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The Association of Methylation Status and Expression Level of *MyoD1* with *DNMT1* Expression Level in Breast Cancer Patients

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ABSTRACT

Background: Breast cancer (BC) is the most common malignancy in women worldwide. The methylation status of *MyoD1*, a tumor suppressor gene, is enrolled in various cancers, i.e., BC. Various studies showed the impact of *MyoD1* epigenetic dysregulation in BC. This study aimed to investigate the methylation status and expression level of *MyoD1* in BC patients and its association with the expression of *DNMT1*.

Materials and Methods: This case-control study was conducted on 30 cases (pathology-confirmed ductal carcinoma) and 18 controls (fibroadenoma and fibrocystic masses), referred to Velayat Hospital, Qazvin, Iran. The expression of the *MyoD1* and *DNMT1* and the promoter methylation of the *MyoD1* were evaluated in tissue blocks of BC patient masses using qRT-PCR and MS-PCR assays, respectively. SPSS 24.0 was used to analyze the data.

Results: The *MyoD1* promoter is hypermethylated in BC patients compared to controls ($p = 0.001$). The expression level of *MyoD1* in BC patients was significantly reduced compared to controls (fold change =0.13, $p = 0.042$). In addition, in BC patients, the reduced expression level of *MyoD1* was significantly associated with methylation of the *MyoD1* promoter ($p = 0.001$). There is no significant difference between the expression level of *DNMT1* in BC patients and controls ($p = 0.197$). A significant association is found between the expression of *DNMT1* and the methylation status of the *MyoD1* promoter ($p = 0.038$).

Discussion: The expression level of *MyoD1* is affected by the methylation status of the promoter of this gene. Moreover, the expression level and methylation status of *MyoD1* are correlated with clinical parameters.

Keywords: Methylation; Breast cancer; Epigenetics; Gene expression

INTRODUCTION

Breast cancer (BC) is the most common malignancy in women, while the odds of developing BC increases with age. A total of 20% to 25% of BCs are hereditary[1]. Approximately 1 in 8 U.S. women (about 13%) will develop invasive BC over the course of their lifetime; in 2023, an estimated 287,850 new cases of invasive BC are expected to be diagnosed in women in the U.S, along with 51,400 new cases of non-invasive (in situ) BC. Moreover, about 43,250 women in the U.S. are expected to die in 2023 from BC. BC represents nearly a quarter (23%) of all cancers [2]. The risk of development of BC is affected by age, genetic family history, reproductive, social, and economic factors, lifestyle, environmental encounters, etc. Furthermore, a late diagnosis, a poor lifestyle, and medical impediments may place women at the highest risk level [3, 4].

In addition to genetic changes, i.e., the mutation in oncogenes and tumor-suppressor genes, epigenetic changes, including DNA methylation, histone modifications, and noncoding RNAs, can also lead to the incidence, induction, and metastasis of BC[5, 6]. Dysregulated epigenetic control is a feature of cancer that is involved in tumorigenesis and the progress of the disease [7]. DNA methylation is one of the main epigenetic mechanisms enrolled in cancer[8]. The methylation of CpG islands in regulatory sites and gene promoters causes gene silencing. The hypermethylation of CpG islands in the tumor-suppressor promoter genes involved in the cell cycle is a critical occurrence in the progress of cancers[9]. DNA methylation is catalyzed by DNA methyltransferase (DNMT)[10, 11]. Cheray et al. have shown an increase in *DNMT1* expression in neoplastic cells, leading to the increased proliferation of cells, tumorigenesis, and tumor progress. Deviations in DNMT expression and disorders in the DNA methylation pattern are closely associated with various types of cancer [12]. Due to the reversibility of the methylation process, demethylating agents (Azacytidine, 5-aza deoxycytidine, 5-aza-2'-deoxycytidine, and Zebularine) are the greatest inhibitors of DNMT1 that delay tumor growth [13, 14].

