# Detection of Hypermethylation BRCA1/2 Gene Promotor in Breast Tumors Among Moroccan Women

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**Research Article**

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# Abstract

**Background:** The promoter region is a key element of gene expression regulation. In mammals, most of the genes present, at the level of their promoter, a large number of islands CpG. Age is seen as another factor promoting breast cell cancer and the tumor stage.

**Aim:** this study aimed to explore the hypermethylation of the BRCA1/2 promoter gene in breast cancer women and correlation with age and tumor stage.

**Materials and methods**: fifty biopsies were derived from Moroccan women treated for breast carcinoma, the DNA extracted was treated by bisulphite and the targeted BRCA1/2 Amplicons were amplified by specific methylation primers (MSP).

**Results:** the result shows that 62% of the samples were BRCA1 methylated in addition and negative result for BRCA2, these positive epigenetic factor were remarkable in women over 47 years and at the stage of malignant tumor.

**Conclusion:** these results show that half of the methylated samples are positive and the majority are over 47 years old, and confirms the impact of age on methylation and might be other factor of breast cancer development.


# Introduction

Any stable alteration during cell proliferation, without any modification of the gene sequence defined as an epigenetic phenomenon. Methylation of DNA is one of the highly responding epigenetic factors in the mammalian genome at a rate of 1% [1]. The methylation profile is uniform on DNA, from regions of non-methylated segments interspersed with methylated regions [2]. It is a reversible covalent chemical modification defined by the addition of a methyl (CH3) group to the carbon 5 of the cytosine followed by a guanine (5' CG 3'), also called dinucleotide Cytosine-phosphate-Guanine (CpG) [3], which occurs throughout the genome with a frequency of about 6% [4]. Nevertheless, studies have predicted that except 5% of the predicted frequency is remarkable because of the hypermutability of condensed methylcytosine in a given region. These small regions of DNA, called CpG islands, ranging from 0.5 to 5 kb and occurring on average every 100 kb, have distinctive properties. These regions aren't methylated but are rich in GC (60–70%) with a CGI ratio of at least 0.6 [4]. About half of all genes in humans have CpG islands, and these are present on DNA repair genes and expressed genes in a specific tissue [5]. DNA methylation can alter gene activity without changing the sequence. Normal cells gradually transform into malignant cells by damage to the genome, which could be a gain, a loss of genetic information leading to a predisposition to cancer [6]. Cancers generally characterized by overall hypomethylation and hypermethylation of tumor suppressor genes. In eukaryotes, the regulation of gene expression is a complex process. It all starts at the transcription level; the genes are expressed in protein via the ribosomes that attach to the promoting region producing a protein-coding for a given function. This transcription is activated and suppressed by a variable rate of methylation in this region. Increased

methylation in the region of a gene promoter leads to reduced expression [7]. In Morocco, breast cancer is ranked first cancer according to the global burden of cancer report – International Agency for Research on Cancer (GLOBOCAN-IARC), which shows 7% of the prevalence of breast cancer in Africa [8]. Several tumor suppressor genes have made the study of mutations in this type of cancer the BRCA1 gene in the first place. BRCA1 is the first genetic predisposition gene (17q21.31) and contains 22 exons coding 1,683 amino acids [9, 10]. More than 500 mutations or sequence variations have already been described in the world [11]. The prevalence of mutations in the BRCA1 gene depends on the ethnic origin of the populations and the activation of the various proteins involved in several cellular processes. The objective of this study is to show the relationship between BRCA1 promoter (LOC111589215) hypermethylation and BRCA2 associated with patient age and cancer stage.

## Materials And Methods

## • Breast tissue specimens

Fifty breast biopsies were derived from hospital university centre (CHU) Ibn Rochd in Casablanca, Morocco, between December 2018 and June 2019, belong women already diagnosed with breast cancer. The age of patients at diagnosis ranged from 31 to 70 years, with a median of 47 years. Along with every sample, the age and tumor stage were recorded. Required Ethical approval was obtained from the committee of biomedical research ethics in Morocco (No. 3/2018/30 April/2018-Morocco).

## • DNA extraction

DNA extraction from breast cancer fresh biopsies performed by using the Kit PureLink™ Genomic DNA (Invitrogen). Aliquots of 25 mg of samples were digested with 20 µl of proteinase K and 180 µl of Digestion buffer at 55ºC, for 1 to 4 hours. DNA precipitation was performed by adding 200 µl of ethanol (90%). DNA was eluted in 25 to 50 µl of Elution buffer and stored at -20ºC until further use. DNA was quantified using the Nanodrop spectrophotometer. Samples with a DNA concentration of 20–50 ng/µl or above were selected to perform the polymerase chain reaction.

