Promoter Methylation Status and Protein Expression of BRCA2 in Patients with Epithelial Ovarian Cancer

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Abstract

Background: Ovarian cancer is the leading cause of death among gynecological cancers. Changes in the methylation of BRCA1 and BRCA2 may be an effective mechanism for breast and ovarian cancer. This study evaluates the protein expression and methylation status of BRCA2 in Iranian patients.

Methods: We assessed 60 Mullerian-type ovarian cancers by methylation-specific PCR assays and immunohistochemistry.

Results: According to methylation status analysis, there were 7 of 60 (11.66%) methylated cases that had low protein expression.

Conclusion: We concluded that changes in the methylation status of BRCA2 cannot be used as an appropriate biomarker to ascertain the development of ovarian cancer.

Keywords: Ovarian cancer, Tumor suppressor, Methylation

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Introduction

Ovarian cancer is the main cause of death among gynecological malignancies. It is the sixth most diagnosed cancer in the world. The median age of patients with ovarian cancer is 60 years, and the average lifetime risk for women is about 1 in 70.[1-3] This cancer varies extensively in its prevalence among different

geographic regions and ethnic groups.[3,4] The majority of cases are sporadic and only 5%-10% of ovarian cancers are familial. Epigenetic alterations are one of the main risk factors for ovarian cancer.[4] Recent studies have shown that epigenetics play an important role in cancer biology, viral infections, and the activity of mobile elements.[5] Changes in mammalian genome methylation are a common epigenetic event. An epigenetic event is a covalent chemical modification that results in the addition of a methyl ($CH3$) group at the carbon 5 position of the cytosine ring. The human genome is not methylated uniformly and contains regions of un-methylated segments interspersed by methylated regions. In contrast to the remainder of the genome, smaller regions of DNA (called CpG islands) that range from 0.5 to 5 kb and occur on average of every 100 kb have distinctive properties. Approximately half of all human genes have CpG islands; these are present on both housekeeping genes and genes with tissue-specific patterns of expression.[5,6] DNA methylation at CpG sites in the promoter region of a gene can alter gene and protein expression, which has recently been considered a significant characteristic of tumor development and progression.[7-9] The inactivation of tumor-suppressor genes due to CpG island methylation has been implicated as one of the major pathways instigated during cancer progression.[10] Methylation is a key silencing mechanism of breast and ovarian cancer susceptibility BRCA1, however less is known about BRCA2.[11] Promoter methylation usually leads to a decreased expression of the protein.[12] The BRCA2 protein is important in preserving genomic stability by ensuring high fidelity repair of double-strand DNA breaks.[13]

The incidence of ovarian cancer varies among different geographic regions and racial groups, which may reflect alterations in methylation status of tumor suppressor genes. Therefore we designed assays based on previous studies[14] to investigate the methylation status of BRCA2 in ovarian cancer samples. We propose that BRCA2 may provide a

basis for a new biomarker for both the identification of patients at risk and early diagnosis of ovarian cancer through the analysis of Mullerian origin tissues. 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

In this study, we selected 60 samples of Mullerian ovarian cancer and 60 matched adjacent normal ovarian tissue samples from the same patient. The ovarian cancer specimens were provided as paraffin sections after examination and staging by a pathologist. The methylation status and protein expression of BRCA2 was studied using methylation specific PCR and immunohis-tochemistry for both the cancer tissue cases and matched controls.

DNA extraction

We extracted DNA from the 5 µm-thick paraffin-embedded tissue by the addition of new lysis buffer that consisted of 700 µl of 0.1 M NaOH, 1% SDS, and 10 pellets of chelexe granules (Merk, Germany), after which it was incubated in boiling water for 20-40 min, and subsequently centrifuged at 12000 rpm for 10 min. The liquid phase was transferred to a new tube and the same volume of phenol and chloroform-isoamyl alcohol (1:1) was added and
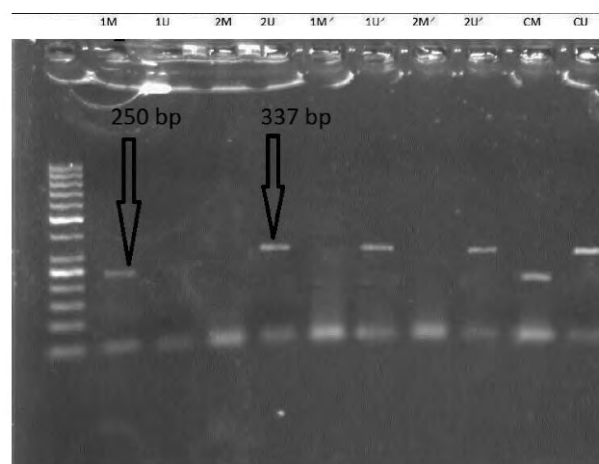

Figure 1. Promoter methylation of BRCA2. I Case group, promoter methylation (M) is 250bp and unmethylation (U) is 337bp. M' and U' are control group. CM and CU are positive control of methylated and unmethylated.

