Methylation of the *BMP6*, *BRCA1*, *ESR2*, *GSTP1* and *TIMP3* genes in Mexican women with breast cancer and benign disease

SAMANTHA REBECA DE LA TORRE-GUZMÁN[1,2], ANDREA MARLENE GARCÍA MURO[1], BRENDA PELAYO-CHÁVEZ[1], CAROLINA VÁZQUEZ-GASTÉLUM[1,2], MARTHA PATRICIA GALLEGOS-ARREOLA[1], ERNESTO SOTO-REYES[3], SERGIO OSVALDO MEZA-CHAVOLLA[4] and JOSEFINA YOALY SÁNCHEZ-LÓPEZ[1]

[1]Genetics Division, Western Biomedical Research Center, Mexican Social Security Institute, Guadalajara, Jalisco 44340, Mexico; [2]University Center for Health Sciences, University of Guadalajara, Guadalajara, Jalisco 44340, Mexico; [3]Department of Natural Sciences, Autonomous Metropolitan University-Cuajimalpa (UAM-C), Mexico City 05348, Mexico; [4]Breast Detection and Diagnosis Unit, Mexican Social Security Institute, Guadalajara, Jalisco 44300, Mexico

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Abstract. Breast cancer (BC) is the main cause of cancer-related mortality in women and is influenced by genetic, environmental and lifestyle factors. The inactivation of tumor suppressor genes, such as bone morphogenetic protein 6 (*BMP6*), breast cancer susceptibility gene 1 (*BRCA1*), estrogen receptor 2 (*ESR2*), glutathione S-transferase π1 (*GSTP1*) and tissue inhibitor of metalloproteinase 3 (*TIMP3*), via methylation may contribute to the development of BC. The present study analyzed the methylation status of these genes in 60 BC and benign disease (BD) tissue samples from Mexican women and the association with clinical and histopathological variables. DNA was modified with sodium bisulfite, followed by methylation-specific PCR and electrophoresis, and analyzed using χ^2 test, Fisher's exact test, logistic regression, odds ratio (OR) with 95% confidence interval and Kaplan-Meier curves. The results indicated that the average age was higher in the BC group compared with that in the BD group (55 vs. 42 years; P<0.00001), with ≥50 years being a significant risk factor (OR, 5.44; P=0.00002). Only *BRCA1* demonstrated a significant difference in methylation status, increasing the susceptibility to BC (OR, 2.50; P=0.0001), and tending to be associated with increased proliferation and lymph node metastasis. *TIMP3* methylation was significantly associated with distant metastasis (OR, 8.65; P=0.028). Methylation of *BRCA1* and *TIMP3* demonstrated a significant association with poor prognosis in BC. *BRCA1* was methylated more frequently in women with BC and *TIMP3* was associated with a higher risk of distant metastasis. The present study highlighted the potential of these genes as prognostic biomarkers and the need to further explore DNA methylation for diagnostic and therapeutic purposes in future research.

Correspondence to: Dr Josefina Yoaly Sánchez-López, Genetics Division, Western Biomedical Research Center, Mexican Social Security Institute, 800 Sierra Mojada, Colonia Independencia, Guadalajara, Jalisco 44340, Mexico
E-mail: yosalo1795@yahoo.com

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Introduction

Breast cancer (BC) can originate in the lobules, ducts or connective tissue; these cancer cells can spread through blood or lymphatic vessels and if these cancer cells spread to other tissues, metastasis may occur. A total of 2.3 million cases of BC were diagnosed in 2022 in women worldwide, and 670,000 mortalities from the disease were recorded (1,2). BC occurs due to the interaction of factors such as lifestyle (obesity, physical inactivity), environmental (alcoholism, smoking), biological (age >50 years, female) and genetic mutations (in genes such as *BRCA1* and *BRCA2*) (1,2). Among genetic factors, DNA alterations, such as chromosomal rearrangements, variants and epigenetic changes (including promoter methylation), can prevent transcription by preventing the binding of transcription factors or allowing the binding of proteins to methyl groups (3).

DNA methylation status has served as an indicator of BC; Vietri *et al* (4) reported that hypermethylation in the promoter region of a tumor suppressor gene causes inactivation, which can be used to assist early identification of BC. Radpour *et al* (5) reported higher methylation in tumor suppressor genes such as breast cancer susceptibility gene 1 (*BRCA1*), bone morphogenetic protein 6 (*BMP6*), glutathione S-transferase π1 (*GSTP1*), estrogen receptor 2 (*ESR2*) and tissue inhibitor of metalloproteinase 3 (*TIMP3*) in breast tumors and each gene has been associated with a certain clinical or histopathological characteristic. For example, *BMP6* methylation has been associated with the presence of lymph node metastasis and has been proposed as a biomarker of metastasis (6). *BRCA1* methylation causes its inactivation, which is common in women with triple-negative BC (P=0.043) (7), and this subtype is associated with increased metastatic risk and a poor prognosis (7). The methylation status of *ESR2* allows for the early detection of BC as it is present in cancerous tissue but not normal tissue (8,9).

GSTP1 methylation is associated with lymph node metastasis and other aggressive clinical features (P=0.02) (10,11). Lastly, *TIMP3* methylation has been associated with a higher tumor grade (II-III) (2) and hormone positivity, reinforcing its role in BC progression (12).

The aim of the present study was to determine whether the methylation status of the *BMP6*, *BRCA1*, *ESR2*, *GSTP1* and *TIMP3* genes is different in patients with BC and women without cancer. The present study also aimed to analyze whether a higher methylation level could be associated with clinical and histopathological variables that are associated with the poor prognosis of patients.

