Clinical research

The molecular significance of BRCA1 promoter methylation in peripheral blood cells in women with breast cancer in West Algeria

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Abstract

Introduction: Epigenetic alterations, in particular DNA methylation, are the most important mechanism that silences tumor suppressor genes during carcinogenesis. Methylation of the BRCA1 promoter in peripheral blood is associated with an increased risk of breast cancer. In the present study, we aimed, for the first time in western Algeria, to investigate the role of BRCA1 CpG promoter methylation in peripheral blood cells (PBCs) among breast cancer patients, as a potential biomarker for detecting breast cancer risk at an early age.
Material and methods: The methylation profile of the BRCA1 gene promoter and its frequency in PBCs, derived from 39 breast cancer patients, was explored by methylation-specific PCR (MSPCR). The association between methylation profiles and clinicopathological features was evaluated with SPSS statistics software to estimate a convenient biomarker for early detection of breast cancer.
Results: MSPCR results demonstrated that the methylation frequency of the BRCA1 gene promoter detected in PBCs is significantly higher than in other populations. It was observed in 23 of 39 (58.97%) breast cancer patients.
Conclusions: Our study showed that the methylation of the BRCA1 gene promoter detected in PBCs' DNA could have potential utility in clinical diagnostics as a new biomarker for breast cancer risk in Algerian women and for early detection.

Key words: breast cancer, BRCA1 promoter methylation, peripheral blood cells, methylation-specific PCR, western Algeria.

Introduction

In 2022, Global Cancer Observatory statistics showed that breast cancer (BC) in Algeria ranked as the first common malignant cancer in both sexes with 14 601 new cases (22.6% of total cancer cases), and was the first cause of cancer-related mortality with 4893 deaths (13.7% of total cancer deaths) [1]. Therefore, early detection is necessary to treat and improve the diagnosis and prognosis of breast cancer patients.

Epigenetic alterations such as hypermethylation are an emerging mechanism that plays a crucial role in carcinogenesis. Several studies

have demonstrated that alterations in epigenetic markers such as modifications of CpG methylation at gene promoters result in transcriptional silencing of active genes or activation of silent genes, leading to similar gene mutations [2, 3]. Aberrant methylation is commonly found in breast cancer tumors, with more than 70 genes shown to be inactivated by hypermethylation of the promoter CpG island [4]; among these genes is BRCA1 [5, 6].

Breast cancer associated gene 1, BRCA1, is a tumor suppressor gene identified in 1994 as the first gene associated with a familial predisposition to breast cancer. Only 5–10% of breast cancer patients are linked to an inherited germline mutation in the BRCA1 gene, and it occurs at an early age [7]. However, sporadic breast tumors, which dominate 90-95% of malignant breast cancers, have lower BRCA1 expression, but no mutated BRCA1 gene [8, 9]. Nevertheless, there are multiple possible alternative molecular mechanisms driving a permanent decrease in BRCA1 levels in tumor cells and silencing their functions, including allelic loss of heterozygosity (LOH) and hypermethylation of the BRCA1 gene promoter region [9, 10].

It has been reported by many researchers that DNA methylation was the major cause of transcriptional silence of BRCA1, ranging from 13% to 40% in sporadic breast cancer [11, 12].

After many studies, it is widely accepted that tumor DNA can be found in the peripheral blood of cancer patients and that this DNA often contains the same genetic and epigenetic modifications as DNA extracted from tumor tissue [13, 14]. These findings may explain the useful detection of tumor aberrant DNA in peripheral blood and may serve as an early and useful biomarker for breast cancer diagnosis and prognosis.

Furthermore, a recent 2020 meta-analysis study confirmed that there was no statistically significant difference in the methylation frequency of the BRCA1 gene promoter between peripheral blood and matched tumor tissue samples in breast cancer of the same patients, supporting the theory that the identification of BRCA1 promoter methylation in peripheral blood can be a rapid tool for monitoring the occurrence of breast cancer [15]. Previous research was conducted to obtain a more accurate estimate of the association between BRCA1 promoter methylation and sporadic breast cancer. These studies showed that methylation of the BRCA1 promoter in PBCs has emerged as a more accessible predictive biomarker for breast cancer [16].

For the first time in Algeria we used the methylation-specific PCR technique to examine the methylation status of the BRCA1 promoter in 39 PBCs from women with breast cancer and to identify the risk of breast cancer at an early age. Additionally, in order to determine whether BRCA1 promoter hypermethylation serves as a biomarker for early diagnosis of breast cancer, we studied the relationship between BRCA1 promoter methylation profiles and the clinicopathological characteristics of breast cancer.

