

Identification and frequency of the rs12516 and rs8176318 *BRCA1* gene polymorphisms among different populations

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Abstract. Genetic mutation of breast cancer 1 (*BRCA1*) is one of the most notable factors responsible for a proportion of breast cancer cases. *BRCA1* encodes a 1,863-amino acid protein and functions as a negative regulator of tumor growth. Thus, investigation of the underlying mechanisms that regulate *BRCA1* gene expression provide further insight into possible targets for breast cancer therapy. Previous studies have demonstrated that the genetic variants in the *BRCA1* 3' untranslated region (3'UTR), in addition to the cytosine-phosphate-guanine (CpG) islands in the promoter region, are significantly associated with breast cancer risk; however, the role of single nucleotide polymorphisms (SNPs) in the *BRCA1* 3'UTR remains unclear. The present study aimed to investigate the association between SNPs and *BRCA1* mRNA expression levels. Bioinformatics analysis demonstrated that 2 SNPs in the *BRCA1* 3'UTR (rs12516 and rs8176318 with putative microRNA binding sites) were significantly correlated with mRNA expression in lymphoblastoid cell lines ($P=2.55 \times 10^{-4}$ and $P=8.78 \times 10^{-5}$, respectively). Furthermore, the genotype frequency distribution varied between populations worldwide. In addition, 3 CpG islands and several transcription factor binding sites in the *BRCA1* promoter region were established. The identification of such polymorphisms and CpG islands may aid in designing improved therapeutic strategies to treat patients with *BRCA1*-associated breast cancer.

Introduction

According to the current literature, breast cancer is the most commonly diagnosed form of cancer and is the principal cause of cancer-associated fatality among females. The disease accounts for 14% of all cancer mortalities, particularly in

economically-developing countries, and 23% of all cancer cases (1). The breast cancer 1 (*BRCA1*) gene is ~100 kb in length and is localized to chromosome 17q21; it is composed of 24 exons, with 22 of these translating into a 1,863 amino acid protein. It is currently well-established that mutations in the *BRCA1* gene result in a significantly increased lifetime risk for the development of breast cancer (2). Thus, *BRCA1*, functioning as a tumor suppressor, may be regarded as a strong candidate gene for breast cancer susceptibility.

The abnormal methylation of cytosine-phosphate-guanine (CpG) islands in gene promoter regions is the predominant epigenetic mechanism by which gene transcription is effectively silenced; therefore, the transcriptional activity of genes with CpG island promoters is suppressed upon methylation (3). Notably, a previous study concluded that microRNAs (miRNAs/miRs) may function as oncogenes and tumor suppressors depending on the genetic variants in the 3'UTR binding sites, regulating gene expression post-transcriptionally (4). For example, miR-146a was observed to bind to the 3'UTR of *BRCA1* and *BRCA2* mRNA, potentially modulating their mRNA expression, and a genetic polymorphism in the miR-146a gene (rs2910164) was associated with a young diagnostic age of familial ovarian and breast cancer (5). However, the function of genetic variants in miRNA binding sites of *BRCA1* remains unclear. In the current study, the hypothesis that the *BRCA1* 3'UTR variants are associated with its mRNA expression was tested by performing bioinformatics analysis. In addition, the CpG islands and transcription factor binding sites (TFBSs) were predicted.

Materials and methods

Prediction of the CpG islands and TFBSs in the promoter region. The human *BRCA1* promoter region was obtained by searching the UCSC Genome Bioinformatics online database (www.genome.ucsc.edu/). The core promoters were identified by Neural Network Promoter Prediction (NNPP; www.fruitfly.org/seq_tools/promoter.html) and Promoter2.0 (www.cbs.dtu.dk/services/promoter/), and the CpG islands were predicted using the bioinformatics tool MethPrimer (www.urogene.org/methprimer/). The criteria used to define a CpG island was as follows: Island size, >100 bp; GC percentage, >50.0; and observed/expected ratio, >0.6. In addition, the TFBSs were predicted by JASPAR (jaspar.genereg.net/).

