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Original Article

Methylation Analysis of 5'UTR Promoter Region of DBC2 as a Biomarker in the Peripheral Bloods of Some Iranian Women with Sporadic Breast Cancer

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ABSTRACT

Background and Objective: **The DBC2 (deleted in breast cancer 2) or RhoBTB2 (Located on 8p21) is a tumor suppressor gene associated with tumorigenesis. Mutational studies of DBC2 at its promoter region in breast cancer revealed an important role for epigenetic changes contributing to its low expression. Epigenetic changes through hypermethylation of the promoter can cause the inactivation of DBC2 gene. The purpose of this study was to investigate methylation pattern of DBC2 gene in the peripheral blood of 40 Iranian women with breast cancer and its comparison with healthy women.**

Material & Methods: **We used peripheral blood samples from 40 patients with sporadic breast cancer and 40 normal individuals. Analysis of the methylation statues of DBC2 promoter region was done by MSP (Methylation Specific PCR) technique on the DNA extracted from the blood samples. The results were validated by sequencing. The methylation status was then correlated with the clinicopathological parameters of breast cancer patients.**

Results: **Methylation pattern was detected in 60% of the patients, whereas 25% of the normal individuals demonstrated a positive methylation pattern ($P \leq 0.01$, odd ratio : 2.143). No significant correlation was obtained between methylated DBC2 and cliniclpathological parameters.**

Conclusion: **Aberrant hypermethylation was observed preferentially in the patients. These findings along with the previous studies, propose that abnormal methylation pattern in DBC2 promoter region may be one of the main reasons for low expression of DBC2 in breast cancer and this hypermethylation pattern could play a fundamental role in the breast tumorigenesis.**

Keywords: **Breast Cancer, Methylation, DBC2 Protein,Women, Iran**

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Introduction

Breast cancer is one of the most common cancers among females worldwide. According to global statistics, the incidence of breast cancer is increasing annually specially in countries with a low incidence rate. Various reports have shown that almost from 9 American women, one develops breast cancer during their lifetime. In Iran, this is also one of the most prevalent deadly cancers among women (1-3).

Two common genes (BRCA1 and BRCA2) have been identified in the hereditary breast cancer syndromes. However the majority of breast cancer types are sporadic and the hereditary susceptibility seems to play a small role. Multiple genetic, epigenetic and environmental factors contribute to the development of breast cancer, which is generally considered a multifactorial disease (4). The term epigenetic refers to the inheritance of information based on gene expression, which in contrast to genetic information is not transmitted on the basis of gene sequence. A major epigenetic modification in human is DNA-methylation at cytosines located 5'to guanosines. CpGdinucleotides have been depleted from the eukaryotic genome owing to the conversion of methyl cytosine to uracil. Most remaining CpGs are methylated. However, there are CG rich regions termed CpG islands, which are often located near the promoter regions of approximately 50% of human genes and are normally unmethylated. DNA methylation in cancer has become the topic of intensive investigation. Global loss of methylation and hypermethylation of promoters of some CpG islands in cancers can lead to the silencing of cancer genes such as tumor suppressor genes (5-8). DBC2 is one of these tumor suppressor genes located on the short arm of chromosome 8. This gene is deleted in 3.5% of breast cancers and silenced in 50% of breast and lung cancers (9-13). DBC2 influences cell cycle pathways, apoptosis, cytoskeleton and membrane-trafficking (14,15).RhoBTB2(DBC2) is a Rho GTPases that contains an N-terminal Rho domain, two tandem BTB/POZ (bric-a-brac tram track broad complex/ pox viruses and zinc fingers) domains and a unique C-terminal region with unknown function (16). DBC2 is a tumor suppressor gene whose activity has been altered in human breast cancer through deletion or loss of heterozygosity, methylation or point mutation (17-19).

In this study, we attempted to find the methylation status of the DBC2 promoter in both breast cancer and normal peripheral blood samples by MSP. The methylation status was then correlated with the clinicopathological parameters of breast cancer samples.