Subjects and methods

Subjects. In total, 60 patients with BC and 60 individuals with benign disease (BD) were included in the present study, samples were collected between August 2017 and August 2023. All individuals signed informed consent forms, and blood and breast tissue samples were obtained at the Mexican Social Security Institute-Breast Clinic in Guadalajara (Mexico). The present study was approved by the Bioethics Committee of Western Biomedical Research Center, Mexican Social Security Institute (approval no. R-2023-1305-024; Guadalajara, Mexico). The inclusion criteria for the BC group were as follows: i) Women ≥18 years of age; ii) patients with a histopathologically confirmed diagnosis, at any clinical stage according to the TNM classification (2) and with comorbidities; iii) patients who provided informed consent; while the exclusion criteria in the BC group were participants whose BC was a secondary tumor. The inclusion criteria for the BD group were as follows: i) Women ≥18 years of age; ii) individuals with benign breast lesions with Breast Imaging-Reporting and Data System (BI-RADS) (13) category 4 diagnosis in whom malignancy was ruled out using histopathological examination; and iii) individuals who provided informed consent; in the BD group, the exclusion criterion was a previous history of cancer.

DNA methylation analysis. Tissue DNA was extracted using the DNeasy® 96 kit (Qiagen GmbH) and 500 ng of tissue DNA was used for sodium bisulfite conversion using the EpiTect® Bisulfite kit (Qiagen GmbH). The methylation status was analyzed using methylation-specific PCR (MS-PCR), the reaction conditions of which are presented in Table I. Primers for the *BMP6*, *BRCA1*, *GSTP*, *TIMP3* (promoter) and *ESR2* (exon 1) genes (Table II) were designed using Methyl Primer Express™ (version, 1.0; Applied Biosystems; Thermo Fisher Scientific, Inc.), the software designed the primers in exon 1 specifically for *ESR2*, as this is where the highest number of CG sites are located. Negative controls and commercial methylated/unmethylated DNA (Qiagen GmbH) were included in the present analysis. Each reaction (12.5 μl) contained 10X PCR buffer, 50 mM $MgSO_4$, 10 mM dNTPs, 10 pM primers, Taq polymerase (1 U) (Invitrogen; Thermo Fisher Scientific, Inc.) and converted DNA (50-100 ng/μl), using reagents sourced from Invitrogen (Thermo Fisher Scientific, Inc.). The products were separated via 2% agarose gel electrophoresis, stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Inc.) and visualized using a UV transilluminator. Sanger sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Inc.) was used as a confirmatory method in certain samples for the *BMP6*, *BRCA1*, *ESR2* and *TIMP3* genes, except for the *GSTP1* gene due to a lack of reagents and resources.

Table I. PCR conditions for the *BMP6*, *BRCA1*, *ESR2*, *GSTP1* and *TIMP3* genes.

Gene	Stage	Temperature, ˚C	Time	Cycles
BMP6	Denaturalization	94.0	5 min	35
		94.0	1 min	
	Alignment	58.5	30 sec	
	Extension	72.0	30 sec	
		72.0	3 min	
		4.0	5 min	
BRCA1	Denaturalization	94.0	5 min	30
		94.0	1 min	
	Alignment	57.0	1 min	
	Extension	72.0	45 sec	
		72.0	5 min	
		4.0	5 min	
ESR2	Denaturalization	94.0	5 min	30
		94.0	30 sec	
	Alignment	58.0	1 min	
	Extension	72.0	30 sec	
		72.0	5 min	
		4.0	5 min	
GSTP1	Denaturalization	94.0	10 min	28
		94.0	30 sec	
	Alignment	63.5	30 sec	
	Extension	72.0	30 sec	
		72.0	7 min	
		4.0	5 min	
TIMP3	Denaturalization	94.0	10 min	30
		94.0	30 sec	
	Alignment	56.5	30 sec	
	Extension	72.0	30 sec	
		72.0	7 min	
		4.0	5 min	

BMP6, bone morphogenetic protein 6; *ESR2*, estrogen receptor 2; *GSTP1*, glutathione S-transferase π1; *TIMP3*, tissue inhibitor of metalloproteinase 3.

Statistical analysis. The clinical and histopathological variables evaluated in the BC group included age, body mass index (BMI), clinical stage, histological grade, histology, molecular subtype, Ki-67 status, lymph node and distant metastasis, progression, recurrence and survival; in the BD group, age, BMI and BI-RADS were considered. The gene methylation profile was analyzed using descriptive statistics. The normality of the quantitative data was assessed using the Shapiro-Wilk's test and the distribution was assessed with the Student's t-test, which was unpaired. Methylation frequencies were compared

Table II. Primer sequences used for amplifying methylated and unmethylated fragments of studied genes.