## • Intrenal control

The samples were checked by internal control for β-globin gene by PCR. Using the primers PCO4 (5’CAACTTCATCCACGTTCACC3’) and GH20 (5’GAAGAGCCAAGGACAGGTAC 3’)which flanks a sequence of about 300 pb [12]. Only the samples that were positive for human ß-globin gene are the subjected to research to the DNA methylation research.

## • Sodium bisulfite conversion and methylation-specific PCR

The conversion of DNA by sodium bisulfite was performed as described previously [13]. Bisulfite treatment was performed using an EZ DNA Methylation Gold Kit (ZYMO Research) according to the manufacturer's protocol. Briefly, 1–2 µg of genomic DNA was denatured with 2 M NaOH (final concentration of 0.2 M) at 50°C for 20 min, followed by incubation with freshly prepared 2.5 M sodium

bisulfite/1 M hydroquinone (pH 5.0) in a total volume of 520 ml, at 70°C for 18 hours. The modification of the DNA completed by the addition of 5 ml of 3 M NaOH at room temperature for 10 min. The precipitation of the modified DNA was carried out through the addition of 75 ml of ammonium acetate 5 M (pH 7.0) and 350 ml of ethanol. The bisulfite-modified DNA was resuspended in 100 ml of sterile water and stored at 20°C or immediately used for MS-PCR analysis.

## • Methylation-specific PCR (MSP)

The PCR amplification was performed with treated DNA as template in a total volume of 25 ml containing 0.25 mM of each primer pair, 200 mM of each dNTP, Tris–HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 1 unit of Taq DNA polymerase (Bioline). For methylated test; the primers: forward (3'-TCG TGG TAACGG AAA AGC GC-5'), reverse (3'-AAA TCTCAA CGA ACT CAC GCC G-5') and targeting 75 pb fragment size at methylation temperature 62°C in 50 uL total PCR tube.For unmethylated test; the primers: forward (5'-TTG GTT TTT GTG GTAATG GAA AAG TGT-3'), reverse (3'-CAA AAAATC TCA ACA AAC TCA CAC CA -5') and targeting 85 bp fragment size at methylation temperature 58°C in 50 uL total PCR tube. The BRCA2 promoter methylation primers were forward: 5'-CGGTTAGATATTGACGGTTGGGA-3' and reverse: 5'-ACCCGCGCTAACCACGTATCGC-3'. Primers specific for unmethylated BRCA2 promoter were forward: 5'-GGTTAGATATTGATGGTTGGGA-3' and reverse: 5'-ACACCA CAAACCCACACTAACCAC-3'. Products sizes were 145 bp for both BRCA2 primer sets.

Cycling conditions were as follows: denaturation at 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, for 30 s at the specific temperature and 30 s at 72°C, The reaction was finished with a 10 min extension at 72°C.

PCR products were electrophoresized in 2% agarose gels containing 3 µL of ethidium bromide and visualised in UV light. The Standard PCR precautions and procedures were used to avoid contamination.

## • Statistical analysis

Statistical analyses Statistical analyses were performed using the Fisher exact chi square test, which facilitates comparison between very small numbers.

# Results

The biopsies studied belong to Moroccan women. The patient's age ranges from 31 to 70 years, with a median of 47 years. DNA extracts are treated with bisulphite, which converts non-methyl cytosines, while methyl cytosines remain unchanged. Methylation of the BRCA1 gene promotor in breast cancer biopsies in Moroccan women is revealed by MS-PCR in a series of 50 breast carcinomas in the Casablanca region and 10 normals tissus.

Methylation of the BRCA1 region was detected in 24 cases (48%) of the 50 tumors studied, 28% of which were negative. The result shows that the methylation profile in the BRCA1 promotor region is very high in

women over 47 years of age (Table 1) with a 58,33% of the 48% positive, while the under 47 years of age group has only 41,66% of the positive results.

Table 1
Methylation and unmethyltion BRCA1 profil in breast cancer biopsies.

| | | BRCA1 Methylated | | | BRCA1 Unmethylated | | |
|---|---|---|---|---|---|---|---|
| | N | Positive cases | % | p-value | Positive cases | % | p-value |
| **Age** | | | | | | | |
| ≤ 47 years | 20 | 10 | 50 | *0,896428* | 10 | 50 | *0,896428* |
| ≥ 47 years | 30 | 14 | 46,66 | | 16 | 53,33 | |
| **Tumor stage** | | | | | | | |
| Metastasis | 22 | 8 | 36,36 | *0,381639* | 14 | 63,63 | *0,414743* |
| Malignant tumor | 28 | 16 | 57,14 | | 12 | 42,85 | |

*All comparisons for statistical significance were performed by use of Fisher's exact test, and chi-square test. The result is not significant at $p < .05$.