Material and methods

Sample collection and DNA preparation

Blood samples for analysis were obtained from the Specialized Hospital Foundation for Oncology, EMIR EAk El-HASSY, Oran, Algeria, between January 2022 and June 2023. The patients selected with breast cancer, who are from different regions of Western Algeria, were interviewed and signed informed consent. Patients' clinicopathological features were obtained by interviewing each patient and filling out a questionnaire, completed by medical records, and 39 blood samples were collected from patients diagnosed with invasive breast cancer. The age at diagnosis of cases ranged from 29 to 64 years, with a median age of 43. All cases were graded according to the modified SBR system, and the clinical stage of the disease was determined according to the TNM classification of the International Union against Cancer. The blood samples collected were frozen at –20°C until the extraction of DNA.

DNA extraction

The manufacturer's instructions were followed to extract whole blood DNA using a commercial kit for blood genomic DNA extraction (Neo-Biotech; France). Blood for analysis was collected from patients within 3 months of the date of diagnosis and before taking any chemotherapy or cure. The DNA extracted quality was verified using horizontal gel electrophoresis (APELEX, France) and quantified using a UV spectrophotometer (BIOCHROM, WPA Lightwave II, England) and stored at –20°C until processing for analysis.

Methylation-specific PCR amplification (MSP)

The methylation status of BRCA1 was evaluated using methylation-specific polymerase chain reaction (MSP-PCR) to amplify bisulfite treated DNA with primers that distinguish methylated (M) and unmethylated (U) DNA. Methylation-specific PCR (MSPCR) has been utilized to detect hypermethylation of the areas of interest in the CpG islands of the BRCA1 promoter by many investigators [17–19].

Bisulfate modification

DNA methylation of the BRCA1 promoter region was assessed by MSP. Genomic DNA (1 µg) was treated with sodium bisulfate and purified using the imprint DNA modification kit (sigma, Aldrich, USA), according to the manufacturer's recommendation. During the treatment, the unmethylated cytosine of the genomic DNAs were converted to uracils, but the methylated cytosine remained unchanged [20].

Analysis of BRCA1 promoter methylation

Purified and converted DNA was processed to methylation-specific PCR (MSPCR). Modified DNA was amplified in a total volume of 25 µl of solution comprising 1X PCR buffer, 2 mM $MgCl_2$, 200 ng of each primer, 0.2 mM of each dNTP, 2 U of Hot Start Taq DNA polymerase, and 14 µl of target DNA. The primers and conditions for the methylation-specific PCR were first reported by Esteller *et al.* [17], followed by additional publications [21–23]. The primer sequences of the BRCA1 promoter for the unmethylated and methylated reactions are presented in Table I.

The unmethylated reaction's sense primer begins at 1536 bp, while the methylated reaction's sense primer begins at 1543 bp in the BRCA1 promoter (GenBank sequence U37574); this region crosses the major transcription start site of the BRCA1 gene [24]. The amplified product of the unmethylated PCR is 86 bp and that of the methylated product is 75 bp. Lymphocyte DNA treated with bacterial SssI methylase CpG methylase (M.SssI, Zymo Research, USA) served as a positive control for the methylated alleles Figures 1 A, B, and DNA from normal lymphocytes served as a negative control for the unmethylated alleles.

The PCR cycling parameters were as follows: 95°C for 10 min, then 40 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and the final extension at 72°C for 10 min. PCR products were electrophoresed on 2% agarose gel, stained with ethidium bromide and visualized on a UV transilluminator (VILBERT LOURMAT, France).

Statistical analysis

The associations between individual promoter BRCA1 methylation status and clinicopathological

Table I. Primer sequences for the unmethylated (u) and methylated (m) BRCA1 gene promoters

Primers name	Sequence (5′-3′)	Amplicon size [bp]
BRCA1 unmethylated (Forward) (U1)	5-TTG GTT TTT GTG GTA ATG GAA AAG TGT-3	86
BRCA1 unmethylated Reverse (U2)	5-CAA AAA ATC TCA ACA AAC TCA CAC CA-3	86
BRCA1 methylated Forward (M1)	5-TCG TGG TAA CGG AAA AGC GC-3	75
BRCA1 methylated Reverse (M2)	5-AAA TCT CAA CGA ACT CAC GCC G-3	75bp

U – unmethylated, M – methylated, bp – base pair.

