Methylation-related mutations in the *BRCA1* promoter in peripheral blood cells from cancer-free women

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Abstract. Early-onset breast cancer is one of the most common malignancies and causes of death among young women, and its incidence is increasing. In the present study, we aimed to investigate the epigenetic modifications of the breast cancer type 1 susceptibility gene (*BRCA1*) in breast tissues and blood cells derived from women with breast cancer and women without breast cancer. *BRCA1* promoter methylation was examined by methylation-specific PCR in 47 breast cancer tissues and in peripheral blood cells derived from 7 breast cancer patients and 73 healthy women. Subsequently, the methylation status of the *BRCA1* promoter was confirmed and analyzed at high resolution by sodium bisulfite genomic sequencing. *BRCA1* promoter methylation was detected in 13 primary sporadic breast cancer tissues (27.3%) and in 2 blood cell samples derived from breast cancer patients (28.5%). A strong association (p-value, 0.0038) was found between *BRCA1* methylation and young age (≤40 years) at diagnosis. The *BRCA1* promoter was also methylated in blood cells from 8 women without breast cancer (10.9%) and 2 breast cancer patients (28%). The methylation pattern of the *BRCA1* promoter CpG island was similar in the blood cells from healthy women as well as in women with breast cancer. Moreover, we report for the first time, the observation of methylation-related mutations leading to the formation of non-CpG methylation, as well as the formation of novel methylated CpG sites in the 5' regulatory region of the *BRCA1* gene in the peripheral blood cells from cancer-free women. These results suggest the possible implication of *BRCA1* promoter methylation in the early onset of breast cancer and propose the use of this epigenetic modification as a powerful molecular marker for detecting women potentially predisposed to cancer.

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Introduction

DNA methylation is an important epigenetic mechanism that occurs in CpG and non-CpG sites and is involved in the regulation of gene expression. The CpG sites are clustered into islands, which are typically found in the promoter regions of genes (1,2). In human cancers, this heritable yet non-genetic modification is a powerful mechanism responsible for the inhibition of different types of genes, including tumor suppressors (3). Besides, the process of non-CpG methylation in GC-rich regions is proposed as an early event in carcinogenesis (4).

The identification of the breast cancer type 1 susceptibility gene (*BRCA1*) as the first major gene associated with breast cancer was a breakthrough towards understanding the molecular bases of this disease (5). Since then, germline mutations of *BRCA1* have been found to be related with the hereditary type of breast cancer, which accounts for about 5-10% of all breast cancers. Individuals carrying germline *BRCA1* mutations are more likely to develop aggressive breast tumors at an early age (<50 years) (6). These tumors are characterized by aneuploidy, a high grade, poor histological differentiation and hormone receptor negativity. These aggressive pathological features were also found to be present in sporadic early-onset breast cancers, suggesting that alterations in *BRCA1* or *BRCA1*-related pathway(s) could also play a role in non-hereditary forms of breast cancer. However, no somatic mutations of *BRCA1* have been found in the sporadic form of the disease (7,8), which accounts for about 90% of breast cancer cases. Nevertheless, *BRCA1* mRNA and protein levels were found to be reduced in a subset of sporadic human breast cancers (9). Therefore, gene silencing by epigenetic mechanisms has been suggested to be an alternative mechanism for *BRCA1* inactivation during sporadic carcinogenesis (10). Results from various studies using different methods of detection have revealed that 9-44% of sporadic breast cancer samples harbor the hypermethylated *BRCA1* promoter (10,11). These tumors display similar pathological features to *BRCA1*-mutated hereditary breast cancer. Indeed, both occur at an early age and present poor histological differentiation, aneuploidy, as well as estrogen receptor (ER) and progesterone receptor (PR) negativity in addition to the similarities in their global gene

expression profiles (12,13). Furthermore, the presence of *BRCA1* promoter methylation has been detected in DNA from both peripheral blood mononuclear cells (PBMCs) and tumor tissues in 3 out of 7 breast cancer patients from breast-ovarian cancer families (14). This suggested an association between the development of *BRCA1*-like breast cancer and the detection of promoter methylation of *BRCA1* in normal tissues of the body. Therefore, it was hypothesized that the inactivation of *BRCA1* by promoter methylation could occur as a germline or as an early somatic event that predisposes to breast cancer with a phenotype similar to that associated with *BRCA1* germline mutations (14).

