

BRCA1 Promoter Methylation Status in Ovarian Cancer

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DOI: 10.1309/LM36TEMLUWNZJOTZ

Abstract

Objective: To evaluate the methylation status of the breast cancer 1, early onset (*BRCA1*) promoter in ovarian cancer in Iranian patients.

Methods: The *BRCA1* promoter methylation status of tissue from 60 patients with Müllerian-type ovarian cancer and matched

benign ovarian tissue from the same patients was evaluated using bisulfate-modified DNA in methylation-specific polymerase chain reaction (MSP CR) assays.

Results: Analysis of *BRCA1* promoter methylation status showed that 8 of 60 cases (13%) were methylated and 11 of 60 cases (18%) were unmethylated.

Conclusion: Methylation of the *BRCA1* promoter may be a risk factor or cause of ovarian cancer.

Keywords: ovarian cancer, tumor suppressor, methylation

Ovarian cancer is the sixth most diagnosed cancer in women worldwide and is the most lethal gynecological malignant neoplasm.¹⁻³ Its prevalence varies extensively among geographic regions and ethnic groups; of note, it is highly prevalent in northern Europe and the United States. Ovarian cancer can be successfully treated in its early stages; however, due to the lack of early biomarkers and screening, more than two-thirds of patients are diagnosed as having the advanced stage of the disease.⁴ Most cases are sporadic; only 5% to 10% of ovarian cancers are familial.⁵

Despite the elevated incidence and fatality rates of ovarian cancer, its etiology is poorly understood. Epigenetic alterations, age, and family history of the disease are risk factors.¹ Most variants of this cancer are epithelial and arise from the surface of the ovary. Other types develop from the ova (ie, germ cell tumors) or the supporting tissue cells (ie, sex cord/stromal type).^{6,7} Abdominal pain or back pain and unusual vaginal bleeding are common but nonspecific symptoms in women with ovarian cancer.⁸⁻¹⁰

Inherited germline or somatic mutations within tissue may influence the expression and activity of tumor suppressor genes or enhance the function of proto-oncogenes.^{11,12} Carcinogenesis can also result from alteration in genomic DNA methylation, including hypermethylation or hypomethylation of the promoter.^{13,14} Inactivation of tumor suppressor genes often results in abnormal proliferation or aberrant cell survival,

resulting in malignant transformation.¹³⁻¹⁵ DNA methylation at CpG sites (CpG sites or CG sites are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases along its length) in the promoter region of a gene can alter the gene and protein expression; recent research¹³⁻¹⁵ has indicated this alteration to be a significant characteristic of tumor development and progression.

Inactivation of tumor-suppressor genes, such as breast cancer 1, early onset *BRCA1* (GenBank accession no. U14680; OMIM# 113705) due to methylation of CpG islands has been implicated as one of the major pathways involved in the progression of cancer.⁴ Low expression of *BRCA1* protein may occur through promoter methylation; a previous study showed that promoter methylation of *BRCA1* usually leads to decreased expression of its protein in ovarian tumors.¹⁶⁻¹⁸ The *BRCA1* protein is important in preserving genomic stability by ensuring high-fidelity repair of double-strand DNA breaks. This process is facilitated by arresting the cell cycle to facilitate DNA repair so that daughter cells inherit faithful copies of DNA. Failure to correctly regulate cell cycle checkpoints often causes cells to accumulate genetic alterations and chromosomal abnormalities.¹⁹

The incidence of ovarian cancer varies among different geographic regions and racial groups, which may reflect alterations in the methylation status of tumor suppressor genes. Therefore, we designed assays based on the information in a previous study²⁰ (The frequency of *BRCA1* promoter methylation as an epigenetic means of *BRCA1* inactivation was determined for a large, population-based cohort of ovarian cancer patients. *BRCA1* promoter methylation was observed in some ovarian tumors) to investigate the methylation status of *BRCA1* in ovarian cancer samples from Iranian patients. We believe that doing so may establish a new biomarker for the identification of patients at risk and for early diagnosis of ovarian cancer through analysis of tissue containing Müllerian-type embryologic origin. Researchers could then use this information to develop assays that could be used to reduce the incidence and mortality of ovarian cancer.

