RESEARCH ARTICLE

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The Relationship between the Methylation of Promoter Regions of Tumor Suppressor Genes *PTEN* and *APC* with Endometrial Cancer

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Abstract

Background: Endometrial neoplasms is one of the most typical gynecologic diseases with harmful effects. Promoter hypermethylation is an important mechanism of the inactivation of tumor suppressor genes in endometrial neoplasms. Epigenetic changes of the *PTEN* and *APC* genes have shown to be present in various cancers. Therefore, in this study, we have investigated the association between the promoter hypermethylation of *PTEN* and *APC* genes with endometrial neoplasms. **Methods:** For this study, 28 patients with endometrial neoplasms as well as 22 controls were studied. Analysis of the promoter methylation regions of *PTEN* and *APC* genes were performed by Methylation-Specific PCR. **Results:** The frequency of *PTEN* and *APC* genes promoter methylation was 28.57% and 17.86% in tumor tissues, and 11.54% and 3.85% in blood samples, respectively. We found a significant relationship between blood and tissue in *PTEN* methylation ($p = 0.0353$). Additionally, we determined a closely significant difference between normal tissue and tumor tissue of the *PTEN* gene ($p = 0.0787$) and blood and tissue samples of the *APC* gene in methylated promoter regions ($p=0.0623$). Furthermore, these results suggest that there is no significant relationship between the promoter methylation of *PTEN* and *APC* with clinical characteristics. **Conclusion:** DNA methylation deficiency is a well known highlighted factor in tumorigenesis, therefore the promoter hypermethylation of *PTEN* and *APC* can be indicated as a risk factor in endometrial neoplasms.

Keywords: Endometrial neoplasms, DNA methylation- *APC*- *PTEN*

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Introduction

One of the most common forms of cancers in females, which is usually recognized at early stages, is endometrial cancer (EC) (Markowska et al., 2014). Collected information has revealed that the prevalence of endometrial cancer with stage I and II was 73% and 10%, respectively (Trimble et al., 2005; Creasman et al., 2006). There are two variants of endometrial cancer with diversity in their clinicopathologic features. Type I tumors (endometrioid caners) are completely differentiated and estrogen-related. On the other hand, type II tumors (non-endometrioid cancers) are non-estrogen-related (Tao and Freudenheim, 2010). Major risk factors in endometrial cancer include the estrogen element, mismatch repair disorders, microsatellite instability, and epigenetic variation (Banno et al., 2014; Mutter et al., 1996). Similar to other types of cancer, endometrial carcinoma can be caused by the aggregation of genetic mutations (Feng et al., 2012).

Genomic DNA methylation depends on the addition of methyl groups at CpG sites by DNA methyltransferase. DNA methylation is normally visible in promoters and is important for gene expression (Muraki et al., 2009). The inactivation of tumor suppressor genes is one of the reasons of tumor formation (Herman and Baylin, 2003). Phosphatase and tensin homologue (*PTEN*) is a type of tumor suppressor gene that is in *10q23.31*. It is vital for the inhibition of cell migration. *PTEN* is a type of lipid 3-phosphatase and can moderate different types of cell-survival pathways (Waite and Eng, 2002). The PI3K–AKT pathway is negatively regulated by the *PTEN* protein. 34–55% of endometrial cancers are reported with mutations in *PTEN* (Risinger et al., 1997; Kong et al., 1997). The inactivation of *PTEN* in endometrial cancer could be investigated by promoter hypermethylation, instability of the protein, and a change in the regulation of the gene (Zhang and Yu, 2010). Many surveys have reported that 20% of type I endometrial cancers are methylated in the promoter region of the *PTEN* and *APC* genes (Macdonald et al., 2004; Salvesen et al., 2001). *PTEN* is a second messenger of phosphatidylinositol 3-kinase (PI3K) that negatively regulates serine/ threonine kinase Akt. The phosphorylation of Akt modifies the

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activities of many downstream proteins that regulate the growth of cells and inhibit apoptosis (Oda et al., 2005; Velasco et al., 2006; Dubrovska et al., 2009; Rychahou et al., 2008).