Table 1. Methylation status of BRCA2.

Variant		Number	Unchanged methylation u/u m/m	Hyper methylated u\m	Hypo methylated u\m	*P* value
Age		60	47(78.33%)	7(11.66%)	6(10%)	0.769
	Upper of 50	32	28(46.66%)	-	4(6.66%)	0.06
	Lower of 50	28	19(31.66%)	7(11.66%)	2.(3.33%)	

m/m (methylated control/methylated case); u/u (methylated control/methylated case); u/m (methylated control/methylated case); m/u (methylated control/methylated case)

centrifuged at 12000 rpm for 10 min. The aqueous phase was transferred to a fresh tube and mixed with an equal volume of 3 M (isopropanol–sodium acetate) (10:1) and centrifuged at 14000 rpm for 10 min. The supernatant was discarded and 500 µl of 70% alcohol was added. After mixing, tubes were centrifuged at 14000 rpm for 10 min and the alcohol was discarded. Tubes were incubated at room temperature until the pellets dried. Then we added 50 µl of DNase-free water to dissolve the DNA pellet.[15]

DNA modification

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 un-methylated cytosines to uracil nucleotides, but does not modify methylated cytosine nucleotides.

Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) is extremely sensitive and uniquely useful and specific for the methylation of virtually any block of CPG sites in a CPG island. DNA that was not treated with bisulfate (unmodified) failed to amplify with either set of methylated or un-methylated specific primers. Bisulfate-modified DNA was amplified with PCR specific primers that distinguished between methylated (M) and un-methylated (U) DNA. The methylated primer was as follows: forward: GACGGTTGGGATGTTTGATAAGG and reverse: AATCTATCCCCT-CACGCTTCTCC. The un-methylated primer was as follows, forward: AGGGTG-GTTTGGGATTTTTAAGG, and, reverse:

TCACACTTCTCCCAACAACAACC. These primers amplify 250-bp methylated and 337-bp un-methylated products (Figure1). PCR was performed in a 20 µl mixture that contained 14 µl H2O, 10 mM tris-HCl (pH 8.3), 30 mM KCl, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase, 250 µM of dNTP, 1 µl of each primer, and 2.5-3 µl of bisulphate-treated template DNA.

For methylated and un-methylated primers, the PCR amplification protocol used was as follows: 94°C for 5 min for one cycle, 35 cycles of 94°C for 30 s, 62°C for 30 second for methylated and 56°C for 30 second for un-methylated, 72°C for 30 s and 72 °C for 4 min. Fully methylated and un-methylated DNA was used as positive controls for the methylated and un-methylated reaction. PCR products were analyzed on a 2% agarose gel.[14-16]
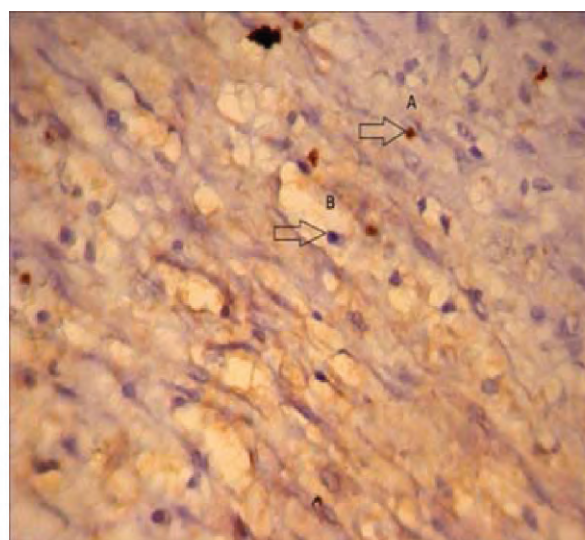

Figure 2. Immunuhistochemistry staining of BRCA2 . A brown color indicates protein expression (A) and blue is not (B).

