin expression is reduced during tumor progression and metastasis, and associated with poor prognosis in a variety of cancers (38–40). Based on these data, analysis of methylation status of E-cadherin gene promoter would be of great importance to understand the implication of this gene silencing in cervical cancer development. Our results show that promoter methylation of *E-cadherin* gene was observed in three cell lines (HeLa, SiHa, and C33A) and 45.5% of cervical cancer samples. These results are consistent with previous studies showing that E-cadherin promoter is methylated in less than 50% of invasive cervical cancer (24,28,41). E-cadherin is a major adhesion component of epithelial cells, which plays an important role as an invasion suppressor gene. Decrease or loss of E-cadherin expression is a common feature of many human epithelial cancers, including cervical cancer, although a decreased expression of this molecule has been described in metastasis, but not primary tumors (42). Promoter hypermethylation has been proposed as an explanation for the decrease of *E-cadherin* expression (24,43) and was even suggested as a potential marker for identifying cervical cancer patients at high risk for relapse (32).

Consistent with previous studies (44,45), our results

did not show any methylation of both $p16^{INK4}$ and E-cadherin gene promoters in normal tissues. In contrast, methylation of either $p16^{INK4a}$ or E-cadherin gene promoters was detected in 86.3% (19/22) of cervical cancers.

Some of the cervical cancer samples had both methylation- and unmethylation-specific bands (five cases with minor unmethylation-specific bands for $p16^{INK4a}$ and one case for E-cadherin gene). If we assume that the tumor developed from the same clone of epithelial cells, the minor unmethylation-specific bands are likely attributable to contamination by stroma cells. Another possibility is that in some cervical cancer samples, only one allele of the gene is hypermethylated. Indeed, MSP is a simple, highly sensitive and nonquantitative test. A minimal degree of methylation can be registered as "positive methylation" depending on the original DNA concentration, primer sets, and the number of cycles used for PCR (46,47).

No association was found between the status of methylation of $p16^{INK4a}$ and E-cadherin gene promoters, suggesting that these methylation abnormalities may occur independently from each other in cervical cancer. Furthermore, association analysis demonstrates that hypermethylation of CpG islands in the promoter regions of $p16^{INK4a}$ and E-cadherin genes was not correlated with clinicopathological parameters (HPV infection, phenotypic distribution, and stage of the disease). However, our findings suggest that E-cadherin methylation in cervical cancer is age related. In previous studies, age-related methylation of E-cadherin was detected in bladder neoplasms, gastric cancer, and nasopharyngeal carcinoma (48–51). Silencing of E-cadherin gene would be an age-related common phenomenon in human cancer. The incidence of cervical cancer rises up in older women and it is usually accompanied by metastasis. Thus, hypermethylation of $p16^{INK4a}$ and E-cadherin gene promoters is a frequent epigenetic event in cervical carcinoma, suggesting an abnormality of the methylation mechanism in the cancer cells. Our results demonstrate that 5′-CpG islands hypermethylation of $p16^{INK4a}$ and E-cadherin genes cannot be an effective marker for cervical cancer diagnosis. However, dietary or pharmaceutical agents that can inhibit this epigenetic event may prevent or delay the development of cervical cancer.

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