Original Study

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Decreased Expression of Proapoptotic Genes *Caspase-8*- and *BCL2*-Associated Agonist of Cell Death (*BAD*) in Ovarian Cancer

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Abstract

The study was performed to evaluate the expression of some proapoptotic genes for early prognosis of ovarian cancer. Twenty-four fresh frozen ovarian tumor and 9 normal ones were considered for real-time polymerase chain reaction. *CASP8* and *BAD* genes were decreased in tumoral tissues. Downregulation of *CASP8* and *BAD* genes in ovarian cancer may be an important cause for ovarian cancer.
Background: Ovarian cancer as the most lethal gynecologic malignancy in women is poorly detected during early stages of carcinogenesis. Therefore, there is an emergent need to look for specific and sensitive biomarkers for early diagnosis of ovarian cancer. **Materials and Methods:** In this study, we performed real-time polymerase chain reaction (PCR) to evaluate the expression of six proapoptotic genes, *CASP8*, *BAK*, *APAF1*, *BAX*, *BID*, and *BAD*, which contain CpG islands in their promoter regions. Afterward, the significantly downregulated genes were investigated by HpaII-PCR and methylation-specific PCR (MSP) to determine the methylation status between tumoral and adjacent normal tissues. **Results:** The real-time PCR results in 24 tumoral and 9 normal adjacent tissues showed decreased expression of *CASP8* and *BAD* genes in tumoral relative to normal samples. Furthermore, the methylation analysis showed no significant methylation between tumoral and normal samples. **Conclusion:** Taken together, this could be concluded that downregulation of *CASP8* and *BAD* genes in ovarian cancer may be as important causes for ovarian cancer carcinogenesis via inducing resistance to apoptosis; however, the downregulations are not due to promoter hypermethylation.

Introduction

Ovarian cancer is the most lethal gynecologic cancer and the seventh leading cause of cancer related death in females worldwide.[1] The epithelial ovarian cancer (EOC) that makes up to 90% of all human ovarian cancers arises from the modified peritoneal mesothelium that covers the surface of the ovary. Because of the lack of either specific symptoms or early detection strategies, ovarian cancer is the most deadly malignancy in women.[2] More than 70% of patients with ovarian cancer are being diagnosed at stages III and IV, and 20% to 35 % of them will have 5 year survival rates, whereas in the case of detection in stage I, up to 90% of patients can be cured with chemotherapy.[2,3]

Currently, transvaginal sonography and biochemical serum markers such as CA125 have been investigated for early detection of ovarian cancer.[4] However, the high rate of false positives makes the CA125 an unsuitable biomarker for EOC diagnosis.[5] In addition, from medical surgery perspective, there is growing evidence about laparoscopic surgery benefits for management of early stage ovarian cancer that by overcoming some limitations can be considered as a new and beneficial technique for treatment of ovarian cancer but with much less postsurgical complications.[6] Interestingly, long term follow up after ovarian cancer laparoscopy has revealed a promising result that would be a new era for ovarian cancer therapy.[7]

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Obviously, early detection of ovarian cancer could significantly improve the survival rate of women at risk of developing ovarian cancer; therefore, finding the sensitive and specific biomarkers for noninvasive diagnosis of this cancer is highly demanded. Many efforts have been made for identification of molecular biomarkers which including gene expression variations, proteins, and DNA methylation markers. DNA methylation as an epigenetic mark which occurs at CG dinucleotides in CpG islands is considered as substantial and promising biomarker for early detection of different cancers including EOC.[8] Many genes and markers such as *TGFBI*, *IFFO1*, *HIN 1*, *CHFR*, *SPARC*, *THBS2*, and *RASSF1A* have been reported to be hypermethylated in ovarian cancer.[9 15]

