The Study of DNA Methylation of *bax* Gene Promoter in Breast and Colorectal Carcinoma Cell Lines

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Abstract

Background: The Bcl-2 protein family members have known as essential controllers of mitochondrial pathway of apoptosis. Bax is a proapoptotic member of Bcl-2 protein family, which is well known to play crucial roles for apoptosis control. *bax* has been implicated as potential tumor suppressor in certain solid tumors such as breast and colorectal carcinoma. DNA methylation of promoter associated CpG islands has known as a common mechanism for gene inactivation in tumor cells.

Methods: The Methylation Specific PCR (MSP) has used to find the methylation profile of the *bax* gene promoter CpG islands in colorectal and breast cancer cell lines.

Results: We have not detected any kind of "CpG islands hypermethylation" in promoter region of the *bax* gene in T47D, MCF7 (as ER positive), MDA-MB-231 and MDA-MB-468 (as ER negative) breast carcinoma-derived cell lines and colorectal cancer cell lines H29 and Caco II.

Conclusion: It seems that CpG island methylation could not play the main role in down-regulation of *bax* gene in breast and colon cancers.

Keywords: CpG island; DNA methylations; Polymerase Chain Reaction (PCR); Breast cancer; Colorectal cancer

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Introduction

The concept of apoptosis has originally introduced by Kerr et al. to introduce a type of cell death with specific morphological features [1]. It is a normal and physiological (as opposed to pathological) control mechanism, but its control could become deficient then lead to numerous pathologies, such as cancer [2]. Apoptotic signaling events depending on initiation mechanism would generally divided into two pathways: the intrinsic pathway, which mostly relies on mitochondrial changes, and the extrinsic pathway, activated by extracellular signals and acted through death receptors [3]. The Bcl-2 family proteins are among the most important regulators of intrinsic pathway of apoptosis. These proteins could determine whether the mitochondria initiate the apoptosis and release proapoptotic factors such as cytochrome c. The Bcl-2 family members have categorized either proapoptotic or antiapoptotic, depending on cellular context [4]. Bax is the first death-promoting member of the Bcl-2 family which should be detected. It is a proapoptotic member of this family that has been considered as a potential tumor suppressor gene [5].

The cell investigation has shown: how much critical are there for cell death beginning through the intrinsic pathway. We have derived these cells of knocked out mice, with both lack of *bax* and *bak*.

The human *bax* gene is on chromosome 19q, has composed of six exons and four variants [6].

P53 protein, in response to genotoxic damage like radiation, plays a key role for apoptosis induction by changing the expression level of necessary genes involved in mitochondria -mediated (e.g. *bax*, *bak*, *bid*) pathway of apoptosis [7]. In a plenty of tumors, decreased concentrations of Bax have associated to mutations in the *p53* gene. The proapoptotic protein Bax has involved in breast and colon carcinogenesis too. *bax* expression has obviously decreased in various human breast and colorectal cancer cell lines

Table 1. Oligodeoxy nucleotide primers used in MSP analysis of *bax* gene DNA methylation status [21]

Primer	Sequence (5′—3′)	Gen Bank accession no.	CGI Location	T_{anneal}, °C	Amplicon size (length in bp)
Sense	M- GAGGTAGGTGCGGTTACGTG	NC_000019.9	5′+ body	56	102
Antisense	M- AATCACGTAAAAACCCCGCT				
Sense	U- GGTGTTGTGGGGTAGTGGTT			63	118
Antisense	U- ACCACCTCTCACCAAATCCA				

M: Methylated sequences; U: Unmethylated sequences; CGI Location: location of the CpG island with respect to the transcription start site of its associated gene.

[8, 9] and mutational study of the *bax* and *p53* genes have detected mutations of the *p53* gene but no mutations of the *bax* gene [10, 11].