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Selection of polymorphisms in the 3'UTR and analysis of the genotype-phenotype association. The SNPs in the human *BRCA1* 3'UTR were identified using the National Center for Biotechnology Information SNP database (www.ncbi.nlm.nih.gov/SNP/). Subsequently, the distribution of all *BRCA1* genotypes among 11 distinctive populations was calculated. The 11 populations studied were as follows: Utah residents with Northern and Western European ancestry from the Center for the Study of Human Polymorphisms collection (CEU); Japanese individuals in Tokyo, Japan (JPT); members of the Yoruba tribe in Ibadan, Nigeria (YRI); Han Chinese individuals in Beijing, China (CHB); members of the Luhya tribe in Webuye, Kenya (LWK); individuals of Mexican ancestry in Los Angeles, California (MEX); Gujarati Indians in Houston, Texas (GIH); Chinese individuals in Metropolitan Denver, Colorado (CHD); individuals of African ancestry in Southwest USA (ASW); members of the Maasai tribe in Kinyawa, Kenya (MKK); and Tuscan individuals in Italy (TSI). SNP Function Prediction (snpinfo.niehs.nih.gov/snpinfo/snpfunc.htm) was utilized to predict the possible miRNA binding sites in the *BRCA1* gene. Additionally, SNPs not in linkage disequilibrium (LD; $r^2 < 0.8$) were selected and LD maps of these SNPs in the *BRCA1* gene were plotted using the online program (snpinfo.niehs.nih.gov/snpinfo/snpntag.htm). Additional data was utilized regarding *BRCA1* genotypes and mRNA levels, which was available from the HapMap database (<http://app3.titan.uio.no/biotools/tool.php?app=snpexp>), for the genotype-phenotype association analysis (6). The genotyping data from the HapMap phase II release 23 dataset consists of ~4 million SNP genotypes from 270 individuals belonging to 4 different populations (7).

Statistical analysis. The genotype and phenotype correlation was analyzed using the χ^2 test. Statistical analysis was performed on SPSS version 21 software (IMB SPSS, Armonk, NY, USA) All statistical tests were two-sided, and $P < 0.05$ was considered to indicate a statistically significance difference.

Results

CpG islands and TFBSs in the BRCA1 promoter region. The online tools NNPP and Promoter2.0 indicated that there were several core promoter regions located in the target sequence. A total of 3 core promoter regions were determined by NNPP, whilst Promoter2.0 identified 2 core promoter regions. The bioinformatics software MethPrimer was used to predict the range of the CpG islands in the *BRCA1* gene. The results determined that there were 3 CpG islands in the *BRCA1* promoter region (Fig. 1; Table I), which may serve a crucial role in the expression of the *BRCA1* gene. In total, 31 transcription factors and 45 binding sites were predicted in the target sequence regions when limiting the relative profile score threshold to $>95\%$.

BRCA1 3'UTR selected variants and their putative miRNA binding sites. A total of 28 SNPs were identified in the human *BRCA1* 3'UTR; 13 of these SNPs had available minor allele frequency (MAF) values, 2 of which (rs12516 and rs8176318) had MAF values >0.05 . SNP Function Prediction demonstrated that these 2 SNPs exhibited putative miRNA binding

Table I. CpG island prediction results.

CpG island	Size, bp	Start	End
1	129	412	540
2	244	608	851
3	221	950	1170

Criteria used were as follows: Island size, >100 bp; GC percent, >50.0 ; observed to expected ratio, >0.6 . CpG, cytosine-phosphate-guanine.

sites (Table II). As presented in Table II, the C-to-thymine (T) transition mutation, rs12516, had 6 potential miRNA binding sites, namely, hsa-miR-188-5p, hsa-miR-502-5p, hsa-miR-557, hsa-miR-623, hsa-miR-637 and hsa-miR-639. The G-to-T transition mutation, rs8176318, was observed to share hsa-miR-639 with rs12516, and contain 4 other miRNA binding sites, namely, hsa-miR-1182, hsa-miR-149, hsa-miR-345 and hsa-miR-544.

