Promoter Methylation Status of the Retinoic Acid Receptor-Beta 2 Gene in Breast Cancer Patients: A Case Control Study and Systematic Review

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Keywords

Breast cancer · *RARβ2* · Hypermethylation · Epigenetic · MSP · Iranian population

Abstract

Background: We aimed to determine the promoter methylation status of the retinoic acid receptor-beta 2 (*RARβ2*) gene among breast cancer patients and to review relevant studies in this field in various populations. **Methods:** We analyzed 400 samples which comprised blood specimens from 102 breast cancer patients, 102 first-degree female relatives of patients, 100 cancer-free females, 48 breast cancer tissues, and 48 adjacent normal breast tissues from the same patients. The *RARβ2* methylation status was determined using methylation-specific polymerase chain reaction (MSP) and DNA sequencing methods. **Results:** The presence of combined partially methylated (MU) and fully methylated (MM) forms of the *RARβ2* gene (MU+MM) in the blood of patients was associated with susceptibility to breast cancer (odds ratio = 4.7, $p = 0.05$). A significantly higher frequency of the MM genotype was observed in cancer tissue (10.4%) compared to matched adjacent normal breast tissue (0%) ($p = 0.02$). **Conclusion:** We found a higher frequency of *RARβ2* gene methylation in the blood and cancer tissues of patients compared to the blood of controls and adjacent normal breast tissues. The survey of studies on various populations demonstrated a higher *RARβ2* methylation frequency in breast cancer patients compared to normal individuals, and many reports suggest a significant association between hypermethylation of the gene and susceptibility to breast cancer.

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Introduction

Breast cancer is a heterogeneous malignancy in women associated with high morbidity and mortality [1, 2]. Due to the considerably increased incidence rate of breast cancer, it could be considered as one of the most common health problems across different populations [3, 4]. In developing countries including Iran, patients diagnosed with breast cancer are 10 years younger compared to those in developed countries [3]. Both genetic and epigenetic changes are involved in the individual susceptibility to breast cancer [5]. Epigenetic modifications alter gene expression through DNA hypermethylation, histone modification, and microRNA mechanisms [6]. In tumor cells, hypermethylation of CpG islands of tumor-related genes can effectively inactivate the gene, whereas in normal cells, CpG islands are not methylated which may lead to gene transcription [7].

Retinoic acid (RA) as a main signaling biomolecule derived from vitamin A is implicated in the control of main developmental processes such as differentiation and cell growth. Based on antiproliferative and differentiation effects of retinoids, RA is considered as the suppressor of cancer cell growth [7, 8]. RA binds and activates 2 types of nuclear receptor heterodimers of the retinoic acid receptor (RAR) and the retinoid X receptor (RXR). Among them, the retinoic acid receptor-beta 2 (*RARβ2*) has a critical role in the chemopreventive effects of retinoids. The tumor suppressor gene *RARβ2* is located on chromosome 3p24 and its loss is associated with RA resistance [7, 8].

Reduced expression of *RARB2* by genetic or epigenetic mechanisms is linked to many human cancers such as cervical [9, 10], head and neck [11], non-small cell lung [12], prostate [13], bladder, brain, and breast cancers [4, 14–16]. Knowledge of the DNA methylation status in the promoter regions of some candidate genes such as *RARB2* may be useful for early detection, prognosis, and drug response prediction in breast cancer patients [6, 17]. The aim of the present study was to evaluate the methylation status of the *RARB2* gene in the CpG islands of its promoter and to detect whether it is associated with the risk of breast cancer in an Iranian population with Kurdish ethnic background. Furthermore, we reviewed studies that have previously investigated the methylation status of *RARB2* in breast cancer patients.