Gene	Primer sequence (5'-3')[a]	Primer sequence size, bp	PCR product size, bp
BMP6			
Methylated	F: GGTTTAGAGTGAT**CGCG**TC	19	108
	R: **CGCG**CAAATCTCTAAAAAC	19	
Unmethylated	F: GAGGTTTAGAGTGAT**TGTG**TT	21	108
	R: CT**CACA**CAAATCTCTAAAAAC	21	
BRCA1			
Methylated	F: ATAGGTAG**CG**ATTTTGATTTTC	22	154
	R: AATCTACCCC**CG**AATAA**CG**	19	
Unmethylated	F: ATAATAGGTAG**TG**ATTTTGATTTTT	25	154
	R: CCCAATCTACCCC**CA**AATAA**CA**	22	
ESR2			
Methylated	F: AGTTGTAGGAGGTG**CG**TTC	19	144
	R: **CG**AAAAAA**CG**CTTACCTTACAA	22	
Unmethylated	F: TGAGTTGTAGGAGGTG**TG**TTT	21	144
	R: AA**CA**AAAAAA**CA**CTTACCTTACAA	24	
GSTP1			
Methylated	F: CGGTTAATATGGTGAAATTTC	21	155
	R: TACAATAA**CGCG**ATCT**CG**	18	
Unmethylated	F: GTTTGGTTAATATGGTGAAATTTT	24	155
	R: TACAATAA**CACA**ATCT**CA**A**CA**	21	
TIMP3			
Methylated	F: TTT**CG**TTT**CG**T**CG**GGTATTC	20	123
	R: CTCCAAAATTAC**CG**TA**CGCG**	20	
Unmethylated	F: TTATTT**TG**TTT**TG**TT**G**GGTATTT	23	123
	R: TCTCCAAAATTAC**CA**TA**CACA**CC	23	

[a]Dinucleotides marked in bold are the CpG sites identified by the designed primer. The forward and reverse primer that amplifies for the methylated state identifies CG dinucleotides, because upon conversion with sodium bisulfite the cytosines remain as cytosines. The first forward that amplifies for the unmethylated state identifies the TG dinucleotides, because the unmethylated cytosines in the conversion were converted to uracils but in PCR they are observed as thymines, and the unmethylated reverse primer identifies CA dinucleotides, that is, it is identifying TG dinucleotides, this is because at the time of conversion, the cytosines were converted to uracils and in the PCR they are observed as thymines but this primer is the complementary of the original sequence (5'-3'). F, forward; R, reverse; bp, base pairs; *BMP6*, bone morphogenetic protein 6; *ESR2*, estrogen receptor 2; *GSTP1*, glutathione S-transferase π1; *TIMP3*, tissue inhibitor of metalloproteinase 3.

using the χ^2 test. The methylation frequencies were analyzed according to BC molecular subtype using χ^2 test and Fisher's exact test. The association between clinical characteristics and methylation status was evaluated using odds ratio (OR) with 95% confidence intervals (CIs). Furthermore, binary logistic regression was applied to analyze the relationship between methylation and the clinical and histopathological characteristics. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS statistical software (version, 24.0; IBM Corp.) and Kaplan-Meier curves were plotted with RStudio (version, 4.3.2; Posit Software).

Results

Clinical and histopathological characteristics of the BC and BD groups. Table III presents the clinical and histopathological characteristics of both BC and BD groups. A statistically significant difference was observed in the age of the patients in the BC and BD groups, with an average of 55 and 42 years, respectively ($P<0.00001$). The OR for the ≥50 years subgroup of the BC group compared with BD group was 5.44 (95% CI, 2.49-11.88; $P=0.00002$). While no significant difference was observed for BMI, the average BMI in the BC and BD group was 28.41 and 27.69 kg/m^2, respectively ($P=0.378$), with both groups being overweight.

Methylation frequency of the BMP6, BRCA1, ESR2, GSTP1 and TIMP3 genes in the BC and BD groups. Table IV presents the methylation results observed for each of the genes analyzed and the differences between the BC and BD groups. The gene with the lowest methylation percentage in both groups was *BMP6*, while the gene with the highest methylation percentage in both groups was *GSTP1*; however, only *BRCA1* demonstrated a higher frequency of methylation in the BC group (32.1%) compared with the BD group (16.6%)

Table III. Clinical and histopathological characteristics of the BC and BD group.

Characteristics	BC (n=60)	BD (n=60)	OR (95% CI)	P-value
Age, years				
Mean ± SD	55±11.60	42±11.64	-	0.00001
Range	37-88	22-70	-	-
<50 years, n (%)	18/60 (30)	42/60 (70)	-	-
≥50 years, n (%)	42/60 (70)	18/60 (30)	5.44 (2.49-11.88)	0.00002
BMI, kg/m^2				
Mean ± SD	28.41±4.58	27.69±5.51	-	0.378
Range	19.30-37.66	18.31-40.79	-	-
Normal, n (%)	16/60 (26)	15/60 (25)	-	1.0
Overweight, n (%)	22/60 (37)	32/60 (53)	0.50 (0.24-1.05)	0.098
Obesity, n (%)	22/60 (37)	13/60 (22)	2.09 (0.93-4.69)	0.108
Clinical stage, n (%)			-	-
In situ	1/60 (2)	NA		
I	7/60 (12)	NA		
II	21/60 (35)	NA		
III	29/60 (48)	NA		
IV	2/60 (3)	NA		
Histological grade, n (%)			-	-
I	11/60 (18)	NA		
II	26/60 (44)	NA		
III	23/60 (38)	NA		
Histology, n (%)			-	-
Ductal	56/60 (93)	NA		
Lobular	4/60 (7)	NA		
Molecular subtypes, n (%)			-	-
Luminal A	26/60 (43)	NA		
Luminal B	15/60 (25)	NA		
HER2	10/60 (17)	NA		
TN	9/60 (15)	NA		
ER, n (%)			-	-
Positive	38/60 (63)	NA		
Negative	22/60 (37)	NA		
PR			-	-
Positive	31/60 (52)	NA		
Negative	29/60 (48)	NA		
HER2, n (%)			-	-
Positive	19/60 (32)	NA		
Negative	41/60 (68)	NA		
Ki-67, n (%)			-	-
<20%	25/60 (42)	NA		
≥20%	35/60 (58)	NA		
Proximal metastasis, n (%)			-	-
Positive (≥1)	40/60 (67)	NA		
Negative (0)	20/60 (33)	NA		
Distant metastasis, n (%)			-	-
Yes	4/60 (7)	NA		
No	56/60 (93)	NA		
Progression, n (%)			-	-
Yes	7/60 (12)	NA		
No	53/60 (88)	NA		

Table III. Continued.