Frequency distribution of selected variants among distinct populations. As previously reported, the derived alleles at rs12516 and rs8176318 in the *BRCA1* 3'UTR demonstrated a positive association with familial ovarian and breast cancer in Thai women, and the 2 SNPs were in strong linkage disequilibrium in populations and varied by ethnicity (8). In the present study, in order to better evaluate the global genotypes of rs12516 and rs8176318 in *BRCA1*, the frequency distribution data of these variants across 11 worldwide populations were summarized (Tables III and IV). The 2 SNPs presented differences in genotype frequency distribution among the worldwide populations. For rs12516, the genotype frequencies of CC, CT and TT were highest in YRI (69.0%), CHD (57.6%) and GIH (18.2%). The frequency distributions of the GG, GT and TT genotypes of rs8176318 ranked first among LWK (84.4%), CHD (56.6%) and GIH (18.2%). The T allele frequencies of rs12516 in the various populations ranged between 44.9% in GIH and 17.3% in YRI. Notably, the frequency distributions of the GG, GT and TT genotypes of rs8176318 were the same as the data for CC, CT and TT genotypes of rs12516 among the CEU and CHB populations.

LD of all SNPs in the BRCA1 gene calculation. SNPs not in LD ($r^2 < 0.8$) were selected and LD maps of those SNPs in the *BRCA1* gene were plotted using the SNP Function Prediction program to identify the potential functional relevance of all selected SNPs (Fig. 2). The degree of pairwise LD between all SNPs was estimated as quantified by the disequilibrium coefficient (D') and r^2 , which represented the proportion of disequilibrium and the maximum possible disequilibrium given for observed allele frequencies, respectively. The higher the D' value, the greater the association between the two loci being studied. The color of each SNP spot reflects its D' value, which changes from red to white as it decreases. The MAF of each aforementioned allele was >0.05 . As for the SNPs in the *BRCA1* 3'UTR, only rs12516 and rs8176318 were included in the LD plot, but neither were the predicted tag SNP.

Table II. Selected SNPs of 3'UTR and putative miRNA binding sites.

SNP	Alleles	MAF, %	Putative miRNA binding sites
rs8176320	G>A	0.51	hsa-miR-101, hsa-miR-15a, hsa-miR-15b, hsa-miR-16, hsa-miR-194, hsa-miR-195, hsa-miR-424, hsa-miR-450b-5p, hsa-miR-545
rs184237074	C>T	0.05	NA
rs189382442	T>C	0.05	NA
rs182218567	A>G	0.05	NA
rs12516	C>T	31.18	hsa-miR-188-5p, hsa-miR-502-5p, hsa-miR-557, hsa-miR-623, hsa-miR-637, hsa-miR-639
rs111791349	C>T	0.51	NA
rs185966495	G>C	0.05	NA
rs8176319	C>T	0.69	NA
rs138782023	T>C	0.28	NA
rs141850147	G>A	0.05	NA
rs8176318	G>T	29.06	hsa-miR-1182, hsa-miR-149, hsa-miR-345, hsa-miR-544, hsa-miR-639
rs56108540	T>C	0.23	hsa-miR-125a-3p, hsa-miR-224, hsa-miR-499-3p, hsa-miR-539, hsa-miR-548c-3p, hsa-miR-767-5p
rs137892861	G>A	0.14	NA

SNP, single nucleotide polymorphism; 3'UTR, 3' untranslated region; miRNA/miR, microRNA; G, guanine; A, adenine; C, cytosine; T, thymine; MAF, minor allele frequency; hsa, *Homo sapiens*; NA, not available.

Table III. Genotype frequency of the breast cancer 1 gene rs12516 polymorphism in different populations.

