

RESEARCH COMMUNICATION

Lack of Significance of the BRCA2 Promoter Methylation Status in Different Genotypes of the MTHFR a1298c Polymorphism in Ovarian Cancer Cases in Iran

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Abstract

Objective: Promoter methylation, which can be regulated by MTHFR activity, is associated with silencing of genes. In this study we evaluated the methylation status (type) of the BRCA2 promoter in ovarian cancer patients carrying different genotypes of the MTHFR gene (A or C polymorphisms at position 1298). **Methods:** The methylation type of the BRCA2 promoter was evaluated using bisulfate-modified DNA in methylation-specific PCR and the MTHFRa1278c polymorphism was assessed by PCR-RFLP. **Results:** Analysis of the BRCA2 promoter methylation type of cases showed that 7 out of 60 cases (11.7%) were methylated while the remaining 53 (88.3%) were unmethylated. In methylated cases, one out of the 7 cases had a CC genotype and the remaining 6 methylated cases had an AC genotype. The AA genotype was absent. In unmethylated cases, 34, 18, and one out of these had AC, AA and CC genotype, respectively. **Conclusion:** There was no significant relationship between the methylation types of the BRCA2 promoter in different genotypes of MTHFRa1298c polymorphism in ovarian cancer; $p=0.255$. There was no significant relation between the methylation types of the BRCA2 promoter in different genotypes of the MTHFRa1298c polymorphism in ovarian cancer.

Keywords: Methylation - ovarian cancer - tumor suppressor

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Introduction

Ovarian cancer is the most common cause of death from gynecological malignancies. In the early stages, women are generally asymptomatic or have non-specific symptoms, making early stage ovarian cancer difficult to diagnose (Permuth-Wey & Sellers, 2009; Swisher et al., 2009; Asadollahi et al., 2010). It is the ninth most common malignancy and the fifth most common cause of death from female cancers in the United States. In 2009, the American Cancer Society estimated that 21,550 women will be diagnosed with ovarian cancer. This disease often is sporadic (Holschneider & Berek, 2000; An et al., 2010). In ovarian cancer, at least 15 oncogenes, 16 candidate tumor suppressor genes and more than seven signaling pathways have been diagnosed. Aberrations in cell propagation, apoptosis and autophagy and alterations in cell sticking and motility all imply to disease progress and metastasis. Epigenetic events lead to changes in gene expression other than by means of DNA sequence alteration and include DNA methylation and histone modifications. From what is presently known, epigenetic have more and more become to be implied in the growth and progression of

ovarian cancer, and their gradual accumulation is related to advancing grade and stage of disease (Asadollahi et al., 2010).

DNA methylation at CpG sites in the regulatory region of a gene can alter gene expression, which has recently been considered an important characteristic of tumor growth and progression (Olek et al., 1996; Paige, 2003; Motoyama & Naka, 2004). DNA methylation refers to the covalent addition of a methyl group resulting from S-adenosyl-l-methionine to carbon 5 of the cytosine ring by DNA methyl transferases (Asadollahi et al., 2010). In the promoter of genes, aberrant methylation of CpG islands that often outcomes in atypical propagation or aberrant cell survival has been implicated as one of the main pathways involved in the progress of cancers, including ovarian cancer (Esteller et al., 2001; Widschwendter et al., 2001).

The importance of the role of unusual methylation in the development of cancer has become clear with the growing of genes that have been shown to be vulnerable to inactivation by promoter methylation (Costello et al., 2000; Kanai & Hirohashi, 2007). The BRCA2 protein is important in preserving genomic stability by interfere in repair of double-strand DNA breaks (Gudmundsdottir

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& Ashworth, 2006). Methylene tetrahydrofolate reductase (MTHFR) can catalyse the reduction of 5, 10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate. This product remethylates homocysteine to methionine which is a system used in the methylation of DNA. A polymorphism which changes an A to C at position 1298 (a1298c) in MTHFR is a reasonably common variant of the gene. Neoplastic cells increase DNA-methyltransferase activity suggesting that methylation imbalance may contribute to tumor evolution (Lin et al., 2004; Jakubowska et al., 2007).

Because of the main role that MTHFR plays in the methylation status of genes, we designed assays to investigate the methylation status of the BRCA2 promoter and investigated any potential correlation with the MTHFRa1298c polymorphism in ovarian cancer samples with a view that it may provide a basis for a new biomarker for both the identification of patients at risk and early diagnosis of ovarian cancer. If a correlation could be established, a significant benefit would be the analysis of MTHFRa1298c polymorphism in tissue or blood, potentially leading to a non-invasive cancer susceptibility or cancer screening test. The ultimate goal of these studies would be to develop assays which could be used to reduce the incidence and mortality of ovarian cancer.

