Original Article
Middle East Journal of Cancer; October 2017; 8(4): 179-186

Evaluation of Promoter Hypermethylation of Tumor-Suppressor Genes *p14* and *p16* in Iranian Endometrial Carcinoma Patients

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Abstract

Background: Endometrial cancer is a common gynecological malignancy with good prognosis in the early stages of the disease. The CpG island in the promoter region of tumor-suppressor genes are frequently methylated in various types of human cancers. In the present study, we have examined the methylation status of the *p16INK4a* and *p14ARF* genes in endometrial cancer and healthy endometrium with the aim to identify correlations between promoter hypermethylation, disease risk, and clinicopathological parameters.

Methods: We collected 28 formalin fixed paraffin embedded samples and 26 blood samples from endometrial cancer patients and 22 controls. Methylation-specific PCR was applied to analyze the promoter methylation status of the *p16INK4a* and *p14ARF* genes in the studied population. The results were analyzed with SPSS software version 20.

Results: There was a significant difference between the study groups and the presence of promoter CpG hypermethylation status in the *p14* ($P<0.0001$) and *p16* ($P<0.05$) genes. *p14* hypermethylation in the blood samples was associated with depth of myometrial invasion in endometrial cancer ($P=0.03$). A significant association existed between *p16* methylation in tissue with endometrial cancer grade ($P=0.06$). No statistically significant difference existed between the *p16INK4a* and *p14ARF* promoter hypermethylations in blood ($P=0.177$) and formalin fixed paraffin embedded ($P=0.221$) samples. An association existed between *p16INK4a* and *p14ARF* gene hypermethylations in blood and tissue with diabetes.

Conclusion: Our results have confirmed that epigenetic mechanisms play an important role in endometrial cancer incidence. They can be utilized as prognostic biomarkers for endometrial cancer. The lack of a significant difference between the *p16INK4a* and *p14ARF* promoter hypermethylations in blood and formalin fixed paraffin embedded samples has indicated that methylation status of a blood sample can be an early, non-invasive diagnostic marker in endometrial cancer.

Keywords: Endometrial cancer, Epigenetic, *p14ARF*, *p16INK4a*

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Middle East Journal of Cancer

Received: January 11, 2017; Accepted: April 8, 2017

Introduction

Endometrial cancer (EC) is the most common gynecologic malignancy in the Western world and the fourth most prevalent cancer in women after breast, lung, and colorectal cancer.[1] More than 90% of cases occur in women older than 50 years of age, with an average age of 63 years.[2] The major etiological factors for development of EC - age, elevated weight, and postmenopausal hormone therapy have been identified.[3] Most EC patients are identified at an early stage; however, about 30% are diagnosed in advanced stages. The frequency and mortality rate of EC has greatly increased in the past few years.[4]

During the last decade, epigenetic changes have been explained in many cancers and are now recognized to be at least as common as genetic changes.[5] DNA methylation, a significant epigenetic modification that primarily occurs at C5 of the cytosine ring within cytosine-guanine (CpG) dinucleotides is commonly located at gene regulatory sites such as promoter regions.[6] DNA methylation changes may functionally alter EC.[7] Hypermethylation of promoters in tumor suppressors and oncogenes involved in various signaling pathways frequently occur, and the pattern differs for different tumors.[8] Many individual genes are abnormally methylated in EC.[9-13]

The INK4a/ARF locus located on chromosome 9p21 has the distinctive feature of encoding two tumor suppressor genes: *p14ARF* and *p16INK4a*.[14,15] The protein products of the *p16INK4a* and *p14ARF* genes are essential for cell cycle regulation.[16] The *p16* proteins belong to the inhibitor of cyclin-dependent kinase 4 (INK4) family and are known as INK4a [cyclin-dependent kinase inhibitor 2A (*CDKN2A*)].[17] By binding directly to CDK4 and CDK6, *p16* blocks the formation of cyclin D-CDK complexes, which results in the phosphorylation of retinoblastoma (RB) proteins and G1-phase cell cycle arrest.[18] The *p14* gene, which is also known as an alternative reading frame (ARF),[17] controls the cell cycle by an alternative pathway. *p14ARF* acts by binding to mdm2, a negative regulator of *p*53, which causes stabilization of *p*53 and leads to cell cycle arrest.[17,19]