Myogenin is a member of the muscle-specific helix-loop-helix family located on chromosome 11p [15], and the destruction of this gene leads to severe muscle defects and perinatal mortality. The *Myogenic differentiation 1* (*MyoD1*), a tumor suppressor gene, shows a different expression pattern during myogenesis. MyoD1 induces muscle-specific genes and acts as a cell cycle inhibitor[16]. The hypermethylation of *MyoD1* is more prevalent in older epithelial cells and tissues than in their younger counterparts, and the highest methylation level occurs in the middle CpG islands in exon 1 of this gene[17]. *MyoD1* methylation has been assessed in various cancers. Many studies have been conducted on the hypermethylation and silencing of various genes in BC. *MyoD1* expression and *BRCA1* expression are linked in sporadic breast tumors, and a high expression of *MyoD1* and *Cmyb* stimulates BRCA1 expression[18]. Increased methylation of *MyoD1* has been observed in several cancers, and the assessment of *MyoD1* methylation can be regarded as a factor for the diagnosis of cancers. The present study investigates the relationship between *DNMT1* expression and the expression and methylation of the *MyoD1* gene promoter in BC patients compared to a control group.

MTERIALS AND METHODS

Sampling

This case-control study was conducted on 30 pathology-confirmed ductal carcinoma paraffined blocks (confirmed by H&E staining) referred to Velayat Hospital, Qazvin, Iran. Also, 18 samples of benign breast masses (fibroadenoma and fibrocystic masses) were considered in the control group. The subjects in the patient and control groups had not received any supplementary medications due to their gene expression and methylation effects. The patients' data were collected from their medical records, i.e., age, tumor size, marital status, pain status, motility of lesion (the movement of the lesion that is felt by touch), menopausal status, involved breast (left/right), cancer history, change in breast skin, and estrogen/ progesterone-receptor status. This study was submitted and approved by the Ethical Committee of the Qazvin University of Medical Sciences (approval code IR.QUMS.REC.1395.233).

RNA extraction

For each individual, 4 pieces of 10-µm slices were prepared from the subjects' tissue blocks according to the RNeasy FFPE (formalin-fixed, paraffin-embedded tissue sections) Kit (QIAGEN Cat No. 73504, Germany) protocol, and the total RNAs were extracted according to manufacturer protocol. The quality of extracted RNA was evaluated by electrophoresis on 1% agarose gel and OD_{260}/OD_{280} measured by Nanodrop spectrophotometer (ThermoFisher, U.S.).

cDNA synthesis

cDNA synthesis was performed using a high-capacity cDNA reverse transcription kit (ABI Cat No. 10400745, U.S.). For this aim, 2 µl of 10× RT buffer, 3.2 µl of RNase-free H_2O, 1 µl of RNase inhibitor (1u/µl), 0.8 µl of 25× dNTP mix (200 mM), 2 µl of 10× RT Random Primer (100mM; ABI (Applied Biosystems), U.S.), 1 µl of multiscribe™ Reverse Transcriptase (50 u/µl), and 10 µl of extracted RNA (0.5 µg) were prepared and incubated as following steps: 10 min in 25 °C, 2 h in 37 °C, 5 min in 85 °C and 4 °C for storage. The quality of synthesized cDNA was evaluated by OD_{260}/OD_{280} measured by a Nanodrop spectrophotometer (ThermoFisher, U.S.).

Quantitative Real-time PCR

The quantitative real-time PCR (qRT-PCR) was carried out using the SYBR-Green PCR master mix (ABI Cat no: 4309155, U.S.). TAG Copenhagen Company synthesized the designed primers. The following primers were used: *MyoD1*; F: 5'-CCTCCCAACAGCGCTTTAAA-3', R: 5'-GCGAGAAAGCTGAACCTAGC-3', *DNMT1*; F: 5'-GTGGGGGACTGTGTCTCTGT-3', R: 5'-TGAAAGCTGCATGTCCTCAC-3', *GAPDH*; F: 5'-CAATGACCCCTTCATTGACC-3', R: 5'-TGGAAGATGGTGATGGGATT-3'.

A total of 12.5 µl of 2× SYBR-Green master mix, 8 µl of RNase-free water, 1 µl (5 pmol) of the forward primer F, 1 µL (5 pmol) of each primer, and 2.5 µl of the relevant cDNA were added to each cap-strip. *GAPDH* was used as the internal control gene for qRT-PCR. To ensure uncontamination, a negative control sample was used. The thermal cycles were performed by StepOnePlus™ Real-Time PCR system (Applied Biosystems Inc., ABI 7700 Foster, CA, U.S.), as follows (for both *MyoD1* and *DNMT1*, separately): 10 min at 95 °C for pre-denaturation and up to 40 cycles for 15 seconds at 95 °C for denaturation and 60 seconds at 60 °C for annealing/extension. The fold change of mRNA expression level was calculated by the $2^{-\Delta\Delta Ct}$ method.