The adenomatous polyposis coli (*APC*) is a tumor suppressor gene that is located in 5q21-22 (Aoki and Taketo, 2007). Alterations of the *APC* gene has been reported in 80% of colon cancers (Powell et al., 1992; Nagase and Nakamura, 1993). The Wnt pathway is regulated by mutations in the *APC* and β-catenin genes that are also correlated with endometrial cancer (Schlosshauer et al., 2000; Kobayashi et al., 1999). Hypermethylation of the *APC* promoter has been reported in 20–45% of endometrial cancers (Banno et al., 2006; Yang et al., 2006). *APC* is shown to moderate β-catenin levels, whereas, inhibition of *APC* expression will lead to an increase in the levels of β-catenin, which induces the Wnt signaling pathway and eventually increases the transcriptional activity (Fearnhead et al., 2001; Behrens et al., 1996). The purpose of the present study is to evaluate the association between *PTEN* and *APC* promoter hypermethylation with endometrial cancer in the blood and tissue samples in endometrial cancer patients. Also, we aim to investigate the correlation between endometrial cancer and distinct clinical characteristics.

Materials and Methods

Research population

Twenty eight cancerous tissues along with associated clinopathological parameters and twenty six blood samples of the same individual with endometrial cancer were collected from patients who had been referred to the Mahdiyeh Hospital (Tehran, Iran), Firuzgar Hospital (Tehran, Iran), Kowsar Hospital (Ghazvin, Iran), and Pastour Hospital (Ghazvin, Iran), between 2014 and 2016. In addition, twenty-two normal tissues were sampled as the control group. Informed consent was signed by the patients. The average age was 65.5 years for both groups (range, 38–76 years). None of the patients had received chemotherapy. They were examined in the Biology Research Center of Islamic Azad University, Zanjan (Iran).The clinical diagnosis of endometrial cancer was in accordance with the criteria of the International Federation of Gynecology and Obstetrics (FIGO).The clinicopathologic traits were diagnosed by experted gynecologists.

DNA isolation and methylation-specific PCR

Tissue samples were cut into 10 μm segments and used for DNA isolation (Cat.NO.180134, QIAGEN Inc, Valencia, CA) and also genomic DNA was extracted from EDTA-blood samples using the cinnaClon Genomic DNA purification Kit (Cat.No.PR881612) following the manufacturer's instructions. Sodium bisulfite converts unmethylated cytosine to uracil. The effects of bisulfite were performed by the CpG genome DNA Modification Kit (Cat.No.59104, QIAGEN Inc, Valencia, CA) (Yari et al., 2016). Afterwards, the buffer BL/carrier RNA solution was used to increase extracted DNA levels (Cat. No.59104, QIAGEN Inc, Valencia, CA) (Gravina et al., 2015). Forward and reverse primers were designed for unmethylated and methylated promoter regions by Gene runner and meth primer software after obtaining gene sequences from Gene Bank (http://www.ncbi.nlm.nih.gov). The primer sequences are shown in Table 1. Amplification was carried by gradient PCR, using a model of PCR gradient thermo cycler (Eppendorf, Germany). MSP amplification was performed in a final volume of 20 μL containing 1X PCR Master Mix (CinnaGen PCR Master Mix, Iran), 50 ng of template bisulfite converted DNA, and 10 pmol of forward and reverse primers each (Gen Fanavaran, Iran). MSP amplifications was performed as follows: the first denaturation cycle of DNA at 95°C for 5 min followed by 34 cycles, each consisting of 45 s denaturation at 95°C, 45 s annealing at 59°C for *PTEN* (M), 57°C for *PTEN* (U), 54°C for *APC* (M), 57°C for *APC* (U), and 45 s extension at 72°C, with the final extension cycle of 72°C for 5 min. The PCR products were analyzed by the electrophoresis of agarose gel (2.5% agarose), stained with DNA safe stain and visualized by a UV transilluminator. For MSP confirmation and correct determination of methylated, hemimethilated, and non methylated genotypes, modified genomic DNA samples from regulatory CpG islands of both normal endometrium and cancerous tissue were analyzed by sequencing for both *PTEN* and *APC* genes.

Statistical analysis

The statistical comparison was calculated by Pearson's chi-square. The data analysis was performed by SPSS20. P-values <0.05 were statistically significant. The rates of odds ratio and 95% confidence intervals were assayed by logistic regression.