The analysis of the 5' regulatory region of *BRCA1* showed that this gene has a promoter with a complex organization. It contains a CpG island that consists of 30 CpG sites covering the region from -567 to +44 relative to the exon 1A transcription start site. Within this region there is a bi-directional core promoter (-218 to +1) which regulates the transcription of both the *BRCA1* and the NBR2 genes. NBR2 lies in a head-to-head orientation 218 bp from the *BRCA1* gene (15). This short CpG-rich stretch of *BRCA1* at the 5' flanking region contains 11 CpG sites that have previously been shown to contain strong promoter activity (16). This region is unmethylated in normal human mammary epithelial cells (17), but it is a target region for aberrant cytosine methylation in human breast cancer cells and tissues (17,18-21).

Breast cancer among Arab women, as in other parts of the world, is one of the most common malignancies and causes of death, and its incidence is increasing. It is noteworthy that the proportions of breast cancers that develop before the age of 40 represent 26.4% of all female breast cancers compared to 6.5% in the USA (22). The study of *BRCA1* and *BRCA2* mutations in women older than 40 years with breast cancer has indicated the likely contribution of these mutations to the pathogenesis of familial breast cancer (23). However, the underlying biology of sporadic breast cancer in young Arab women still needs to be elucidated.

In the present study, we used the methylation-specific PCR (MSP) technique to examine the methylation status of the *BRCA1* promoter in 47 breast cancer tissues and in blood cells from 73 healthy women. The obtained results provide evidence for the possible implication of the *BRCA1* gene in the early onset of breast cancer in women through epigenetic modifications. In addition, we show for the first time, the presence of methylation-related mutations in the 5' regulatory region of the *BRCA1* gene in the PBMC DNA of healthy women.

Materials and methods

Study samples. Tissue samples from 47 Arab female patients, diagnosed with locally advanced breast carcinoma, were obtained from the Pathology Department, King Faisal Specialist Hospital and Research Centre in Riyadh, Saudi Arabia. The age of the patients at diagnosis ranged from 22 to 80 years. Clinicopathological data (age, histological grade and ER and PR status) were provided by the Department of Pathology. As the control, 73 fresh blood samples were collected from healthy female volunteers with ages ranging from 17 to 76 years. All patients gave written informed consent to participate in the study. The study was approved by the Human Research Ethics Committee of King Faisal Specialist Hospital and Research Centre.

DNA extraction. DNA was extracted from tissue and blood samples by the DNA isolation kit 'Gentra' following the manufacturer's instructions.

MSP. DNA methylation of the *BRCA1* promoter region was assessed by MSP. Genomic DNA (1 μg) was treated with sodium bisulphite and purified using the EpiTect Bisulphite Kit (Qiagen) following the manufacturer's recommendations. Modified DNA was amplified with published PCR primers that distinguish methylated and unmethylated DNA (23,24). The sense methylated and unmethylated primers begin at 1543 and 1507, of GenBank sequence U37574, giving PCR products of 84 and 103 bp, respectively. The methylated primers were: Sense, 5'-TCGTGGTAACGGAAAAGCGC-3' and antisense, 5'-CCGTCCAAAAAATCTCAACGAA-3'. The PCR cycling parameters were 95˚C for 1 min; 40 cycles at 95˚C for 30 sec, 60˚C for 55 sec, 72˚C for 30 sec, and then extension at 72˚C for 7 min (24). The unmethylated primers were: Sense, 5'-TTGAGAGGTTGTTGTTTAGTGG-3' and antisense, 5'-AACAAACTCACACCACACAA-3'. The PCR cycling parameters were 95˚C for 1 min; 40 cycles at 95˚C for 30 sec, 54˚C for 30 sec, 72˚C for 30 sec, and then extension at 72˚C for 7 min (25). The PCR products were electrophoresed on 2% ethidium bromide-stained agarose gels. *Sss*I methylase-treated and -untreated bisulfite-modified lymphocyte DNA was used as the positive and negative controls, respectively. All PCR reactions were carried out in replicate.