DNA extraction

For each sample, 8 pieces of 10-µm slices were used for DNA extraction via QIAamp DNA FFPE Tissue Kit (QIAGEN Cat No. 56404, Germany), according to manufacturer protocol. Deparaffinization of blocks was carried out in the early stages of DNA extraction using Xylene and 100% ethanol. The quality of extracted DNA was evaluated by OD_{260}/OD_{280} measured by a Nanodrop spectrophotometer (ThermoFisher, U.S.).

DNA bisulfite treatment

According to manufacturer protocol, bisulfite treatment was carried out by EpiTect Fast FFPE Bisulfite Kit (QIAGEN Cat No. 59844). For this aim, 2 µg of the purified DNA was treated for each sample.

Methylation-specific PCR

Methylation-specific PCR (MS-PCR) was performed using the EpiTect MSP Kit (QIAGEN Cat No. 59305, Germany). The primers were synthesized by TAG Copenhagen Company. The following primers were used:

MyoD1 (Met-set); F: 5'-GACGGTTTTCGACGGTTT-3', R: 5'-GCCCGAAACCGAATACAC-3' (Product Size (bp) = 184), *MyoD1* (Unmeth-set); F: 5'-ATTTGATGGTTTTTGATGGTTT-3', R: 5'-CACACACATACTCATCCTCACA-3' (Product Size (bp) = 213) [19, 20].

The EpiTect PCR Control DNA Set (QIAGEN Cat No: 59695) was used to control and optimize the MS-PCR reactions. The reactions were performed at the final volume of 25 µl, using 12.5 µl of 2× MS-PCR master mix, 2 µl of bisulfite-treated DNA, 8.5 µl of nuclease-free water, and 1 µl of each primer (forward/reverse; 1 pmol of Meth primers and 3 pmol of Un-meth primers were used in the MS-PCR reactions). The thermal cycles were defined as follows: first, 10 minutes at 95 °C for predenaturation and activation

of Hot star Taq$^{d\text{-}Tect}$. Then, 40 cycles inducing 30 sec at 94 °C (denaturation), 45 sec at 54 °C (annealing), and 45 sec at 72 °C (extension), with a final extension stage at 72 °C for 10 min. The MS-PCR products were analyzed by 1.5%-agarose (Sigma-Aldrich, U.S.) gel electrophoresis.

Statistical analysis

Data were analyzed in SPSS ver. 24 at the significance level of 5%. Mann-Whitney's test was used to compare the expression level of genes in two groups. One-way ANOVA was used to analyze the relationship between the expression level and methylation statuses. Fisher's Exact test was used to assess the qualitative features of methylation and their relationship with the other qualitative data. The relationship between the quantitative data was assessed using Spearman's Correlation Coefficient.

RESULTS

***MyoD1* methylation and expression and *DNMT1* expression statuses**

The Fisher's Exact Test analysis showed that the *MyoD1* promoter is hypermethylated in BC patients compared to controls (p =0.001). In this regard, 17 patients of cases (56.6%) and 13 patients of cases (43.3%) cases showed unmethylated and methylated statuses, respectively, while all control individuals (18 participants) showed unmethylated status in the promoter of *MyoD1*. The results of MS-PCR are demonstrated in Figure 1. The Mann-Whitney's test analysis showed that the expression level of *MyoD1* in the BC patients (ΔCt (mean ±SD) =4.04 ± 5.48) was significantly reduced compared to the expression level of *MyoD1* in controls (ΔCt (mean ± SD) =6.99 ± 12.27) (p =0.042, fold change =0.13). In addition, one-way ANOVA statistical analysis showed that in BC patients, the reduced expression level of *MyoD1* was significantly associated with methylation of the *MyoD1* promoter (p =0.001). Moreover, the Mann-Whitney's test analysis showed that there is no significant difference between the expression level of *DNMT1* in BC patients (ΔCt (mean ±SD) =4.66 ± 6.97) and controls (ΔCt (mean ±SD) =2.12 ± 2.36) (p =0.197). furthermore, one-way ANOVA statistical analysis showed that there was a significant association between the expression of *DNMT1* and the methylation status of the *MyoD1* promoter (p =0.038).