Human cancer initiation and progress has taken place in multi-step process. A large amount of studies focused on this process, has considered the role of direct changes of DNA sequences (mutations). Both new and inherited mutations have well found in tumorigenesis. These changes might cause activation of oncogenes and inactivation of tumor suppressor genes. Investigations have initiated to evaluate the role of epigenetics in tumor development [12]. DNA methylation, has known as the best studied way of epigenetic changes that be able to regulate gene expression [13]. It is a post-replicative modification of DNA that has occurred at the 5′ position of cytosine rings which predominantly, not exclusively, have located in CpG dinucleotide [14]. DNA methylation within the 5′ end of genes, particularly methylation of promoter CpG-island, has been evidently associated to chromatin condensation, and repression of gene transcription [15]. Therefore, methylation of promoter CpG island has been suggested as third way in carcinogenesis, according to Knutson's "two hit hypothesis". In addition, methylation has represented as an important indicator for cancer identification, the same as a prognostic marker [16]. DNA hypermethylation has referred principally to gain methylation in specific sites, but could be changed to unmethylated forms under normal conditions. This aberrant methylation has occurred mainly in promoter CpG islands [17]. Recent whole genome has approached revealing that promoters of vertebrate genes have generally been free of DNA methylation, and then there is an overall inverse correlation between promoter DNA methylation and gene expression [18]. In contrast to global DNA hypomethylation, hypermethylation has observed in specific CpG islands [19]. Since mutations of the *bax* gene are uncommon, then promoter region has crucial role in regulation of gene expression.

In present study, we have tested the relationship of CpG island hypermethylation and altered expression of *bax* gene in breast carcinoma and colorectal carcinoma cell lines, to see if the down regulation of *bax* has related to hypermethylation of its promoter. For this aim, promoter methylation status of *bax* gene in four representative T47D, MCF7 (as ER positive), MDA-MB-231 and MDA-MB-468(as ER negative), in breast carcinoma-derived cell lines and two colorectal cancer cell lines H29 , and Caco II has analyzed by MSP assay.

Materials and Methods

Cell Lines and Cell Culture

Human normal lymphocytes; and four representative T47D, MCF7, MDA-MB-231 and MDA-MB-468 breast carcinoma; and two representative human colorectal cancer cell lines H29 and Caco II have been used in our study. Four human breast carcinoma epithelial cell lines T47D, MCF7, MDA-MB-231 and MDA-MB-468 and two human colorectal cancer cell lines H29 and Caco II have purchased from the National Cell Bank (Pasteur Institute of Tehran, Iran) and have routinely maintained in RPMI 1640 medium fulfilled with 10% heat-inactivated fetal bovine serum, penicillin(100 U/ml), streptomycin(100 µg/ml). All cell cultures have incubated at 37°C humidified atmosphere containing 5% CO2.

Genomic DNA Isolation and Sodium Bisulfite Modification

The total genomic DNA from White Blood Cells (WBCs) and from cell lines has extracted using the Qiagen Genomic DNA Isolation Kit (Qiagen Inc. FlexiGene DNA kit, Germany) and the DNA concentration and quality have determined using a spectrophotometer. After the determination of concentration of each DNA sample with spectrophotometry, sodium bisulfite modification has

