RESEARCH ARTICLE

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Promoter Methylation of BRCA1, DAPK1 and RASSF1A is Associated with Increased Mortality among Indian Women with Breast Cancer

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Abstract

Background: Promoter methylation has been observed for several genes in association with cancer development and progression. Hypermethylation mediated-silencing of tumor suppressor genes (TSGs) may contribute to breast cancer pathogenesis. The present study was conducted to investigate the promoter methylation status of BRCA1, DAPK1 and RASSF1A genes in Indian women with breast cancer. **Materials and Methods:** Promoter methylation was evaluated in DNA extracted from mononuclear cells (MNCs) in peripheral blood samples of 60 histopathologically confirmed newly diagnosed, untreated cases of breast cancer as well as 60 age and sex matched healthy controls using MS-PCR. Association of promoter methylation with breast cancer-specific mortality was analyzed with Cox proportional hazards models. Kaplan-Meier survival analysis was performed for overall survival of the breast cancer patients. **Results:** We observed a significant increase of BRCA1, DAPK1 and RASSF1A promoter methylation levels by 51.7% (P <0.001), 55.0% (P <0.001) and 46.6% (P <0.001), respectively, when compared to healthy controls. A strong correlation was noted between hypermethylation of the tumor suppressor genes BRCA1 (P= 0.009), DAPK1 (P= 0.008) and RASSF1A (P= 0.02)) with early and advanced stages of breast cancer patients. We also found that breast cancer-specific mortality was significantly associated with promoter methylation of BRCA1 [HR and 95% CI: 3.25 (1.448-7.317)] and DAPK1 [HR and 95% CI: 2.32 (1.05-5.11)], whereas limited significant link was evident with RASSF1A [HR and 95% CI: 1.54 (0.697-3.413]. **Conclusion:** Our results suggest that promoter methylation of BRCA1, DAPK1 and RASSF1A genes may be associated with disease progression and poor overall survival of Indian women with breast cancer.

Keywords: Promoter methylation- tumor suppressor genes- MS−PCR- breast cancer

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Introduction

Breast cancer is the leading cause of mortality among women worldwide, but the exact etiology of breast cancer remains unknown. DNA methylation has attracted deep investigation in past several years and it has been seen that methylation regulation of genes related to cancer (Das and Singal, 2004).

Specifically, aberrant promoter methylation takes place in several genes in cancer development and progression (Widschwendter and Jones 2002). BRCA1 (Catteau et al., 1999; Rice et al., 2000), RASSF1A (Agathanggelou et al., 2001), DAPK1 (Dulaimi et al., 2004) are frequently methylated tumor suppressor genes in breast cancer. The process of gene silencing by methylation and its role in cancer pathogenesis is well mentioned, with methylation of tumor suppressor genes, affecting transcriptional activity of the genes, believed to be the most important drivers of carcinogenesis.

Recently, attention is paid to the phenomenon of hypermethylation of disease-related genes in peripheral blood DNA and its involvement in the pathology of cancer and other diseases (Woodson et al., 2001; Widschwendter, et al., 2008; Flanagan et al., 2009; Iwamoto, Yamamoto et al., 2011). This suggested that detection of tumor DNA in the blood may serve as an early and more accessible marker of diagnosis and prognosis of breast cancer. However, the frequency of aberrant methylation in peripheral blood has not been extensively investigated. BRCA1 status may potentially be used as a prognostic marker as several studies have shown that BRCA1 mutated breast cancer is associated with poor survival (Moller et al., 2007). BRCA1 promoter methylation was observed to be significantly associated with breast cancer-specific mortality (Xu et al., 2009, Hsu et al., 2013). DNA methylation markers have been

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used as an alternative approach to molecular profiling of breast cancer. RASSF1A promoter methylation provides important prognostic information in early stage breast cancer patients (Widschwendter et al., 2004; Jezkova et al., 2016). Promoter methylation of DAPK1 gene was also observed to be associated with DCIS, LCIS and all grades and stages of breast cancer patients (Dulaimi et al., 2004). All of these results suggest that DNA methylation correlates with clinical findings in breast cancer and may help in the prediction of therapeutic strategy for breast cancer. Moreover, these results demonstrate that MNCs DNA may be a potential biomarker for analysis of promoter methylation status.