Numerous genetic and epigenetic abnormalities of the *p16* and *p14* genes have been reported in human tumors.[17] *p16* (*CDKN2A* or *INK4a*) is one of the most frequently deleted genes in cancer genomes and the most widely studied.[9] Previous studies have demonstrated that inactivation of the *p16/INK4a* gene is mainly caused by its promoter hypermethylation in EC.[4] Only one study about the association between *p14* gene promoter hypermethylation and EC has been published.[1]

Limited studies have been conducted about the relationship between *p14ARF* and *p16INK4a* gene promoter methylations with EC. Therefore, the current study investigated this correlation to identify whether these genes could be used as prognostic biomarkers for the incidence of EC. We assessed the relationships of aberrant hypermethylation with clinicopathological parameters in EC. Co-promoter methylation status of both genes in normal and cancer tissues were detected. We performed a comparison of the *p14ARF* and *p16INK4a* gene promoter methylations in blood and tissue samples in EC and investigated the impact of epigenetics in carcinogenesis.

Materials and Methods

Patients and tissues

We collected the samples in an ethical manner after consent by the patients. The patients mainly had early disease stage and were seen at 2 hospitals in Tehran, Iran (Mahdiyeh and Firouzgar) and 2 hospitals in Qazvin, Iran (Kosar and Pasteur) during 2014 to 2016. The Zanjan Medical University Ethical Committee approved this study (reference number: ZUM.REC.1395.139). We obtained participants' clinical data that included age, weight, numbers of pregnancies and abortions, infertility, the use of tamoxifen tablets, history of chemotherapy, other cancers, infections, polycystic ovaries, diabetes, hypothyroidism, menstrual disorders, and hypertension. All participants provided written informed consent.

Table 1. Summary of primer sequences and annealing temperatures for *p14ARF* and *p16INK4a* promoter regions and product sizes for MSP- PCR.

Primers	Primer sequences	Temperatures(°C)	Sizes(bp)
P16 (FM)	5'-TTATTAGAGGGTGGGGCGGATCGC-3'	57	150
P16 (RM)	5'-GACCCCGAACCGCGACCGTAA-3'		
P16 (FU)	5'-TTATTAGAGGGTGGGGTGGATTGT-3'	59	151
P16 (RU)	5'-CAACCCCAAACCACAACCATAA-3'		
P14 (FM)	5'-GTGTTAAAGGGCGGCGTAGC-3'	54	122
P14 (RM)	5'-AAAACCCTCACTCGCGACGA-3'		
P14 (FU)	5'-TTTTTGGTGTTAAAGGGTGGTGTAGT-3'	58	132
P14 (RU)	5'-CACAAAAACCCTCACTCACAACAA-3'		

M: Methylated sequence; U: Unmethylated sequence; F: Forward; R: Reverse

The EC patients provided blood samples (n=26) and formalin fixed paraffin embedded (FFPE) samples (n=28). We simultaneously collected blood and FFPE samples from the same 25 patients. In addition, 22 normal samples were collected from healthy women. Endometrial cancer patients ranged in age from 38 to 76 years (median: 65.5 years). A pathologist performed tissue diagnoses and grading.

Analysis of p14ARF and p16INK4a promoter methylation status

Genomic DNA was extracted from peripheral blood lymphocytes with CinnaPure DNA (cat. no. PR881612) and from FFPE tissues using a DNA FFPE Kit (cat. no.180134, Qiagen, Inc., Valencia, CA).

