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Original Research Article

The Diagnostic Implications of Aberrantly-Methylated Tumour Suppressor Genes *RARβ2* and *3-OST-2* – Captured from Cell-Free DNA in the Serum – in Breast-Cancer Diagnosis

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Abstract: Epigenetics has emerged as one of the most-exciting frontiers in the study of the human carcinogenesis. Promoter CpG islands (CGIs) hypermethylation is an important epigenetic change associated with silencing the DNA transcription and has a role in tumour suppressor genes inactivation in many types of cancer. Here, we have investigated aberrant methylation of two tumour suppressor genes (*RARβ2*: retinoic acid receptor gene and *3-OST-2*: heparan sulphate D-glucosaminyl 3-Osulphotransferase-2 gene) which are early markers for breast cancer. A total of 178 individuals was enrolled in this study; they were divided into breast- cancer patients (n=93), patients with benign breast tumour (n=55), and healthy individuals (n=30). Using the relative quantitative methylation specific PCR (RQ-MSP) technique, the methylations of *RARβ2* and *3-OST-2* genes were analysed in the serum samples and compared with traditional tumour markers and clinicopathological factors. The methylat ions of *RARβ2* and *3-OST-2* – which are tumour suppressor genes –were significantly higher in breast-cancer patients than in the benign and control individuals (p<0.0001). Methylated genes were not significantly related to clinicopathological factors apart from pathological types. Both methylated genes were found in all different tumours regardless the grades and stages. Sensitivities and specificities for the candidate genes were together superior to other tumour markers in the detection of early- stage and low-grade breast cancer..
Keywords: Aberrant methylation; breast cancer; early diagnosis; *RARβ2*; *3-OST-2*.

INTRODUCTION

Breast cancer is a heterogeneous disease, manifesting variations at the clinical, biological, histopathological, and molecular levels [1]. Early breast-cancer detection can be curable. Traditional methods used to detect and monitor breast cancer are based on palpation or radiological images [2], however many tumours cannot be detected till being in the late stage. Hence, other sensitive detection methods need to be developed.

Methylated CpG islands in the promoter and 5′ regions of *RARβ2* and *3-OST-2* genes are related to transcription disruption since they are altered in the cancer-related genes **[**3], thus leading to abnormal gene expression, suggesting that these alterations can be recommended as markers for detection of cancer [4]. The retinoic acid receptor (RAR) – a type of nuclear receptor – consists of three receptors (RARα, RARβ, and RARγ differentially expressed throughout development. It has been reported that RARβ contributes in the regulation of epithelial cell and in tumourigenesis [5].

The *RARβ2* is located in chromosomal region 3*p*24 (2477/+392, GenBank accession numbers S82362 and M96016) [6], and it is a tumour suppressor gene expressed in many tissues in the lung, bladder, and gynecological neoplasia, indicating that aberrant *RARβ2* gene methylation participates in the origin of cancer [7].

Also, aberrant methylation of cytosine preceding guanine islands (CGI) in 5′ regions of the heparan sulphate D-glucosaminyl 3-Osulphotransferase-2 (3-OST-2) gene (GenBank Accession number: NM 006043) was reported in several types of cancer. The *3-OST-2* gene encodes Osulphotransferase which is involved in the last modification step of glycosaminoglycan chains of heparin sulphate proteoglycans (HSPGs) [8, 9] including glypicans and

syndecans. Their altered expressions have been detected in breast cancers[10].

The current study aimed to: i) detect the aberrant methylation for the two suppressor genes *RARβ*$_2$ and *3-OST-2* in primarily breast-cancer patients; ii) identify their diagnostic efficacy as compared to other commonly-used tumour markers (CEA and CA15.3); iii) determine the correlation between methylation status and clinicopathological features.

MATERIALS AND METHODS

Studied groups

The present study is comprised of three groups: group (I) primary breast-cancer women (n= 93) (range 23–70 years, mean age=47 years); all of them had no evidence of other cancers based on clinical and radiological examinations. None of them received chemotherapy or radiotherapy prior to blood sample assembly.

Pathological staging and grading were assessed using the Tumour-Node-Metastases classification (TNM) [11], and the modified Scarff-Bloom-Richardson histologic grading system [12], group (II) patients with benign breast lesions (n=55) (range 28 – 58 years, mean age=40 years), group (III) healthy volunteers individuals (n=30) (range 25 – 60 years, mean age = 40 years). After obtaining informed consent from all the participants, full clinical and pathological data were collected and whole blood was drawn from all individuals before surgery, then serum was obtained immediately by centrifugation at 10.000 rpm for 10 min at 4 °C. The samples were stored at -80 °C until analyzed.