Results

Determination of clinicopathological features

The samples consisted of 25 endometrioid carcinomas [endometrial adenocarcinoma:19 FIGO grade 1 (stage IA, IB), 4 FIGO grade 2, 2 FIGO grade 3 (IIIB)], and 3 non-endometrioid carcinomas [serous papillary :3 FIGO grade 3 (stage IIIA)]. Nine of twenty-five (36%) endometrioid carcinomas showed metastasis. In addition, non-endometrioid carcinomas lacked signs of any metastasis.

Analysis of promoter methylation in PTEN and APC genes

Methylation specific PCR was used to assay the role of the methylation status of *PTEN* and *APC* promoters in endometrial cancer. The frequencies of *PTEN* and *APC* methylation in promoter regions have been shown in Table 2. Hypermethylation of *PTEN* was observed in 28.57% of tumor tissues and 4.54% of normal tissues (Figure 1, 3). The results were closely linked to being statistically significant between tumor tissues and normal tissues (OR=2.0765, 95% CI [0.9197-4.6887], p = 0.0787) (Table 2). There was a significant increase of *PTEN* methylation in patients' blood (11.54%) compared to tumor tissues (28.57%). There was a significant difference in the methylation of the *PTEN* promoter between the patients' blood and tumor tissues (OR=2.4377, 95% CI [1.0635-5.5876], p = 0.0353) (Table 2). Promoter methylation

Table 1. Primer Pairs Used for Methylation-Specific PCR

Primer	Primer sequence (5′-3′)	Size (bp)
PTEN promoter methylated (M)	F:GGT TTC GGA GGT CGT CGGC	19
	R:CAA CCG AAT ATT AAC TAC TAC GAC	24
PTEN promoter unmethylated (U)	F:TGG GTT TTG GAG GTT GTT GGT	21
	R:ACT TAA CTC TAA ACC ACA ACC	21
APC promoter methylated (M)	F:TAT TGC GGA GTT CGG GTC	18
	R:TCG ACG AAC TCC CGA CGA	18
APC promoter unmethylated (U)	F:GTG TTT TAT TGT GGA GTG TGG GTT	24
	R:CCA ATC AAC AAA CTC CCA ACA A	22

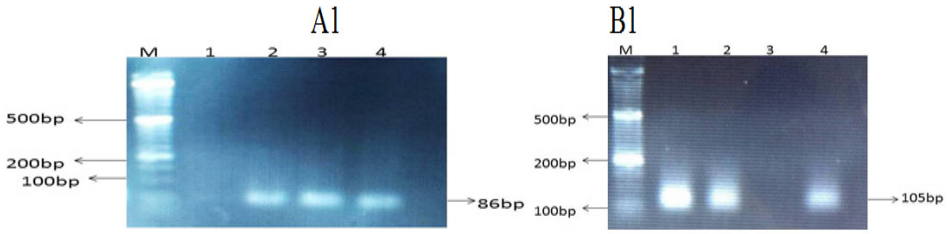

Figure 1. Methylation-Specific *PCR* Analysis of *PTEN* Promoter: A1: using unmethylated promoter primers; Lane M: 50bp ladder; Lane 1: absence of *PCR* products: methylated promoter; Lanes 2, 3, 4:unmethylated *PCR* products (86bp). B1: using methylated promoter primers; Lane M: 50bp ladder; Lanes 1, 2, 4: methylated *PCR* products (105bp); Lane 3: absence of *PCR* products: unmethylated *PCR* products.

was shown in the *APC* gene in 17.86% of tumor tissues and 4.55% of normal tissues (Figure 2, 3). The study showed that *APC* methylation was not significantly correlated between tumor tissues and normal tissues (OR=0.7073, 95% CI [0.2995-1.6703] , p = 0.4296) (Table 2). Among the patients' blood, the frequency of promoter methylation in the *APC* gene was 3.85% (Figure 2, 3). Results indicate that the promoter methylation analysis of the *APC* gene between tumor tissues and patients' blood is statistically significant (OR=0.4047, 95% CI [0.1563-1.0476] , p=0.0623) (Table 2). The association between the methylation of *PTEN* and *APC* promoters with clinicopathological features was analyzed in the endometrial cancer. We found no significant correlations between clinicopathological features, including age, tumor grade, tumor stage, histologic type, depth of myometrial