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Abbreviations

BRCA1, breast cancer 1, early onset; PCR, polymerase chain reaction; MSP, methylation-specific

Material and Methods

Subjects

We obtained 60 tissue samples from ethnic Iranian women with Müllerian-type ovarian cancer and 60 matched control samples taken from the healthy adjacent ovarian tissue of the same patients. Paraffin-embedded tissue sections were examined by a pathologist (Ardeshir Talebi, MD) to confirm the presence of ovarian cancer and its stage. The methylation status of *BRCA1* was studied using methylation-specific polymerase chain reaction (PCR) for the cancer-tissue cases and the matched controls.

DNA Extraction

DNA was extracted from 5- μ m-thick paraffin-embedded tissue by adding lysis buffer (700 μ L of 0.1M sodium hydroxide, 1% sodium dodecyl sulfate, and 10 pellets of Chelex 100 resin granules [Bio-Rad Laboratories, Hercules, CA]); the DNA was incubated in boiling water for 20 to 40 minutes and subsequently centrifuged at 12,000 rpm for 10 minutes. The liquid phase was transferred to a new tube; the same volume of phenol-chloroform and isoamyl alcohol (1:1) was then added and centrifuged at 12,000 rpm for 10 minutes. The aqueous phase was transferred to a fresh tube; it was then mixed with an equal volume (ie, 3M) of isopropanol-sodium acetate (10:1) and centrifuged at 14,000 rpm for 10 minutes. The supernatant was discarded; 500 μ L of 70% alcohol was then added. After mixing their contents, the tubes were centrifuged at 14,000 rpm for 10 minutes; the alcohol was then discarded. Tubes were incubated at room temperature until the pellets were dry; 50 μ L of DNase-free water was added to dissolve the DNA pellet.

Modification of DNA

Cytosine nucleotides were changed to uracil by bisulfate treatment using the EZ DNA Methylation Kit (Zymo Research, Irvine, CA) 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

Methylation-specific (MSP) PCR is sensitive and specific for methylation of virtually any block of CpG sites within a CpG island. The frequency of CpG sites in CpG islands renders this technique useful and extremely sensitive for such regions. DNA not treated with bisulfate (ie, unmodified) failed to amplify with either set of methylated- or unmethylated-specific primers. Bisulfate-modified DNA was amplified with PCR-specific primers (Table 1) that distinguish methylated and unmethylated DNA.²⁰ These primers amplify a 182-bp product.^{20,21} The PCR was carried out in a 25 μ L mixture containing 10 mM tris hydrochloride (pH, 8.3), 50 mM potassium chloride, 1.5 mM magnesium chloride, 5 units of recombinant Taq DNA polymerase, 200 μ M of dNTP, 0.6 μ M of each primer, and 2.5 μ L of bisulfate-treated template DNA.

For methylated (ie, specific for methylated DNA) primers, the PCR amplification protocol used was 94°C for 5 minutes for 1 cycle and 40 cycles of 94°C for 15 seconds, 65°C for 30 seconds, and 72°C for 30 seconds. The amplification protocol for unmethylated (ie, specific for unmethylated DNA) primers was 94°C for 5 minutes for 1 cycle and 35 cycles of 94°C for 15 seconds, 61°C for 30 seconds, and 72°C for 30 seconds. Fully methylated DNA and DNA derived from the lymphocytes of

healthy individuals were used as positive controls for the methylated and unmethylated reaction, respectively. Polymerase chain reaction products were analyzed in a 2% agarose gel matrix.²⁰

Results

The results of methylation status studies of the *BRCA1* promoter in diseased and healthy ovarian tissue samples revealed that 41 of the 60 cases were unchanged regarding promoter methylation status, 8 cases changed from unmethylated to methylated in the promoter, and 11 cases changed from methylated to unmethylated in the promoter (Table 2; results from 2 such patients are shown in Figure 1). No significant relationship was observed between the methylation status of case and controls per χ^2 analysis ($P = .45$). Most cases involving methylated promoters were observed in patients older than 50 years; however, the Fisher exact test demonstrated that the relationship between age and methylation status is not significant ($P = .45$).