Materials and Methods

Sample Collection

In the present case-control study, 400 samples including blood specimens from 102 female patients with breast cancer, 102 first-degree female relatives of patients (mother, sister, or daughter), 100 cancer-free females, 48 breast cancer tissues, and 48 adjacent normal breast tissues from the same patients were investigated. The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences, Iran. All individuals agreed to participate in the study and signed informed consent in accordance with the principles of the Helsinki II declaration.

Genomic DNA Extraction

DNA was extracted from EDTA-treated whole blood and from paraffin-embedded tissue blocks using the phenol-chloroform method [21].

Methylation-Specific Polymerase Chain Reaction

The methylation status of *RARB2* was detected using methylation-specific polymerase chain reaction (MSP) as previously described [4]. The specific band intensity of the MSP product (170 bp) was determined using TotalLab TL120 software (TotalLab Ltd., Newcastle upon Tyne, UK). Based on TotalLab TL120 software analysis, only bands with an intensity higher than 50,000 values were considered. 8 samples from patients that were identified as MM (fully methylated) or UU (unmethylated) genotypes using the MSP method were selected for DNA sequencing (MacroGen Inc., Seoul, South Korea).

Statistical Analysis

The significance of differences in the frequencies of genotypes between the 3 study groups and between breast cancer tissues and adjacent normal breast tissues was determined using the chi-square test. Odds ratios (OR) and 95% confidence intervals (95% CI) were measured using SPSS 16.0 software (IBM Corp., Armonk, NY, USA). Statistical significance was assumed at the $p < 0.05$ level [1, 18].

Literature Study and Data Extraction

We included all relevant studies published in the English language related to DNA methylation of the *RARB2* gene in breast cancer patients, obtained from searching the databases MEDLINE/PubMed and Scopus up to March 2016. Using the keywords 'DNA methylation', '*RARB2*', and 'breast cancer', 21 eligible original articles were identified and included in the systematic review.

Fig. 1. Analysis of a fragment of the *RARB2* gene in the promoter region using a DNA sequencing apparatus (MacroGen Inc., Seoul, South Korea). **A** Promoter sequence of the *RARB2* gene in the NCBI database; **B** obtained sequences from MethPrimer software; **C** sequences obtained by DNA sequencing using a sodium bisulfite-treated sample. Underlined CG represents the CpG islands that have not been modified by sodium bisulfite treatment and remained intact as CG, whereas those C* nucleotides that were not followed by a G nucleotide were modified to T after sodium bisulfite modification (**B**).

Results

The mean age of the patients was 49.8 ± 10.3 years (range 29–79 years). 10 (10.2%) and 28 (28.6%) patients were aged below 30 and 40 years, respectively. The result of direct genomic DNA sequencing from 1 sample is shown in figure 1. The frequencies of *RARB2* combined methylated genotype (MU+MM) in the blood of patients, relatives of patients, and healthy individuals were 8.8, 2.9, and 2%, respectively ($\chi^2 = 6.3$, $p = 0.042$) (table 1). The presence of *RARB2* MU+MM was associated with susceptibility to breast cancer (OR = 4.7, 95% CI 0.99–22.5, $p = 0.05$) (table 1). A higher frequency of fully methylated *RARB2* was observed in cancer tissue (10.4%) compared to matched adjacent normal breast tissue (0%) ($p = 0.02$) (table 2). As indicated in table 2, we did not detect the same frequency of *RARB2* MU+MM in the tumor tissue and white blood cells (WBC) of patients. Our results indicated that the MU+MM genotype of the *RARB2* gene was not correlated with breast cancer prognostic parameters in our population.