Characteristics	BC (n=60)	BD (n=60)	OR (95% CI)	P-value
Recurrence, n (%)			-	-
Yes	10/60 (17)	NA		
No	50/60 (83)	NA		
Pathological response, n (%)			-	-
Complete	35/60 (58)	NA		
No response	25/60 (42)	NA		

BC, breast cancer; BD, benign disease; BMI, body mass index; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; Ki-67, proliferation protein; NA, not applicable; OR, odds ratio; PR, progesterone receptor; SD, standard deviation; TN, triple-negative; CI, confidence interval.

Table IV. Methylation observed for each of the genes analyzed in patients with BC and BD.

Gene	BC, n (%)	BD, n (%)	P-value[a]	OR	95% CI	P-value[b]
BMP6	8/56 (14.3)	2/55 (3.6)	0.093	4.36	0.81-44.16	0.093
BRCA1	17/53 (32.1)	10/60 (16.6)	0.040[c,d]	2.50[d]	1.02-6.42	<0.001[c,d]
ESR2	21/55 (38.2)	15/53 (28.3)	0.378	0.69	0.30-1.56	0.378
GSTP1	48/51 (94.1)	55/57 (96.5)	0.660	1.75	0.28-10.95	0.660
TIMP3	9/48 (18.7)	10/48 (20.0)	0.830	1.11	0.40-3.04	0.830

[a]P-values for comparison between BC and BD groups calculated using χ^2 test; [b]P-values obtained for odds ratio and 95% CI of methylation in each gene; [c]P<0.05; [d]Values that were statistically significant and associated with a risk susceptibility. χ^2 or Fisher's exact test was used as appropriate. BC, breast cancer; BD, benign disease; OR, odds ratio; CI, confidence interval. *BMP6*, bone morphogenetic protein 6; *ESR2*, estrogen receptor 2; *GSTP1*, glutathione S-transferase π1; *TIMP3*, tissue inhibitor of metalloproteinase 3.

Table V. Methylation frequency of the studied genes across breast cancer molecular subtypes.

Gene	LA, n (%)	LB, n (%)	HER2, n (%)	TN, n (%)	P-value[a]
BMP6	5/24 (21)	2/14 (14)	0/8 (0)	1/10 (10)	0.222
BRCA1	8/24 (33)	4/11 (36)	1/8 (13)	4/10 (40)	>0.999
ESR2	8/24 (33)	6/14 (43)	5/9 (56)	2/9 (22)	>0.999
GSTP1	21/24 (88)	11/12 (92)	7/8 (88)	8/8 (100)	>0.999
TIMP3	4/24 (17)	1/10 (10)	2/8 (25)	2/10 (20)	>0.999

[a]P-values calculated using Fisher's exact test. LA, luminal A; LB, luminal B; HER2, human epidermal growth factor receptor 2; TN, triple-negative; *BMP6*, bone morphogenetic protein 6; *ESR2*, estrogen receptor 2; *GSTP1*, glutathione S-transferase π1; *TIMP3*, tissue inhibitor of metalloproteinase 3.

(P=0.040), indicating that methylation of the *BRCA1* gene increased the susceptibility of developing BC in the examined cohort OR of 2.50 (95% CI, 1.02-6.42; P=0.0001). Regarding the other genes analyzed in the present study, although *BMP6* demonstrated higher methylation in the BC group (14.3%) compared with that in the BD group (3.6%), the difference was not statistically significant (P=0.093). Furthermore, methylation frequencies were analyzed according to the BC molecular subtype (including luminal A, luminal B, HER2-enriched and triple-negative BC), with no statistically significant differences observed among the molecular subtypes; however, the *BMP6* gene was mostly methylated in the luminal A subtype, *BRCA1* in the triple-negative subtype, *ESR2* in the HER2-enriched subtype, *GSTP1* in the triple-negative subtype and *TIMP3* in the HER2-enriched subtype (Table V). DNA sequencing yielded results consistent with those of MS-PCR for the *BMP6* and *TIMP3* genes (Fig. 1).

Relationship between BMP6, BRCA1, ESR2, GSTP1 and TIMP3 gene methylation and clinical and histopathological variables. Although the *TIMP3* gene demonstrated no