Cloning of the PCR products. The PCR products from the methylated primers were cloned into the TOPO TA plasmid, and 10 positive recombinants were isolated and sequenced as described below.

High-resolution sodium bisulfite genomic sequencing of the BRCA1 promoter region. The *BRCA1* promoter island was amplified from the bisulfite modified DNA by two rounds of PCR using nested primers specific to the bisulfite-modified sequence as previously described (17). The resultant PCR product was cloned into the TOPO plasmid following the manufacturer's instructions. Twenty to 35 positive recombinants were isolated using the QIAprep Spin Plasmid Miniprep Kit (Qiagen) according to the manufacturer's instructions and sequenced on an ABI automated DNA sequencer. The sequences obtained were compared to the known *BRCA1* sequence for the determination of the methylation status of individual CpG sites. The methylation percentage for each site was determined by dividing the number of methylated CpGs at a specific site by the number of clones analyzed.

Statistics. The Chi-square and Fisher's exact tests were performed to determine the statistical significance for the correlation between *BRCA1* promoter methylation and clinicopathological features. Comparisons between median ages of breast cancer cases and healthy cancer-free individuals were carried out using the Mann-Whitney test. All observed differences were considered to be significant when associated with a value of $p<0.05$.

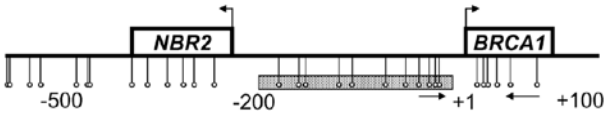

Figure 1. Schematic representation of the *BRCA1* CpG island. The middle hatched box represents the region of the *BRCA1* core promoter. The bent arrows show the transcription start sites and directions. The vertical lines with circles indicate the positions of the 30 CpG sites analyzed. The horizontal arrows show the position of the MSP primers. The numbers refer to the nucleotide positions relative to the *BRCA1* transcription start.

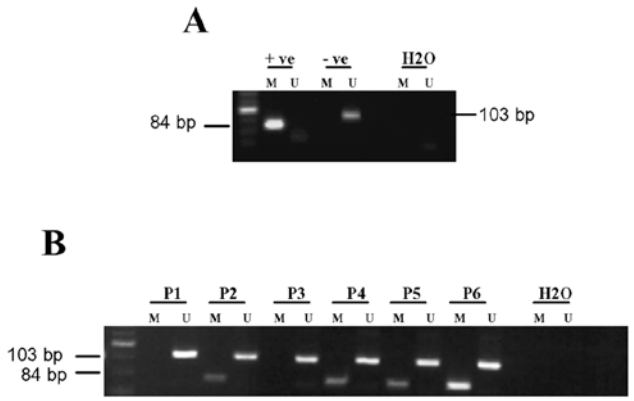

Figure 2. MSP analysis of the *BRCA1* promoter region in breast cancer tumor tissues. (A) *Sss*I methylase-treated and -untreated bisulfite-modified RD39 DNA used as the positive (+ve) and negative (-ve) controls, respectively. (B) MSP products amplified from tumor DNA derived from breast cancer patients (P1-P6). M, methylated product; U, unmethylated product.

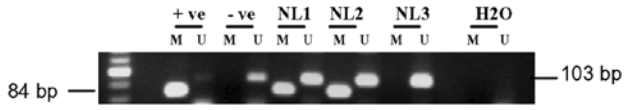

Figure 3. MSP analysis of the *BRCA1* promoter region in peripheral blood cells from women without breast cancer. *Sss*I methylase-treated and -untreated bisulfite-modified RD39 DNA used as the positive (+ve) and negative (-ve) controls, respectively. NL1-3, blood DNA samples from women without breast cancer. M, methylated product; U, unmethylated product.

Results

Methylation of the BRCA1 promoter in breast cancer tissues. In order to study the prevalence of *BRCA1* promoter methylation, genomic DNA was purified from 47 breast cancer tissue samples and analyzed by MSP. The methylated PCR product is a stretch of 84 bases containing 10 CpG sites (Fig. 1). Representative results for MSP are shown in Fig. 2. *Sss*I methylase-treated and -untreated bisulfite-modified lymphocyte DNA was used as the positive and negative controls, respectively (Fig. 2A). Importantly, *BRCA1* promoter methylation was detected in 13 out of 47 (27.6%) primary sporadic breast cancer tissues (Fig. 2B; Table I).