Cancer as a complex disease that results from genetic and epigenetic modifications of tumor suppressor genes or oncogenes can be developed because of alterations of apoptosis signaling pathways.[16] The apoptosis or programmed cell death is initiated via 2 extrinsic and intrinsic pathways that have been proposed to be involved in different cancers. The extrinsic and intrinsic pathways are triggered by the death receptors on cell surface (TRAIL R1 and R2, FAS, and/ or TNF α) and mitochondria, respectively.[17] These pathways consist of many factors that have important roles in initiation and progression of apoptosis. Absence or downregulation of the proapoptotic genes could cause resistance to apoptosis and associated with different cancers.[18] The genes, *CASP8*, *BAK1*, *APAF1*, *BID*, *BAD*, and *BAX*, are essential factors of cell death, and deregulation of these genes is involved in different human malignancies and chemoresistance.[16,19] These genes have CpG islands in their 5′ upstream flanking regions; thus, they are prone to be methylated and transcriptionally silenced. Therefore, the aim of this study was to determine the changes in gene expression of 6 important proapoptotic genes between tumoral and normal adjacent tissues of ovary and to look for DNA methylation in the promoter of the genes which were significantly downregulated.

Materials and Methods

Patients and Specimens

Twenty four fresh frozen ovarian tumor and 9 normal adjacent tissues were provided by the Iran National Tumor Bank (INTB) which is funded by Cancer Institute of Tehran University. The normal tissues were dissected at least 6 cm away from tumor sites. The clinical data including histology, patient history, and family history of all samples were provided by INTB (Table 1). Furthermore, the informed consents were signed by the patients or close relatives.

DNA/RNA Extraction and cDNA Synthesis

The fresh frozen tissues were grinded in liquid nitrogen using mortar and pestle and then transferred instantly into lysis buffer of AllPrep DNA/RNA Mini Kit (Qiagen, Los Angeles, California) for following extraction based on manufacturer protocol. The quality and quantity of extracted DNA and RNA were measured via agarose gel electrophoresis and Biophotometer (Eppendorf, Hamburg, Germany). The cDNA was synthesized using 500 ng of total RNA and PrimeScript RT Reagent Kit (Takara Bio Inc, Shiga, Japan) regarding manufacture protocol.

Real-Time Polymerase Chain Reaction (PCR)

The proapoptotic gene expression levels between normal and adjacent normal tissues were measured by quantitative real time

Table 1	Clinicopathologic Features of Study Samples
Clinicopathologic Features	**Number (%)**
Age (years)	
≤50	15 (62.5)
>50	9 (37.5)
Site of Primary	
Ovarian	24 (100)
Other	0 (0)
Tumor Spreading	
Bilateral	7 (29.2)
Unilateral	17 (70.8)
Tumor Size (cm)	
≤10	10 (41.6)
>10	14 (58.4)
Histologic Subtype	
Mucinous	4 (16.7)
Serous	14 (58.3)
Clear cell	6 (25)
Grade/Differentiation	
G1 G2	6 (25)
G3 G4	7 (29.1)
GX	6 (25)
GB	5 (20.9)

PCR in a Rotor gene 6000 instrument (Corbett life science, Sydney, Australia) using SYBR Premix Ex Taq II (TAKARA, Japan). The total 20 μ reaction volume contained 10 μ SYBR Premix, 1 μ cDNA, 1 μ of forward and reverse primers, and 8 μ ddH$_2$O that was performed in 2 step real time PCR (95°C for 10 seconds and 60°C for 35 seconds). Relative gene expression changes between cDNA samples were determined using Relative expression software tool 9 (REST 9, Qiagen), in which the *GAPDH* gene was used as internal control. The primer sequences for real time PCR were extracted from PrimerBank Web site (http://pga.mgh.harvard.edu/primerbank/; Table 2).

HpaII/MspI-PCR

The HpaII methylation sensitive restriction enzyme that has 5′ CCGG 3′ cutting site in unmethylated cytosine status was performed to evaluate the methylation ratios between tumoral and normal samples. At first, 10 μg of extracted DNAs was digested using HpaII and MspI restriction enzymes separately; then, the PCR was carried out after digestion. The specific primer sets for *BAD* gene promoter regions were designed, wherein the primers flank one CCGG site sequence and amplicons have at least one HpaII/MspI cutting site. The primer sets were designed using Primer 3 software (http://bioinfo.ut.ee/primer3/), and include: F: 5′ CCCACGCTCCTCTCTCCTAT 3′ and R: 5′ GCTATGGGCGGAAGTTTTCT 3′.

