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BRCA1 methylation in breast duct carcinoma, a practical study in Duhok-Iraq

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ABSTRACT

Lacking protein functions of Breast cancer susceptibility gene1 (BRCA1) and Breast cancer susceptibility gene2 (BRCA2), by methylation, represents tissue-specific silent epigenetic regions that tolerate genomic instability and may end in different cancers, mainly breast and ovary. Promoter-CpG island hypermethylation is a common molecular defect in cancer cells. This has prompted us to use MSP for identification of BRCA1 methylation in these groups of women at Duhok, north of Iraq. Genomic DNA was isolated from 96 tumor samples from patients with primary breast cancer and normal tissues which include; 40 non-neoplastic breast tissues (considered as external control) and 40 distant non-cancerous tissues from the same cancerous women (internal control). The extracted DNA was subjected to methylation-specific PCR (MSP) to determine the promoter methylation status of BRCA1 and its correlation with study parameters including protein expression level of ER, PR, Her2/neu, and Ki67 receptors. The study revealed 10.4% complete BRCA1 methylation and 66.6% partial methylation (PM) among the cancerous samples. Partial methylation was observed in 95% of internal control and 20% of the external control. Complete methylation was negative in both control groups. Compared with negative methylation, positive BRCA1 promoter methylation was significantly high among triple negative (ER-, PR-, Her2-) cases with high proliferative index. There was also a methylation trend toward cases with T2 and higher staging status, although didn't reach the level of significance. BRCA1 promoter complete methylation was exclusive for cancerous tissues. With management of the above concerns, this line of research gains a strength point including the prevalence of DNA methylation changes among sporadic breast cancer (i.e. not restricted to the inherited type). Considering partial or focal BRCA1 methylation, caution has to be taken as this epigenetic alteration, which may progress to complete methylation status, was detected in non-neoplastic breast tissues adjacent to the cancerous ones and even normal breasts. This triggers application of extended screening programs, including BRCA1 methylation, for identification of women at risk, and can benefit from early intervention.

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Introduction

Across continents, breast carcinoma remains the top cancer among women in terms of prevalence and mortality in most countries (1). To reduce disease burden, it is important to identify their etiologic factors and those that predict survival and response to therapy. The etiologic factors comprise variable incorporation of genetic abnormalities and environmental factors (2,3). Breast Cancer 1 (BRCA1) and Breast Cancer 2 (BRCA2) genes inactivation confers a lifetime risk of breast cancer (4,5). These gene proteins participate in DNA repair and homologous recombination. A cell with a lack of BRCA1 or BRCA2 protein functions, in addition to the ability of decreased DNA damage pair, represents tissue-specific silent regions that tolerate instability at the epigenetic genomic which may end in cancer of breast, ovary, and pancreas (5, 6,7). Promoter-CpG island hypermethylation is a widespread molecular defect in cancer cells. It has been considered as an alternative mechanism to BRCA1 silencing in breast cancer where its somatic mutations are rare. In breast cancer, BRCA1 promoter region methylation is found to be incriminated with tumorigenesis of breast cancer and related to particular biological and clinicopathological features and tumor progression and acts as a promising prognostic and therapeutic target (3,4,5,7,8). Epigenetic silencing manifests itself as complete or partially methylated domains of genes. The partially methylated domains reflect reduced average DNA methylation levels that cover poor gene transcriptional inactivation and tend to be heterochromatic (9). As well, the genomic distribution of partially or completely methylated tissue can be missed by the routine PCR techniques applied for genetic counseling. MSP may allow every laboratory to access the DNA methylation marker. Hypermethylation of cytosine residues in CpG islands within the promoter of many tumor suppressor genes is correlated strongly with the loss of gene function. BRCA1 protein expression was found to be absent or decreased markedly in the majority of the BRCA1 methylated tumors, suggesting epigenetic gene silencing in these tumors (6). BRCA1 DNA methylation has become the most attractive marker due to its specificity sensitivity and applicability to a variety of clinical specimens (10).

A limited number of data-based sets have been reported about BRCA1 methylation among patients with cancerous and non-cancerous tissues of the same breast and among women with no breast cancer, and MSP techniques applied

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for the detection of this methylation. This has prompted us to use MSP for the identification of BRCA1 methylation in these groups of women at Duhok, north of Iraq. This may help to identify women at risk of developing breast cancer, as these women might benefit from supplemental screening modalities such as mammography, and MRI, which may target families with breast cancer susceptibility genes.