In current study, we investigated the promoter methylation status of BRCA1, DAPK1 and RASSF1A genes in relation to clinicopathological features and breast cancer survival in breast cancer patients.

Materials and Methods

Study Population

The current study was performed on 60 histopathologically confirmed newly diagnosed, untreated cases of North Indian breast cancer patients and 60 age-matched female healthy volunteers. Samples were collected from Department of Surgery, Lok Nayak Jaiprakash Hospital, New Delhi during January 2012 to December 2013. 5ml of peripheral blood sample was collected from each patient as well as healthy volunteer and stored at -80^{o}C.

The study was ethically approved by Institutional Ethics Committee, Maulana Azad Medical College, New Delhi. Written informed consent was taken from each study subjects. Demographic data of patients and controls are shown in Table 1.

Patient data collection and Follow-up

Patient follow-up was done through the hospital records and confirmed by direct patient contact. Tumor characteristics and treatment information was obtained from the patient at the time of diagnosis and/or during the regular visit and verified with hospital record. The questionnaires were administrated to evaluate the demographic features and breast cancer-related features of patients. Patients with a history of any other malignancy or metastasized cancer from any other sites were excluded. The total follow-up period was 45 months and mean follow-up time was 30.98.

DNA extraction and bisulfite modification

DNA extraction was performed on peripheral blood mononuclear Cells (PBMNCs) using Blood DNA extraction kit (Geneaid) by following manufacturer's instructions. DNA concentrations were measured and 1µg of DNA was used for bisulfite modification. DNA bisulfite modification was performed using Bisulflash DNA modification kit (Epigenetek) according to the manufacturer's instructions. Bisulfite treated DNA was immediately stored at -20^{o}C.

Methylation Specific- Polymerase Chain Reaction (MS-PCR) Analysis

After bisulfite conversion, Qualitative methylation status of different genes were analyzed by Methylation-Specific Polymerase Chain Reaction (MS-PCR). Primers for MS-PCR were as shown in previous studies (Estellers et al., 1999; Baldwinet al., 2000; Burbee et al., 2001) and also shown in Table 2. PCRs were run in a volume of 25 µl, containing 2ul bisulfite-modified DNA, 12 µl of 2x Hot Start PCR Mastermix (Fermentas), 0.25µl sense primer (25 pM), 0.25 µl antisense primer (25 pM), and 12.5µl H2O. The PCR profile was 95°C for 10 minutes, 40 cycles at 95°C for 45 seconds, primer annealing at 56°C to 60°C for 45 seconds, 72°C for 45 seconds, and a final extension step at 72°C 10 minutes. The amplified PCR products were further electrophoresed on 2% agarose gels and evaluated under ultraviolet light (Figure 1).

Statistical Analysis

SPSS 16 and GraphPad Statistical software were used for statistical analysis of the study. Methylation frequencies between the patients and healthy volunteers were analyzed using the Chi-square test and values less than 5 were analyzed by Fisher exact test. The Cox proportional hazard regression (Hosmer, 1999) was used to calculate the hazard ratio (HR) and 95% confidence interval (CI) for the association between gene promoter methylation status and breast cancer-specific mortality. Kaplan-Meier survival analysis was performed for overall survival of breast cancer patients. The p-value less than 0.05 was considered to be statistically significant.

Results

Patient Characteristics

Among the cases, 26(43.3%) were age ≤ 45 age group and 34(56.7%) >45 years group. Menopausal status shows that 21(35%) patients were in premenopausal status and 39(65%) patients were in postmenopausal status. TNM staging reveals that 32(53.3%) patients were in early stages (I and II) and 28(46.7%) patients were in advanced stages (III and IV). Histological grading of the patients shows that 4(6.6), 33(55%) and 23(38.4%) were in well differentiated, moderately differentiated and poorly differentiated, respectively. Lymph node status shows that 29(48.4%) cases were positive for lymph node metastasis. Hormone receptor status shows that 11(18.3%) patients were positive for Estrogen receptor (ER), 9(15%) patients were positive for Progesterone receptor (PR) and 23(38.4%) were HER2/neu positive. Of the total breast cancer cases, 3(5%) patients having distant metastasis.