Populations	n	Genotype frequency, n (%)			Allele frequency, %		HWP
		CC	CT	TT	C	T	
CEU	226	100 (44.2)	102 (45.1)	24 (10.6)	66.8	33.2	1.000
JPT	170	82 (48.2)	76 (44.7)	12 (7.1)	70.6	29.4	0.527
YRI	226	156 (69.0)	62 (27.4)	8 (3.5)	82.7	17.3	0.752
CHB	82	38 (46.3)	32 (39.0)	12 (14.6)	65.9	34.1	0.403
LWK	180	112 (62.2)	62 (34.4)	6 (3.3)	79.4	20.6	0.655
MEX	98	62 (63.3)	26 (26.5)	10 (10.2)	76.5	23.5	0.100
GIH	176	50 (28.4)	94 (53.4)	32 (18.2)	55.1	44.9	0.479
CHD	170	46 (27.1)	98 (57.6)	26 (15.3)	55.9	44.1	0.150
ASW	98	44 (44.9)	50 (51.0)	4 (4.1)	70.4	29.6	0.150
MKK	286	172 (60.1)	104 (36.4)	10 (3.5)	78.3	21.7	0.403
TSI	174	64 (36.8)	88 (50.6)	22 (12.6)	62.1	37.9	0.527

CEU, Utah residents with Northern and Western European ancestry from the Center for the Study of Human Polymorphisms collection; JPT, Japanese individuals in Tokyo, Japan; YRI, members of the Yoruba tribe in Ibadan, Nigeria; CHB, Han Chinese individuals in Beijing, China; LWK, members of the Luhya tribe in Webuye, Kenya; MEX, individuals of Mexican ancestry in Los Angeles, California; GIH, Gujarati Indians in Houston, Texas; CHD, Chinese individuals in Metropolitan Denver, Colorado; ASW, individuals of African ancestry in Southwest, USA; MKK, members of the Maasai tribe in Kinyawa, Kenya; TSI, Tuscan individuals in Italy; HWP, Hardy-Weinberg probability; C, cytosine; T, thymine.

BRCA1 mRNA expression by genotypes in lymphoblastoid cell lines. The present study took advantage of the available HapMap-complementary DNA expression database for the correlation analysis between *BRCA1* genotype and mRNA

expression in 270 lymphoblastoid cell lines. Excluding the 6 cell lines with unavailable values for rs12516, 20 (7.6%) cell lines exhibited the TT genotype, 108 (40.9%) cell lines exhibited the CT genotype and 136 (51.5%) cell lines exhibited

Table IV. Genotype frequency of the breast cancer 1 gene rs8176318 polymorphism in different populations.

Populations	n	Genotype frequency, n (%)			Allele frequency, %		HWP
		GG	GT	TT	G	T	
CEU	226	100 (44.2)	102 (45.1)	24 (10.6)	66.8	33.2	1.000
JPT	172	88 (51.2)	72 (41.9)	12 (7.0)	72.1	27.9	0.752
YRI	226	180 (79.6)	42 (18.6)	4 (1.8)	88.9	11.1	0.584
CHB	82	38 (46.3)	32 (39.0)	12 (14.6)	65.9	34.1	0.403
LWK	180	152 (84.4)	26 (14.4)	2 (1.1)	91.7	8.3	0.655
MEX	98	62 (63.3)	28 (28.6)	8 (8.2)	77.6	22.4	0.251
GIH	176	52 (29.5)	92 (52.3)	32 (18.2)	55.7	44.3	0.584
CHD	170	48 (28.2)	96 (56.5)	26 (15.3)	56.5	43.5	0.200
ASW	98	56 (57.1)	42 (42.9)	0 (0)	78.6	21.4	0.251
MKK	286	206 (72.0)	72 (25.2)	8 (2.8)	84.6	15.4	0.752
TSI	176	68 (38.6)	88 (50.0)	20 (11.4)	63.6	36.4	0.479