DNA Extraction and Purification

Cell-free DNA in serum was extracted using the QIAamp DNA Blood Mini Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol.

DNA Treatment and Bisulphite Conversion

Bisulphite conversion was based on the principle that bisulphite treatment of DNA converts unmethylated cytosine residues to uracil; whereas methylated cytosine residues would remain unmodified. Thus, after bisulphite conversion, methylated and unmethylated DNA sequences would be distinguishable by sequence-specific primers. Each DNA sample was treated with sodium bisulphite using the EpiTectPlus DNA Bisulphite Kit (QIAgen, Hilden, Germany). DNA was converted and purified using the EpiTect Bisulphite Kit (QIAgen, Hilden, Germany) and all the converted DNA samples were assessed for their DNA purity and quantified on a Q-5000 Spectrophotometer (Quawell Technology, Inc., San Jose, USA), then stored at -20 °C.

Methylation-Specific PCR

Methylation-specific PCR (MSP) was performed using the EpiTect MSP Kit (QIAgen, Hilden, Germany) according to the manufacturer's protocol. Sense and antisense primer sequences for methylated and unmethylated *RARβ*$_2$ are as follows: [methylated : 5'-TCG AGA ACG CGA GCG ATT CG - 3'(sense), 5'-GAC CAA TCC AAC CGA AAC GA -3' (antisense), which amplify a 702-bp product, unmethylated *RARβ*$_2$: 5'- TTG AGA ATG TGA GTG ATT TGA -3'(sense), 5'- AAC CAA TCC AAC CAA AAC AA -3'(antisense), which amplify a 696-bp product]. In case of *3-OST-2* [methylated : 5'- CGG TTG TTC GGA GTT TTA TC - 3' (sense), 5'- GTA ACG CTA CCA CGA CCA CG -3' (antisense), which amplify a 260-bp product, unmethylated *3-OST-2*: 5'- TGG AGT TTT ATT GTT TAG GAT T -3'(sense), 5'- AAA ACT CAC ATA ACA CTA CCA CA -3' (antisense),which amplify a 172-bp product] gene promoter regions [13]. PCR was performed in a thermal cycler (SureCycler 8800, Agilent Technologies, Santa Clara, USA). A master mix consists of**:-**1.25 mM dNTP, 16.6 mM (NH4) $2SO_4$, 67 mMTris, pH 8.8, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 1U Red*Taq* genomic DNA polymerase (Sigma-Aldrich, Inc., St. Louis, MO), 25 pmol each of the forward and reverse primers specific to the methylated and unmethylated DNA sequences and 2µl of bisulphite-treated DNA. Methylated and unmethylated primers were tested in separate reactions. PCR conditions were as follows for both genes: activation at 95°C for 5 minutes; then 35 cycles of denaturation at 94°C for 1 minute; annealing at 50°C for 1 minute; and extension at 72°C for 1 minute, followed by final extension at 72°C for 5 minutes and hold at 4°C. The amplified PCR products were electrophoresed through a 2% agarose and visualized by ethidium bromide staining, and gel photos were captured using ultraviolet Gel Doc analyzer (G: Box F3, Court Suite, Frederick, USA) [14].

Relative Quantitation gene expression analysis of *RARβ*$_2$ and *3-OST-2*

To control the test efficiency and to normalize for sample to sample variation in DNA amount as well as to quantify the methylated and unmethylated level of the investigated genes relatively to the expression of *β*-actin as a housekeeper in each sample, *β*-actin-specific primers were used as follows: (sense primer: 5\-GCGGGAAATCGTGCGTG-3\;and antisense primer 5\-CAGGGTACATGGTGGTGCC-3\) with generation of a 309 base-pair fragment, PCR conditions were as follows: activation at95°C for 5 minutes; then 35 cycles as follows: denaturation at 94°C for 1 minute; annealing at 58°C for 30 seconds; and extension at 72°C for 30 seconds, followed by final extension at 72°C for 10 minutes and hold at 4°C. The signal intensities in agarose gel for either *RARβ*$_2$ and *3-OST-2* in each sample were determined relative to that of *β*-actin in the

same sample using Gel-pro ([version 3.1] Media cybernetics, USA) software consequently conclude the relative amount of the different samples. The fragments' sizes of the PCR products were estimated by comparison with DNA molecular weight marker (1 kb) (provided by Promega, GE Healthcare Bio-science, UK limited), (Figure 1).