Promoter hypermethylation and clinicopathological features of breast cancer patients

Of the three tumor suppressor genes tested, All three genes (BRCA1, DAPK1 and RASSF1A) were found significantly hypermethylated (P <0.001) in cases than the healthy controls. Their methylation levels were 31/60(51.66%) (P <0.001), 33/60(55%) (P <0.001), 28/60(46.6%) (P <0.001) respectively (Table 3).

We found a significant difference between tumor

Table 1. Demographic Features of Breast Cancer Patients and Healthy Controls

Parameters	Cases (%)	Healthy Controls (%)
Patients	60 (100%)	60 (100%)
Age at diagnosis		
Age ≤ 45	26 (43.3)	25 (41.7)
Age > 45	34 (56.7)	35 (58.3)
Mean±SD	49.2 ± 12.47	48.69±12.25
Menopause		
Pre	21 (35)	
Post	39 (65)	
TNM Stages		
I	3 (5)	
II	29 (48.3)	
III	25 (41.7)	
IV	3 (5)	
Tumor Grading		
I	4 (6.6)	
II	33 (55)	
III	23 (38.4)	
Lymph Node Status		
Positive	29 (48.4)	
Neative	31 (51.6)	
Chemotherapy		
Adjuvant	14 (23.3)	
Neo-Adjuvant	46 (76.7)	
ER Status		
Positive	11 (18.3)	
Neative	49 (81.7)	
PR Status		
Positive	9 (15)	
Neative	51 (85)	
HER2/neu Status		
Positive	23 (38.4)	
Neative	37 (61.6)	
Distant Metastasis		
Positive	3 (5)	
Neative	57 (95)	

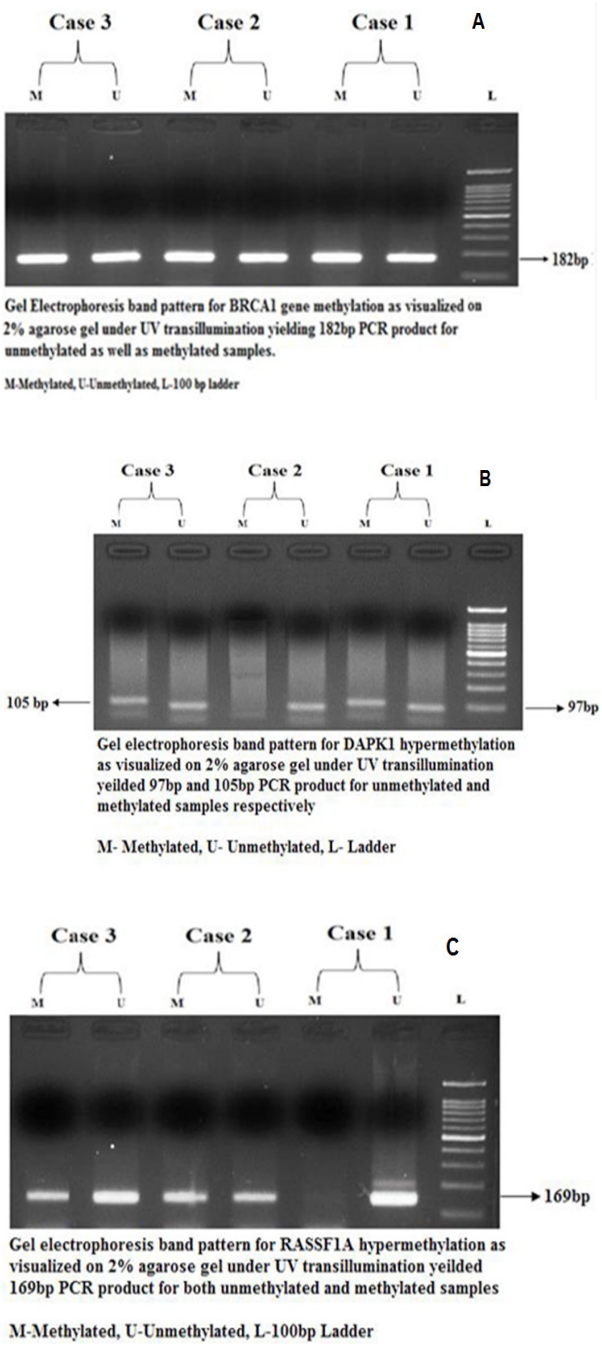

Figure 1. Representative Results of MS-PCR Analysis for (A) BRCA1, (B) DAPK1 and (C) RASSF1A in Breast Cancer Patients. Lanes M and U correspond to methylated and unmethylated samples respectively and Last Lane to a 100bp ladder as molecular weight marker.