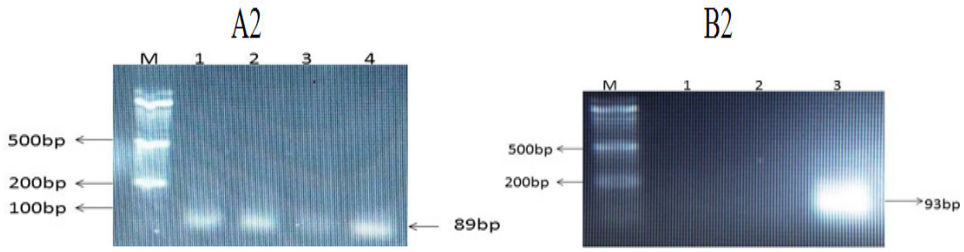

Figure 2. Methylation-Specific *PCR* Analysis of *APC* Promoter: A2: using unmethylated promoter primers ; Lane M: 50bp ladder; Lanes 1,2, 3, 4:unmethylated *PCR* products(89bp). B2: using methylated promoter primers ; Lane M: 50bp ladder; Lanes 1, 2: absence of *PCR* products: unmethylated *PCR* products; Lane 3: methylated *PCR* products(93bp).

Table 2.The Methylation Status of the Promoter Region of *PTEN* and *APC* Genes in the Study Population

Gene	Study population (number)	Methlyted number (%)	Hemi-methlyted number (%)	Non-methlyted number (%)	OR	95%CI	P-value
PTEN	Normal tissues (22)	1 (4.54%)	13 (59.10%)	8 (36.36%)			
	Tumor tissues (28)	8 (28.57%)	13 (46.43%)	7 (25%)	2.0765	0.9197-4.6887	0.0787
	Patients' blood (26)	3 (11.54%)	23 (88.46%)	0 (0%)	2.4377	1.0635-5.5876	0.0353
APC	Normal tissues (22)	1 (4.55%)	4 (18.18%)	17 (77.27%)			
	Tumor tissues (28)	5 (17.86%)	5 (17.86%)	18 (64.28%)	0.7073	0.2995-1.6703	0.4296
	Patients' blood (26)	1 (3.85%)	7 (26.92%)	1 (69.23%)	0.4047	0.1563-1.0476	0.0623

Table 3. Relationship between *PTEN* and *APC* Promoter Methylation and Clinicopathological Features in Endometrial Carcinoma Patients

Parameters	Blood samples		Tissue samples	
	Number of samples (%)		Number of samples (%)	
	PTEN	*APC*	*PTEN*	*APC*
Total (tissue:n= 28, Blood: n=26)				
Age (yr)				
<50 (4)	2 (40%)	1 (25%)	1 (25%)	2 (50%)
>50 (24)	12 (57.1)	3 (14.3%)	13 (54.2)	6 (25%)
	p=0.49	P=0.75	p=0.28	p=0.31
Tumor grade				
G1 (19)	9 (52.9%)	3 (17.6%)	9 (50%)	3 (16.7%)
G2 (4)	3 (75%)	1 (25%)	3 (60%)	2 (40%)
G3 (5)	3 (60%)	1 (20%)	3 (60%)	1 (25%)
	P=0.72	p=0.95	p=0.88	p=0.53
Tumor stage				
IA (17)	9 (56.3%)	4 (25%)	9 (52.9%)	3 (17.6%)
IB (2)	1 (50%)	0 (0%)	0 (0%)	0 (0%)
II (4)	2 (66.7%)	1 (33.3%)	2 (50%)	1 (25%)
IIIA (3)	2 (66.7%)	1 (33.3%)	2 (67%)	2 (67%)
IIIB (2)	2 (100%)	0 (0%)	2 (100%)	0 (0%)
	P=0.81	p=0.81	p=0.37	p=0.30
Histologic type				
Endometrioid type (25)	13 (56.5%)	4 (17.4%)	13 (52%)	5 (20%)
Nonendometrioid type (3)	2 (66.7%)	1 (33.3%)	2 (66.7%)	1 (33.3%)
	p=0.74	p=0.51	p=0.63	p=0.60
Depth of myometrial invasion				
Negative (3)	2 (66.7%)	0 (0%)	2 (66.7%)	2 (66.7%)
<50 % (15)	8 (57.1%)	3 (21.4%)	7 (46.7%)	2 (13.3%)
>50 % (10)	6 (66.7%)	2 (22.2%)	5 (50%)	3 (30%)
	P=0.88	p=0.67	p=0.82	p=0.14
Metastase				
Negative (19)	10 (55.6%)	4 (22.2%)	11 (57.9%)	4 (21.1%)
Positive (9)	5 (62.5%)	1 (12.5%)	4 (44.4%)	2 (22.2%)
	P=0.74	P=0.56	P=0.50	P=0.94