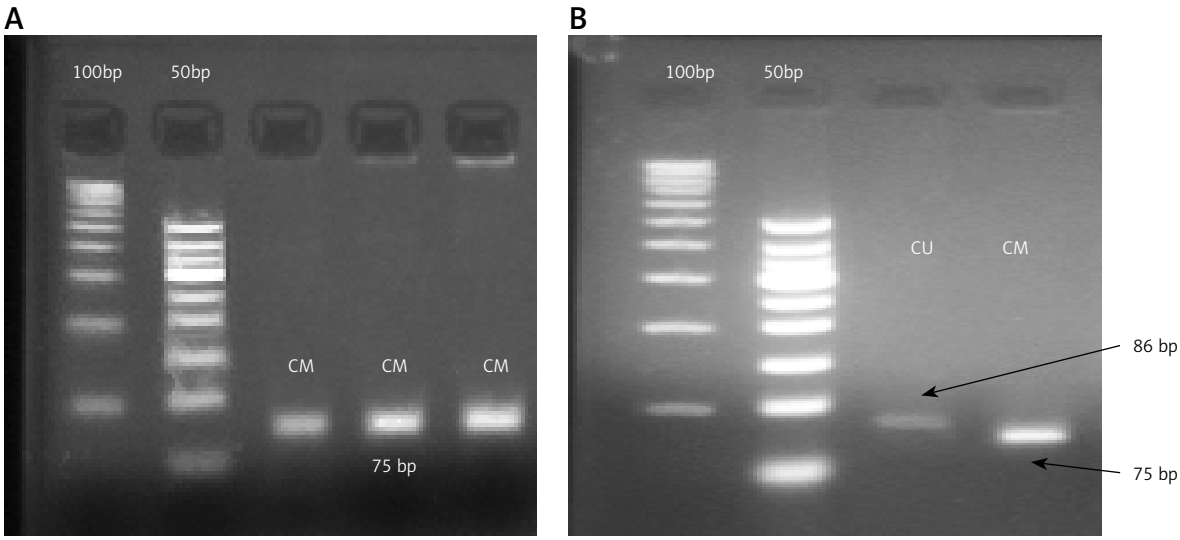

Figure 1. A – Specificity controls for the MSPCR reaction (CM). **B** – (CU) universally unmethylated bisulfite-converted genomic DNA and (CM) universally methylated bisulfite converted

characteristics were examined by use of Pearson's χ^2 test and Fisher's exact test. All statistical analyses were done using SPSS statistics software version 25.0 for Windows. A *p*-value of 0.05 or less was considered statistically significant.

Results

Gel electrophoresis results

The results for each experiment are presented here for 39 patients with breast cancer who were examined for BRCA1 promoter methylation in their peripheral blood by the MSPCR method. The clinical characteristics of the 39 breast cancer patients are summarized in Table II. Among these patients, the median age was 43 years (ranging from 29 to 64 years).

Our results demonstrate the methylation level of the BRCA1 promoter in peripheral blood. DNA was detected in 23 of 39 breast cancer patient women (58.97%), in which 12 PBCs were positive only for the methylated reaction (homozygous) and 11 were positive for both unmethylated and methylated reactions (heterozygous). Figure 2 shows the representative methylation status of the BRCA1 promoter by methylation-specific PCR.

U-labeled lanes represent PCR products amplified with unmethylated primers (86 bp). M-la-

Table II. Association between BRCA1 gene promoter methylation profiles in peripheral blood and clinicopathological characteristics of breast cancer patients

Characteristic	Total	BRCA1 promoter methylation		*P*-value
		Unmethylated	Methylated	
Age:				
≤ 45	24 (61.5%)	7 (43.8%)	17 (73.9%)	$p = 0.057$ $R = 0.3$ $\chi^2 = 3.627$
> 45	15 (38.5%)	9 (56.3%)	6 (26.1%)	
Family history:				
Yes	19 (48.7%)	8 (50%)	11 (47.8%)	$p = 0.894$ $\chi^2 = 0.018$
No	20 (51.3%)	8 (50%)	12 (52.2%)	
Histology type:				
Invasive ductal carcinoma	29 (74. 4%)	13 (81.3%)	16 (69.6%)	Fisher exact, $p = 0.480$
Others	10 (25.6%)	3 (18.8%)	7 (30.4%)	
Grade:				
I	1 (2.6%)	0 (0%)	1 (4.3%)	Fisher exact, $p = 0.848$
II	23 (59%)	9 (56.3%)	14 (60.9%)	
III	15 (38.5%)	7 (43.8%)	8 (34.8%)	
ER:				
Positive (+)	30 (76.9%)	14 (87.5%)	16 (69.6%)	Fisher exact, $p = 0.262$
Negative (–)	9 (23.1%)	2 (12.5%)	7 (30.4%)	
PR:				
Positive (+)	29 (74.4%)	12 (41.4%)	17 (58.6%)	Fisher exact, $p = 0.939$
Negative (–)	10 (25.6%)	4 (40%)	6 (60%)	
HER:				
Positive (+)	27 (69.2%)	10 (62.5%)	17 (73.9%)	Fisher exact, $p = 0.498$
Negative (–)	12 (30.8%)	6 (37.5%)	6 (26.1%)	
Molecular subtype:				
Luminal A	06 (15.4%)	4 (25%)	2 (8.7%)	Fisher exact, $p = 0.599$
Luminal B	26 (66.7%)	10 (62.5%)	16 (69.6%)	
HER	1(2.6%)	0 (0%)	1 (4.3%)	
Triple (–)	6 (15.4%)	2 (12.5%)	4 (17.4%)	
Metastasis:				
Yes	21 (53.8%)	7 (43.8%)	14 (60.9%)	$p = 0.291$ $\chi^2 = 1.113$
No	18 (46.2%)	9 (56.3%)	9 (39.1%)	
Nodular invasion	28 (71.8%) 11 (28.2%)	11 (68.8%) 5 (31.3%)	17 (73.9%) 6 (26.1%)	Fisher exact, $p = 0.734$