suppressor gene, BRCA1 (P= 0.009), DAPK1 (P= 0.008) and RASSF1A (P= 0.02)) hypermethylation with early and advanced stages of breast cancer patients (Table 3). No significant association was found between tumor suppressor genes (BRCA1, DAPK1 and RASSF1A) and Age at diagnosis, Menopausal status, histological grading, Lymph node status, Chemotherapy, Estrogen receptor (ER), Progesterone receptor (PR), HER2/neu and Distant metastasis.

Promoter Hypermethylation and survival analysis of breast cancer patients

Among total 60 cases of breast cancer, 25 patients died during the follow-up period. We found that all 25 cases died due to the advancement of the disease. Table 4 shows the association of methylation status of BRCA1, DAPK1 and RASSF1A with breast cancer-specific mortality in Indian population. At the end of follow-up, Compared to the cases with unmethylated promoter of BRCA1, cases with methylated promoter having highest risk (HR: 3.25(1.448- 7.317)) of death due to breast cancer. In cases of promoter methylation of DAPK1, we found comparatively low but significant risk (HR: 2.32(1.05-5.11)) of breast cancer-specific mortality than BRCA1 promoter methylation. In comparison of

Table 2. Primer Sequence for Methylation- Specific Polymerase Chain Reaction used for BRCA1, DAPK1 and RASSF1Agenes

Gene	Primer Name	Sense Primer	Antisense Primer	Annealing Temp (°C)	Size (bp)
BRCA1	Unmethylated	GGTTAATTTAGAGTTTTGAGAGATG	TCAACAAACTCACACCACACAATCA	56	182 bp
	Methylated	GGTTAATTTAGAGTTTCGAGAGACG	TCAACGAACTCACGCCGCGCAATCG	56	182 bp
DAPK1	Unmethylated	GGAGGATAGTTGGATTGAGTTAATGTT	CAAATCCCTCCCAAACACCAA	60	105 bp
	Methylated	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCGA	60	97 bp
RASSF1A	Unmethylated	GGAGGATAGTTGGATTGAGTTAATGTT	GGTTTTTGTGAGTGTGTTTAG	60	169 bp
	Methylated	GCTAACAAACGCGAACCG	CCCTCCCAAACGCCGA	60	169 bp

BRCA1 and DAPK1 promoter methylation with survival, RASSF1A promoter methylation having lowest risk ((HR: 1.54(0.697-3.413)) of breast cancer-specific mortality.

Discussion

To effectively reduce the disease burden of breast cancer, it is important to identify etiologic factors of the disease as well as factors that predict survival. We studied promoter methylation of three tumor suppressor genes previously found to be associated with breast cancer-specific mortality (Cho et al., 2012).

In present study, we found a significant difference between promoter methylation of cases than controls for

Table 3. Association between Promoter Methylation of Tumor Suppressor Genes and Clinico- Pathological Features