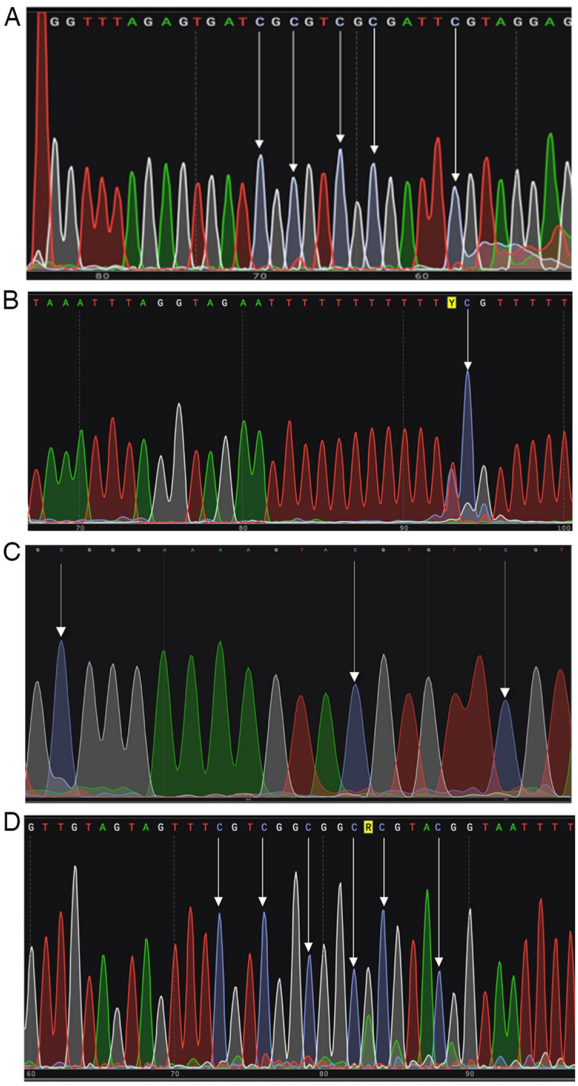

Figure 1. (A) Partial electropherogram of a DNA sample positive for methylation in the *BMP6* gene. The first three arrows indicate three CG sites (methylated) detected by the forward primer 5'-GGTTTAGAGTGAT**CGCGTC**-3' (bold) using MS-PCR (100% concordance with DNA sequencing at these 3 sites) and the remaining two arrows point to other (methylated) CG sites that were visualized via DNA sequencing. The sequence described in NCBI Reference Sequence: NC_000006.12 was used as a reference. (B) Partial electropherogram of a DNA sample positive for methylation in the *BRCA1* gene. The arrow indicates a methylated CG site not located within the primer sequence; in this case, as the primer sequence is not visible, it is not possible to determine the correspondence between the sites observed by MS-PCR and sequencing. (C) Partial electropherogram of a DNA sample positive for methylation in the *ESR2* gene. The arrows indicate three CG sites (methylated) not located within the primer sequence; in this case, as the primer sequence is not presented, it is not possible to determine the correspondence between the sites observed by MS-PCR and sequencing. The sequence described in NCBI Reference Sequence: NG_011535.1 was used as a reference. (D) Partial electropherogram of a DNA sample positive for methylation in the *TIMP3* gene. The arrows indicate six CG sites (methylated); the first three indicate sites not located in the primer sequence, whilst the fourth, fifth and sixth arrows indicate other sites located in the primer reverse 5'-GG**CGCGT**A**CG**GTAATTTTGGAG-3' (bold) detected by MS-PCR (100% concordance at these 3 sites). MS-PCR, methylation specific-PCR; *BMP6*, bone morphogenetic protein 6; *BRCA1*, breast cancer susceptibility gene 1; *TIMP3*, tissue inhibitor of metalloproteinase 3; *ESR2*, estrogen receptor 2; NCBI, National Center for Biotechnology Information.

significant differences in methylation frequency between the BC (18.7%) and BD (20%) groups, methylation of the *TIMP3* gene indicated a significant relationship with distant metastasis with an OR of 8.65 (95% CI, 1.29-57.9; P=0.028); while methylation of the other genes exhibited no association with clinical histopathological characteristics (Table VI). However, a trend was observed between *BRCA1* gene methylation and ≥20% Ki-67 protein levels (P=0.057) as well as lymph node metastasis (P=0.059) (Table VI).

Relationship between BMP6, BRCA1, ESR2, GSTP1 and TIMP3 gene methylation and BC prognosis. A progression-free survival (PFS) analysis was performed using Kaplan-Meier curves (Figs. 2-6) and the log-rank test, which evaluated the probability of survival rate over 12, 36 and 60 months in patients with BC according to the baseline methylation status of the target gene (methylated vs. unmethylated). Baseline promoter methylation status was determined using the baseline sample from each patient. Median PFS was estimated from time to progression and event status, that is, from the time of diagnosis confirmed using histopathology until 5 years of follow-up.

The Kaplan-Meier curve for *BMP6* promoter methylation demonstrated no significant difference (P=0.72) in survival and progression time. The median PFS was also calculated, demonstrating a time of 60 months in both the methylated and unmethylated groups; therefore, there was no statistically significant difference (P=0.72; Fig. 2).

BRCA1 promoter methylation resulted in no significant difference (P=0.16) in survival and progression time. The median PFS time was also calculated, which was 60 months in the methylated group and 48 months in the unmethylated group. This finding indicated that patients with *BRCA1* promoter methylation progressed 1 year later compared with those without methylation; however, the difference between the two groups was not statistically significant (P=0.16; Fig. 3).

ESR2 promoter methylation resulted in no significant difference (P=0.31) in survival and progression time. The median PFS time was also calculated, which was 60 months in the methylated group and 48 months in the unmethylated group. This finding demonstrated that patients with *ESR2* promoter methylation progressed 1 year later compared with those without methylation; however, the difference between the two groups was not statistically significant (P=0.31; Fig. 4).

GTSP1 promoter methylation resulted in no significant difference (P=0.18) in survival and progression time. The median PFS was also calculated, with a time of 60 months observed in both groups; therefore, there was no statistically significant difference (P=0.18; Fig. 5).