BRCA1 promoter methylation is associated with the early onset of breast cancer. We then investigated the potential association between *BRCA1* promoter methylation and the clinicopathological features of breast tumors. A strong association was found between *BRCA1* methylation and young age (≤40 years) at diagnosis (9/12) 75% (p=0.0038), (Table I). No association of *BRCA1* methylation was found with ER status (p=0.190), or PR receptor status (p=0.734). However, a high proportion (8/12; 67%) of tumors with methylated *BRCA1* were of grade 3.

Table I. Clincopathological features of sporadic *BRCA1*-methylated and -unmethylated breast tumors.

Feature	Breast tumors (n=47)		
	BRCA1-methylated (n=13)	*BRCA1*-unmethylated (n=34)	P-values
Age at diagnosis			
≤40	9 (75%)	8 (23.5%)	0.0038
>40	3 (25%)	26 (73.4%)	
ND/NA	1		
Histological grade			
G3	8 (62%)	19 (56%)	
G2	4 (31%)	15 (44%)	
ND/NA	1		
TP53 mutations			
Mutated	4 (31%)		
Wild-type	9 (69%)		
ND/NA			
Estrogen receptor			
Negative	7 (58%)	12 (35%)	0.190
Positive	5 (42%)	22 (65%)	
ND/NA	1		
Progesterone receptor			
Negative	8 (67%)	19 (56%)	0.734
Positive	4 (33%)	15 (44%)	
ND/NA	1		

ND/NA, no data/not available.

Methylation of BRCA1 promoter in peripheral blood DNA from women with breast cancer and healthy women. In order to examine *BRCA1* promoter methylation in non-cancerous tissue, we made use of blood cells from both breast cancer, as well as from healthy women. Genomic DNA was purified from PBMCs from 7 breast cancer patients, other than the 47 patients used above, whose tumors showed *BRCA1*-like characteristics, and from 73 healthy individuals. Hypermethylation was detected in 2 out of 7 (28.5%) blood cells from breast cancer patients. Unexpectedly, the methylated *BRCA1* promoter was also detected in 8 out of 73 (10.9%) normal blood samples (Fig. 3). To confirm these results, the methylated PCR products of 3 normal blood DNA samples were cloned and sequenced and 10 clones were examined from each sample. In 2 out of the 3 samples, methylated