Material and Methods

Patients and blood samples

Peripheral blood samples were collected from 40 patients diagnosed with sporadic breast cancer. The patients were not under any treatment. The age range for the patients was 31 to 84 years with an average age of 51.There was no evidence of familial cancer in any of the patients and the normal individuals for 2-3 generations. The blood samples were obtained from Mehrad Hospital in Tehran-Iran. The clinicopathological data of the patients were collected from the patients' files. The Peripheral blood samples from 40 normal individuals were used as normal controls. Blood specimens were collected in EDTA containing tubes. The study was approved by the ethical committee of the University of Social Welfare and Rehabilitation Sciences. An informed consent was obtained from all the participating individuals under the guidelines of the ethical committee.

DNA extraction and bisulfate modification

DNA of the samples was extracted using salting out method. Bisulfate modification of DNA was carried out as follows:1)10 µl of DNA was mixed in 40 µl of water and 5.5 µl of denaturizing 2 M

sodium hydroxide for 20 min at 37℃. 2) Thirty microliters of 10 mM hydroquinone and 520 µl of 3 M sodium Bisulfate at pH 5 (both freshly prepared) were added and mixed.
3) Samples overlaid with mineral oil and were incubated at 50°C for 16 hrs.4) Modified DNA was purified using High Pure PCR Template Purification Kit according to the manufacturer (Roche). 5)Modification was completed by NaOH (3M) treatment for 5 min at room temperature, followed by ethanol precipitation.6) DNA was resuspended in water and used immediately or stored at -20°C.The DNA samples were used for PCR reactions.

Nested MSP analysis and sequencing

The design of Nested primer design was done by Methyl Primer Express soft-ware V1.0 (ABI, Foster City, CA) using GenBank AF315385 (DBC2). The nucleotide sequences for the primers are listed below:

External primers:

Sense:
5'-GGTGGTTTATTTGGTGATATTG-3'
Antisense:
5'-CCTACAACCTTACCTCCTAACAC-3'(439 bp)

Internal Primers:

Methylated primers:
Sence:
5'-GCGAGTTGGTATGTTATGTC-3'
Antisense:
5-'TAATCTTACCCACGACGTTA-3'(144bp)
Unmethylatedprimers:
Sense:
5'-GGTGAGTTGGTATGTTATGTT-3'
Antisense:
5'-CTAATCTTACCCAC AACATTA-3' (144 bp)

In the first round of amplication, 25 uL of the reagent mix was used. PCR conditions are chosen as follows: 94°C for 3 min, then 35 cycles of 94°C for 30 s, 60°C for 1min, 72°C for 50 s, continued by a final extension of 72°C for 10 min. After the first PCR reaction, all of the PCR products were used for the second PCR reaction by using methylated and unmethylated primers. The second PCR conditions are chosen as follows: 94°C for 3 min, then 30 cycles of 94°C for 30 s, 57°C for 1min, 72°C for 50 s, continued by a final extension of 72°C for 10 min. PCR products were identified on 1.5% agarose gel electrophoresis. Amplifications with methylated primer showing relevant bands were considered methylation positive, and amplifications with both methylated primers and unmethylated primers were considered as methylation negative. H2O was used as negative control.
The MSP products were prepared for sequencing using ABI sequence analysis.

Statistical analysis

Fisher's exact and $\chi 2$ tests (SPSS 16.0) were used to analyze the data and the correlation between the DBC2 gene promoter hypermethylation and clinicopathologicalparameters of the patients. *P*-value of ≤ 0.05 was considered statistically significant.

Results

Methylation status of DBC2 gene in the peripheral blood samples of patients with breast cancer and normal individuals

In this study we analyzed the methylation status of the DBC2 promoter in the peripheral blood samples of 40 patients with breast cancer and 40 normal individuals by MSP (Fig.1). We detected the methylation pattern in 60% of the patients while the negative methylation pattern was just seen in 40٪ of patients. In the majority of normal control women (75%) negative methylation pattern was seen, while possitive methylation pattern was just seen in 25٪ of normal women (Table 1). Aberrant methylation was observed

preferentially in the patients ($P \leq 0.01$). Analysis of sequencing validated our findings: Due to bisulfate modification, the MSP products which had the methylated band in electropherogram showed unconverted cytosines in CpG Island and converted-thymines in the non-CpG region. Thus, Bisulfate changes only the non methylatedcytosines in CpG islands and converts them to thymine but no conversion would occur in methylated-cytosine in CpG islands.