Lastly, *TIMP3* promoter methylation resulted in no significant difference (P=0.99) in survival and progression time. The median PFS time was also calculated, which was 48 months in the methylated group and 60 months in the unmethylated group. This finding indicated that patients with *TIMP3* promoter methylation progressed slightly earlier (48 months) compared with those without methylation (60 months). Although the methylated group demonstrated a lower PFS trend, the difference between the two groups was not statistically significant (P=0.99; Fig. 6).

Table VI. Association between clinicopathological characteristics and gene methylation status analyzed using binary logistic regression.

Clinicopathological characteristics	*BMP6*	*BRCA1*	*ESR2*	*GSTP1*	*TIMP3*
Age (≥50 years)	0.997	0.095	0.704	-	1.000
BMI	0.998	0.744	0.976	-	0.998
PR (positive)	1.000	0.920	0.650	-	0.998
ER (positive)	0.899	0.999	0.764	-	0.999
HER2 (positive)	0.974	0.999	0.562	-	0.999
Clinical stage	0.997	0.973	0.390	-	0.998
Ki-67 (≥20%)	0.996	0.057[a] (OR, 6.55; 95% CI, 1.01-41.8)	0.674	-	1.000
Lymph node metastasis	0.997	0.059[a] (OR, 6.47; 95% CI, 1.01-43.7)	0.626	-	0.998
Distant metastasis	0.144	0.275	1.000	-	0.028[b] (OR, 8.65; 95% CI, 1.29-57.9)

[a]P-values indicate variables displaying a trend toward susceptibility associated with the *BRCA1* gene. [b]P-values indicate a statistically significant association with increased risk susceptibility for the *TIMP3* gene. -, analysis was not performed due to identical results across all variables; BMI, bone mass index; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal receptor 2; *BRCA1*, breast cancer susceptibility gene 1; *TIMP3*, tissue inhibitor of metalloproteinase 3; *ESR2*, estrogen receptor 2; *GSTP1*, glutathione S-transferase π1.

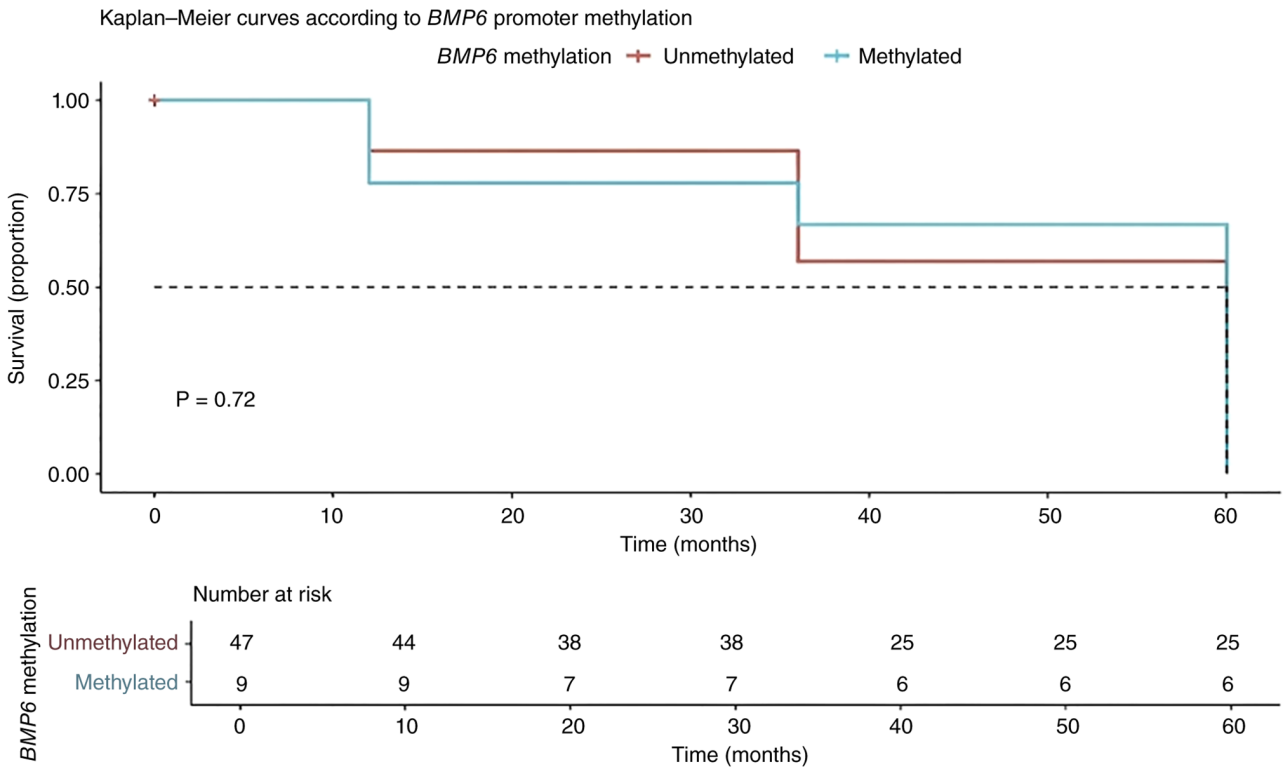

Figure 2. Kaplan-Meier curve according to *BMP6* promoter methylation. Median PFS was estimated from time to progression and event status, that is, from the time of diagnosis confirmed by histopathology up to 5 years of follow-up. The probability of survival over the course of 12, 36 and 60 months was evaluated using the log-rank test (Mantel-Cox). The blue line represents patients with methylated *BMP6* gene and the red line represents unmethylated *BMP6* gene. *BMP6*, bone morphogenetic protein 6; PFS, progression-free survival.