CEU, Utah residents with Northern and Western European ancestry from the Center for the Study of Human Polymorphisms collection; JPT, Japanese individuals in Tokyo, Japan; YRI, members of the Yoruba tribe in Ibadan, Nigeria; CHB, Han Chinese individuals in Beijing, China; LWK, members of the Luhya tribe in Webuye, Kenya; MEX, individuals of Mexican ancestry in Los Angeles, California; GIH, Gujarati Indians in Houston, Texas; CHD, Chinese individuals in Metropolitan Denver, Colorado; ASW, individuals of African ancestry in Southwest, USA; MKK, members of the Maasai tribe in Kinyawa, Kenya; TSI, Tuscan individuals in Italy; HWP, Hardy-Weinburg probability; C, cytosine; T, thymine; G, guanine.

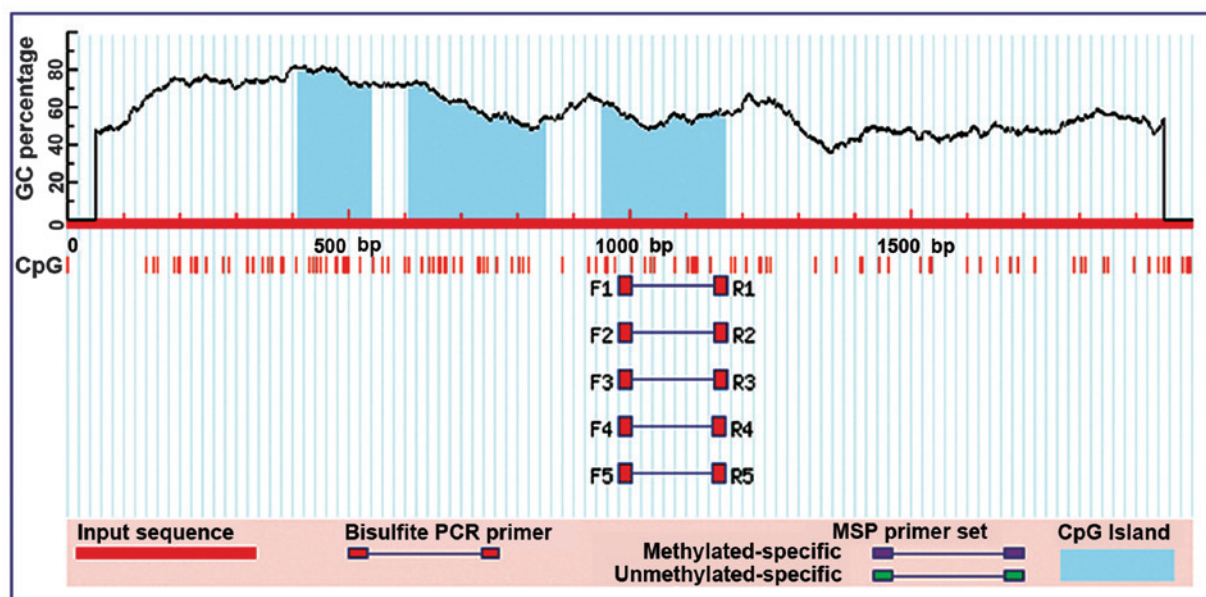


Figure 1. CpG island prediction results in the breast cancer 1 gene promoter region. Criteria used to define a CpG island were as follows: Island size, >100; GC percentage, >50.0; and observed/expected ratio, >0.6. CpG, cytosine-phosphate-guanine; PCR, polymerase chain reaction; MSP, methylation-specific PCR.

the CC genotype. For rs8176318, 19 (7.0%) cell lines exhibited the TT genotype, 95 (35.2%) exhibited the GT genotype and 156 (57.8%) exhibited the GG genotype. The effect of the rs12516 and rs8176318 genotypes on *BRCA1* mRNA expression levels is presented in Fig. 3. For rs12516, the *BRCA1* mRNA expression level was significantly decreased in the CC genotype compared with the CT and TT genotypes ($P=2.55 \times 10^{-4}$; Fig. 3A). Similarly, the rs8176318 GG genotype had a significantly lower expression level than the GT and TT genotypes ($P=8.78 \times 10^{-5}$; Fig. 3B).