Bisulfite Conversion and Methylation-Specific PCR (MSP)

Sodium bisulfite conversion of genomic DNA was carried out using EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). The

Table 2	The Primer Sets for Real-Time Polymerase Chain Reaction		
Gene Name	**Primer Sequence**	**Gene Name**	**Primer Sequence**
CASP8	F: AGAGTCTGTGCCCAAATCAAC	*APAF1*	F: AAGGTGGAGTACCACAGAGG
	R: GCTGCTTCTCTCTTTGCTGAA		R: TCCATGTATGGTGACCCATCC
BID	F: CCTTGCTCCGTGATGTCTTTC	*BAK*	F: ATGGTCACCTTACCTCTGCAA
	R: GTAGGTGCGTAGGTTCTGGT		R: TCATAGCGTCGGTTGATGTCG
BAD	F: CCCAGAGTTTGAGCCGAGTG	*GAPDH*	F: AAGGTGAAGGTCGGAGTCAAC
	R: CCCATCCCTTCGTCGTCCT		R: GGGGTCATTGATGGCAACAATA
BAX	F: CCCGAGAGGTCTTTTTCCGAG		
	R: CCAGCCCATGATGGTTCTGAT		

human control DNA containing both bisulfite converted methylated and unmethylated DNA sets (EpiTect PCR Control DNA Set, Qiagen, Hilden, Germany) was used for MSP control reactions. The specific primer pair for methylated and unmethylated DNA (Table 3) and PCR condition was extracted from previously published literature.[20]

Results

Patient Samples

The samples composed of 24 tumoral, 9 normal adjacent, and 9 normal with no diagnosed cancer. The patients aged between 22 and 71 years (mean = 49), with both unilateral (68%) and bilateral (32%) tumors. In addition, the tumor samples sizes were ranged between 4 and 20 cm and different grades and different TNM pathologic staging (Table 1).

Real-Time PCR Results

The real time PCR was carried out (Table 2), and the results showed downregulation of *BAD* gene in 9 tumoral samples relative to their normal adjacent controls. When we totally compared the relative expression of the genes between 24 tumoral and 18 normal tissues (9 normal adjacent samples and 9 normal noncancerous people), the *CASP8* gene showed significant decreased expression in tumoral compared to normal samples (Figure 1).

HpaII/MspI-PCR Result

The PCR after digestion with HpaII and MspI enzymes showed no significant methylation difference between tumoral tissues and normal samples (Figure 2). In this study, methylated and unmethylated digested and intact undigested DNAs were used as controls.

MSP Result

The MSP for *CASP8* gene promoter methylation (Table 3) was performed to verify our HpaII/MspI PCR results. The MSP assays
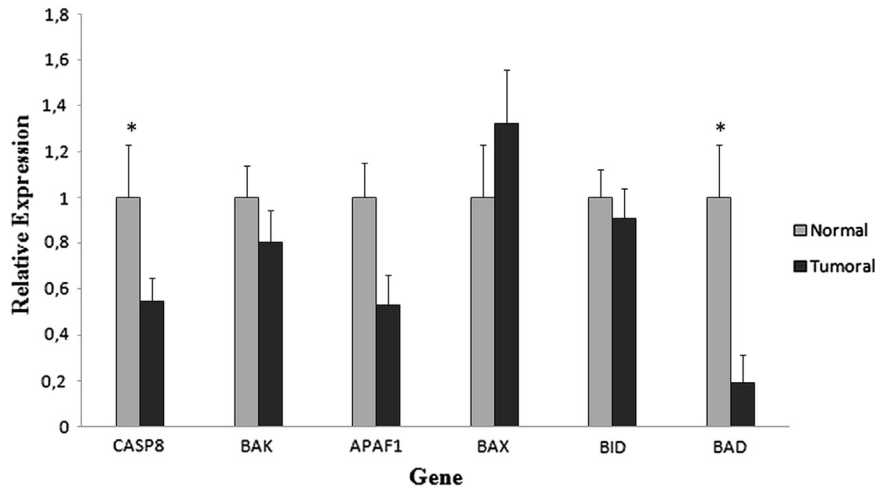
confirmed our result and showed no significant methylation in tumor samples compared to normal samples (Figure 3).