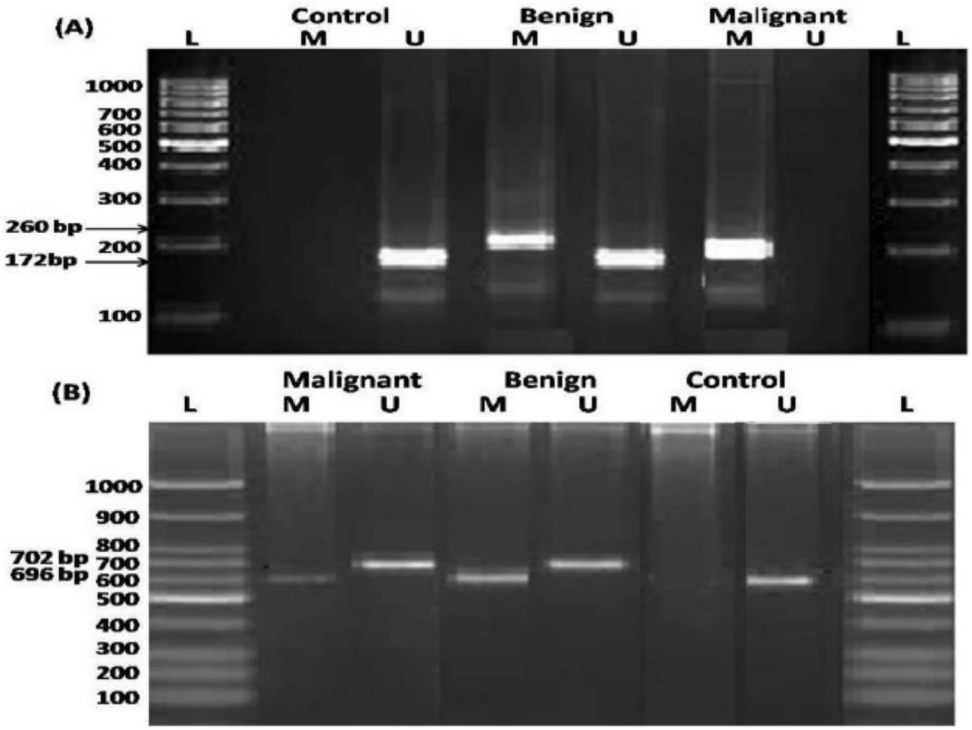

Fig-1: PCR products methylation of cancer-related genes *3-OST-2* (A) and *RARβ2* (B) genes in serum by agarose gel electrophoresis stained ethidium bromide. Each sample was represented by two successive lanes, one for unmethylated (U) band and the second for methylated (M) band. *3-OST-2* Positive bands for both unmethylated and methylated bands are shown at 172 and 260 bp., respectively; and *RARβ2* bands for both unmethylated and methylated bands are shown at 296 and 702 bp, respectively.

Statistical analysis

The Kruskal-Wallis ANOVA test was used to study differences in methylation levels among the three groups (malignant, benign and control). Receiver operating characteristic curve was plotted [true-positive fraction (sensitivity %) *versus* false-positive fraction (100- specificity %)] to calculate the best cut-off points for the two investigated genes [15]. We used Pearson's X^2 to compare between their positivity rates. A $p< 0.05$ was considered a statistically-significant difference. All of the statistical analyses were performed using SPSS Software (version 10.0).

RESULTS

This study dealt with 178 individuals who were divided into three groups according to their clinical and histopathological database into primary breast-cancer patients (n=93), patients with benign breast tumour (n=55) and healthy individuals (n=30). The entire group of breast-cancer patients consisted of 68 patients with early stage and 25 with late stage. Regarding their histological grading; breast-cancer patients were divided into 74 patients with low grade and the remaining were with high-grade cancer.

Methylation pattern of the investigated genes among the studied groups

Relative quantification was determined based on dividing the threshold copies of the investigated genes of interest *RARβ*$_2$ and *3-OST-2* by the internal reference gene *β*-actin. As shown in Table "1", methylation patterns were significantly detected among the three studied groups; median levels for CEA, CA15.3, *RARβ*$_2$ and *3-OST-2* were increased 6.1 fold, 1.75 fold, 1.26 fold and 1.21 fold, respectively in the malignant group compared to the benign one, 6.25 fold, and 1.02 fold for CEA, and 2.17 fold and 1.24 fold for CA15.3 in the malignant compared to the control, and the benign compared to the control, respectively. Regarding the median levels of *RARβ*$_2$ and *3-OST-2*, they were not detected in the control individuals ($p< 0.001$).