According to the age groups of breast cancer patients in Morocco, the most significant age group is between 47 and 60 years, followed by the age group between 50 and 60 years (Fig. 1).

Depending on the stage of cancer, malignant tumor predominate in women over 47 years of age with a 66,66 %. This percentage is divided into 41,66 % in the 40 to 50 year age group followed by the 50 to 60 year age group indicates 20,83 %, and the group of age [50–60] is the 33,33 % with a small percentage among women over 60 years of age of 4,16 % (Fig. 1).

Conversely, the relationship between clinicopathological features of the tumor stages and the BRCA1methlation promoter in the fresh biopsies of breast cancer patients did not show a statistically significant difference for any of the parameters (Table 2).

Table 2
Clinical Characteristics of patients with breast cancer.

| | **BRCA1 Hypermethylated (%)** |
|---|---|
| **Nombre** | 50 |
| **Age** | |
| < 47 years | 10 (41,66 %) |
| ≥ 47 years | 14 (58,33 %) |
| **Tumor stage** | |
| Metastasis | 8 (33,33 %) |
| Malignant tumor | 16 (66,66 %) |
| **Tumor size** | |
| ≤5 cm | 4 (16,66 %) |
| ≥ 5cm | 20 (83,33 %) |
| **Tumor grade** | |
| I | 4 (16,66 %) |
| II | 6 (25 %) |
| III | 14 (58,34 %) |
| **ER** | |
| ER+ | 21 (87,5 %) |
| ER- | 3 (12,5 %) |
| **Therapy** | |
| Chemotherapy | 20 (83,33 %) |
| Untreated | 4 (16,67 %) |
| *All comparisons for statistical significance were performed by use of Fisher's exact test, and chi-square test. The result is not significant at $p < .05$. | |

Breast cancer is a disease that primarily affects women with or without a history of cancer and may be hereditary. This cancer mainly affects women over 47 years of age and presents malignant tumors in the majority of cases.

The result of test khi2 present a significant result in methylation of the promotor region between normal and cancer tissues. The khi2 test is 4.95 a result which means that the hypermethylation of the promoter

of the BRCA1 gene is a biomarker, confirmed by a significant p-value 0.026.

# Discussion

The loss of function of a tumour suppressor gene is induced on the one hand by epigenetic modifications including the hypermethylation mechanism of the promoters of these genes [14]. Several studies have shown that the hypermethylation of CpG islands of certain tumour suppressor genes is responsible for the loss of expression of these genes [15].

BRCA1/2 is a tumor suppressor gene involved in the detection and repair of double strand breaks in DNA. Certain molecular events, such as methylation, lead to inactivation of these genes and thus to alteration of cellular functioning. Biochemically, methylation is a vital metabolic process, which consists of the transmission of one methyl group (-CH3) from one molecule to another [16]. This process ensures a multitude of functions in the human body, primarily the modulation of the expression of certain tumor suppressor gene.

BRCA1, which is a typical tumour suppressor gene, contributes to the regulation of transcriptional activation, DNA repair, apoptosis, control of cell cycle control points and chromosomal remodeling [17]. This study reported that the hypermethylation of the gene promoter BRCA1 was present in 48% of breast cancer patients, which was significantly associated with the age range greater than 40 and a malignant tumour stage.

Our results are showing that BRCA1 promoter hypermethylation was involved in breast carcinoma as an epigenetic change. Our study also supports the findings of Hanahan D. et al., 2000 as they studied that tumour of breast cancer patients showed promoter hypermethylation of BRCA1 gene [18], these results were similar to our studied reults, that in BRCA1 gene in breast cancer patients, there is an altrations of methylation pattern, which may the causative agent for the breast cancer and is also in support of the findings of Turnbull et al., 2008 [19], they studied in their study that alterations in BRCA1 have been estimated to be responsible for about 50% of familial breast cancer.

This led us to speculate that the hypermethylation of the promoter of the BRCA1 gene seemed to confer an advantage for tumor cell invasion, and can be used as a biomarker. The results of this study demonstrated that expression of BRCA1 decreased significantly in patients with BRCA1 hypermethylation, consistent with results reported by Shilpa et al., [20]. In contrast, the promoter of the BRCA2 gene was unmethylated in the study patients despite the known interaction between BRCA1 and 2.

These epigenetic markers like hypermethylation in the promoter region have the double interest of being highly sensitive (a reliable prediction of more than 96%) but also very specific to the tissues in which they are found [21].

# Conclusion

Several factors influence DNA methylation, such as the age of the cancer stage. The interraction between tumor suppressor genes is very extensive which leads to searching for the methylation profile in different genes succeptible to induce breast cancer. In Morocco, BRCA1 is very remarkable in the tissues subject to the study but negative results are for BRCA2.This shows that epigenetics in terms of methylation is accelerated with age, and promotes cancer, so it can be considered as a better biomarker for prognostic therapy.