Materials and Methods

Subjects

This study was undertaken as part of the completion of a PhD thesis in anatomy. It was done in Department of Anatomy and Molecular Biology of Isfahan University of Medical Sciences from 2009 to 2011. In this study, 60 samples of ovarian cancer and 60 matched adjacent normal ovarian tissue samples with müllerian origin from the same patient were chosen in pathology wards of Isfahan hospitals. The methylation status of BRCA2 was studied using methylation specific PCR for both the cancer tissues and the matched controls. Polymorphisms of the MTHFR gene (a1298c) were evaluated using PCR-RFLP.

DNA extraction

DNA was extracted from 5-10 µm thick paraffin-embedded tissue by adding lysis buffer (700 µl of 0.1M NaOH, 1% SDS and 10 pellets of chelex granules (Merk, Germany)) and incubated in boiling water for 20-40 minutes and subsequently centrifuged at 12,000 rpm for 10 minutes. The liquid phase was transferred to a new tube and an equal volume of phenol, chloroform-isoamyl alcohol (1:1) was added and centrifuged at 12,000 rpm for 10 minutes. The aqueous phase was transferred to a fresh tube and mixed with an equal volume of isopropanol-3 M sodium acetate (10:1) and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded and 500 µl of 70% alcohol was added to the pellet. After mixing, the tubes were centrifuged at 14,000 rpm for 10 minutes and the aqueous supernatant was discarded. Tubes were incubated at room temperature until the pellets were dry and 50 µl of DNase free water was added to dissolve the DNA pellet.

The assessment of the MTHFR a1298c polymorphism

The PCR-RFLP method was used to determine the MTHFR a1298c polymorphism. The a1298c nucleotide substitution creates a novel MboII restriction site. PCR was performed using specific primers (forward: CTTTGG GGA GCT GAA GGA CTA CTA C

And reverse: CAC TTT GTG ACC ATT CCG GTT TG). The PCR mixture included 2.5-3 µl of template DNA, 10 mM tris-HCl (pH 8.3), 30 mM KCl, 1.5 mM MgCl₂, 1 units of recombinant Taq DNA polymerase, 250 µM of each dNTP and 1 µl of each primer, made up to a total of 20 µl by adding distilled water. The DNA amplification was performed using the following program: 5-min at 94 °C, 35 cycle of (94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s) and 5 minutes at 72 °C. 10 µl of PCR product was mixed with 10U of HinfI for digestion at 37 °C overnight. For the analysis of the MTHFRa1298c polymorphisms, a CC genotype produced 84, 31, 30 and 18 bp fragments, AC genotypes produced 84, 56, 31, 30, 28 and 18 bp fragments while the AA genotypes produced five fragments of 56, 31, 30, 28 and 18 bp. Digestion products were visualized after electrophoresis on a 3% agarose gel stained with ethidium bromide (Lin et al., 2004; Jakubowska et al., 2007).

Modification of DNA

Cytosine nucleotides were changed to uracil by bisulfate treatment using the EZ DNA methylation kit (Zymo Research, USA) according to the manufacturer's guidelines. In brief, this procedure modifies unmethylated cytosines to uracil nucleotides but does not modify methylated cytosine nucleotides.

Methylation specific PCR

Bisulfate-modified DNA was amplified with PCR specific primers (Table 1) that distinguish methylated (M) and unmethylated (U) DNA. Amplicon is 337 and 250 bp in length for unmethylated and methylated BRCA2, respectively. Contents of the PCR mixture included 2.5-3 µl of bisulfate treated template DNA, 10 mM tris-HCl (pH 8.3), 30 mM KCl, 1.5 mM MgCl₂, 1 units of recombinant Taq DNA polymerase, 250 µM of each dNTP and 1 µl of each primer, made up to a total of 20 µl by adding distilled water. Thermocycling program is 94 °C for 5 min. followed by 35 cycles (94 °C for 30 seconds, 62 °C for methylated primer and 56 °C for unmethylated primer for 30 seconds, 72 °C for 30 second and 72 °C for 4 minutes. Fully unmethylated and methylated DNA after bisulfite conversion were used as unmethylated and

Table 1. Methylated and Unmethylated Specific Primers Used for the Evaluation of the BRCA2 Promoter

Gene	Primer	Size	Annealing
BRCA2 Methylated- specific	F: gacggttgggatgtttgataagg	250 bp	62°C
	R: aatctatcccctcacgcttctcc		
BRCA2 Unmethylated-specific	F: aggggtgtttgggattttaagg	337 bp	56°C
	R: tcacactctcccacaacaacc		

methylated controls. PCR products were analyzed on a 2% agarose gel (Hilton et al., 2002; Varinderpal, 2004). The methylation status of the BRCA2 promoter in the different genotypes of MTHFR was assessed and the results are shown in Table 2 (The table shows methylation status of the BRCA2 promoter compared to the MTHFR polymorphism (AA, AC or CC). "Methylation" indicates those patients that had no detectable methylation in the BRCA2 promoter taken from normal tissue however; the tumor sample from the same patient was methylated. "Unmethylated" indicates a change in the promoter from methylated, in normal tissue, to unmethylated in the tumors sample.)