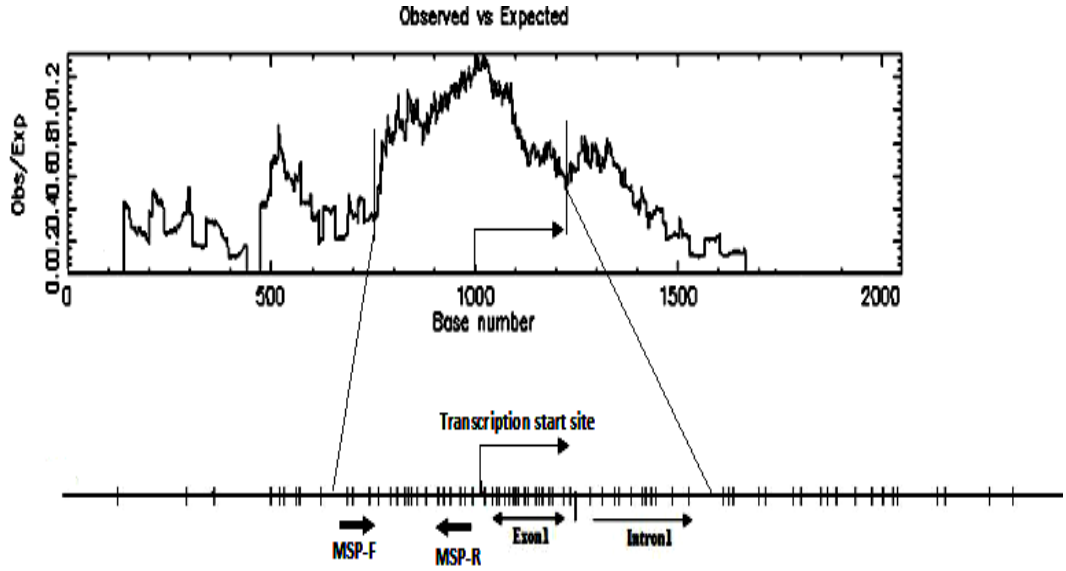

Figure 1. Analysis of the CpG island; consisting of promoter, exon 1 and 5' end of intron 1 regions of *bax* gene. MSP-F and MSP-R primers anneal near transcription start site. Each vertical mark indicates a CpG pair. The transcription start site has indicated by the bent arrow.

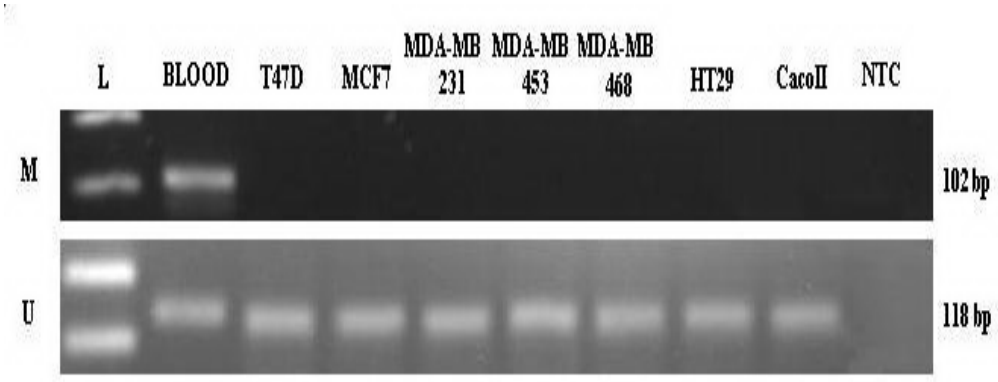

Figure 2. Methylation analysis of the *bax* gene in breast and colorectal cancer cell lines. Blood (universally positive control for methylated and unmethylated DNA); NTC (No template DNA); L: Molecular marker, M: Methylated control, U: Unmethylated control.

performed using the Imprint DNA Modification Kit (Sigma-Aldrich, Inc. UK) according to the manufacturer's protocol with minor adjustments. DNA has used immediately or should be stored at -20°C. Results from different sequences of methylated and unmethylated DNA bisulfite treatment has followed by MSP as described by Herman et al. [20].

Methylation Specific PCR (MSP) Assay

Analysis of the methylation status of *bax* gene promoter region has done by Methylation-Specific Polymerase Chain Reaction (MSP) assay, as described before [21]. This method is highly specific for the analysis of methylation of CpG dinucleotides in a CpG island. In brief, DNA methylation patterns of the CpG island existing in promoter sequence of the *bax* gene have determined by chemical modification of unmethylated but not the methylated cytosines to uracil and subsequent PCR using primers specific for either methylated or the modified unmethylated DNA. MSP has performed in a thermal cycler (Verriti, ABI, USA) with the following cycling conditions: The amplifications have consisted of a Taq activation step at 95°C for 5min followed by 35 amplification cycles (95°C for 30 s, annealing temperature for 45 s, and 72°C for 30 s) and final incubation at 72°C for 5min. The PCR mixture contained 100–200 ng of bisulfite-treated DNA, 1×