Discussion

MSP is a rapid, most widely used, and cost-effective method for gene methylation analysis [19]; however, it is not a quantitative method [20]. Hence, the results obtained in our study could be confirmed using a quantitative MSP method. However, a number of studies have

Table 1. *RARβ2* methylation status in the blood of healthy individuals, relatives of patients, and patients

<i>RARβ2</i> genotype	Patients, n (%) (n = 102)	Relatives of patients, n (%) (n = 102)	Healthy individuals, n (%) (n = 100)	χ^2 , p value	Odds ratio, 95% confidence interval, p
UU	93 (91.2)	99 (97.1)	98 (98)	7.67, 0.1 ^a	
MU	7 (6.9)	3 (2.9)	2 (2)		
MM	2 (1.9)	0 (0)	0 (0)		
MU+MM	9 (8.8)	3 (2.9)	2 (2)	4.56, 0.033 ^b 0.18, 0.66 ^c	4.7, 0.99–22.5, 0.05 ^b

^aOverall χ^2 comparing 3 genotypes between 3 groups was 7.67, $p = 0.1$; overall χ^2 comparing MU+MM versus UU genotype between 3 groups was 6.3, $p = 0.042$.

^bComparing patients and healthy individuals.

^cComparing relatives of patients and healthy individuals.

UU = Unmethylated; MU = partially methylated; MM = fully methylated.

suggested that MSP has a high sensitivity and specificity for the identification of breast cancer cells [21, 22]. A frequency of 10–42% for the methylation of *RARβ2* in the promoter region of breast cancer tissue has been reported [23]. Among our studied population, the frequency of methylated *RARβ2* was found to be 18.8% in cancer tissues, which is lower than that reported for the population of Tehran (36.4%), the capital and a cosmopolitan city of Iran [24]. However, similarity between the frequency of *RARβ2* methylation in breast cancer tissue in the present study (18.8%) and some Asian countries such as India (15%) [25] could be attributed to genetic admixture since the Kermanshah province is historically known as the gate of Asia through which passed the important silk road [26]. Also, the type of examined sample (blood or tissue) could be a reason for discrepancy in the reported methylation rates of *RARβ2* among various populations. The presence of discordance in the methylation status of the *RARβ2* promoter between whole blood samples and tumor tissue has been reported. Further, the frequency of hypermethylation of the gene might be ethnic-dependent. In this study, we identified a low methylation frequency of the *RARβ2* gene (2.9%) among first-degree relatives of the patients similar to that among healthy individuals (2%). We detected a significantly higher frequency of fully methylated *RARβ2* in cancer tissue (10.4%) compared to matched adjacent normal breast tissue (0%) ($p = 0.02$) and also blood of breast cancer patients (8.8%) compared to healthy individuals (2%), and this was associated with susceptibility to breast cancer (OR = 4.7, 95% CI 0.99–22.5, $p = 0.05$).

There are controversial reports regarding the methylation rate of the *RARβ2* gene and its association with susceptibility to breast cancer among different populations (table 3). In many populations, including American [27–

Table 2. *RARβ2* methylation status in cancer and adjacent normal breast tissues and blood of patients

<i>RARβ2</i> genotypes	Cancer tissue, n (%) (n = 48)	Normal tissue, n (%) (n = 48)	Blood of patients, n (%) (n = 48)
UU	39 (81.3)	44 (91.7)	42 (87.5)
MU	4 (8.3)	4 (8.3)	4 (8.3)
MM	5 (10.4)	0 (0)	2 (4.2)
			overall $\chi^2 = 5.7$, $p = 0.22^a$
MU+MM	9 (18.8)	4 (8.3)	6 (12.5)
			overall $\chi^2 = 2.3$, $p = 0.3^a$

^a $\chi^2 = 5.3$, $p = 0.07$ comparing 3 genotypes between breast cancer tissue and adjacent normal breast tissue; $\chi^2 = 5.3$, $p = 0.02$ comparing MM versus UU between breast cancer tissue and adjacent normal breast tissue; $\chi^2 = 1.39$, $p = 0.49$ comparing 3 genotypes between cancer tissue and blood of patients; $\chi^2 = 2.05$, $p = 0.35$ comparing 3 genotypes between normal breast tissue and blood of patients.

UU = Unmethylated; MU = partially methylated; MM = fully methylated.