Correlation between *MyoD1* methylation and expression and *DNMT1* expression with clinical data

Our results show that there is no correlation between *MyoD1* methylation, *MyoD1* expression, and *DNMT1* expression with aging in the case group. The Mann-Whitney's test analysis showed that the increase in Hb level is associated with a higher *MyoD1* expression level (p =0.011) and lower *DNMT1* expression (p =0.016) in the control group. Increasing *MyoD1* expression leads significantly to more motility of lesions in the case and control groups (p =0.012).

Moreover, Fisher's Exact test analysis showed that more methylation in the *MyoD1* promoter is significantly correlated to more estrogen-receptor positivity in malignant patients (p =0.024) (Tables 1 and 2).

Other clinical correlations in data

The results of the Mann-Whitney test analysis between the data of participants in both groups showed that mass size is increased within aging in the case group (p =0.005), while hemoglobin level is reduced within aging (p =0.003). The mean age was higher in the cases than in the controls, and the age difference between the two groups was significant (p <0.001). Fisher's Exact Test analysis showed that there were more premenopausal individuals in the case group (60% of all studied individuals), and a larger number of the cases were post-menopausal than in the control group (p =0.049). Moreover, the tumor size was bigger in the cases compared to the controls (p =0.006), and the tumors had more motility in the benign breast lesions compared to the malignant cases of cancer (p <0.001) (Table 3).