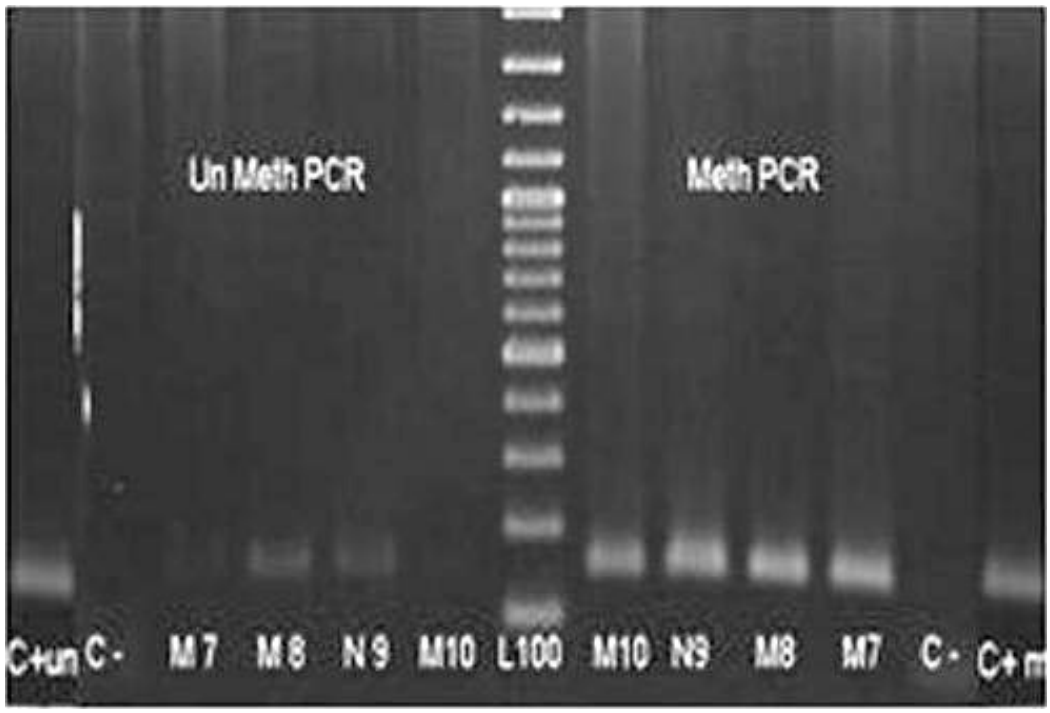

Fig. 1: MSP Gel Picture of patient and normal DNA.

M: patient sample. N: normal sample. Methylated Gel Picture is on the right of ladder and UnMethylated Gel Picture is on the left. C+un: Positive Control for unmethylated PCR. C+m: Positive Control for methylated PCR. C - ; H2O as negative Control. Numbers of M and N are the code of the samples.

Table 1- DBC2 methylation status in blood samples of breast cancer patients and normal individuals (n=80)

Status of Methylation	Methylated DBC2 gene	Nonmethylated DBC2 gene
No. of Normal Individuals	10/40(25%)	30/40(75%)
No. of Patients	24/40(60%)	16/40(40%)

Fisher›s exact and $\chi 2$ tests were used to compare patients and normal individuals. The differences in the methylation pattern between them were significant ($P \leq 0.01$).

Association between methylation pattern of DBC2 and clinicopathological features

The association between methylation pattern of *DBC2* gene and clinicopathological data of the patients was investigated (Table 2). There was no significant association between methylated *DBC2* and clinicopathological data of age, tumor size, estrogen, progesterone receptor and P53 in the patients (Table 3).