30], Chinese [31], Indian [25], Portuguese [32], and Belgian [33], a significant association between the methylation status of the *RARβ2* gene and breast cancer incidence has been observed. Further, a meta-analysis by Fang et al. [34] reported that the methylation rate of *RARβ2* is significantly increased among breast cancer patients compared to cancer-free controls. In a report from Russia, methylated *RARβ2* was found to be 3 times more frequent in breast cancer patients than in fibroadenoma patients [35]. In contrast, a lack of association between the meth-

Table 3. *RARβ2* methylation status and breast cancer in different populations

Population	Year of publication	Methylation method	Specimen	Methylation, %	Main finding	Reference
American	2001	MSP	breast cancer tissue white blood cells normal breast tissue	37.5 3.6 0	high percent of methylated <i>RARβ2</i> in carcinomas ($p < 0.01$)	[28]
American	2003	MSP	invasive carcinomas in situ carcinomas benign breast tissue	64 60 33	high frequency of methylated <i>RARβ2</i> in both benign lesions and invasive carcinomas ($p = 0.05$)	[38]
American	2003	MSP	breast cancer tissue normal breast tissue	46 0	frequent hypermethylation in breast cancer tissue compared to normal tissue ($p = 0.01$)	[27]
American	2005	MSP	breast cancer tissue normal tissue of controls	32 9	methylation of <i>RARβ2</i> was associated with increased breast cancer risk ($p = 0.002$)	[29]
American	2005	MSP	primary breast tumor sentinel lymph node tumor metastasis	24 48	association of <i>RARβ2</i> hypermethylation with macroscopic SLN metastasis ($p < 0.001$)	[39]
American	2009	quantitative MSP	breast cancer tissue	27.5	hypermethylation of <i>RARβ2</i> was inversely associated with histological grade of breast cancer ($p = 0.13$)	[40]
American	2010	quantitative MSP	blood of breast cancer patients blood of women with benign breast disease blood from healthy controls	6.7 2.3 1.1	absence of association of <i>RARβ2</i> methylation with breast cancer risk ($p > 0.05$)	[41]
American	2011	quantitative MSP	breast cancer tissue	27.5	absence of association between <i>RARβ2</i> methylation status with intake of vitamin B complex ($p > 0.05$)	[42]
American	2015	MSP	blood of breast cancer patients breast cancer tissue blood of healthy individuals	1.5 44.5 1.5	association between WBC DNA methylation of the <i>RARβ2</i> and breast cancer risk (OR: 0.67; 95% CI: 0.55–0.81)	[30]
Australian	2006	MSP	breast cancer tissue	26	<i>RARβ2</i> methylation is linked to poor histological tumor differentiation ($p = 0.03$)	[23]
Belgian	2009	quantitative MSP	breast cancer tissue benign breast tissue	29 0	significantly higher frequency of methylated <i>RARβ2</i> in inflammatory (53%) than in non-inflammatory breast cancer (23%) ($p = 0.006$)	[33]
Chinese	2010	MSP	blood of breast cancer patients blood of healthy individuals	42 4	association of methylation status of <i>RARβ2</i> with sporadic breast cancer ($p < 0.05$)	[31]
Egyptian	2015	MSP	blood of breast cancer patients blood of breast benign lesion cases blood of healthy subjects	95.9 14.5 0	association of <i>RARβ2</i> methylation with breast cancer risk but not with clinicopathological factors ($p < 0.0001$)	[22]
Japanese	2012	OS-MSP	breast cancer tissue normal breast tissue	78 0	high percentage of methylated <i>RARβ2</i> in carcinomas (p value not reported)	[43]

Table 3 (continued)