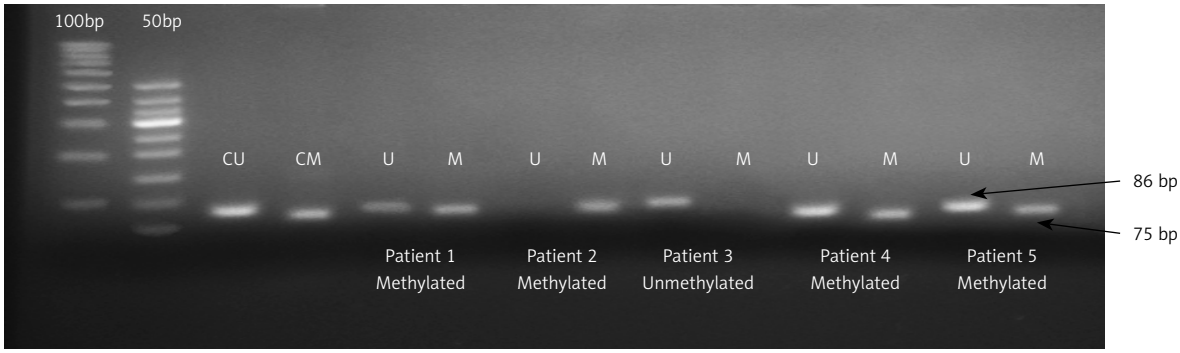

Figure 2. DNA methylation profiles of the BRCA1 promoter region in peripheral blood cells from 5 breast cancer patients, determined by MSPCR

beled lanes represent PCR products amplified with methylated primers (75 bp). Patient 3 only shows unmethylated reaction (negative). Patients 1, 4, 5 show the presence of a PCR product in both reactions, indicating BRCA1 methylated (positive). Patient 2 show methylated reaction (positive).

The association between BRCA1 promoter hypermethylation in peripheral blood and clinicopathological characteristics of breast cancer patients

The clinical significance of BRCA1 promoter methylation detected in peripheral blood was determined by investigating the relationship between the methylation profile of the BRCA1 promoter gene and clinicopathological characteristics of breast cancer patients. We presented the results in Table II.

Chi-square analysis revealed that although there was no significant difference between status of BRCA1 promoter methylation in ER receptors (p = 0.262), or PR receptor (p = 0.939), a high proportion of tumors with methylated BRCA1 in PBCs were of grade II (14/23; 60.9%) and classified luminal B (16/23; 69.6%) beside the histology type of invasive ductal carcinoma (16/23; 69.6%). Although with these results no significant correlation was found between the three parameters and methylation profile of BRCA1 promoter (p = 0.848; p = 0.599; p = 0.480), we observed an association between the methylation profile of BRCA1 promoter and metastasis (14/23), but this association was not statistically significant (p = 0.291). Furthermore, our results show equality between absence/presence of family history and hypermethylation of BRCA1 promoter (12/23 and 11/23) without any significant correlation. There was no association of BRCA1 promoter methylation with the presence of lymph node invasion (p = 0.734). However, a borderline statistically significant association was found between BRCA1 promoter methylation and early age (≤ 45) at diagnosis of breast cancer in this unselected group of patients (p = 0.057).

Discussion

The effect of BRCA1 promoter methylation status in PBCs DNA from breast cancer patients in western Algeria was investigated in order to reveal a novel epigenetic biomarker of breast cancer risk at an early age.