Discussion

Since the identification of the *BRCA1* gene 20 years ago (9), the biological basis underlying the high breast cancer risk in women with *BRCA1* mutations has gained increasing attention. *BRCA1* operates in a series of cellular processes, including DNA repair, chromatin remodeling, protein ubiquitination, regulation of transcription, apoptosis and cell cycle checkpoint control. As observed in carriers of germline *BRCA1* mutations, the disruption of any of the aforementioned processes may result

BRCA1 gene regulation on a transcriptional level, the present study predicted 3 CpG islands and several putative TFBSs in the *BRCA1* promoter region using bioinformatics analysis.

Previous studies have demonstrated that *BRCA1* 3'UTR miRNA binding site variants are associated with breast cancer risk post-translationally. For example, miR-24 was observed to directly target the 3'UTR of *BRCA1* and resulted in significant repression of the *BRCA1* gene (17). In addition, it was demonstrated that the *BRCA1* polymorphism, rs799917, was associated with breast cancer risk (18). By contrast, Hasan *et al* (19) reported that rs799917 demonstrated no significant association with breast cancer in 100 patients with breast cancer in Saudi Arabia (19). Therefore, the role of genetic variants in the *BRCA1* 3'UTR and its post-transcriptional regulation remains unclear. Such differences may be due to reproductive patterns, in addition to exposure to particular environmental carcinogens, different lifestyles and different genetic backgrounds (19). The present study focused on the polymorphisms in the *BRCA1* 3'UTR, and identified that rs12516 and rs8176318 had potential miRNA binding sites. Furthermore, rs12516 and rs8176318 exhibited differences in genotype frequency distribution among populations worldwide. Each SNP had a significant association with *BRCA1* mRNA expression, therefore implying that these SNPs may partially contribute to *BRCA1* post-transcriptional regulation.

In conclusion, the current study predicted 3 GpG islands, 45 TFBSs located in the promoter region and 13 SNPs located in the 3'UTR of the *BRCA1* gene. A total of 4 SNPs (rs8176320, rs12516, rs8176318 and rs56108540) were confirmed to have putative miRNA binding sites, among which only 2 (rs12516 and rs8176318) had MAF values >0.05. These 2 SNPs, rs12516 and rs8176318, each demonstrated a significant association with *BRCA1* mRNA expression. The results from the present study have raised the possibility that rs12516 and rs8176318 may be associated with an increased risk of breast cancer by altering the *BRCA1* mRNA level. However, such results require substantiation by experimental data, therefore further investigations are warranted to confirm the function of these polymorphisms. In addition, further functional analysis is necessary to validate the promoter CpG islands and SNPs in the 3'UTR to allow for investigation of *BRCA1* gene regulation as a potential therapy for breast cancer.