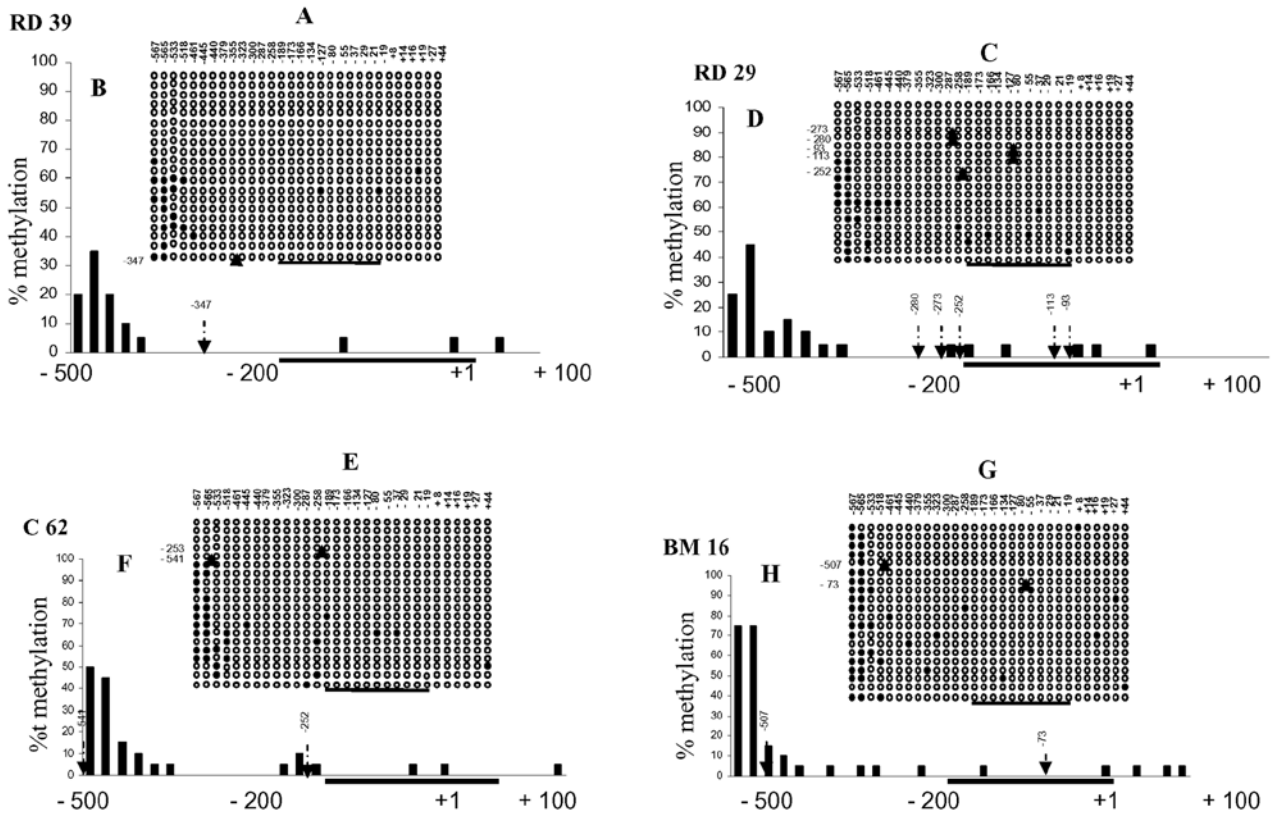

Figure 4. Cytosine methylation status of the *BRCA1* CpG island in the normal peripheral blood cell DNA samples. The 611 bases (-567 to +44) of the *BRCA1* promoter region as depicted on Fig. 1 were cloned into the TOPO TA plasmid. For each cloned sample, 20 to 35 clones were isolated and sequenced. (A, C, E and G) Allelic patterns of cytosine methylation of the samples (Rd39, RD29, C62 and BM16, respectively). Each row of circles represents the cytosine methylation pattern obtained for individual clones of the *BRCA1* CpG island PCR products. The position of each CpG site relative to the transcription start site is shown. Open circles indicate unmethylated CpG sites, and filled circles indicate methylated CpG sites. The arrow heads indicate the position of the methylation-related newly-formed CpG sites. (B, D, F and H) Graphical representations of the allelic patterns of cytosine methylation described above for the samples (Rd39, RD29, C62 and BM16, respectively). The methylation percentage of each site was determined by dividing the number of methylated CpG sites at a specific site by the total number of clones analyzed. The line beneath each graph designates the *BRCA1* core promoter region. The dotted arrows indicate the position of methylation-related newly-formed CpG sites.

cytosines were present in 9 out of the 10 CpG sites (-37, -29, -21, -19, +8, +14 +16, +19, +27 and +44) (Fig. 1), while in the third one, all 10 clones had methylated cytosines present in the 10 CpGs, which confirmed the methylation seen by the MSP assay.

The methylation pattern of the BRCA1 promoter CpG island is similar in blood cells from healthy women and women with breast cancer. To further elucidate the methylation of the *BRCA1* promoter, the methylation pattern of the 30 CpG sites in the *BRCA1* promoter region was analyzed by high resolution sodium bisulfite sequencing of DNA from peripheral blood cells obtained from healthy women as well as from women with breast cancer. Six hundred and eleven bases (-567 to +44) of the *BRCA1* promoter region (Fig. 1) were cloned into the TOPO TA plasmid for sequencing. For each cloned sample, 20 to 35 clones were isolated and sequenced. Fig. 4A and B shows the pattern of cytosine methylation in the *BRCA1* CpG island of the sample (Rd39) used as the negative control for methylation. The sample showed low levels of cytosine methylation detected only at few sporadic sites. On the contrary, in the positively methylated *BRCA1* samples, RD29, C62 and BM16, several CpG sites were methylated in the *BRCA1* core promoter region as well as throughout the entire CpG island (Fig. 4C and D, E and F, and G and H, respectively). These results showed an allelic pattern of cytosine methylation with a low percentage (5 to 15%) of the DNA molecules showing methylation throughout the promoter region with the remainder being largely unmethylated.