First, in this study, we estimated the probability of considering that the methylation status of the BRCA1 promoter detectable in PBCs is correlated with breast cancer risk. In order to carry out this work, we evaluated the frequency of methylation in an unselected group of Algerian women breast cancer patients.

In our study, we detected a high frequency (23/39-58.97%) of the hypermethylated BRCA1 gene promoter in PBCs among breast cancer women in western Algeria. A Polish study by Gupta *et al.* showed similar results; a significant association between BRCA1 promoter methylation in peripheral blood and breast cancer risk with a frequency of 22.7% (15/66) was reported in patients compared to control with 5.6% (OR = 12.1, 95% CI: 2.5–59.0) [25].

This observation is in disagreement with Japanese and Saudi Arabian studies of an unselected breast cancer patient group. BRCA1 promoter methylation in PBCs was found in the first study in 21.5% of cases and 13.5% of controls (OR = 1.73, p = 0.05) [26]; in the second study, performed by Al-moghrabi *et al.* (2011), BRCA1 promoter methylation was detected in 28.5% vs. 10.9% [27].

The frequency of aberrant promoter methylation in peripheral blood DNA as a potential biomarker of risk has been extensively investigated [28–33]. However, the results have been conflicting, because the previous research differed in sample size [14, 26], selection of study groups [29], and methods and techniques used for methylation analysis, and several did not include a control group [28]. These studies failed to detect any association with breast cancer risk [26–33].

Considering these previous observations in comparison with our findings, we can suggest that the methylation promoter BRCA1 gene detectable

in peripheral blood DNA from Algerian women could be potentially used as a new biomarker which is significantly associated with a risk of developing breast cancer.

One of the strengths of our study is the potential association between the methylation profile of the BRCA1 promoter and the early age at diagnosis of breast cancer (≤ 45 years) (p = 0.057). This finding is consistent with numerous published results in Saudi Arabia reported since 2014 by Al-Moghrabi *et al.* They indicated a strong association between the presence of methylated BRCA1 promoter in PBCs and early onset of breast cancer (< 50 years) (12.90%) [34]. These results are in concordance with previous studies, which showed that BRCA1 epimutations were significantly enriched in woman with early-onset breast cancer [28]. Similar findings were observed in research by Bosviel *et al.*; they found, in blood-derived DNA of affected women aged over 70 years, that BRCA1 promoter methylation was associated with age (p = 0.017) [30].

These observations suggest that BRCA1 promoter methylation found in peripheral blood at an early age may represent a heritable germline epimutation or a somatic mutation occurring in the early developmental stages, and this may not be the case later in life when heavily influenced by environmental factors.

This study sought to determine whether the possible contribution of the methylated BRCA1 promoter to the early onset of breast cancer in Algerian women, in addition to the high prevalence of the methylated BRCA1 promoter in the peripheral blood DNA of these patients, raises the possibility of its potential use as a biomarker for detecting predisposed individuals at a much earlier age. No association of the frequency of BRCA1 promoter methylation in PBCs with age was observed in studies by Iwamoto *et al.* and Kontorovich *et al.* [26, 29].

The results of our study did not show any significant correlation between the clinicopathological features and the profile of BRCA1 promoter methylation. In agreement with our results, Bosviel found that BRCA1 promoter methylation in PBCs was not associated with tumor grade and size, histological type or lymph node metastasis [30].

In contrast to previous studies, other studies that showed that the presence of BRCA1 promoter methylation in peripheral blood may be associated with pathologic features of breast cancer, i.e., estrogen (ER) and progesterone (PR) status [14, 27, 33].

Reports on the relationship between methylation of the BRCA1 promoter region and clinicopathological features have been conflicting. However, a recent study conducted in Saudi Arabia in 2024 indicated that BRCA1 epimutations significantly contribute to the development of breast cancer in Saudi cancer patients. These blood-based biomarkers could help identify female patients at high risk of developing triple negative breast cancer (TNBC) at an early age [34].

Our results demonstrated the lack of a significant correlation between histologic invasive ductal carcinoma and methylation status. However, Gupta *et al.* reported that methylation of the promoter of the BRCA1 gene detectable in peripheral blood DNA could be a marker for increased susceptibility to triple-negative or medullary breast cancer [25].

In conclusion, the present study provides evidence for the first time in Algeria that the aberrant methylation of the BRCA1 gene promoter is involved in at least the tumorigenesis of breast cancer.

Our findings show the possibility of using BRCA1 promoter methylation detected in peripheral blood as a significant biomarker for detecting women predisposed to breast cancer at an early age in western Algeria. These findings could lead to simple and accessible measures of DNA methylation to help identify women at high risk for breast cancer.

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Ethical approval

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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