The benign and healthy normal groups were combined in a non-malignant group, and the best cut-off values for the investigated parameters were calculated by the ROC curve as 5ng/ml, 15 ng/ml, 6.438 copies and 0.99 copies for CEA, CA15.3, *RARβ*$_2$ and *3-OST-2*, respectively (Figure 2). With regard to these cut-off values, methylated genes, as well as traditional tumour markers, revealed a significant difference between the three investigated groups ($P < 0.001$) (Table 1).

Correlation between investigated markers and clinicopathological factors

The correlation between two traditional tumour markers (CEA and CA15.3) and aberrant methylated *RARβ*$_2$ and *3-OST-2* genes among clinicopathological factors was reported in Table "2". Both the tumour markers CA15.3 and *RARβ*$_2$ showed significant correlation with the pathological types. Also, CEA and *RARβ*$_2$ showed significant correlation with lymph node involvement. Although all the investigated markers were highly detected in early stages; a significant level was not reached.

Table-1: Analysis of methylation status for *RARβ*$_2$, *3-OST-2* and traditional tumor markers among investigated groups.

Investigated groups	CEA		CA15.3		*RARβ*$_2$		*3-OST-2*	
	Median	> 5 ng/ml	Median	> 15 ng/ml	Median	> 6.438 copies	Median	> 0.99 copies
Control (n=30)	0.80[a]	0 (0%) [b]	9.2 [a]	0 (0%) [b]	0 [a]	0 (0%) [b]	0 [a]	0 (0%) [b]
Benign (n=55)	0.82	1 (1.8%)	11.45 [a]	6 (10.9%)	5.54	11 (20%)	0.99	25 (45.5%)
Malignant (n=93)	5	31 (33.3%)	20	53 (57%)	7	81 (87.1%)	1.2	91 (97.8%)

Significant level at P< 0.0001 using [a] ANOVA test and [b] Chi-square test.

Table-2: Positivity rates of traditional tumor markers and methylated pattern of *RARβ* and *3-OST-2* among breast cancer patients.

Clinicopathological factors	CEA N (%)	CA15.3 N (%)	*RARβ*$_2$ N (%)	*3-OST-2* N (%)
Age (years)				
< 45 years (n=42)	13 (31%)	26 (61.9%)	36 (85.7%)	42 (100%)
≥ 45 years (n=51)	15 (29.4%)	27 (52.9%)	45 (88.2%)	49 (96.1%)
Pathological type				
NIDC (n=41)	14 (34.15%)	18 (43.9%)	39 (95.1%)	40 (97.6%)
IDC (n=52)	14 (26.9%)	35 (67.3%)	42 (80.1%)	51 (98.1%)
		X^2=5.12, P=0.024	X^2=4.2, P=0.04	
Histological grades				
Low grade (n=74)	22 (29.7%)	41 (55.4%)	64 (86.5%)	73 (98.6%)
Late grade (n=19)	6 (31.6%)	12 (63.2%)	17 (87.5%)	18 (94.7%)
Clinical Stages				
Early stage (n=68)	22 (32.4%)	41 (60.3%)	58 (85.3%)	67 (98.5%)
Late stage (n=25)	6 (24%)	12 (48%)	23 (92%)	24 (96%)
Lymph node involvement				
-ve (n=25)	14 (56%)[a]	14 (56%)	25 (100%)	25 (100%)
+ve (n=68)	14 (20.6%)	39 (57.4%)	56 (82.4%)	66 (97.1%)
	X^2=10.9, P<0.01		X^2=5, P=0.024	
ER				
-ve (n=52)	11 (21.2%) [a]	28 (53.8%)	44 (84.6%)	51 (98.1%)
+ve (n=41)	17 (41.5%)	25 (61%)	37 (90.2%)	40 (97.6%)
	X^2=4.5, P=0.034			
PgR				
-ve (n=41)	11 (26.8%)	25 (61%)	34 (82.9%)	39 (95.1%)
+ve (n=52)	17 (32.7%)	28 (53.8%)	47 (90.4%)	52 (100%)
HER-2/neu				
-ve (n=38)	11 (28.9%)	20 (52.6%)	31 (81.6%)	37 (97.4%)
+ve (n=55)	17 (32.9%)	33 (60%)	50 (90.9%)	54 (98.2%)

NIDC, non invasive duct carcinoma; IDC, invasive duct carcinoma, ER, estrogen receptor; PgR, progesterone receptor, HER-2/neu, human epidermal growth factor receptor 2.