Table 2 -Clinicopathological data for the patients

	No.	%
Age:< 50 years	14	51.9
≥ 50 years	13	48.1
Tumor size :< 2 cm	8	29.6
≥ 2 cm	19	70.4
ER*:Positive	20	74.1
Negative	7	25.9
PR:Positive**	17	63
Negative	10	37
P53:Positive	8	29.6
Negative	19	70.4
Total No.	27	

* Estrogen Receptor **Progestrone Receptor

Table 3- Association between methylation status of DBC2 and clinicopathological data

Clinicopathological data	No. of patients with/without Methylated DBC2 (%) +	-	*P*-value
Age:< 50 years	11(79)	3(21)	0.236
≥ 50 years	7(54)	6(46)	
Tumor size :< 2 cm	7(88)	1(12)	0.201
≥ 2 cm	11(58)	8(42)	
ER*:Positive	14(70)	6(30)	0.653
Negative	3 (43)	4 (57)	
PR**:Positive	11(65)	6(35)	1.000
Negative	7 (70)	3 (30)	
P53:Positive	3(38)	5(62)	0.07
Negative	15 (79)	4 (21)	

Fisher's exact and χ^2 tests were used to analyse the correlation between thehypermethylation status of the promoter of DBC2 gene and clinicopathological features

* Estrogen Receptor **Progestrone Receptor

Discussion

DBC2 gene is inactivated in the majority of breast cancer patients. DBC2 is a tumor suppressor gene and its function has been altered by deletion or loss of heterozygosity, down regulation or point mutation in human breast cancer. DBC2 is a Rho GTPase that contains an N-terminal Rho domain, two tandem BTB/POZ domains and a C-terminal region (12, 20).

As many studies have shown, abnormal epigenetic changes play a fundamental role in down regulation of many genes related to cancers. The expression is altered in many genes in breast cancer. Tumor suppressor genes are subject to aberrant methylation in CpG islands. The mechanism of CpG island methylation in cancers is unknown. It is obvious that loss of expression of tumor suppressor genes is involved in regulation of cell growth, differentiation, metastasis, and genetic stability. Thus the inactivation of DBC2, a tumor suppressor gene, will contribute to carcinogenesis.

In our study, DNA of the peripheral blood sam-

ples was used for MSP technique. We detected methylated DBC2 in the DNA of blood samples of breast cancer individuals ($P \leq 0.01$) and obtained an odd ratio of 2.143 compared to that in the normal individuals. This ratio indicates that individuals that have methylated DBC2 gene are approximately two times subjected to breast cancer.

We also observed methylated DBC2 in 25% of normal women. However, the aberrant methylation changes in normal individuals should be studied more. These women are probably predisposed to breast cancer, pending the influence of other factors. In analyzing clinicopathological data we found no significant correlation between methylated DBC2 and these parameters. Methylation pattern and clinicopathological and immunohistochemical parameters should be studied for a larger sample size (4,21-22).

Reports on epigenetic role of DBC2 gene in breast cancer are limited. Hypermethylation of the promoter region in plasma and serum has also been shown in patients with cancers of the lung, colon, and breast (8, 23-25). In a similar study by Mirzaei *et al.,* 2012, on 100 Iranian women with sporadic breast cancer, DBC2 methylation was observed in the peripheral blood specimens of 46% of the patients and 16.6% of normal individuals (17).

More studies would possibly result in a better DBC2 role in the development of breast cancer. These studies could identify DBC2 or other related proteins as targets for better therapy for the breast cancer patients (9).

Conclusion

Our study provides evidence that hypermethylation of the DBC2 gene is involved in the tumorigenesis of sporadic breast cancer. Since DNA methylation is known to be a potential marker for tumor diagnosis and prognosis, our findings will be useful in the detection and epigenetic therapy of breast cancer. However, additional investigations are needed in order to demonstrate the potential of DBC2 gene methylation analysis for clinical applications.

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