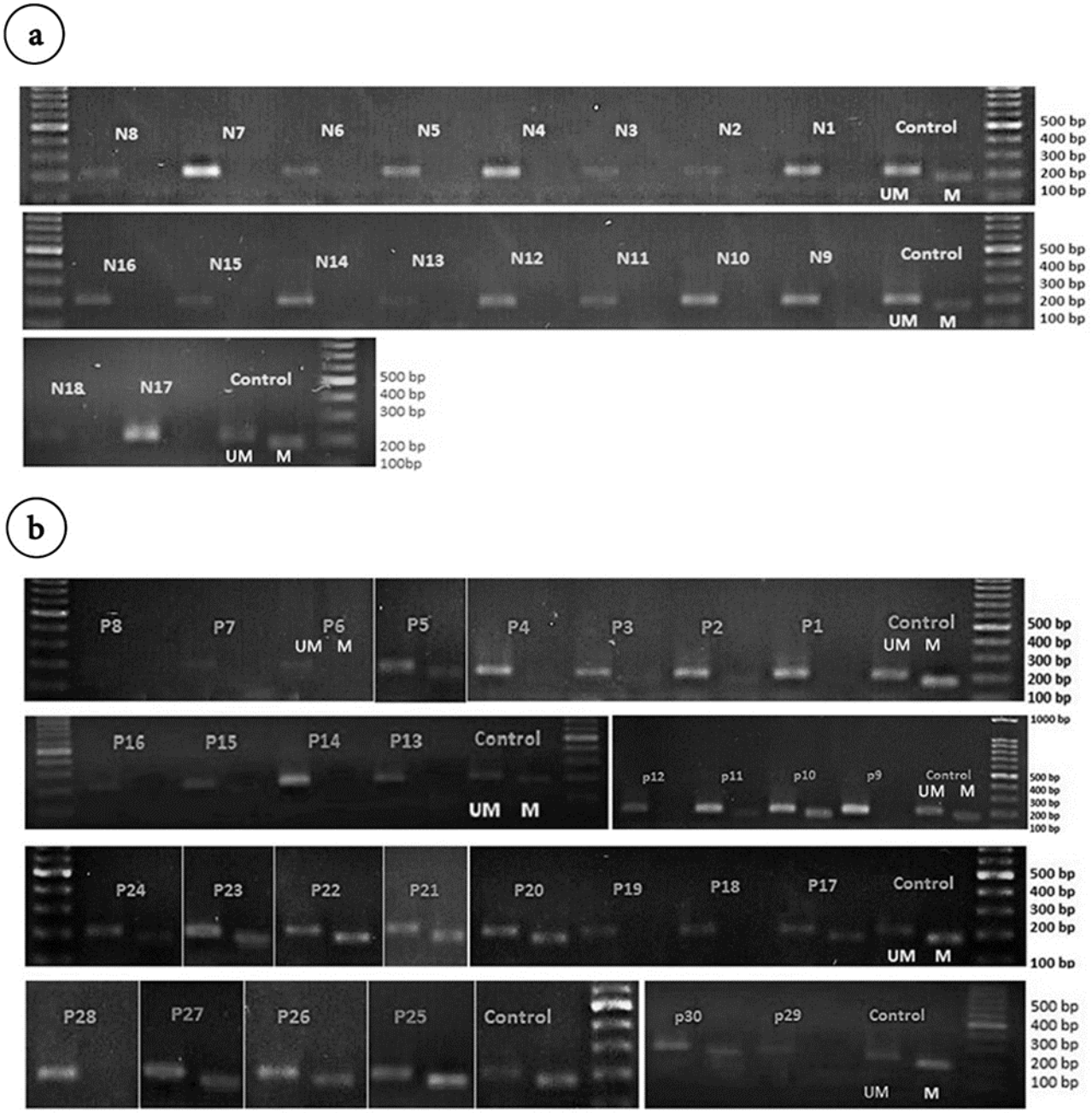

Figure 1. Methylation status of *MyoD1* promoters in control benign tumors (A) and BC samples (B) determined by MS-PCR assay
UM (unmethylated), M (methylated)

Table 1: Quantitative demographic data and their correlation with methylation status of *MyoD1*, expression level of *MyoD1*, and expression level of *DNMT1*

Quantitative data	Group	Mean ±SD	P-value†	P-value ‡		
				***MyoD1* Methylation**	***MyoD1* expression**	***DNMT1* expression**
Age (year)	Cases	52 ±15	**<0.001**	0.934	0.127	0.447
	Controls	31 ±10		N.M.*	0.134	0.647
				0.032	0.169	0.744
Hemoglobin	Cases	12.1 ±1.4	0.117	0.263	0.398	0.681
	Controls	12.8 ±1.0		NM.	**0.011**	**0.016**
				0.745	0.699	0.404
Tumor size (mm)	Cases	9.15 ±2.75	**0.006**	1.000	0.515	0.964
	Controls	2.95 ±2.75		NM.	0.357	0.584
				0.226	0.325	0.641

*NM: not measurable due to the unmethylated status of *MyoD1* promoter in all control samples
† P determining significance difference of quantitative demographic data (e.g., age, hemoglobin, and tumor size) in case and control groups
‡ P determining association of methylation status of *MyoD1*, expression level of *MyoD1*, and expression level of *DNMT1*, with quantitative demographic data in each case and control groups

Table 2: Correlation between the evaluated methylation and expressions with descriptive demographic data

Demographic data	Group	Data status	*MyoD1* Methylation status		P		
			Met	Un Met	*MyoD1* methylation	*MyoD1* expression	*DNMT1* expression
Marriage	Cases	Yes	12	17	0.433	0.733	0.400
		No	1	0			
	Controls	Yes	0	14	N.M.*	0.101	0.574
		No	0	4			
		Total			1.000	0.305	0.922
Pain	Cases	Yes	5	8	0.721	0.198	0.094
		No	8	9			
	Controls	Yes	0	5	N.M.	0.924	0.849
		No	0	13			
		Total			1.000	0.383	0.170
Motility of lesion	Cases	Yes	2	0	0.179	0.115	0.460
		No	11	17			
	Controls	Yes	0	16	N.M.	0.327	0.641
		No	0	2			
		Total			0.092	0.012	0.949
Post-menopausal	Cases	Yes	5	7	0.590	0.200	0.884
		No	8	10			
	Controls	Yes	0	2	N.M.	0.837	0.732
		No	0	16			
		Total			0.480	0.212	0.847
Involved breast	Cases	Left	9	9	0.465	0.602	0.723
		Right	4	8			
	Controls	Left	0	11	N.M.	0.479	0.151
		Right	0	7			
		Total			0.522	0.454	0.628
Cancer history	Cases	Yes	1	3	0.613	0.391	0.930
		No	12	14			
	Controls	Yes	0	0	N.M.	N.M.	N.M.
		No	0	18			
		Total			1.000	0.706	0.986
Change the skin of breast	Cases	Yes	1	4	0.355	0.787	0.627
		No	12	12			
	Controls	Yes	0	0	N.M.	N.M.	N.M.
		No	0	18			
		Total			1.000	0.974	0.719
Estrogen-receptor	Cases	Positive	12	9	0.024	0.929	0.304
		Negative	1	8			
	Controls		N.A.**		N.M.	N.M.	N.M.
		Total			NM.	NM.	NM.
Progesterone-receptor	Cases	Positive	9	7	0.123	0.822	0.984
		Negative	4	10			
	Controls		N.A.		N.M.	N.M.	N.M.
		Total			NM.	NM.	NM.