The promoter methylation statuses of *p14ARF* and *p16INK4a* were evaluated using methylation specific (MSP) PCR. Genomic DNA extracted from blood and FFPE samples was modified by sodium bisulfite treatment with an Epitect Bisulfite kit (cat. no. 59104, Qiagen, Inc., Valencia, CA) according to the manufacturer's recommendations. After genomic DNA purification and bisulfite modification, we performed MSP of the *p14* and *p16* genes with primers designed for the methylated and unmethylated promoter regions. The PCR reaction mix (20 µl) consisted of 1X Taq premix (10 µl; lot. no. 1536, Parstous), 1 µl upstream primer (10 mol/µl), 1 µl downstream primer (10 mol/µl), 2 µl, template DNA (100 ng), and 6 µl sterilized distilled water. PCR cycling conditions were: initial denaturation at 95ºC for 5 min, followed by 35 cycles of 95ºC denaturation for 45 s, annealing for 45 s (primer specific temperatures are listed in Table 1), 72ºC extensions for 45 s, and a final extension at 72ºC for 5 min. The MSP products were separated by electrophoresis on 2.5% agarose gel and stained with safe stain (Figure 1).

Statistical analysis

All statistical analyses were performed with the Statistical Package for Social Sciences (SPSS, version 20). The differences between gene

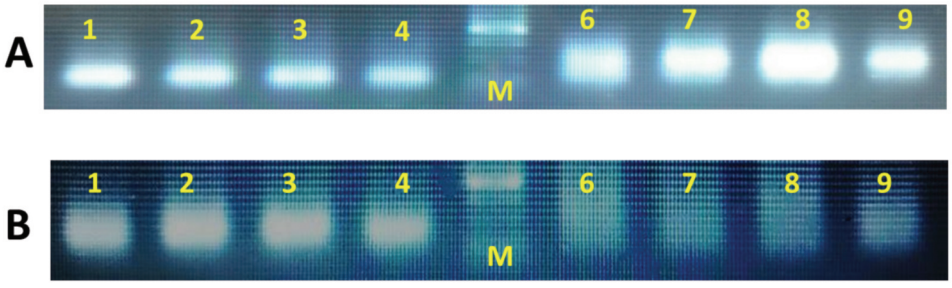

Figure 1. Detection of methylation status of *p14ARF* and *P16* promoter region using methylation-specific PCR (MSP).
A: *P14ARF*; lanes 1-4 positive methylated PCR products (122 bp); M: 50bp ladder; lanes 5-8 unmethylation-positive PCR products (132bp).
B: *P16*; lanes 1-4 positive methylated PCR products (151 bp), M: 50bp ladder, lanes 5-8 unmethylation- negative.

Table 2. Relationship of aberrant hypermethylation in endometrial cancer (EC) to clinicopathological parameters.

Parameters (n)	Blood n (%)		Tissue n (%)	
	p14ARF	*p16INK4a*	*p14ARF*	*p16INK4a*
Total (28 tissue, 26 blood)				
Age (yr)				
<50 (4)	2 (50)	4 (100)	3 (75)	3 (75)
>50 (24)	14 (63.6)	19(86.4)	16 (66.7)	20 (83.3)
	P=0.6	*P*=0.4	*P*=0.7	*P*=0.6
Menopausal status				
Premenopausal (4)	2 (50)	4 (100)	3 (75)	3 (75)
Postmenopausal (24)	14 (63.6)	19 (86.4)	16 (66.7)	20 (83.3)
	P=0.6	*P*=0.4	*P*=0.7	*P*=0.6
Tumor grade				
G1 (18)	11 (64.7)	15 (88.2)	16 (88.9)	16 (88.9)
G2 (5)	3 (75)	1 (25)	4 (80)	4 (80)
G3 (5)	2 (40)	3 (60)	4 (80)	2 (40)
	P=0.5	*P*=0.7	*P*=0.7	*P*=0.06
Tumor stage				
IA (17)	11 (68.7)	14 (87.5)	12 (70.5)	14 (82.3)
IB (2)	1 (50)	2 (100)	2 (100)	2 (100)
II (4)	1 (50)	1 (50)	2 (50)	3 (75)
IIIA (3)	1 (33.3)	3 (100)	2 (66.6)	3 (100)
IIIB (2)	1 (50)	2 (100)	1 (50)	1 (50)
	P=0.8	*P*=0.4	*P*=0.6	*P*=0.7
Histologic type				
Endometrioid type (25)	14 (60.8)	21 (91.3)	17 (68)	21 (84)
Nonendometrioid type (3)	1 (33.3)	3 (100)	2 (66.7)	2 (66.7)
	P=0.5	*P*=0.4	*P*=0.9	*P*=0.
Depth of myometrial invasion				
Negative (3)	1 (33.3)	1 (33.3)	2 (66.7)	2 (66.7)
<50% (15)	12 (92.3)	12 (92.3)	14 (93.3)	12 (80)
>50% (10)	9 (90)	9 (90)	9 (90)	7 (70)
	P=0.03	*P*=0.12	*P*=0.3	*P*=0.2
Metastasis				
Negative (19)	1 (33.3)	14 (82.4)	13 (68.4)	16 (84.2)
Positive (9)	6 (66.7)	9 (100)	6 (66.7)	7 (77.8)
	P=0.6	*P*=0.1*P*	*P*=0.9	*P*=0.6