Materials and Methods

Study design and research sample

This is a cross sectional study conducted during the period extended from August 2021 to August 2022 in the Duhok Medical Research Center, College of Medicine. Ninety-six archived surgically excised primary breast duct carcinoma tissue specimens were collected from histopathology units in General Central Laboratory and Vin Private Laboratory in Duhok-Iraq for five years (from January 2018 to December 2022). These cases included 96 surgically excised tissues for breast carcinoma without a family history of breast or ovarian carcinoma (sporadic type). Other, 40 non-neoplastic breast tissues (considered as external control) and age-matched cases were also included. Patients' age range was 18 - 83 years; median: 50.5 years. The other 40 samples were taken as external control from women with normal breast tissue removed from women for plastic purposes (aged 28 -71 years; median: 49.5). From the same cancerous women, 40 distant non-cancerous tissues (internal control) were taken. Clinical information about the patient's age, sex, tumor size, and lymph nodes was obtained from the available histopathological reports and the patient's files in the Central and Vin Private Laboratories. The study was approved by scientific committees at the Duhok College of Medicine the University of Duhok the and Duhok Directorate of Health. Three mm-thick tissue sections were taken and stained again with Hematoxylin and Eosin (H&E) and sections were re-examined by the pathologist for definite tumor diagnosis and grading. Of these, 96 cases of grade 3 infiltrative duct carcinoma were enrolled in the study.

Immunohistochemistry (IHC) test

Unstained representative tumor sections were subjected to immunohistochemistry (IHC). They were cut and mounted on poly- *l* -lysine-coated slides and processed according to the manufacturer's instructions (Dako Denmark, A/S), using the autostainer (Dako Denmark, Link48). The markers used included Estrogen Receptors (ER: Era EP1) and Progesterone Receptors (PR: PgR, 636). More than 5% of tumor cells with brown-stained nuclei were considered positive, and cytoplasmic-stained cells were ignored. Other markers included Her-2/neu (c-erbB-2) receptors. Hercept test-kit was applied to evaluate the results as follows: 0 or +1 (negative) when there was no or faint staining of tumor cell membrane respectively; +2 (borderline) when >10% of tumor cells showed a weak to moderate membranous staining, and +3 (strongly positive) when >10% of tumor cells showed a strong and complete brown stained membrane (11,12).

The proliferative index was tested in tumor sections using Ki67 (MIB1) status, the assessment of which was estimated as the percentage of positively stained cancer cell nuclei. Cases with more than 14% stained nuclei were considered as high proliferative index while those with equal or less than 14% positive nuclei were reported as low (11). Appropriate positive controls were run parallel with each set of IHC techniques, using internal non-neoplastic breast acini for ER and PR; Her2-strongly positive breast cancer for Her2/neu; and a lymph node with Burkitt's lymphoma for Ki67. Negative controls were accomplished by incubating non-stained sections with buffer solution instead of the primary antibodies (11).

DNA extraction and bisulfite modification

For molecular study, tissue sections were paraffin blocks then extraction of DNA which was performed by using proteinase K digestion Isolation, Genomic DNA concentration and purity were quantified in duplicate using NanoDrop spectrophotometer and Gel electrophoresis. DNA samples were stored at -20^{o}C after that and treated with Sodium Bisulfate to convert the DNA with the use of EpiTect Bisulfite from Qiagen for the conversion and cleanup of the DNA for methylation analysis.

Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) methods used where the CpG sites residing within the primer sets were used as a proxy for the methylation status of the region of interest. In the used MSP, the target (BRCA1 tumor suppressor genes) DNA was amplified with the mutation-specific primers in the promoter region of BRCA1 (MF: 5'-TCGTGGTAACGGAAAAGCGC-3' and MR: 5'-AAATCTCAACGAACTCACGCCG-3', PCR product size: 75 bp). The unmethylated DNA sequence was amplified using a primer specific to the unmethylated-bisulfite-converted DNA sequence, in which the C's (cytosines) in the template should be treated as T's (UF: 5'-TTGGTTTTTGTGGTAATGGAAAAGTGT-3' and UR: 5'-AAAAAATCTCAACAAACTCACACCA-3', PCR product size: 86 bp). The methylation and unmethylation-specific primers were adapted from (13,14). MS-PCR reactions were performed in 20µl total volume which contained 0,5µl MF primer and 0,5µl MR primer (synthesized by Macrogen, South Korea), 2x Hot reaction Master mix (Addbio, Korea), and 2µl template of bisulfite converted DNA and this was made up to a final volume with DNase-free water.. The cycling conditions were initial denaturation at 96^{o}C for 4 minutes followed by 35 cycles of denaturation at 95o C for 50 seconds, annealing at 62^{o} C for 30 seconds, and extension at 72^{o}C for 30 seconds, followed by a final extension at 72^{o}C for 5 minutes, using a thermocycler (Applied Biosystems USA). The PCR products were separated in 2% agarose gel electrophoresis after staining with Prime Safe Dye. The molecular findings were directly visualized under UV illumination.