Discussion

The main objective of the present study was to analyze the methylation status of the *BMP6*, *BRCA1*, *ESR2*, *GSTP1* and *TIMP3* genes in Mexican women with BC or BD and the association with clinical and histopathological variables associated with prognosis. The clinical and histopathological variable results demonstrated that an age of ≥50 years conferred an

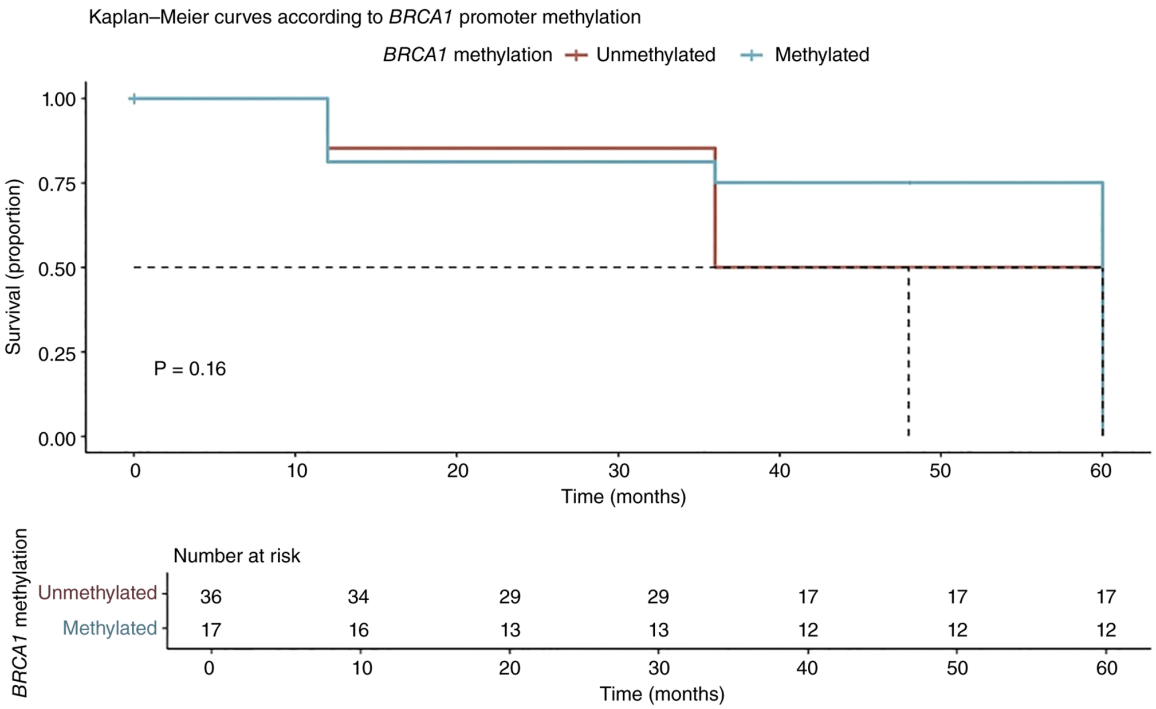

Figure 3. Kaplan-Meier curve according to *BRCA1* promoter methylation. Median PFS was estimated from time to progression and event status, that is, from the time of diagnosis confirmed by histopathology up to 5 years of follow-up. The probability of survival over the course of 12, 36 and 60 months was evaluated using the log-rank test (Mantel-Cox). The blue line represents patients with methylated *BRCA1* gene and the red line represents unmethylated *BRCA1* gene. PFS, progression-free survival; *BRCA1*, breast cancer susceptibility gene 1.