	BRCA1 Positive n(%)	p-value	DAPK1 Positive n(%)	p-value	RASSF1A Positive	p-value
Cases (60)	31 (51.66)	<0.001	33 (55)	<0.001	28 (46.6)	< 0.001
Controls (60)	0 (0)		0 (0)		00 (0)	
Age at Diagnosis						
Age ≤ 45 (26)	11 (42.3)	0.3	15 (57.7)	0.92	12 (46.2)	0.8
Age > 45 (34)	20 (58.80)		18 (53)		16 (47.1)	
Menopause Stages						
Pre (21)	8 (38.1)	0.2	12 (57)	0.9	8 (38.1)	0.4
Post (39)	23 (59)		21 (53.8)		20 (51.2)	
TNM Stages						
Early (I&II) (32)	11 (34.3)	0.009	12 (37.5)	0.008	10 (31.3)	0.02
Advanced	20 (71.4)		21 (75)		18 (64.3)	
(III&IV) (28)						
Histological Grading						
I (4)	1 (25)	0.2	1 (25)	0.38	1 (25)	0.24
II (33)	15 (45.4)		20 (60.6)		13 (39.4)	
III (23)	15 (65.2)		12 (52.2)		14 (60.9)	
Lymph Nodes						
Positive (31)	19 (61.3)	0.19	20 (60.6)	0.3	16 (55.2)	0.5
Negative (29)	12 (41.3)		13 (44.8)		12 (44.4)	
Chemotherapy						
Adjuvant (14)	4 (28.6)	0.06	8 (57.1)	0.9	5 (37.8)	0.5
Neoadjuvant (46)	27 (58.7)		25 (54.3)		23 (50)	
ER Status						
Positive (11)	6 (54.6)	0.9	6 (54.5)	0.76	6 (54.6)	0.8
Negative (49)	25 (51.0)		27 (55.1)		23 (46.9)	
PR Status						
Positive (09)	5 (55.5)	1	5 (55.5)	0.63	5 (55.5)	0.5
Negative (51)	26 (51)		28 (54.9)		24 (47.1)	
HER2/neu						
Positive (23)	12 (52.2)	0.8	14 (60.8)	0.64	11 (47.8)	0.9
Negative (37)	19 (51.4)		19 (51.3)		17 (45.9)	
Distant Metastasis						
Positive (03)	3 (100)	0.2	3 (100)	0.2	3 (100)	0.09
Negative (57)	28 (49.2)		30 (52.6)		25 (43.9)	

Table 4. Hazard Ratios (HRs) and 95% Confidence Intervals (CIs) for the Associations of Gene Promoter Methylation Status and Mortality among Indian Breast Cancer Patients

Genes	No of Cases	No of Deaths	Hazard ratio (95%CI)
BRCA1			
Unmethylated	29	7	1.00 (Ref)
Methylated	31	18	3.25 (1.448- 7.317)
DAPK1			
Unmethylated	27	7	1.00 (Ref)
Methylated	33	18	2.32 (1.05-5.11)
RASSF1			
Unmethylated	33	11	1.00 (Ref)
Methylated	27	14	1.54 (0.697-3.413)

Table 5. Number of Methylated Genes in Relation to Breast Cancer- Specific Mortality among Indian Breast Cancer Patients

No. of genes methylated	No. of Cases	No. of Deaths	HR (95% CI)
0	9	2	1.00 (ref.)
1	21	4	0.81 (0.13-4.73)
2	20	12	2.50 (0.82-7.66)
3	10	7	4.12 (1.09-15.57)

all three genes. Frequencies for the methylation of these three genes (BRCA1, DAPK1 and RASSF1A) were 51.66%, 55%, 46.6% respectively. Similarly, significant results were also seen in the previous studies analyzed these three genes in different populations (Bagadi et al., 2008; Ahmed et al., 2010; Cho et al., 2012; Spitzwieseret al., 2015).

A similar study was performed on the similar population by Sharma et al., (2009) found comparatively higher frequency for RASSF1A, but lower frequency for BRCA1 promoter methylation. Another study by Dulaimi et al., (2004) found almost similar frequency for DAPK1 gene in serum of breast cancer patients. This discrepancy in results was found may be due to various reasons like sample size, race, treatment status, dietary intake, family history etc. While analyzing the number of methylated genes and survival of the patients in a dose-dependent manner, we found significant decrease in overall survival with increase in number of promoter methylated genes (Table 5).

Additionally, we found significant correlation between promoter methylation of all three genes and early and advanced stages of breast cancer patients, which demonstrate an increase in promoter methylation level with the advancement of disease. Several previous studies are in support of our findings (Singh et al., 2011; Tserga et al., 2012). Apart from TNM stages, we are not able to find any correlation between promoter methylation of these tumor suppressor genes and other clinico-pathological features of breast cancer patients.