Statistical analysis

Version 22 of IBM SPSS Statistics software was used to analyze data. The data were arranged using frequencies and percentages. Chi-square test was used for analyzing statistical associations and differences. When Chi-square assumption is violated, the results of Fisher's Exact test were recorded. Statistically significant P value equal to or less than 0.05 was considered. When a cell had a zero value, 0.5 was used to all cells to correct for undefined findings (IBM Corp., 2013).

Results

As demonstrated in Table (1), the Methylation-Specific PCR (MSP) used to investigate BRCA1 promoter methylation status in cancer cases revealed 10 (10.4%) cases of complete methylation (CM) and 64 (66.6%) cases of partial methylation (PM). None of the external or internal controls showed complete methylation. However, partial methylation was observed in 95% of the internal control and 20% of the external control. Statistically, the difference was significant between the control groups. Of the cancerous women with total methylation, 58.1% were above 50 years and the remainder (41.9%) were less than 50 years. The age difference was statistically not significant.

Compared with negative methylation, cases with positive BRCA1 promoter methylation were significantly high among ER-, PR-, Her2-, and high proliferative index (Table 2). Regarding T-status, methylation trended toward cases with T2 and higher status but didn't reach the level of significance (Table 2). There was a trend toward cases higher than T1 but didn't reach the level of significance.

After grouping of the tested markers, BRCA1 methylation was high among ER-/PR-/Her2- ($p< 0.05$). However, adding the proliferative index value, the difference didn't reach the level of significance with $p= 0.6$ (Table 3).

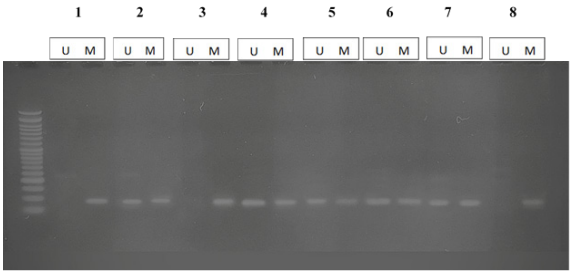

Figure 1. Methylation-specific PCR of the BRCA1 promoter region in tumor samples. Electrophoresis of Amplification Products Specific Promoter Region BRCA1 from Bisulfite-Treated DNA in Human Tumor Tissue. Each line reflects amplification products from methylated (M), 75bp or unmethylated (U), 86bp primer. The presence of a visible PCR product in lane (U) indicates the presence of unmethylated BRCA1 genes, and the presence of a product in lane (M) indicates the presence of methylated BRCA1 genes. Sample no. 1, 3, and 8 have complete methylated (CM) promoter region of the BRCA1 gene and sample no. 2, 4, 5, 6, and 7 has partial methylated (PM) promoter region of the BRCA1 gene. The ladder used in the first line is 50bp.

Discussion

Among the wide spectrum markers capable of diffe-

Table 1. BRCA1 Methylation status among cancerous and control tissues.

	BRCA1 methylation					
		Positive			Negative	p
	Total	CM (%)	PM (%)	CM+PM No. (%)	Negative No. (%)	
Malignant	96	10 (10.4)	64 (66.6)	74 (77)	22 (23)	
Internal control	40	0	38 (95)	38 (95)	2 (5)	< 0.001
External group	40	0	8 (20)	8 (20)	32 (80)	

The CM and PM methylation results of some cases are shown in Figure 1.

Table 2. BRCA1 methylation and the studied parameters.

Variable		BRCA1 methylation					
		Positive			Negative	Total	*P*
		CM (%)	PM (%)	Total positive methylation No. (%)	Total negative methylation No. (%)	No. (%)	
ER	positive	2 (5)	33 (82.5)	35(87.5)	5(12.5)	40	
	negative	8 (14.2)	31 (55.4)	39 (69.6)	17 (30.3)	56	0.02
PR	positive	2 (5.1)	32 (82.1)	34 (87.2)	5 (12.8)	39	
	negative	8 (14.1)	32 (56.1)	40 (70.2)	17 (29.8)	57	0.03
Her2/neu	positive	3 (17.6)	7 (41.2)	10 (58.8)	7 (41.2)	17	
	negative	7 (8.9)	57 (72.2)	64 (81.1)	15 (19)	79	0.04
Ki67	high	7 (8.7)	54 (68.4)	61 (77.2)	18 (22.8)	79	
	low	3 (17.6)	10 (58.8)	13 (76.5)	4 (23.5)	17	0.05
TN	T1N1	2 (14.3)	11 (78.6)	13 (92.9)	1 (7.1)	14	
	T1N2	0	2 (66.7)	2 (66.7)	1 (33.3)	3	
	T1N3	0	2 (66.7)	2 (66.7)	1 (33.3)	3	
	T2N1	0	1 (100)	1 (100)	0	1	
	T2N2	2 (8.7)	17 (73.9)	19 (82.6)	4 (17.4)	23	
	T2N3	3 (15)	10 (50)	13 (65)	7 (35)	20	
	T3N1	0	9 (69.2)	9 (69.2)	4 (30.8)	13	0.549
	T3N2	2 (40)	3 (60)	5 (100)	0	5	
	T3N3	1 (7.1)	9 (64.3)	10 (71.4)	4 (28.6)	14	