Finally, Statistical analyses, including chi-square tests, were performed using SPSS for Windows, version 16.0. All statistical tests were two sided, and P values less than .05 were considered statistically significant.

Results

Forty-seven of the 60 cases were unchanged in promoter methylation when comparing normal tissue to those taken from the same patients tumor, while 7 of them changed from unmethylated to methylated promoters and 6 cases changed from methylated to unmethylated in the promoter (Table 3) (Figure 1).

7 out of 60 cases (11.66%) were methylated and 53 out of these (88.33%) were unmethylated in promoter. In methylated cases, one out of seven cases had a CC genotype and the remaining 6 methylated cases had an AC genotype. The AA genotype was absent. In unmethylated cases, 34, 18, and one out of these had AC, AA, and CC genotypes, respectively (Table 2). There was no significant relationship between the methylation type of the BRCA2 promoter in different genotypes of MTHFRa1298c polymorphism in ovarian cancer; $p=0.255$.

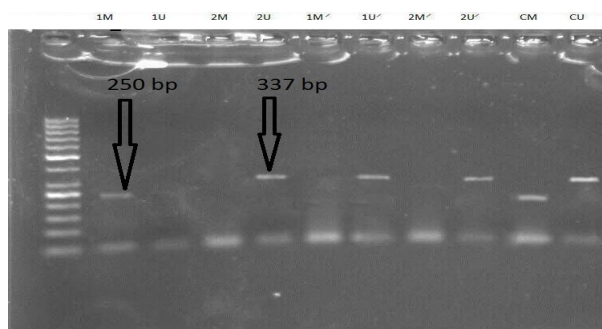


Figure 1. Methylated (M) and Unmethylated (U) BRCA2 Bound for Several Case and Control Samples. Positive Methylated and Unmethylated control (c) Exists as Well. Size of M and U Product is 250 bp and 337 bp

Table 2. Methylation Status of BRCA2 in Different Genotype Variants of the MTHFR Gene in Cases

Genotypes of MTHFR Polymorphism	Methylation statuses of BRCA2	
	Methylation	Unmethylation
AA	-	18
AC	6	34
CC	1	1

Table 3. Methylation Status of BRCA2 Promoter in the Assessed Cohort (n=60)

Variant	Unchanged methylation u/u m/m	Hypermethylation u/m	Hypomethylation m/u
	47	7	6
AGE:			
Upper of 50	28	-	4
Lower of 50	19	7	2

U/u, indicates the patient had an unmethylated control tissue and unmethylated tumor tissue. This was scored as an overall unchanged methylation pattern; M/m, indicates the patient had methylated control tissue and methylated tumor tissue. This was scored as an overall unchanged methylation pattern, U/m, indicates the patient had unmethylated control tissue however, the cancer tissue was methylated. This was scored as methylation, M/u indicates that normal tissue was methylated; however the tumor sample was unmethylated. This was scored as unmethylation

Discussion

ADNA methylation is a distinctive and important process because it involves the covalent modification of a cell's genetic substance. Methylation as a mechanism for gene inactivation can occur in some gene promoter tumors. MTHFR plays a central role in the metabolism of folate and has been implicated in the etiology of cancer via its effects on DNA methylation (Lin et al., 2004; Jakubowska et al., 2007).

We studied the methylation status of the BRCA2 promoter in patients with different genotypes of the MTHFR gene (A or C nucleotides at position 1298) because of the importance that this gene may have in the development of ovarian carcinogenesis. We speculate that in our specimens, the methylation of the BRCA2 promoter can lead to gene silencing and that this may be responsible for the development of cancer in at least some of our patients. It should be mentioned that inactivation of BRCA2 may occur by mechanisms other than promoter methylation and this may also influence expression in our cohorts.

Methylation status of the gene promoter shows different results in numerous investigations (Hilton et al., 2002; Paige, 2003). One factor influencing the results could be caused by contamination of specimens with cells from nearby tissues during analysis and isolation of tumor samples. Unmethylated DNA from the normal cells might compromise the results of the methylation levels of the tumor tissue (Hilton et al., 2002). We arrange to determine if differences in the BRCA2 promoter methylation could be correlated to different genotypes of the MTHFR gene in cases (a1298c polymorphism). No significant correlation was observed between the different genotypes of MTHFR and the methylation type of the BRCA2 promoter. However, we observed the different genotypes of the MTHFR gene we found no correlation with changes in BRCA2 promoter methylation. Therefore, we concluded other factors affect the rate of promoter methylation of BRCA2 in the cohort studied here.

In conclusion, there was no relation between

methylation types of the BRCA2 promoter in different genotype of MTHFRa1298c polymorphism in ovarian cancer.

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