Discussion

To decrease the risk of ovarian cancer, it is essential to understand and characterize the etiologic factors of the disease. We studied *BRCA1* promoter methylation in CpG islands because the importance of the gene has been well documented in ovarian cancer.²² *BRCA1* is a tumor suppressor gene important in the development and progression of ovarian cancer.²⁰ Methylation of promoters can influence the normal expression of genes; therefore, we investigated the methylation status of the *BRCA1* promoter region.

Table 1 Methylated- and Unmethylated-Specific Primers Used for Evaluation of the *BRCA1* Promoter^a

<i>BRCA1</i> Gene Type	Primer	Annealing Temperature
Methylated-Specific	Forward: ggtaatttagagtttcgagagacg	65°C
	Reverse: tcaacgaactcagccgcgcaatcg	
Unmethylated-Specific	Forward: ggtaatttagagtttcgagagatg	61°C
	Reverse: tcaacaaactcacaccacacaatca	

BRCA1, breast cancer 1, early onset.

^aEach product was 182 bp in size.

Table 2 Methylation Status of the *BRCA1* Promoter^a

	Unchanged Methylation Status ^b	Changed to Methylation ^c	Changed to Unmethylation ^d
Variant, No (%)	41 (68)	8 (13)	11 (18)
Age, y			
≥ 50	23 (38)	6 (10)	5 (8)
< 50	18 (30)	2 (3)	6 (10)

BRCA1, breast cancer 1, early onset.

^a $n = 60$. Percentages may not equal 100 because of rounding.

^b unmethylated control tissue and unmethylated tumor tissue; this was scored as an overall unchanged methylation pattern. Others had methylated control tissue and methylated tumor tissue. This was scored as an overall unchanged methylation pattern.

^c Unmethylated control tissue and methylated tumor tissue; this was scored as methylated.

^d Control tissue was methylated and tumor tissue was unmethylated; this was scored as unmethylated.

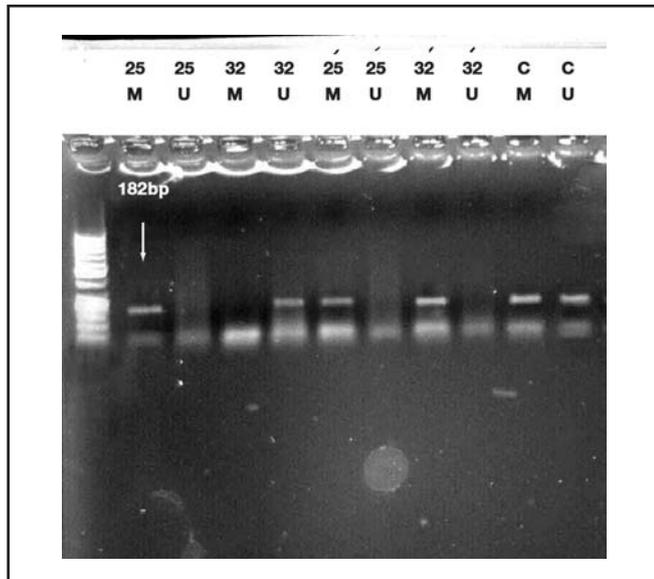


Figure 1 An ethidium bromide-stained gel of polymerase chain reaction (PCR) products showing analyses of methylated (M) and unmethylated (U) breast cancer 1, early onset (*BRCA1*) promoters. Samples from patients 25 and 32 are shown. The promoter of the *BRCA1* gene was tested in the tumor samples (lanes 25M, 25U, 32M, and 32U) and compared with healthy tissue extracted adjacent to the tumor (lanes 25'U, 32'M, and 32'U). In each case, the promoter was tested for methylation status using PCR primers specific to the methylated status (such as 25M) or primers specific to the unmethylated status (such as 25U).