CM: Complete methylation, PM: Partial methylation.

Table 3. Positive BRCA1 methylation, Ki67 association with hormone receptors groups.

Hormone Receptor Groups	CM	PM	Positive BRCA1 methylation (CM+PM)
ER+/PR+/Her2/neu+	1 (1.4%)	6 (8.1%)	7 (9.5%)
ER+/PR+/Her2/neu+/High Ki67	1 (1.4%)	6 (8.1%)	7 (9.5%)
ER+/PR+/Her2/neu+/low Ki67	0 (0%)	0 (0%)	0 (0%)
ER+/PR+/Her2/neu-	8 (10.8%)	20 (27.0%)	28 (37.8%)
PR+/PR+/Her2/neu-/High Ki67	1 (1.4%)	22 (29.7)	23 (31.1%)
PR+/PR+/Her2/neu-/low Ki67	1 (1.4%)	4 (5.4%)	5 (6.8%)
ER-/PR-/Her2/neu+	3 (4.1%)	1 (1.4%)	4 (5.5%)
ER-/PR-/Her2/neu+/High Ki67	2 (2.8%)	0 (0%)	2 (2.8%)
ER-/PR-/Her2/neu+/low Ki67	1 (1.4%)	1 (1.4%)	2 (2.8%)
ER-/PR-/Her2/neu-	5 (6.8%)	30 (40.5%)	35 (47.3%)
ER-/PR-/Her2/neu-/High Ki67	4 (5.4%)	25 (33.8%)	29 (39.2%)
ER-/PR-/Her2/neu-/low Ki67	1 (1.4%)	5 (6.8%)	6 (8.2%)
ER-/PR+/Her2/neu+	0 (0%)	0 (0%)	0 (0%)
ER-/PR+/Her2/neu+/High Ki67	0 (0%)	0 (0%)	0 (0%)
ER-/PR+/Her2/neu+/Low Ki67	0 (0%)	0 (0%)	0 (0%)
ER-/PR+/Her2/neu-	0 (0%)	0 (0%)	0 (0%)
ER-/PR+/Her2/neu-/High Ki67	0 (0%)	0 (0%)	0 (0%)
ER-/PR+/Her2/neu-/Low Ki67	0 (0%)	0 (0%)	0 (0%)
ER+/PR-/Her2/neu-	0 (0%)	1 (1.4%)	1 (1.4%)
ER+/PR-/Her2/neu-/High Ki67	0 (0%)	1 (1.4%)	1 (1.4%)
ER+/PR-/Her2/neu-/Low Ki67	0 (0%)	0 (0%)	0 (0%)
ER+/PR-/Her2/neu+	0 (0%)	0 (0%)	0 (0%)
ER+/PR-/Her2/neu+/High Ki67	0 (0%)	0 (0%)	0 (0%)
ER+/PR-/Her2/neu+/Low Ki67	0 (0%)	0 (0%)	0 (0%)

p-value = 0.05 based on Fisher's exact test.