Population	Year of publication	Methylation method	Specimen	Methylation, %	Main finding	Reference
Indian	2008	MSP	breast cancer tissue normal breast tissues	15 0	association of <i>RARβ2</i> methylation with older age at onset of disease ($p = 0.04$)	[25]
Indian	2012	MSP	blood of patients blood of healthy women breast carcinoma tissue adjacent normal tissues	20 0 26 6.6	absence of association between <i>RAR-β2</i> hypermethylation and loss of protein expression ($p = 0.70$)	[44]
Iranian	2010	MSP	breast cancer tissue	36.5	association of hypermethylated <i>RARβ2</i> with younger age at diagnosis and negative family history of breast cancer ($p = 0.033$)	[24]
Present study–		MSP	breast cancer tissue normal breast tissue blood of breast cancer patients blood of relatives of patients blood of healthy women	16.3 10.2 8.7 2.9 2	significantly higher frequency of methylated <i>RARβ2</i> in tissues and blood of patients than in the blood of 2 control groups ($p < 0.05$)	
Italian	2004	MSP	invasive breast tissue benign breast tissue	20 0	methylated status of <i>RARβ2</i> and its inverse correlation with the <i>BRCA1</i> promoter ($p < 0.03$)	[45]
Portuguese	2008	quantitative MSP	breast cancer tissue breast normal tissue	53 25	significantly higher <i>RARβ2</i> methylation percentage in breast cancer tissue than in normal tissue ($p < 0.01$)	[32]
Russian	2006	MSP	blood of breast cancer patients blood of healthy individuals	15 0	<i>RARβ2</i> methylation in circulating DNA is useful for the detection of malignant tumors (p value not reported)	[21]
Russian	2008	MSRA	breast cancer tissue breast normal tissue	46 4	absence of association of <i>RARβ2</i> methylation with breast cancer risk ($p > 0.05$)	[46]
Senegalese	2006	quantitative MSP	blood of breast cancer patients blood of healthy individuals	26 8	absence of association of <i>RARβ2</i> methylation with breast cancer risk ($p = 0.09$)	[37]
Tunisian	2010	MSP	breast cancer tissue	66.6	frequent <i>RARβ2</i> methylation in breast cancer tissue ($p = 0.043$)	[35]
Turkish	2010	MethyLight assay	breast cancer tissue adjacent normal tissue blood of breast cancer patients	25 25.9 10	frequent <i>RARβ2</i> hypermethylation in tumor and adjacent tissues ($p < 0.05$)	[36]

MSP = Methylation-specific polymerase chain reaction; OS-MSP = one-step MSP; MSRA = enzyme-linked methylation-sensitive restriction analysis; SLN = sentinel lymph node; WBC = white blood cells.

ylation status of *RARβ2* and breast cancer risk has been reported in Russian [21], American [35], Turkish [36], and Senegalese [37] populations. Also, some studies found a lack of concordance between *RARβ2* methylation in tumor tissue and paired WBC, suggesting that there might not be a direct link between WBC gene methylation and development of breast cancer (table 3).

Conclusion

Our study results indicated that the presence of hypermethylation of the *RARβ2* gene in the blood of breast cancer patients was associated with susceptibility to breast cancer. This suggests that *RARβ2* hypermethylation in cancer tissue might be an epigenetic marker for

susceptibility to breast cancer. The strength of the present research was that the *RARβ2* methylation status was studied in both the blood and tissues of patients and that methylation was further determined in relatives of the patients. Despite this strength, the low sample size of studied tissues represents a limitation of our study. Among various populations, higher frequencies of *RARβ2* methylation in breast cancer patients compared to cancer-free individuals have been reported, and in many studies, a significant association between hypermethylation of the gene and susceptibility to breast cancer has been observed. Some studies reported an association between the gene methylation rate and histological differentiation and tumor metastasis. The presence of inconsistent findings might be due to sample size, type of studied specimen (blood or tissue), breast cancer subtype, and ethnicity.

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Contribution

K.Y. performed experiments and wrote the first draft of the manuscript. Z.R. designed the study and critically revised the manuscript.

Disclosure Statement

The authors declare no potential or actual conflicts of interest in relation to the current article.

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