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References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D: Global cancer statistics. *CA Cancer J Clin* 61: 69-90, 2011.
- Ayoub N, Lucas C and Kaddoumi A: Genomics and pharmacogenomics of breast cancer: Current knowledge and trends. *Asian Pac J Cancer Prev* 12: 1127-1140, 2011.
- Zeisberg EM and Zeisberg M: The role of promoter hypermethylation in fibroblast activation and fibrogenesis. *J Pathol* 229: 264-273, 2013.
- Hook L, Hancock M, Landais I, Grabski R, Britt W and Nelson JA: Cytomegalovirus microRNAs. *Curr Opin Virol* 7: 40-46, 2014.
- Shen J, Ambrosone CB, DiCioccio RA, Odunsi K, Lele SB and Zhao H: A functional polymorphism in the miR-146a gene and age of familial breast/ovarian cancer diagnosis. *Carcinogenesis* 29: 1963-1966, 2008.
- Holm K, Melum E, Franke A and Karlsen TH: SNPexp - A web tool for calculating and visualizing correlation between HapMap genotypes and gene expression levels. *BMC Bioinformatics* 11: 600, 2010.
- International HapMap Consortium: The International HapMap Project. *Nature* 426: 789-796, 2003.
- Pelletier C, Speed WC, Paranjape T, Keane K, Blitzblau R, Hollestelle A, Safavi K, van den Ouweland A, Zelterman D, Slack FJ, *et al*: Rare *BRCA1* haplotypes including 3'UTR SNPs associated with breast cancer risk. *Cell Cycle* 10: 90-99, 2011.
- Narod SA, Feunteun J, Lynch HT, Watson P, Conway T, Lynch J and Lenoir GM: Familial breast-ovarian cancer locus on chromosome 17q12-q23. *Lancet* 338: 82-83, 1991.
- Yang ES and Xia F: *BRCA1* 16 years later: DNA damage-induced *BRCA1* shuttling. *FEBS J* 277: 3079-3085, 2010.
- Dacheux E, Vincent A, Nazaret N, Combet C, Wierinckx A, Mazoyer S, Diaz JJ, Lachuer J and Venezia ND: *BRCA1*-Dependent Translational Regulation in Breast Cancer Cells. *PLoS One* 8: e67313, 2013.
- Williamson EA, Dadmanesh F and Koeffler HP: *BRCA1* transactivates the cyclin-dependent kinase inhibitor p27(Kip1). *Oncogene* 21: 3199-3206, 2002.
- Iwamoto T, Yamamoto N, Taguchi T, Tamaki Y and Noguchi S: *BRCA1* promoter methylation in peripheral blood cells is associated with increased risk of breast cancer with *BRCA1* promoter methylation. *Breast Cancer Res Treat* 129: 69-77, 2011.
- Xu X, Gammon MD, Zhang Y, Bestor TH, Zeisel SH, Wetmur JG, Wallenstein S, Bradshaw PT, Garbowski G, Teitelbaum SL, *et al*: *BRCA1* promoter methylation is associated with increased mortality among women with breast cancer. *Breast Cancer Res Treat* 115: 397-404, 2009.
- Hsu NC, Huang YF, Yokoyama KK, Chu PY, Chen FM and Hou MF: Methylation of *BRCA1* promoter region is associated with unfavorable prognosis in women with early-stage breast cancer. *PLoS One* 8: e56256, 2013.
- Wu L, Wang F, Xu R, Zhang S, Peng X, Feng Y, Wang J and Lu C: Promoter methylation of *BRCA1* in the prognosis of breast cancer: A meta-analysis. *Breast Cancer Res Treat* 142: 619-627, 2013.
- Lal A, Navarro F, Maher CA, Maliszewski LE, Yan N, O'Day E, Chowdhury D, Dykxhoorn DM, Tsai P, Hofmann O, *et al*: miR-24 Inhibits cell proliferation by targeting E2F2, MYC and other cell-cycle genes via binding to 'seedless' 3'UTR microRNA recognition elements. *Mol Cell* 35: 610-625, 2009.
- Huo X, Lu C, Huang X, Hu Z, Jin G, Ma H, Wang X, Qin J, Wang X, Shen H and Tang J: Polymorphisms in *BRCA1*, *BRCA1*-interacting genes and susceptibility of breast cancer in Chinese women. *J Cancer Res Clin Oncol* 135: 1569-1575, 2009.
- Hasan TN, Shafi G, Syed NA, Alsaif MA, Alsaif AA and Alshatwi AA: Lack of association of *BRCA1* and *BRCA2* variants with breast cancer in an ethnic population of Saudi Arabia, an emerging high-risk area. *Asian Pac J Cancer Prev* 14: 5671-5674, 2013.