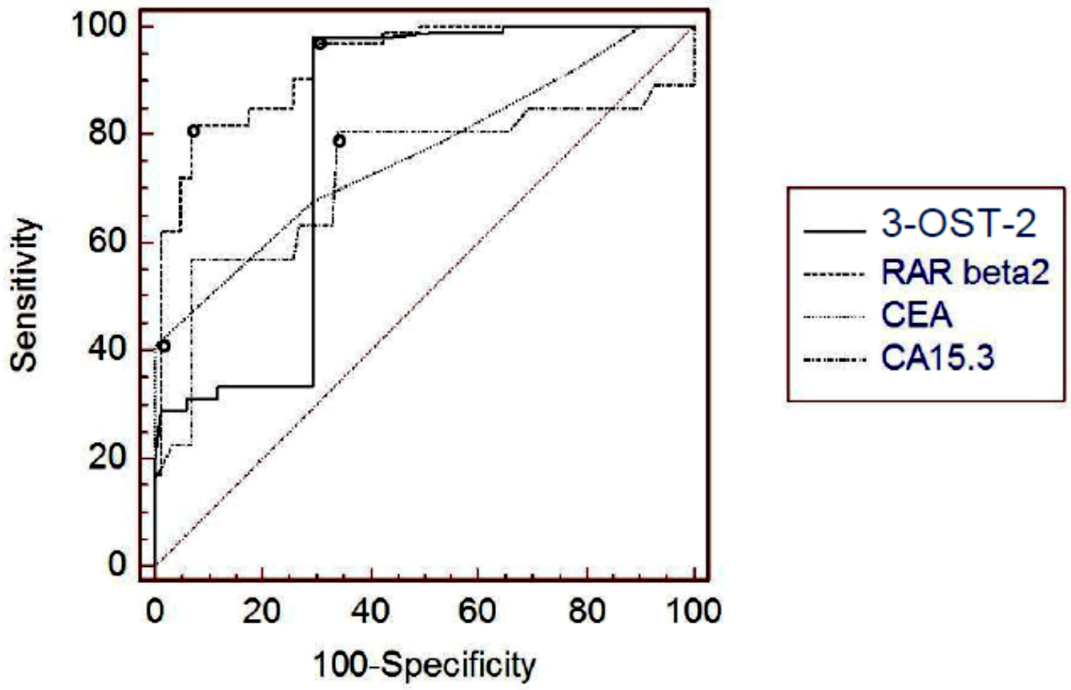

Fig-2: ROC curve analysis for tumour markers (CEA and CA15.3), *RARβ₂* and *3-OST-2* genes to discriminate between malignant and non-malignant groups. Open circles denote best cutoff points of CEA as 5 ng/ml [sensitivity = 33.3% and specificity = 98.8%. Area under the curve (AUC) [SE] = 0.76 [0.0356], 95% confidence limits range = 0.691 – 0.821, *P* < 0.0001], CA 15.3 as 15 ng /ml [sensitivity = 57% and specificity = 92.9%. Area under the curve (AUC) [SE] = 0.724 [0.0377], 95% confidence limits range = 0.652 – 0.788, *P* < 0.0001], *RARβ₂* gene as 6.348 copies [sensitivity = 88% and specificity = 86.1%. Area under the curve (AUC) [SE] = 0.93 [0.0197], 95% confidence limits range = 0.691 – 0.821, *P* < 0.0001], and *3-OST-2* gene as 0.99 copies [sensitivity = 97.8% and specificity = 70.6%. Area under the curve (AUC) [SE] = 0.794 [0.0334], 95% confidence limits range = 0.727 – 0.851, *P* < 0.0001].

Table 3: Overall sensitivity, specificity, PPV, NPV, and accuracy of aberrant methylated genes and traditional tumor markers

	CEA	CA15.3	*RARβ₂*	*3-OST-2*
Breast cancer detection				
Sens.	33.3	57	88	97.8
Spec.	98.8	92.9	86.1	70.6
PPV	96.9	89.8	88	78.4
NPV	57.5	66.4	87.1	96.8
Accuracy	64.6	74.8	87	84.8
Early Stage				
Sens.	33.8	60.3	85.3	98.5
Spec.	98.2	89.1	80	54.5
PPV	95.8	87.2	84.1	72.8
NPV	54.5	64.5	81.5	96.8
Accuracy	62.6	73.2	83	78.9
Low grade breast cancer				
Sens.				
Spec.	35.1	55.4	86.5	98.6
PPV	98.2	89.1	80	54.5
NPV	96.3	87.2	85.3	74.5
Accuracy	52.9	59.8	81.5	96.8
	62	69.8	84	79.8

Sens., sensitivity; spec., specificity; PPV, positive predictive value; NPV, negative predictive value.