n: number; (%): percent

methylation status and clinicopathologic characteristics were assessed using Pearson's chi-square test. The association between hypermethylation of the genes and risk of EC was estimated by computing odds ratios (ORs) and 95% confidence intervals (CI) using the chi-square test and Fisher's exact test. *P*-values less than 0.05 were considered statistically significant.

Results

We investigated the patients' clinicopathological characteristics. Pathological diagnosis showed that 3 patients had serous papillary and 25 had endometrial adenocarcinoma. According to the International Federation of Gynecology and Obstetrics (FIGO) staging system, patients had the following tumor stages: IA (n=17), IB (n=2), II (n=4), IIIA (n=3), and IIIB (n=2). The majority of cases had stage IA, grade I adenocarcinoma and were postmenopausal. There was metastasis in 9 of 28 cases. A total of 85.7% of patients were in the >50 years age group, while 14.3% of patients were <50 years of age. The majority of patients were overweight and the depth of myometrial invasion was less than 50%.

Table 3. Relationship of aberrant hypermethylation in endometrial cancer (EC) to clinical parameters.

Parameters (n)	Blood n (%)		Tissue n (%)	
	p14ARF	*p16INK4a*	*p14ARF*	*p16INK4a*
Diabetes				
Negative (17)	8 (47.1)	9 (52.9)	8 (47.1)	9 (25.9)
Positive (11)	8 (88.9)	8 (88.9)	10 (90.9)	10 (90.9)
	P=0.03	*P*=0.06	*P*=0.01	*P*=0.03
Obesity				
Negative (9)	5 (44.4)	7 (55.6)	5 (44.4)	8 (66.7)
Positive (19)	13 (82.4)	16 (94.1)	16 (84.2)	17 (89.5)
	P=0.2	*P*=0.1	*P*=0.21	*P*=0.9
High blood pressure				
Negative (20)	12 (60)	17 (85)	12 (60)	16 (80)
Positive (8)	4 (66.7)	5 (83.3)	7 (87.5)	7 (87.5)
	P=0.7	*P*=0.6	*P*=0.1	*P*=0.6
Menstrual disorder				
Negative (25)	13 (56.5)	20 (87)	16 (64)	20 (80)
Positive (3)	2 (66.7)	3 (100)	3 (100)	3 (100)
	P=0.7	*P*=0.5	*P*=0.2	*P*=0.3
Hypothyroidism				
Negative (26)	14 (58.3)	22 (91.7)	17 (65.4)	22 (84.6)
Positive (2)	1 (50)	2 (100)	1 (50)	1 (50)
	P=0.2	*P*=0.6	*P*=0.21	*P*=0.6