Discussion

Our findings suggest that downregulation of *CASP8* and *BAD* in ovarian tumoral versus normal adjacent samples might have possible roles in ovarian carcinogenesis. These 2 genes have roles in initiation and regulation of apoptosis. The protein encoded by *BAD* gene forms heterodimers with BCL xL and BCL 2 proteins and reversing their death repressor activity and therefore positively regulates and initiates cell apoptosis.[21,22] The function of BAD protein is regulated through its phosphorylation in some serine residues, in which modulate its protein–protein interactions and cellular localization.[23] On the other hand, the caspase 8 is activated after the formation of death inducing signaling complex (DISC) and cleaves different apoptotic proteins, resulting in execution of apoptosis.[24] This caspase is involved in both extrinsic and intrinsic pathways of apoptosis and acts via caspase 3 and Bid proteolytic cleavage. It was indicated that caspase 8 is inactivated in many human cancers, which may cause tumor progression and resistance to chemotherapeutics.[25] On the basis of our results that showed downregulation of *BAD* and *CASP8* in ovarian tumoral samples compared to normal tissues, we may explain that lack or decreased rates of Bad or caspase 8 proteins in ovarian epithelial cells could possibly prevent intrinsic and extrinsic apoptosis signaling pathways. Accordingly, the decrease or resistance to apoptosis in epithelial ovarian cells might be an important step in ovarian carcinogenesis.

In a previous study, it has been shown that overexpression of *BAD* inhibits the growth of A549 lung adenocarcinoma cells through inhibiting cell proliferation and inducing of apoptosis.[21] Another study reported that reduction of *BAD* expression by RNA interference leads to apoptosis prevention that suggests integration of *BAD* in the antiapoptotic effects of EGFR and PI3K pathways.[26] The decreased levels of phosphorylated BAD were shown during the development of hepatocellular tumorigenesis as well, which propose its important role in apoptosis and cancer.[27] In addition, other studies have shown the involvement of caspase 8 in ovarian apoptosis resistance. In a study on the ovarian cancer cell line model of acquired cisplatin resistance, it was shown that the increased caspase 8 protein level is involved in the initiation of apoptosis via sensitivity to rhTRAIL induced apoptosis.[28] Moreover, acquired rhTRAIL resistance in SW948 colon carcinoma cell line was shown to be caused by decreased ratio of procaspase 8/c FLIP.[29]
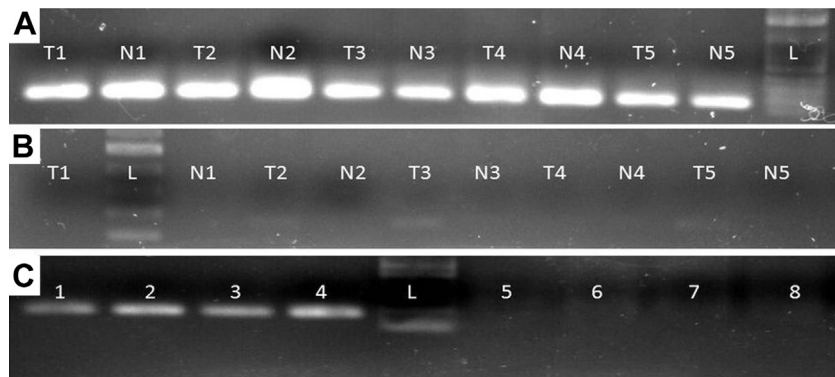
Table 3	Methylation-Specific Polymerase Chain Reaction Primer Sets for Methylated and Unmethylated *CASP8* Promoter	
Primer	**Sequence**	
Casp 8 methylated forward	TAGGGGATTCGGAGATTGCGA	
Casp 8 methylated reverse	CGTATATCTACATTCGAAACGA	
Casp 8 unmethylated forward	TAGGGGATTTGGAGATTGTGA	
Casp 8 unmethylated reverse	CCATATATCTACATTCAAAACAA	