invasion, and metastasis in endometrial cancer (P>0.05) (Table 3). In addition, the correlation between promoter hypermethylation of the *PTEN* and *APC* genes with clinical characteristics, including diabetes, high weight, high blood pressure, and menstrual disorder was evaluated. Results show there was no significant difference between clinical characteristics and promoter hypermethylation of the *PTEN* and *APC* genes in endometrial cancer (P>0.05) (Table 4).

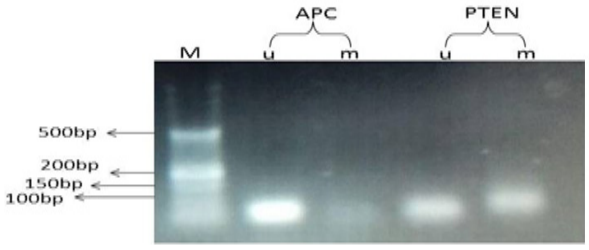

Figure 3. Methylation-Specific PCR Analysis of *APC* and *PTEN* Promoters: *APC* was unmethylated, *PTEN* was hemi-methylated; M, 50bp ladder ; u, reactions in unmethylated promoter primers; m, reactions in methylated promoter primers.

Discussion

Aberrant DNA hypermethylation of tumor-suppressor genes is a prevalent molecular change in the early stages of cancers and it can be highlighted as a biomarker in the detection and treatment of tumors (laird., 2003). Epigenetic deficiencies with promoter hypermethylation have an important role in the inactivation of tumor suppressor genes in cancer (Salvesen et al., 2001). This study investigated the methylation of the promoter regions of the *PTEN* and *APC* genes in endometrial cancer. Considering circulating tumor cells are present

Table 4. Association between Clinical Characteristics of the Study Groups with Eendometrial Cancer

Parameters	Blood samples		Tissue samples	
	Number of samples (%)		Number of samples (%)	
	PTEN	*APC*	*PTEN*	*APC*
Diabetes				
Negative (17)	8 (47%)	4 (23.5%)	9 (52.9%)	4 (23.5%)
Positive (11)	7 (63.6%)	2 (18.2%)	6 (54.5%)	2 (18.2%)
	p=0.390	P=0.736	p=0.934	p=0.736
High weight				
Negative (9)	5 (55.6%)	2 (22.2%)	4 (44.4%)	2 (22.2%)
Positive (19)	10 (52.6%)	3 (15.8%)	10 (52.6%)	9 (47.4%)
	P=0.885	p=0.678	p=0.686	p=0.203
High blood pressure				
Negative (20)	9 (45%)	3 (15%)	8 (40%)	4 (20%)
Positive (8)	6 (75%)	2 (25%)	6 (75%)	2 (25%)
	P=0.150	p=0.533	p=0.094	p=0.771
Menstrual disorder				
Negative (25)	20 (80%)	6 (24%)	15 (60%)	6 (24%)
Positive (3)	3 (100%)	2 (66.7%)	2 (66.7%)	1 (333%)
	p=0.393	p=0.122	p=0.823	p=0.724