*NM: not measurable, **NA: not accessible

Table 3: Qualitative demographic data of case and control groups

Quality data		Group				P
		Control		Case		
		Number	Percent	Number	Percent	
Marriage	Yes	14	78%	29	97%	0.059
	No	4	22%	1	3%	
Pain	Yes	13	72%	13	43%	0.363
	No	5	28%	17	57%	
Motility of lesion	Yes	16	89%	2	3%	< 0.001
	No	2	11%	28	97%	
Post-menopausal	Yes	2	11%	12	40%	0.049
	No	16	89%	18	60%	
Involved breast	Left	11	61%	18	60%	1.000
	Right	7	39%	12	40%	
Cancer history	Yes	0	0%	4	13%	0.282
	No	18	100%	26	87%	
Change the skin of breast	Yes	0	0%	5	17%	0.142
	No	18	100%	25	83%	
MyoD1 Methylation	Met	0	0%	13	43%	0.001
	Un Met	18	100%	17	57%	

DISCUSSION

Epigenetic changes occur more frequently compared to genetic changes and only in specific parts of the genes, which are reversible with medication[21]. Shreds of evidence suggest that aberrant DNA methylation can be evaluated in early BC malignancies, even on small amounts of DNA[22]. Studies have examined the methylation pattern of many genes in BC that have a vital role in DNA restoration, apoptosis, and cell cycle. The regulation of the methylation of tumor-suppressor genes using methyltransferase inhibitors can be considered an excellent medical objective [21]. MyoD1 regulates cell differentiation and growth, which has been demonstrated to be involved in cell apoptosis through the regulation of caspase-3 [23]. According to recent studies, *MyoD1* expression is reduced in many cancers, and the assessment of the methylation of this gene can be regarded as a prognostic factor in cancers. The present study examined the expression level and methylation status of *MyoD1* and its association with *DNMT1* expression level (as the main methylation enzyme).

Our results showed that the expression level of *DNMT1* in BC patients is partially reduced in BC patients compared to controls, but this difference is not significant. A study conducted by Cha et al. showed that the expression of *DNMT1* increases in type-A luminal invasive lobular cancer, and DNMT inhibitors could suppress the growth of tumors and are therefore medically valuable [24]. Li et al. found that the signaling pathway of AKT-NF*k*B and STAT3 can increase the expression of *DNMT1*, and the out-of-control activity of this pathway leads to a bit methylation in the DNA of tumor-suppressor genes and causes gastric cancer[25]. In assessing the expression of *DNMT1* and $DNMT_{3b}$, the increased expression of *DNMT1* was found to be linked to carcinogenesis and the survival of cancer cells and to lead to an impairment in the methylation pattern and the histochemical analysis results obtained by a group of researchers showed that the increase in the expressions of *DNMT1* and $DNMT_{3b}$ is linked to the hypermethylation of tumor-suppressor genes in squamous carcinoma[26]. Although our present findings align with the results of many other studies, the results obtained by several studies disagree with most articles published to date. For instance, a study on the expression of *DNMT1* in peripheral nucleated blood cells in ductal carcinoma of BC showed that the expression of this enzyme increases with the progress of cancer [27].