Very limited studies were done to investigate the prognostic role of promoter methylation of these tumor suppressor genes in Indian breast cancer patients. In

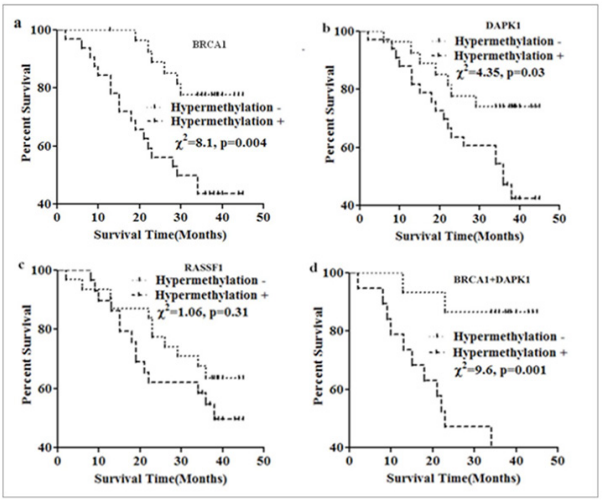

Figure 2. Kaplan – Meier Survival Plot for Breast Cancer Patients by (a) BRCA1, (b) DAPK1, (c) RASSF1A and (d) BRCA1 + DAPK1 Promoter Methylation Status in Peripheral Blood Samples

our study, we have seen a strong association between BRCA1 and DAPK1 promoter methylation with poor prognosis of breast cancer patients. BRCA1 and DAPK1 shown to be significantly associated with poor overall survival (Figure 2a and 2b respectively). For RASSF1A promoter methylation, we have seen a weak association with overall survival (Fig 2c). Furthermore, we have analyzed the combined effect of BRCA1 and DAPK1 methylation in survival of breast cancer patients; we found a significant decrease in breast cancer survival (Figure 2d). A previous study Xu et al., (2009) also found similar association between BRCA1 promoter methylation in breast cancer patients with poor survival. Another study by Cho et al.,(2012) (found similarly weak association between RASSF1A promoter methylation and breast cancer survival.

Few studies of BRCA1 promoter methylation in normal breast tissues have identified it in 8.3–22% of these tissues (Bean et al., 2007). However, these studies did not confirm the absence of tumor cells and benign proliferative lesions in the analyzed tissues (Bean et al., 2007; Vasilatos et al., 2009). Pu et al., (2003) observed that promoter methylation of RASSF1A was found to be more commonly in healthy female predicted to have a high risk of breast cancer.

In conclusion, we found a significant association between BRCA1, DAPK1 and RASSF1A gene promoter methylation with North Indian breast cancer patients compared to healthy controls. Promoter methylation of these three tumor suppressor genes individually and in combined significantly multiply the risk of breast cancer progression. Moreover, we also observed that promoter methylation of these genes associated with high TNM stages and Poor survival of breast cancer patients. Our results indicate that promoter methylation of BRCA1, DAPK1 and RASSF1A genes in PBMNC DNA may be associated with breast cancer progression and poorer overall survival. A large pooled study on Indian breast cancer cases is required to confirm our finding.

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Conflicts of interest

The authors declare that there is no conflict of interests concerning this article.

References

Agathanggelou A, Honorio S, Macartney DP, et al (2001). Methylation associated inactivation of RASSF1A from region 3p21.3 in lung, breast and ovarian tumours. *Oncogene*, **20**, 1509-18.

Ahmed IA, Pusch CM, Hamed T, et al (2010). Epigenetic alterations by methylation of RASSF1A and DAPK1 promoter sequences in mammary carcinoma detected in extracellular tumor DNA. *Cancer Genet Cytogenet*, **199**, 96-100.

Bagadi SA, Prasad CP, Kaur AS, et al (2008). Clinical significance of promoter hypermethylation of RASSF1A, RARbeta2, BRCA1 and HOXA5 in breast cancers of Indian patients. *Life Sci*, **82**, 1288-92.

Baldwin RL, Nemeth E, Tran H, et al (2000). BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. *Cancer Res*, **60**, 5329-33.

Bean GR, Ibarra Drendall C, et al (2007). Hypermethylation of the breast cancer-associated gene 1 promoter does not predict cytologic atypia or correlate with surrogate end points of breast cancer risk. *Cancer Epidemiol Biomarkers Prev*, **16**, 50–6.

Burbee DG, Forgacs E, Zochbauer-Muller S, et al (2001). Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression. J *Natl Cancer Inst*, **93**, 691-9.

Catteau A, Harris WH, Xu CF, et al (1999). Methylation of the BRCA1 promoter region in sporadic breast and ovarian cancer: correlation with disease characteristics. *Oncogene*, **18**, 1957-65.