CpG islands are typically found in the promoter or regulatory regions of genes. Normally, unmethylated CpG islands (ie, regions of rich CG content) are observed in the promoters of expressed genes, whereas methylated promoters are usually associated with genes with low or reduced transcriptional rates. However, the normal methylation status can be changed in neoplastic cells, possibly due to increased DNA-MTase activity and/or through local shielding mechanisms. Hypomethylation of regulatory DNA sequences can sometimes activate transcription of proto-oncogenes,¹³⁻¹⁵ potentially giving them oncogenic function; this can occur after neoplastic progression. Other promoters can become methylated in normal cells through the aging process. This latter alteration may promote susceptibility to neoplasia.^{2,14,25} Methylation, as a mechanism for gene inactivation, has been speculated to occur in some *BRCA1* tumors. However, it should be noted that *BRCA1* may also be inactivated post-translationally by aberrant phosphorylation or other modifications.²²

The current study investigated the methylation status of the *BRCA1* promoter in tumor samples by comparing them with that of benign tissue obtained from a region adjacent to the tumor lesion. When the methylation status of the case (ie, cancerous sample) and control (ie, patient-matched sample) was the same, the methylation status of cases was scored as unchanged methylation. Forty-one of 60 cases displayed unchanged methylation status. When the promoters of the case and control samples were methylated and unmethylated, respectively, the methylation status was scored as methylated. Eight of the 60 cases were scored as having displayed methylation in the promoter. In previous reports, the *BRCA1* promoter was methylated in 12 of 81 ovarian tumors¹⁴ and 15 of 30 ovarian tumors,²² respectively. As reported by a previous study,²⁰

BRCA1 promoter methylation was observed in some ovarian tumors, methylation of the *BRCA1* promoter is considered an epigenetic factor that can lead to gene silencing in ovarian and other cancer types; this may be a contributing factor to the development of ovarian cancer.

Of note, inactivation of *BRCA1* may occur via mechanisms other than promoter methylation; this occurrence may also have influenced expression in our cohort. Investigation of the methylation status of the *BRCA1* promoter produced different results in other reports.^{4,22} Several factors may be responsible for these differences. For example, the methods used for the evaluation of methylation status were different between the reported investigations; however, it is undetermined whether different procedures can yield different results within the same tumor. One factor influencing the results could be contamination of specimens with cells from nearby tissue during the dissection and isolation of tumor samples. Unmethylated DNA from the healthy cells might compromise the results of the methylation levels of the tumor tissue.

Methylation occurs in a stepwise process during tumor maturity and progression; therefore, higher methylation levels could be a result of more advanced tumor stages at the time of diagnosis. Thus, it is possible that the variation in methylation occurrence reported in the literature²² could be due to the variation in the distribution of cases and the stage of diagnosis.

Although the differences between the age groups examined in our study regarding the methylation status of the *BRCA1* promoter were not significant, methylation status analysis cannot be excluded as a marker of future susceptibility to ovarian cancer. Our study reports on a total of 60 ovarian cancer samples; age was not a factor in selecting patients for the study. In the future, larger studies with additional clinical parameters will be required to determine if a susceptibility gene or polymorphism is associated with *BRCA1*-promoter methylation and subsequent progression of ovarian cancer. LM

Acknowledgments: This study was undertaken as part of the fulfillment of a doctoral thesis (PhD) in anatomy (Ahmad Shabanizadeh). We thank all personnel from the anatomy and molecular biology groups involved in this study.

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