The pattern of methylated cytosines obtained for the normal peripheral blood DNA was compared to that of the 2 *BRCA1*-methylated breast cancer peripheral blood DNA samples, C1 and C2 (Fig. 5A and B, and C and D, respectively). It should be noted that the two patterns exhibited high similarity.

Presence of methylation-related mutations in the BRCA1 promoter CpG island. Intriguingly, the high resolution analysis by DNA sequencing of the methylated *BRCA1* promoter region detected in DNA from both normal as well as breast cancer peripheral blood cells, revealed the presence of methylation-related mutations. In these types of mutations, T to C transitions were associated with cytosine methylation leading to the formation of non-CpG-methylated sites, such as the site -147 (Fig. 6), and novel CpG-methylated sites, such as the site -113 (Fig. 6). Several of these methylation-related mutations were detected in the *BRCA1* core promoter region as well as throughout the entire CpG island (Figs. 4 and 5). In

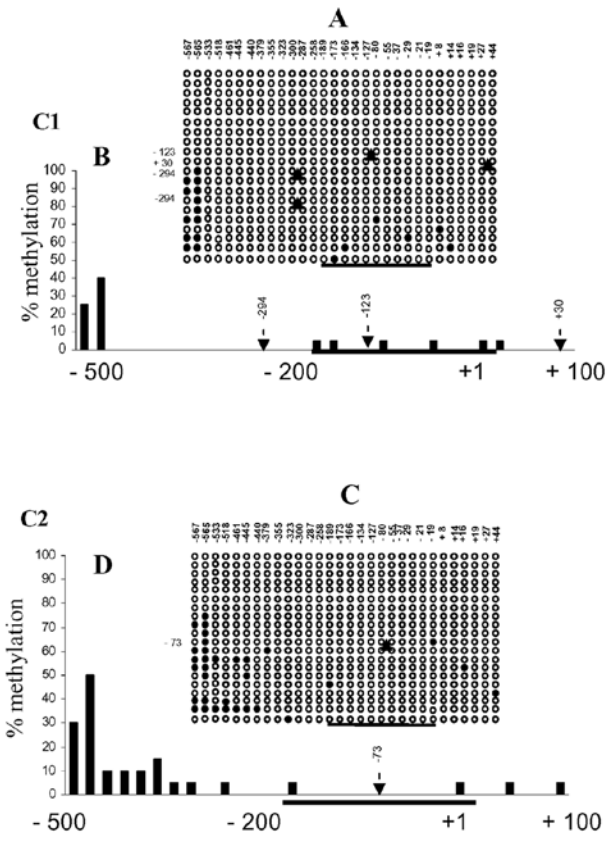

Figure 5. Cytosine methylation status of the *BRCA1* CpG island in peripheral blood cell DNA from breast cancer patients. (A and C) Allelic patterns of cytosine methylation. (B and D) Graphical representations of the allelic patterns of cytosine methylation for the samples (C1 and C2, respectively).

contrast, only one such novel CpG site was detected outside the core promoter region in the *BRCA1* methylation-negative sample (Rd39).

Age similarities between breast cancer patients and healthy women harboring BRCA1 promoter methylation. The Mann-Whitney test was used to compare the median age at diagnosis for the 12 *BRCA1*-methylated breast cancer cases with that of the 8 healthy individuals harboring the *BRCA1*-methylated promoter. As can be seen in Fig. 7, the two groups showed similar median age values of 38.5 and 32 years, respectively (p=0.4363).