# Abreviation

**BRCA1**

breast cancer 1

**BRCA2**

breast cancer 2

**CpG**

cytosine-phosphate-guanine

**DNA**

disoxyribonucleotide

**CGI**

cytosine-guanine island

**bp**

base pairs

# Declarations

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### Conflict of interest

The authors declare no conflict of interest

### Ethics Approval

The study was approved by the Biomedical Research Ethics Committee of the School of Medicine and Pharmacy of Casablanca.

### Consent to Participate

All patients consented to participate in the study according to the ethical standards.

### Consent for publication

All authors approved and consented to publish the presented results

### Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**The project respects the agreement of the ethics committee (**No. 3/2018/30 April/2018-Morocco**).**

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# Figures

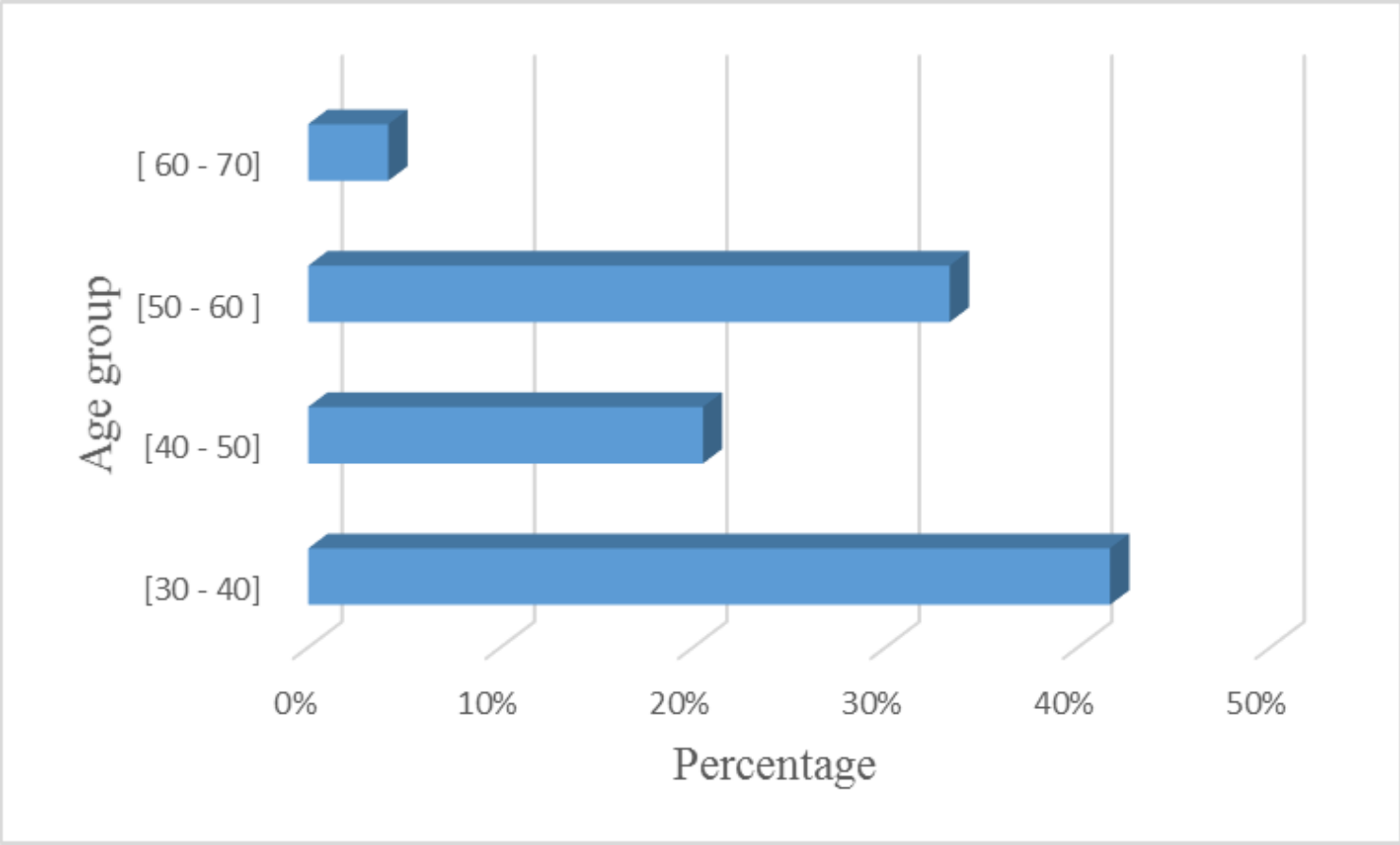


**Figure 1**

presentation percentage depending age group in tissue breast cancer.