n: number; (%): percent

Methylation analysis

We analyzed the methylation status of the promoter region for the *p16INK4a* and *p14ARF* genes in the blood samples (n = 26) and formalin fixed paraffin embedded (FFPE) samples (n=28) were collected from endometrial cancer patients that 25 of the patients' blood and FFPE samples were collected from the same patients simultaneously. Also, 22 normal samples were collected from healthy women. Analysis of the samples by MSP showed that the *p16INK4a* and *p14ARF* gene promoters in normal FFPE samples were methylated (m+/u–) in 32% (7/22) and 0.0% of samples. They were hemimethylated (m+/u+) in 54% (12/22) in both, and non-methylated in 14% (3/22) and 46% (10/22) of samples, respectively. The patient FFPE samples were methylated in 64% (18/28) and 36% (10/28) of samples, hemimethylated in 36% (10/28) and 61% (17/28), and non-methylated in 0.0% and 3% (1/28) of samples. These findings illustrated that hypermethylation of the *p16* ($P<0.0001$) and *p14* ($P<0.05$) genes significantly correlated with EC patients. Our results indicated that the *p16* and *p14* gene hypermethylations could play an important role in EC.

We compared the relationships of aberrant hypermethylation in blood and tissue samples. A total of 21/26 (81%) blood samples and 18/28 (64%) tissue samples were positive for *p16INK4a* methylation. Also 5/26 (19%) blood samples and 10/28 (36%) tissue samples had *p14ARF* promoter methylation. There was no statistically significant difference in the occurrence of *p16INK4a* or *p14ARF* promoter hypermethylation in blood ($P=0.177$) and tissue ($P=0.221$) from cancer patients. Evaluation of methylation status in the blood offered a non-invasive approach.

In this study, we measured the co-promoter methylation status of the *p14ARF* and *p16INK4a* genes in normal and cancer tissues. The results showed a significant association between the simultaneous methylation of *p14* and *p16* genes with endometrial cancer ($P=0.045$). This study showed that the methylation of these two genes (*p14* or *p16*) has positive influence on the methylation process of the other gene. In another words, the methylation of one gene stimulated the

methylation of another one.

We compared the clinicopathological parameters of EC with the frequency of *p14ARF* and *p16INK4a* gene promoter methylations (Table 2). Our results from this analysis indicated that hypermethylation of *p16* in patient tissue correlated with tumor grade in EC (P=0.06). Additionally, hypermethylation of *p14* in patients' blood samples correlated with depth of myometrial invasion in EC (P=0.03). The *p16INK4a* and *p14ARF* gene hypermethylations in blood and tissue were associated with diabetes in EC (Table 3). There was no significant correlation observed between hypermethylation in any genes with age, tumor stage, histologic type, and metastases in EC.

We evaluated the normal and patient groups in terms of obesity, hypertension and diabetes. The findings showed that obesity (P=0.01) and diabetes (P=0.027) were risk factors for EC.

Discussion

Several tumor suppressor genes in malignant cells are inactivated by aberrant DNA methylation in promoter CpG islands, which suggests that aberrant DNA methylation may lead to carcinogenesis.[20] In EC, detection of aberrant methylation of specific genes has been studied in sputum, blood, and urine samples.[21] Our results have shown that *p14ARF* and *p16INK4a* gene promoter methylations are associated with EC. Our data demonstrated that hypermethylation of the *p16* and *p14* genes correlated with an increased risk of EC and played a critical role in this cancer.

Numerous molecular modifications are established in tumor cells; for example, DNA mutations and DNA methylation are present in cell-free circulating DNA (circDNA) distributed from the tumor into the blood,[22] thus making circDNA an ideal applicant for a blood-based cancer analysis test.[23] Since DNA methylation is indicated within circDNA,[22] recognition of tumor-specific DNA methylation in patient blood is a reasonable method for a blood-based test. Aberrant circDNA methylation is present in most cancer types and under study for clinical use. In the current study, we have evaluated the methylation status of the *p16* and *p14* genes in EC in blood and FFPE samples. In addition, we compared blood methylation status with tissue in EC. No significant difference existed between *p16INK4a* and *p14ARF* promoter hypermethylation in blood (P=0.177) and FFPE samples (P=0.221). The methylation status of the blood sample could be an early, non-invasion diagnostic marker in EC.