Discussion

In this study, we report a high frequency (27.6%) of the hypermethylated *BRCA1* gene promoter in primary sporadic breast carcinomas in Arab women, which is at the higher end of previously reported frequencies of 7 to 44% (10,11). Importantly, the methylation of the *BRCA1* promoter was found to be strongly associated with the onset of cancer at an early age (≤40 years) (p=0.0038) and was more common in high-grade tumors. This is in agreement with previously published results where it has been shown that, like germline mutations, *BRCA1*-methylated breast cancer tumors occur at an early age (≤50 years) with aggressive pathological features (12,26,27). Although certain studies have shown a high association between ER negativity and *BRCA1* methylation (10), others have shown a high frequency of *BRCA1* promoter methylation among ER-positive tumors, which suggests a complex association (24). In the present study, no association

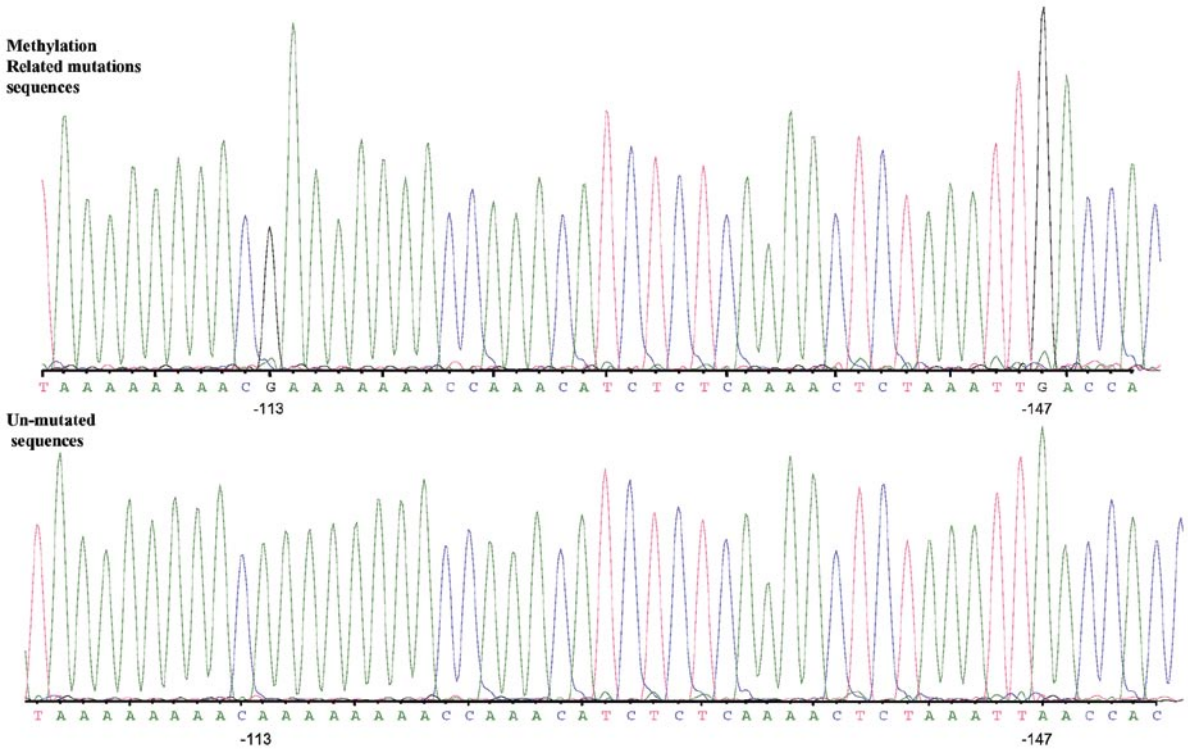

Figure 6. Representative high-resolution sodium bisulfite sequencing of the *BRCA1* promoter 5' region in DNA isolated from peripheral blood cells showing methylation-related mutations. (Site -147) represents a non-CpG methylation site. (Site -113) represents a newly-formed methylated CpG sequence.