Immunohistochemistry

We evaluated BRCA2 protein expression by immunohistochemistry technique, with H-300 antibodies to 60 epithelial ovarian cancers and 60 matched normal ovarian samples. We defined protein expression as follows: low (tumor cells with <10% nuclear staining) reduced (10%-30% nuclear staining), and normal (>30% nuclear staining).[17]

Results

In this study we evaluated both protein expression and the methylation status of the BRCA2 promoter in diseased and normal ovarian samples. The results revealed that 7 of the 60 (11.66%) cases were methylated in the promoter region (Table 1). Of the 60 sporadic epithelial ovarian cancers, 14 (23.33%) had loss,[18] (30%) cases had reduced, and 28 (46.66%) cases had normal BRCA2 protein expression (Figure 2). Of the 14 cases that had a loss of protein expression, 7 were methylated in the promoter region.

Discussion

To decrease the risk of ovarian cancer, it is essential to understand and characterize the etiologic factors of the disease. We have studied protein expression and the promoter methylation status of BRCA2 on CpG islands.

BRCA2 is a tumor suppressor gene that may be effective in suppressing ovarian cancer.

It has been proven that BRCA1 and BRCA2 methylation are altered in breast cancer. BRCA1 methylation status in ovarian cancer has been examined, however the BRCA2 gene is under review.

Therefore, we have researched the BRCA2 methylation status in this study. CpG islands are typically found in the promoter or regulatory regions of genes. Normally, un-methylated CpG islands (regions of rich CG content) are seen in the promoters of expressed genes, whereas methylated promoters are usually associated with genes with low or reduced transcriptional rates. However, 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 the transcription of proto-oncogenes,[7-10] potentially giving them oncogenic function. This can occur after the development of neoplastic progression. Other promoters can become methylated in normal cells throughout the aging process. This latter alteration may give rise to a susceptibility to neoplasia.[19] Methylation, as a mechanism for gene inactivation, has been proposed to occur in some BRCA2 tumors. However, it should also be noted that BRCA2 may also be inactivated post-translationally by aberrant phosphorylation or other post-translational modifications.[18]

This current study investigated the promoter methylation status of BRCA2 in tumor samples by comparing them with benign tissue from an area adjacent to the tumor lesion. When the methylation status of the case (cancer sample) and control (patient matched sample) were the same, the methylation status of the case was scored as 'unchanged methylation'. A total of 47 (78.33%) from 60 cases had unchanged methylation. When the promoter of the control was un-methylated and the case was methylated, we scored the methylation status of the case as 'methylated'. There were 7 (11.66%) cases that had methylation in the promoter region. Of the 60 sporadic epithelial ovarian cancers, 14 (23.33%) cases had loss, 18 (30%) cases had reduction, and 28 (46.66%) cases had normal BRCA2 protein expression. Protein expression decreased in 14 cases, 7 of which were methylated, which suggested that other mechanisms effect a transcriptional silencing of BRCA2. In the previous study, BRCA2 protein expression and promoter methylation status was assessed in 92 ovarian cancers. In those, 12 tumors lacked detectable BRCA2 mRNA, but the BRCA2 promoter was hyper-methylated in only one.[20,21] In the other study, out of 30 ovarian cancers 6 cases had low protein expression and 3 were hyper-methylated.[22]

Based on previous studies, BRCA2 promoter methylation status is not considered an epigenetic

factor that can lead to gene silencing in ovarian and other cancer types. This may not be a contributing factor to ovarian cancer development.[14]

In the current study, chi-square analysis demonstrated no significant relation between the methylation status of cases and controls ($P=$ 0.769). The majority of methylated cases were observed in patients younger than 50 years, but Fisher's exact test demonstrated no significant relation between patients older and younger than 50 ($P=$ 0.06). It should be mentioned that BRCA2 inactivation might occur by mechanisms other than promoter methylation, and this possibly influenced expression in our cohorts.

The methylation status of the BRCA2 promoter has shown different results in various investigations.[10] Several factors may be responsible for these differences. For example, the methods used to evaluate methylation status vary among investigations. Methylation occurs in a step-wise process during tumor maturity and progression, therefore higher methylation levels could result in more advanced tumor stages at the time of diagnosis. Thus, it is possible that the variation in methylation occurrence that has been reported in the literature could be due to the variation in the distribution of cases and the stage of diagnosis.[18]

Based on our results and those of previous studies, it can be concluded that alteration in the methylation of BRCA2 may not be a risk factor for ovarian cancer development in this studied population, and thus it is not an appropriate biomarker for the early diagnosis of ovarian cancer.

Acknowledgements

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