Our data analysis showed a significant association between co-promoter methylation of the *p16INK4a* and *p14ARF* genes and EC in normal and cancer tissues (P<0.05). Both *p14ARF* and *p16INK4a* genes have been described as tumor suppressor genes that repress the proliferation of cells through the direct inhibition of cell cycle progression. According to the common position of these two genes, it can be concluded that methylation of these genes have a synergetic effect on each other's methylation.

Several studies have been conducted about the relationship between *p16* and *p14* gene methylation with EC. Medumi et al. reported that hypermethylation of *p16* had no significant influence on the carcinogenesis of EC in Japanese patients.[24] Guida et al., in a study in Italy, reported that hypermethylation of the *p16* gene had a significant increased frequency in EC,[25] but not benign lesions. Zhang et al. in a Chinese study reported that the frequency of promoter methylation in cancer tissues was 37% for *p16* and 57% for *p14*.[1] Di Domenico et al. observed a statistically significant association between *p16* gene methylation and EC (P<0.05).[26] Hu et al. conducted a meta-analysis on 261 EC patients and reported that the *p16* gene promoter hypermethylation significantly correlated with EC patients (P<0.0001).[27]

We observed 32% hypermethylation in the *p16* gene and 0.0% in *p14* in normal tissue. In comparison, there was a significantly increased hypermethylation rate in patient tissue for *p16* (64%) and *p14* (36%). Race and ethnicity play an important role in the DNA methylation pattern. This might account for the higher frequencies of *p16* and *p14* promoter hypermethylation found in the current study.

No relationship was reported between *p16* promoter methylation and clinicpathological characteristics and EC prognosis from 1999 to 2008.[28,29] Ignatov et al. reported the extent of promoter hypermethylation of 17.4% for the *p16* gene and indicated *p16* alterations were associated with the metastatic possibility of EC.[29] Zhou et al., in China, reported a statistical correlation with methylation of the *p16* gene to histological grade ($P<0.05$).[30] We observed a significant association between the *p16* gene in tissue with grade and *p14* gene promoter hypermethylation in blood with depth of myometrial invasion in EC. Many risk factors have been reported in the development of EC.[3] our findings in normal and patient cases have shown that diabetes and elevated weight are risk factors for the Iranian population.

We have assessed gene promoter methylation status with MSP-PCR, which can identify aberrant hypermethylation with a high grade of sensitivity with lesser amounts of DNA.[31] This technique can be used with numerous biological samples - sputum, plasma, and urine, and has been used to identify aberrant methylation of cancer related genes in endometrial cell samples.[21]

The key difference between epigenetic abnormalities such as DNA methylation and genetic anomalies is that epigenetic changes are alterable and do not contain changes in base sequence. Epigenetic data may result in important molecular targets for treatment.[31] The influence of 5-aza-2-deoxycytidine (AZA) on tumor growth inhibition has been detected in human EC xenografted in nude mice. The tumor inhibition rates were 79.10% in AZA ($P<0.01$).[4] This method may be used as a new target and hope for cancer treatment thought gene therapy. Demethylating agents such as 5-azacytidine and decitabine that inhibit DNMTs efficiently change expressions of previously silenced genes.

Conclusion

Epigenetic changes such as DNA methylation play an important role in carcinogenesis. *p16INK4a* and *p14ARF* promoter hypermethylations are associated with EC. Specific patterns of *p16INK4a* and *p14ARF* methylation may also be useful as predictive indicators or prognosticators of treatment response. In the future, the knowledge about epigenetic changes that occur in the human genome carcinogenesis may facilitate an early diagnosis and enable the introduction of new, more efficient methods of cancer treatment.

Acknowledgment

This work was supported by the Biology Research Center of Islamic Azad University, Zanjan, Iran. We would like to express our appreciation to Mahdiyeh and Firouzgar hospitals in Tehran, and Kosar and Pasteur hospitals in Qazvin for their collaboration. We are grateful to Dr. Farzam and Dr. Elmizade from Qazvin University of Medical Sciences for providing samples. We thank the patients for their participation.

Conflict of interest

No conflict of interest is declared.

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