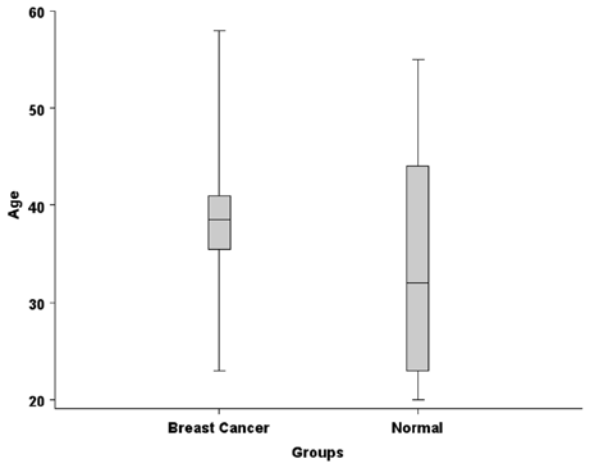

Figure 7. Box-plot graph showing age distribution of both breast cancer cases and healthy cancer-free individuals in relation to *BRCA1* promoter methylation.

of *BRCA1* methylation was found with ER status (p=0.190), or PR status (p=0.734). It is of interest to mention that in a previous study of 867 breast cancer patients in Saudi Arabia, 33.2% were of age 40 or less, with a high proportion of double-negative ER/PR status (22). Consequently, it is very tempting to believe that epigenetic modification of the *BRCA1* gene could play a role in the underlying biology of breast cancer at a young age in Arab women.

The methylated *BRCA1* promoter was also detected in peripheral blood cell DNA from cancer-free women and from breast cancer patients whose tumors showed *BRCA1*-like characteristics. Importantly, the methylation pattern of the *BRCA1* promoter CpG island was found to be similar in the blood cells from healthy women as well as from women with breast cancer. The presence of the methylated *BRCA1* promoter in healthy women may reveal the predisposition of these individuals to develop breast cancer. Indeed, the functional equivalence between the effect and significance of the epigenetic silencing of *BRCA1* and the inheritance of *BRCA1* mutations (26,28,29), has supported the notion that *BRCA1* promoter methylation may serve as a 'first hit', much like an inherited germline mutation (25). Moreover, it has been reported that the inactivation of *BRCA1* by promoter methylation could be an inherited event that predisposes to breast cancer with pathological features similar to those normally associated with *BRCA1* germline mutations including the occurrence of the disease at an early age (14). Indeed, in the present study, methylated *BRCA1* was detected in the peripheral blood cell DNA of healthy women with a median age of 32 years, which was close to the median age of the patients that harbored methylated *BRCA1* in their tumors (38,5 years). The inheritance of a methylated gene has been previously reported for other cancer suppressor genes, where it has been shown that somatic methylation affecting an allele of the MLH1 gene in patients with hereditary non-polyposis colorectal cancer has been detected in all the tissues examined (30-32). Additionally, germline methylation inheritance of the MSH2 gene was described in a family where siblings carrying the MSH2 germline methylation exhibited mosaic methylation in a variety of tissues (33).

To the best of our knowledge, this is the first study reporting the detection of methylation-related mutations leading to non-CpG methylation, as well as to the formation of novel methylated CpG sites in the *BRCA1* promoter region in peripheral blood cell DNA from women with breast cancer and healthy women. Although the functional significance of these mutations is not known, however, our results point to their possible involvement in carcinogenesis, as they may add to the overall increase in methylation of the *BRCA1* promoter region. Indeed, in the *TP53* gene, several methylation-related mutations, including those leading to the formation of new CpG sites, were found to prevail during lung carcinogenesis (4). In addition, non-CpG cytosine methylation has been reported to be associated with viral infection. Indeed, extensive CpG and non-CpG methylation of the CMV-PE, a promoter in adenoviral vectors commonly used in gene therapy, was found to result in the rapid transcriptional silencing of the vector containing the CMV-PE-hfGF-4 gene after its intramuscular delivery into rats (34). Several other studies have also reported an increase of *de novo* methylation as a result of viral infection (35). In a recent study, a significant correlation was found between the presence of SV40 and the promoter methylation of *BRCA1* in breast cancer patients (36).

In conclusion, our study demonstrates the possible contribution of the methylated *BRCA1* promoter in the early onset of breast cancer in Arab women. The high prevalence of the methylated *BRCA1* promoter in the peripheral blood DNA of cancer-free individuals raises the possibility of its potential use as a molecular marker for detecting predisposed individuals at a much earlier age. Further studies are required in order to explore the functional significance of the observed methylation-related mutations.

Acknowledgements

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