Our present findings showed a lower expression level of *MyoD1* in the patients than in the controls and the hypermethylation status of *MyoD1* in the case group compared to the controls. In another study, researchers examined methyl-CpG binding protein 2 (*mecp2*) oncogene is abundantly upregulated in various cancers and showed that there are higher levels of *mecp2* expression in BC tissues than in normal tissues, suggesting a developmental role in BC[28]. Moreover, mecp2 binding to the region methylated on CpG islands of the *MyoD1* promoter leads to an inhibited expression in gastric cancer at the transcription level, and mecp2 suppresses the MyoD1/Caspase-3 signaling pathway and thus leads to the inhibition of apoptosis[29]. Chatterjee et al. argued that PI3K/AKT and p38α MAPK signaling pathways have a key role in establishing a relationship between *MyoD1* and transcription activators[30]. Furthermore, it is shown that the STAT3 signaling pathway activated by IL-6 induces myogenic progress by regulating *MyoD1*[31]. According to the study of Pan et al., the wnt signaling pathway in renal cells can cause the activation of pax3/pax7 and myogenic regulatory factors (MRFs), especially *MyoD1* and *Myf5*. Wnt3 activates the expression of *MyoD1* in the myoblast, and the increased expression of active beta-catenin can induce *MyoD1* expression [32].

A study by Widschwendter et al. showed that *MyoD1* is 60% methylated in the blood cells of patients with BC [9]. Studies have shown that *MyoD1* expression and *BRCA1* expression are linked in sporadic breast tumors, meaning that the high expression of *MyoD1* and *Cmyb* stimulates *BRCA1* expression[18]. The assessment of total methylation by the MethyLight method showed that *MyoD1* methylation in the serum of patients with BC increases with the progress of the malignancy[33]. These studies show that *MyoD1* has been methylated in the BC patient's serum and cell lines BC, and its expression is reduced in the patients compared to the controls; further studies have been recommended on the subject and

in tissue blocks of BC patients. Several scientists investigated the inhibitory effect of *MyoD1* on the growth of cell lines in BC, and this was the first report that showed that *MyoD1* might be considered a suppressor gene in various cancers. They believed that the expression of *MyoD1* is low in normal samples and observed that the expression of *MyoD1* increases when a cell is damaged or becomes cancerous. This difference in the expression of the *MyoD1* gene between the two study groups contradicts the findings of the present study and other studies.

According to previous studies, methylation is related to aging. A study found that the methylation of the *MyoD1* gene promoter in colorectal cancer increases with age and is affected by age [34], while in our study, 69% of *MyoD1* hypermethylation occurs in younger patients (below 55-year-old) in the case group. In line with the results reported by Widschwendter *et al.* on the serum of BC patients[9], the present study found no significant relationships between a family history of BC and the methylation of the *MyoD1* gene promoter in tissue blocks of BC patients. No significant relationships were observed in the present study between the methylation of the *MyoD1* gene promoter and the menopause status, while 61% of the methylation of the *MyoD1* gene promoter appears to occur in premenopausal patients. Müller et al. did not find a significant relationship between the methylation of *APC* promoter and the menopause status of the patients in serum samples of patients with BC[33]. Shan et al. investigated the relationship between the methylation of *P16*, *APC,* and *RASSF1A* and tumor size in the serum of BC patients. They found that the sensitivity of diagnostic mammography and ultrasound tests is associated with tumor size, and these methods fail to detect small tumors, while methylated genes show greater sensitivity for detecting BCs with small tumors, which leads to the diagnosis of early-stage BC[35]. The present study assessed the relationship between the methylation of the *MyoD1* promoter and tumor size. The contradictory results may be due to several factors, including small sampling or problems in the study design.

The importance of an efficient screening program can be priorities for future research, and DNA methylation occurs earlier and is constant during tumorigenesis, the lack of diagnostic markers that can be used for the early diagnosis of cancers is a major problem in the management of patients, and there are no detectable signs in the mammography and ultrasound imaging of patients with primary BC. Alongside directed studies that are recommended to be conducted in the future, the methylation of the *MyoD1* gene promoter can be used as a BC prognostic factor and ultimately for the early diagnosis and treatment of BC.

CONCLUSION

Based on our results, the *MyoD1* promoter is methylated in BC cancer patients compared to controls. Also, the expression level of *MyoD1* was reduced and significantly associated to the methylation status of *MyoD1*. The methylation status and mRNA level of *MyoD1* can be a potential diagnostic factor in BC compared to other breast masses, which can make differential diagnosis more accurate. To investigate the sensitivity and specificity, and avoiding heterogeneity, it is suggested to study on more sample size.

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CONFLICTS OF INTEREST

There are no conflicts of interest/competing interests in this manuscript.

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