in blood and bodily fluids, furthermore, the availability of patients' blood is more convenient compared to tumor tissues (Nagrath et al., 2007; Sharma et al., 2007). For this study, both blood and tissue samples were selected. Hemi-methylated promoter regions of *PTEN* was 88.46% in patients' blood, 46.43% in tumor tissues and methylated regions were 11.54% and 28.57% in patients' blood and tumor tissues, respectively. The results indicated that the frequency of hemi-methylated regions was higher than methylated regions. Our findings support a significant difference in the promoter methylation of the *PTEN* gene in the patients' blood and normal tissues (p = 0.0353). In contrast, the methylated promoter region of *APC* was 3.85% and 17.86% in patients' blood and tumor tissues, respectively and no significant difference was observed in patients' blood (p = 0.0623) and tumor tissues (p=0.4296). This discrepancy might be due to environmental effects and sample size. *PTEN* promoter hypermethylation is a broadly studied genetic mutation in endometrial cancer (Salvesen et al., 2001). Gao et al., (2009) reported that the expression of *PTEN* has decreased during the progression of endometrial cancer (P<0.01). They showed that *PTEN* expression is correlated to the tumor stage, tumor grade, and histologic type (P<0.05), but there was no significant difference in the depth of myometrial invasion (P>0.05). In our study, we observed a significant increase in the hypermethylation of *PTEN* in patients' blood. We found no relationship between clinicopathological features and endometrial cancer. Zuberi et al., (2014) showed that the frequency of *PTEN* promoter hypermethylation was 16% in the blood of patients with ovarian cancer and there was no significant difference in the *PTEN* methylation of the promoter between patients and controls (p=0.09). They found no significant correlations between clinicopathological features, including age, tumor stage, and histologic type, and patients type (P>0.05). In our estimate, the prevalence of *PTEN* methylation was 11.54% and 28.57% in patients' blood and tumor tissues, respectively. QI et al., (2014) showed that the frequency of *PTEN* promoter hypermethylation was 62% in tumor tissues of patients with cervical cancer where the methylation of the *PTEN* promoter was significantly related to the tumor grade, metastasis, and tumor stage (P<0.05). They found a significant association between the promoter methylation of *PTEN* and cervical cancer (P=0.042). They also suggested that promoter hypermethylation could be a key mechanism of *PTEN* inactivation in cervical cancer. We found a meaningful association between the hypermethylation of the *PTEN* promoter and endometrial cancer in patients' blood. The *APC*/β-catenin pathways were initially reported in the field of endometrial cancer (Aoki and Taketo, 2007; Moreno-Bueno et al., 2002). Guo et al., (2014) reported a significant association between the *APC* promoter methylation and non-small cell lung cancer (NSCLC) (OR=3.79, 95% CI [2.22 - 6.45], P < 0.0001). According to Ignatov (2010), 56.9% of endometrial carcinomas show *APC* promoter methylation in tumor tissues. They revealed that there was no relationship between histological type, histological grading, and methylation of the *APC* gene. However, there was a significant reverse relationship between metastasis and *APC* methylation (p= 0.002). In our study, promoter methylation in the *APC* gene was found in 3.85% and 17.86% of patients' blood and tumor tissues, respectively. Richiardi (2009), reported that *APC* hypermethylation had an important role in prostate cancer and there was a significant association between prostate cancer and *APC* promoter hypermethylation (OR=1.49, 95% CI [1.11 to 2.00], P =0.047). Our results closely linked a statistically significant difference in the promoter methylation analysis of the *APC* gene between tumor tissues and patients' blood. Moreover, we found

no significant relationship between clinicopathological features and *APC* methylation (P>0.05). Based on our study, promoter hypermethylation of *APC* and *PTEN* genes may be a biomarker in the diagnosis of endometrial cancer. More studies with larger populations and a suitable election of the case-control study will be essential to understand the role of hypermethylation of the *APC* and *PTEN* genes as biomarkers in endometrial cancer.

In conclusion, promoter methylation of tumor-suppressor genes is a typical epigenetic change in endometrial cancer. Hypermethylation of *PTEN* and *APC* in the promoter region may have a significant effect in the development of endometrial cancer. In our study, there was a significant relationship between blood and tissue in *PTEN* methylation (OR=2.4377, 95% CI [1.0635-5.5876], p = 0.0353). However, there was no evidence to support an association of promoter methylation of the *APC* gene in tumor tissues with endometrial cancer (OR=0.7073, 95% CI [0.2995-1.6703], p = 0.4296). Furthermore, there was a closely significant difference between the methylated regions in blood and tissue samples of the *APC* gene as well as normal tissue and tumor tissue of the *PTEN* gene (OR=0.4047, 95% CI [0.1563-1.0476], p=0.0623); (OR=2.0765, 95% CI [0.9197-4.6887], p = 0.0787), respectively.

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Conflict of Interest

The authors declare no conflict of interest.

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