PCR Buffer (CINAGEN), 1.5 mM MgCl2, 0.2 mM of each dNTP, 20 pmol of each primer set, and 1.25 units of Taq (Sinagen Inc., Tehran, Iran) in a final volume of 25 µl. Amplified products have electrophoresed on 2.5% agarose gels, stained by ethidium bromide and directly visualized under Ultraviolet (UV) illumination. All MSP assays have repeated at least twice. The primer sets for MS-PCR of *bax* gene have listed in Table 1 [21].

Human normal lymphocytes DNA from healthy volunteers has treated in vitro with excess SssI methyl transferase (New England Bio labs, UK) then has subsequently subjected to bisulfite treatment to generate completely methylated DNA at all CpGs and has used as positive control for methylated alleles of each gene, finally normal DNA from human peripheral blood has modified with sodium bisulfite and used as an unmethylation control.

Results

This study has performed on samples, derived from four breast and two colorectal carcinoma cell lines. First, genomic DNA sequence from *bax* gene has downloaded from public databases available and then subjected to CpG island discovery software. A typical CpG island in promoter of *bax* gene showing ≥60% CpG content and an observed vs. expected CpG frequency of ≥0.65 has detected using the freely available CpGPlot/CpGreport website (http://www.ebi.ac.uk/emboss/cpgplot/), with a high probability of being subject to epigenetic control. This CpG island largely spans the core promoter, exon1 and 5'end of intron 1. The CpG islands have sited in promoter regions of genes represent a major target for DNA hypermethylation. A DNA region with an observed/expected CpG ratio of equal or more than 0.65 and a GC content of equal or more than 55% is considered a CpG island [22]. Our results have demonstrated that, the *bax* proximal promoter (-315 to +51 relative to Transcription Start Site) partially overlaps with the CpG island (Figure 1).

Considering the reduced expression of *bax* in various breast cancer cell lines [8- 10], we have interested to study the possible relationship between this reduction and methylation of the *bax* promoter region. Thus, MSP method has used to determine the methylation status of *bax* promoter in the four breast and two colorectal carcinoma cell lines. In this assay, bisulfite modification results in a C to U conversion when the C is unmethylated in the context of a CpG dinucleotide. However, when the cytosine is methylated, bisulfite modification leaves the methylated cytosine unchanged. Therefore, after bisulfite modifications, methylation specific and unmethylation-specific primers have used in MSP. Primers sets have designed to specifically amplify either Methylated (M) or Unmethylated (U) bisulfite-modified sequences in the *bax* promoter-associated CpG islands and promoter methylation status has detected by PCR. Amplification with either the methylated or unmethylated set of primers results, in presence or absence of PCR product, depending on the methylation status of the CpG dinucleotide have interrogated by that primer pair. As shown in Figure 2, when *bax* promoter has studied by MSP, in all selected cell lines a band has seen only with the U-primers that recognize the unmethylated sequence and no bands have achieved with the M-primers. This result illustrated that *bax* promoter is unmethylated in T47D, MCF7, MDA-MB-231, MDA-MB-468 H29 and Caco II cell lines.

Discussion

Cancer research has recently focused on some of challenges, one of the most important is the identification and classification of several tumor associated biomarkers, could help in prognosis, diagnosis and development of therapeutic strategies. A plenty of markers have discovered within the last decades include factors related to different processes, such as cell cycle, apoptosis, invasion and metastasis [23]. Apoptosis is an essential physiological process operating at all stages of mammary gland and rectum remodeling. The process is rapid and precisely a controlled type of cell death, involving many signaling molecules, regulatory proteins (e.g. Bcl-2 death triggers and inhibitors) and mitochondrial intermediaries (cytochrome c, AIF). The major candidates are proapoptotic proteins from Bcl-2 family such as Bid and Bax [24, 25]. *bax* is an apoptotic regulatory gene that induces caspase-dependent DNA degradation after inducing the release of cytochrome c from mitochondrial membranes [26]. Although there is enormous evidence that breast cancer is fundamentally a genetically based disease but epigenetic alterations resulting in aberrant silencing of gene expression, especially DNA methylation, play an important role in breast tumorgenesis [27]. There would be increasing interest in utilization of methylation markers, since promoter CpG island methylation-induced tumor suppressor gene silencing has suggested to be a third way in carcinogenesis according to Knutson's "two hit theory"[28]. Tumor suppressor genes involved in regulation of cell cycle, differentiation, and apoptosis have commonly suppressed in breast cancer cells by CpG island