Aberrant methylation as a suppressor mechanism of gene transcription was frequently reported in promoter regions of tumor suppressors in ovarian cancer.[30] In this study, we investigated the presence of DNA methylation in promoter regions of 2 genes, BAD and CASP 8, in which their expressions were shown to be decreased in tumoral samples compared to normal adjacent ovarian tissues. First, we performed methylation sensitive enzyme HpaII and following PCR to determine the possible methylation; then, the MSP was carried out to confirm our results. The results showed no significant DNA methylation in both investigated gene promoters. Promoter hypermethylation in *BAD* gene was reported only in myeloma,[31] whereas methylation of CASP8 gene has been reported

frequently in many cancers.[32 35] In a previous study on ovarian cancer by Ozdemir et al.,[30] it was reported that among 24 investigated tumor suppressor genes, promoter methylation of 3 genes, CDKN2B, CDH13, and RASSF1, was most frequent, and methylation in *CASP8* gene promoter reported very low in their samples. In another study by Yang et al., it was shown that different genes were methylated in 3 different gynecologic cancers: cervical, endometrial, and ovarian cancers. The *CASP8* gene was just methylated in endometrial cancer and was not methylated in ovarian cancer samples.[36] Thus, our result is in agreement with those previous observations that showed no significant promoter hypermethylation in *CASP8* and *BAD* genes in ovarian cancer.

Figure 3 Methylation-Specific PCR for Analysis of *CASP8* Gene Promoter Methylation Status. The UC and MC Represent Unmethylated and Methylated Control DNA; UT and UN Represent Investigated Tumor and Normal Samples Using Unmethylated Specific Primers; and MT and MN Represent Tumoral and Normal Samples Using Methylated Specific Primers. It Is Clear that There Is No DNA Methylation Exist in *CASP8* Gene Promoter in Ovarian Tumoral and Normal Samples
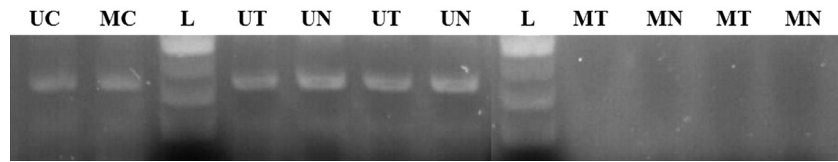
Consequently, the significant downregulation of these 2 genes might be due to other mechanisms such as promoter mutations, other epigenetic mechanisms, and/or upstream alterations of transcription cascade activation. Taken together, our study showed downregulation of *BAD* and *CASP8* genes in ovarian cancer tumoral samples compared to normal ovarian tissues, suggesting the important role for these proapoptotic genes during ovarian carcinogenesis.

Clinical Practice Points

What Is Already Known About This Subject?.

- Regarding our subject of study, it was previously shown that caspase 8 gene is inactivated in many human cancers, and this can lead to tumor progression and also causes resistance to chemotherapeutics. Besides, it was reported that decreased level of *BAD* expression in the cells using RNA interference can prevent apoptosis.

What Are the New Findings?.

- Our findings showed downregulation of *CASP8* and *BAD* genes in tumoral ovarian samples versus normal ones that could lead to decreased levels of Bad or caspase 8 proteins in ovarian epithelial cells. Thereby, this shortcoming could possibly prevent or decrease intrinsic and/or extrinsic apoptosis signaling pathways and has possible roles in ovarian carcinogenesis.

How Might It Impact on Clinical Practice in the Foreseeable Future?.

- The potential clinical impact of these types of findings from clinical research into clinical practice needs confirmatory studies using wider population and functional studies both in vitro and in animal models. The possible applications could be performing gene therapy using vectors expressing these downregulated genes to sensitize them to regular cell apoptosis or along with che motherapies. In addition, expression profiling of different types of tumors might have invaluable application in personal medi cine for chemotherapies with less side effects. We believe that this study will be a promising approach to cancer treatment.

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Disclosure

The authors have stated that they have no conflicts of interest.

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