Cho YH, Shen J, Gammon MD, et al (2012). Prognostic significance of gene-specific promoter hypermethylation in breast cancer patients. *Breast Cancer Res Treat*, **131**, 197-205.

Das PM, Singal R (2004). DNA methylation and cancer. *J Clin Oncol*, **22**, 4632-42.

Dulaimi EJ, Hillinck I, Ibanez R, et al (2004). Tumor suppressor gene promoter hypermethylation in serum of breast cancer patients. *Clin Cancer Res*, **10**, 6189-93.

Esteller MM, Sanchez R, Rosell F, et al (1999). Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res*, **59**, 67-70.

Flanagan JM, Munoz M, Henderson T, et al (2009). Gene-body hypermethylation of ATM in peripheral blood DNA of bilateral breast cancer patients. *Hum Mol Genet*, **18**, 1332-42.

Hosmer D (1999). Applied survival analysis : regression modeling of time to event data. New York: Wiley, pp 410-6.

Hsu NC, Huang YF, Yokoyama KK, et al (2013). Methylation of BRCA1 promoter region is associated with unfavorable prognosis in women with early-stage breast cancer. *PLoS One*, **8**, e56256.

Iwamoto TN, Yamamoto T, Taguchi R, et al (2011). 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.

Jezkova EK, Kajo P, Zubor D, et al (2016). Methylation in promoter regions of PITX2 and RASSF1A genes in association with clinicopathological features in breast cancer patients. *Tumour Biol*, **37,** 15707-18.

Moller PD, Evans G, Reis M, et al (2007). Surveillance for familial breast cancer: Differences in outcome according to BRCA mutation status. *Int J Cancer*, **121**, 1017-20.

Pu RT, Laitala LE, Alli PM, et al (2003). Methylation profiling of benign and malignant breast lesions and its application to cytopathology. *Mod Pathol*, **16**, 1095–101.

Rice JC, Ozcelik H, Maxeiner p, et al (2000). Methylation of the BRCA1 promoter is associated with decreased BRCA1 mRNA levels in clinical breast cancer specimens. *Carcinogenesis*, **21**, 1761-5.

Sharma G, Mirza S, Yang YH, et al (2009). Prognostic relevance of promoter hypermethylation of multiple genes in breast cancer patients. *Cell Oncol*, **31**, 487-500.

Singh AK, Pandey P, Tewari M, et al (2011). Epigenetic silencing of BRCA1 gene associated with demographic and pathologic factors in sporadic breast cancer: a study of an Indian population. *Eur J Cancer Prev*, **20**, 478-83.

Spitzwieser M, Holzweber E, Pfeiler G, et al (2015). Applicability of HIN-1, MGMT and RASSF1A promoter methylation as biomarkers for detecting field cancerization in breast cancer. *Breast Cancer Res*, **17**, 125.

Tserga A, Michalopoulos NV, Levidou G, et al (2012). Association of aberrant DNA methylation with clinicopathological features in breast cancer. *Oncol Rep*, **27**, 1630-8.

Vasilatos SN, Broadwater G, Barry WT, et al (2009). CpG island tumor suppressor promoter methylation in non-BRCA-associated early mammary carcinogenesis. *Cancer Epidemiol Biomarkers Prev*, **18**, 901–14.

Widschwendter M, Apostolidou S, Raum E, et al (2008). Epigenotyping in peripheral blood cell DNA and breast cancer risk: a proof of principle study. *PLoS One*, **3**, e2656.

Widschwendter M, Jones PA (2002). DNA methylation and breast carcinogenesis. *Oncogene*, **21**, 5462-82.

Widschwendter M, Jack KD, Siegmund HM, et al (2004). Association of breast cancer DNA methylation profiles with hormone receptor status and response to tamoxifen. *Cancer Res*, **64**, 3807-13.

Woodson K, Mason J, Choi SW, et al (2001). Hypomethylation of p53 in peripheral blood DNA is associated with the development of lung cancer. *Cancer Epidemiol Biomarkers Prev*, **10**, 69-74.

Xu X, Gammon MD, Zhang Y, et al (2009). BRCA1 promoter methylation is associated with increased mortality among women with breast cancer. *Breast Cancer Res Treat*, **115**, 397-404.