Overall sensitivity, specificity, PPV, NPV, and accuracy of *RARβ2*, *3-OST-2*, CEA and CA15.3

Aberrant methylated *RARβ2* gene reported higher sensitivity and accuracy for early detection of breast cancer, early stage and low grade tumours, followed by *3-OST-2*, then CA15.3; while CEA showed the lowest sensitivity, as shown in Table "3".

DISCUSSION

Circulating tumour derived DNA in blood has enhanced researchers to advance DNA-based assays that can detect cancer using blood samples [16]. The present authors aimed to determine the efficacy of aberrant methylated DNA genes in blood samples for breast-cancer detection.

Other authors investigated aberrant methylation of 5′ gene promoter region which is a major mechanism for silencing the tumour suppressor genes[17]. Thus, in a cohort of 178 individuals the aberrant methylations of the two suppressor genes *RARβ2* and *3-OST-2* were studied in serum samples using RQ-MSP. The important benefit for utilizing RQ-MSP is based on presenting the values as a ratio of specified gene signal divided by the *β*-actin signal in the same sample; also, it can characterize a cut-off point between cancer and non-cancer groups by using the ROC curve. Methylated genes were significantly detected in breast-cancer patients as compared to the benign ones; while they were not detected in the control individuals; this confirms previous reports [12, 18, 19]. The high levels of the methylated genes in breast-cancer patients could be attributed to either aberrant *de novo* methylation due to mutation in DNA-methyl transferase, or to the loss of protection against *de novo* methylation through the loss of a trans-activating factor[20].

The relation between aberrantly-methylated tumour suppressor genes *RARβ2* and *3-OST-2* and other clinicopathological factors are shown in Table "2". DNA methylation of candidate genes was not clinical stage- or grade-dependent, pointing out that their usefulness is not limited to high grade or late stage tumours, and the presence of blood-circulating DNA of tumour cells could be attributed to cellular turn-over, necrosis and apoptosis of tumour cells, and also is due to the presence of tumour-specific hypermethylated genes in the plasma or serum which point to the discharge of adequate amounts of DNA from tumour cells into the blood circulation that is expected to be correlated with the degree of invasiveness[16]. Our results revealed the high frequency of methylated *RARβ2* in NIDC (95.1%) as compared to IDC (80.1%). These findings offer an optimistic issue for the methylation-based screening of breast cancer with different stages and grades. This is conditioned upon using a panel of specific genes for breast cancer, selecting proper MSP primers and optimizing PCR technology.

The idea for using the hypermethylated genes in serum samples as markers for early detection of breast cancer is due to the achievement of high both sensitivity and PPV values. Moreover, the investigated genes (*RARβ2* and *3-OST-2*) revealed higher sensitivity, and specify for detecting superficial and low-grade breast-cancer patient than traditional tumour markers (CEA and CA15.3) as shown in Table "3". Future studies are in great demand to verify the sensitivity and specificity of these and other epigenetic markers.

To conclude our study, the diagnostic efficacy of serum DNA methylation markers (*RARβ2* and *3-OST-2*) was reported and both markers achieved high sensitivity and a reasonable specificity in distinguishing between malignant and non-malignant (benign and healthy individuals). Moreover, using relative Q-MSP for these genes in serum reported a promising new diagnostic tool for the detection of breast cancer.

CONCLUSION

Methylations of *RARβ2* and *3-OST-2* genes are promising serum-based markers for breast-cancer detection.

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Abbreviations

CpG: cytosine preceding guanine; CGIs; cytosine preceding guanine islands; *RARβ2*: retinoic acid receptor gene; *3-OST-2*: heparan sulphate (glucosamine) 3-O-sulphotranspherase 2 gene; RQ-MSP: relative quantitation-methylation specific polymerase chain reaction. ROC curve: receiver operating characteristic curve; IDC: invasive duct-carcinoma; NIDC: non-invasive duct-carcinoma; PPV: positive predictive value; NPV: negative predictive value; HER-2/neu: human epidermal growth factor receptor-2/neuroblastoma.

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