hypermethylation of promoter region [29]. According to previous studies, it seems that hypo- and hypermethylation known as an early event in etiology of breast carcinogenesis, resulting in activation of many oncogenes and silencing of tumor suppressors, respectively, and promote proliferation of abnormal cells [30].

Hence DNA methylation plays a major role in regulation of various genes, which have potentially involved in breast cancer. MSP technique is a specific method for methylation status study of genes in any type of biological samples. In spite of being nonquantitative methodology, MSP remains extremely useful specifically in an initial screen of a large number of tumor samples because of the quickness in which is performed. More detection and characterization of abnormalities in gene expression might eventually result in the treatment strategies that particularly target apoptosis signaling in breast cancer cells, not normal cells, thereby leads to decreased breast cancer death rate. Potential mechanisms for down-regulation of *bax* include genetic changes (mutation or deletion) and epigenetic suppression. In a previous study, no mutation has recognized in the coding sequence of the *bax* gene. As a result, mutations or deletions of the *bax* gene are uncommon and hypermethylation has provided a second explanation to the observed reduced expression of Bax protein in human breast tumors [31]. The *bax* gene contains a large CpG island including the proximal promoter region, exon 1 and part of intron 1 of the gene (Figure 1), that consists of 55 CpG dinucleotides spread over a 470 bp region (freely available online CpGPlot software, http://www.ebi.ac.uk/ emboss/CpGPlot/). For the first time, we have determined the promoter CpG island methylation profile of *bax* gene in four breast cancer cell lines (T47D, MCF7, MDA-MB-231 and MDA-MB-468) and two colorectal cancer cell lines (H29 and Caco II) with the MSP Method. We have found that none of the 6 tested cell lines displayed methylation of *bax* gene, so we have classified *bax* gene as unmethylated in these cell lines. Based on our results it appears that hypermethylation of the *bax* gene does not play a key role in the generation and development of breast and colorectal cancers, even if this might be the case for other malignancies. Although MSP could detect one methylated allele between 1000 unmethylated alleles, however the assay is a qualitative assay and does not give quantitative information about the methylated alleles. Thus, the methylation has detected by MSP assay, does not necessarily mean gene silencing and the vast majority of tumor cells might not harbor CpG island methylation of a given gene [32].

Conclusion

In spite of that all MSP assays have repeated at least twice with methylated and unmethylated controls, the obtained data has shown no methylation of CpG island of *bax* gene in T47D, MCF7, MDA-MB-231 and MDA-MB-468 breast cancer and H29 and Caco II colorectal cancer cell lines. While the mutually related evidences between hypermethylation of CpG islands at the 5' upstream region of genes and silencing of gene transcription have been collecting and the significance of CpG island hypermethylation in carcinogenesis has been more and more recognized, there has been an argument whether CpG island hypermethylation is a cause or secondary event of down regulation of gene expression.

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Conflict of Interest

The authors have no conflict of interest.

Authors' Contribution

Mohsen Alipour, Seyed Jalal Zargar, Shahrokh Safarian and Ebrahim Azizi conceived, designed the study, interpreted the results, drafted the manuscript and carried out the data analyses. Shamileh Fuladdel assisted to perform the experimental techniques. Mohsen Alipour and Seyed Jalal Zargar participated in writing and revising the manuscript too, while Naser Jafargholizadeh revised the final manuscript.

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