rentiating tumors from normal tissue is DNA methylation. The BRCA1 gene is known to be a target for aberrant DNA methylation in breast cancers, particularly high-grade tumors. Identification of this gene methylation is achieved by different techniques (5,15,16,20). In this case-control study, BRCA1 promoter complete methylation was detected in 10.4% of cases. Although this percentage appears to be similar to what has been reported in Australia (7), however, it is at the lowest third among the divergent inter-individual BRCA1 hypermethylation results (Table 4). Such divergent rates may be attributed to the different populations studied with their geographic, ethnic and race heterogeneity (3,7,8,18,19). Also, exclusive testing to certain ages with certain parametric results and distinct breast cancers (inherited, sporadic, or tumors with specific immune profiles) have also an impact on the reported percentages (7,18). The chosen tested sample (blood, fresh tissue, or paraffinized tissue) may also alter the results (2,7,8,18). It is noteworthy to mention that our study samples were tissues taken from sporadic breast duct carcinoma with a negative family history. Another contributing agent is the contamination with normal and non-neoplastic tissues during the processing procedure which might attenuate the methylation levels. This contamination was minimized as much as possible among the study cases because the tumor tissue taken was micro-dissected from selected tumor-stuffed paraffin-embedded sections with aseptic processing steps, using disposable blades and tubes. As well, the assessment technique applied forms an important contributing factor (20). One significant drawback of many methylation research is that they only examine a small group of CpGs, particularly cases with partial or focal methylation. This makes it difficult to pinpoint exactly how many and where methylation loci are present (20). The MSP used in this study is a perfect, widely used technique nowadays for the identification of different methylation statuses by methylated versus unmethylated DNA-specific primer amplification of bisulfite-treated DNA (21,22). Furthermore, the primer sequences, the target regions, and heterogenous methylated loci which are hypervariable in level, size, and distribution, all might display altered detection rates (20,23). Actually speaking, adding the complete methylation (10.4%) with the partial methylation rate (66.7%) will increment our scale (77.1%) to fall within the highest literature rates. In breast cancer, the extent and variations of partial methylation domain detection in primary tumors are hitherto unknown (20).

On the other hand, none of the control cases showed any evidence of complete methylation. However, partial (or focal) methylation was detected in 95% of internal controls and in 20% of external control. Studies have reported variable BRCA1 methylation levels among normal breast tissues, although some are shown to be high, but significantly lower than their matched cancerous tissues (2,7,9,18,20). It is noteworthy that such high levels of detected partial methylation among the non-cancerous tissues of internal controls premises that methylation of the BRCA1 gene promoter might contribute to the initiation of breast carcinoma, probably with silencing of the wild gene allele by time, an event that might be reversible and erased by time in some cases (24). Furthermore, the identification of methylation among both internal controls

Table 4. Reported geographic percentages of breast carcinoma with BRCA1 methylation.

Study, Year	Country	Number	BRCA1 methylation %
Present study, 2023	Duhok-Iraq	164	10.4 (complete) and 66.6 (partial)
Lonning et al, 2022	Bergen, Norway	637	5.5
Stefansson et al, 2020	Iceland	1031	3
Lan et al, 2014	Vietnam	60	11.7
Hsu et al, 2013	Taiwan	139	56
Al-Moghrabi et al, 2011	Saudi Arabia	47	27.6
Xu et al, 2009	USA	851	59
Jing et al, 2008	China	-	32

and normal breast tissues (external controls) may be attributed to the high-quality technique used in this study and the implementing measurements applied in breast cancer clinics of this locality outweighed by the gain in increased risk prediction model accuracy and the more accurate determination of those who stand to gain the most from early detection and intervention. This premises the issue that methylation detection test may become a great additional test to the breast cancer screening strategies that have to be offered for younger aged women.

Interestingly, BRCA1 epigenetic silencing appeared significantly high among triple negative (ER-, PR-, Her2-) cancer cases with a high proliferative index, but not with TN staging status despite the trend toward T2N2. An association between BRCA1 hypermethylation and triple-negative breast cancers has been described predominantly among the germline mutation carriers and high-graded breast carcinoma (4,5,18). However, such an association appears to be complex in the literature (2). Another factor that may be involved is the correlation between the proliferation index (Ki67) and graded breast carcinoma. (11).

Limitations have to be mentioned, a small number of patients and control groups, absence of inherited cases, deficient clinical data like patient's diet, hormonal and other environmental information which may have an impact on BRCA1 promotor methylation, lack of post-therapy follow-up to predict recurrence and survival. All of these may give bias to the results.

In conclusion**,** this limited case-control study showed BRCA1 promoter complete methylation was seen only among cancerous tissues while negative among non-cancerous control groups even tissues adjacent to cancerous cells (internal control). Here caution has to be taken when considering partial or focal BRCA1 methylation, as this epigenetic alteration was detected in normal breasts. With the management of the above concerns, this line of research has several strengths, including the prevalence of DNA methylation changes among sporadic breast cancer (i.e. not restricted to the inherited type). Adding to this, the presence of partial methylation in non-neoplastic samples adjacent to cancerous tissue and in normal breast tissue triggers the application of extended screening programs for the identification of women at risk and can benefit from early intervention on early detection. Further research is required to determine whether constitutional methylation of BRCA1 genes acts as pan-cancer independent risk factors or is incorporated with other aberrant tumor-related genes.

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Interest conflict

The authors declare that they have no conflict of interest.

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