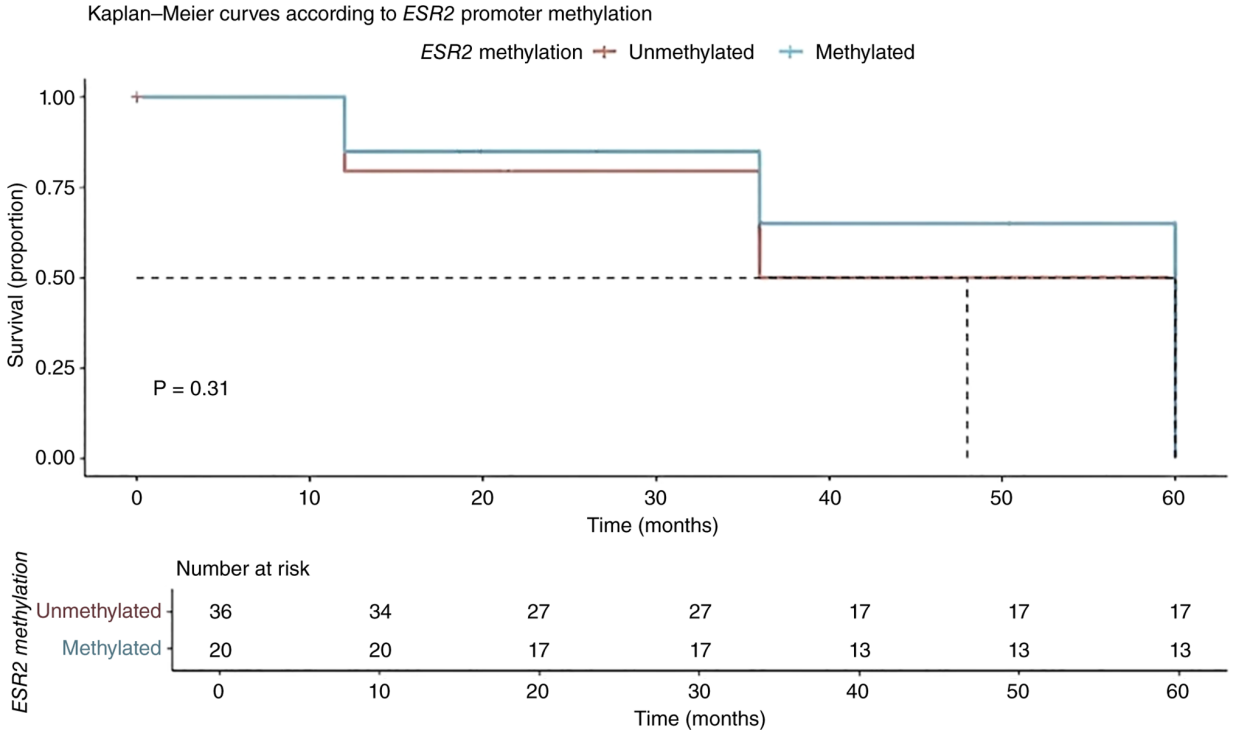

Figure 4. Kaplan-Meier curve according to *ESR2* promoter methylation. Median PFS was estimated from time to progression and event status, that is, from the time of diagnosis confirmed by histopathology up to 5 years of follow-up. The probability of survival over the course of 12, 36 and 60 months was evaluated using the log-rank test (Mantel-Cox). The blue line represents patients with methylated *ESR2* gene and the red line represents unmethylated *ESR2* gene. *ESR2*, estrogen receptor 2; PFS, progression-free survival.

increased susceptibility to BC (OR, 5.44; 95% CI, 2.49-11.88; P=0.00002). The average age was 55 vs. 42 years in the BC and BD groups, respectively (P<0.00001). These findings were consistent with those reported by Segovia-Alvarez *et al* (14) of an average age of 52.29 years for BC cases and 42.81 years for the control group. In Mexican patients with BC, age was

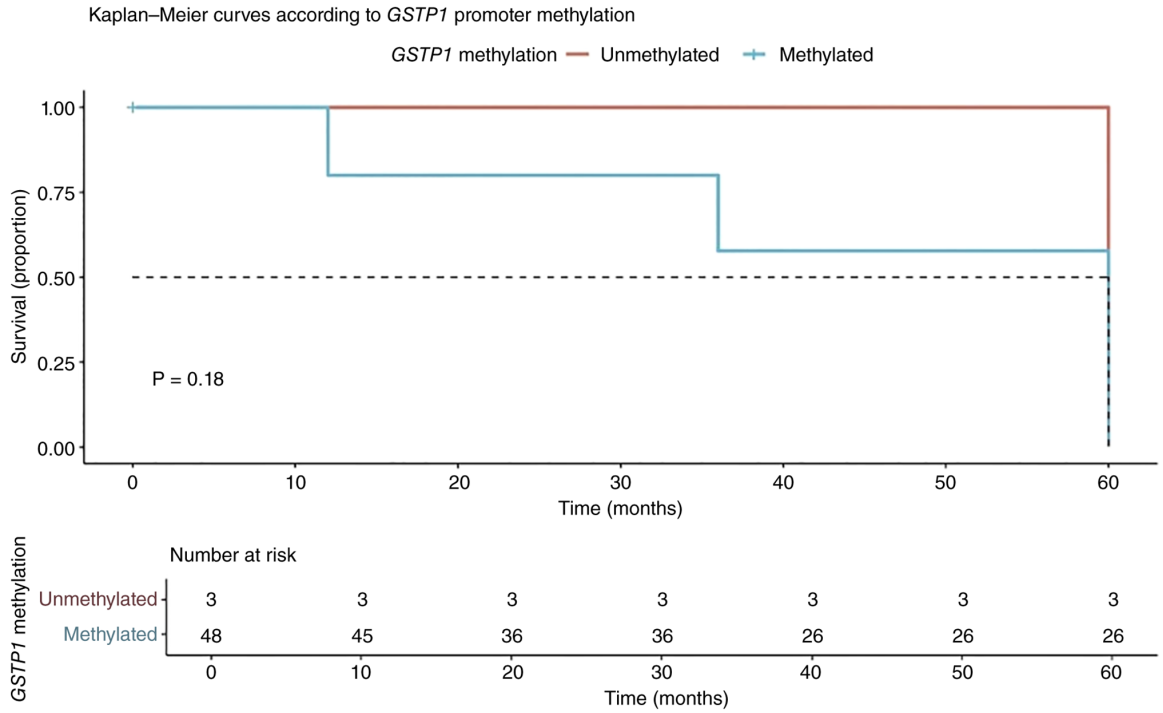

Figure 5. Kaplan-Meier curve according to *GSTP1* promoter methylation. Median PFS was estimated from time to progression and event status, that is, from the time of diagnosis confirmed by histopathology up to 5 years of follow-up. The probability of survival over the course of 12, 36 and 60 months was evaluated using the log-rank test (Mantel-Cox). The blue line represents patients with methylated *GSTP1* gene and the red line represents unmethylated *GSTP1* gene. *GSTP1*, glutathione S-transferase π1; PFS, progression-free survival.

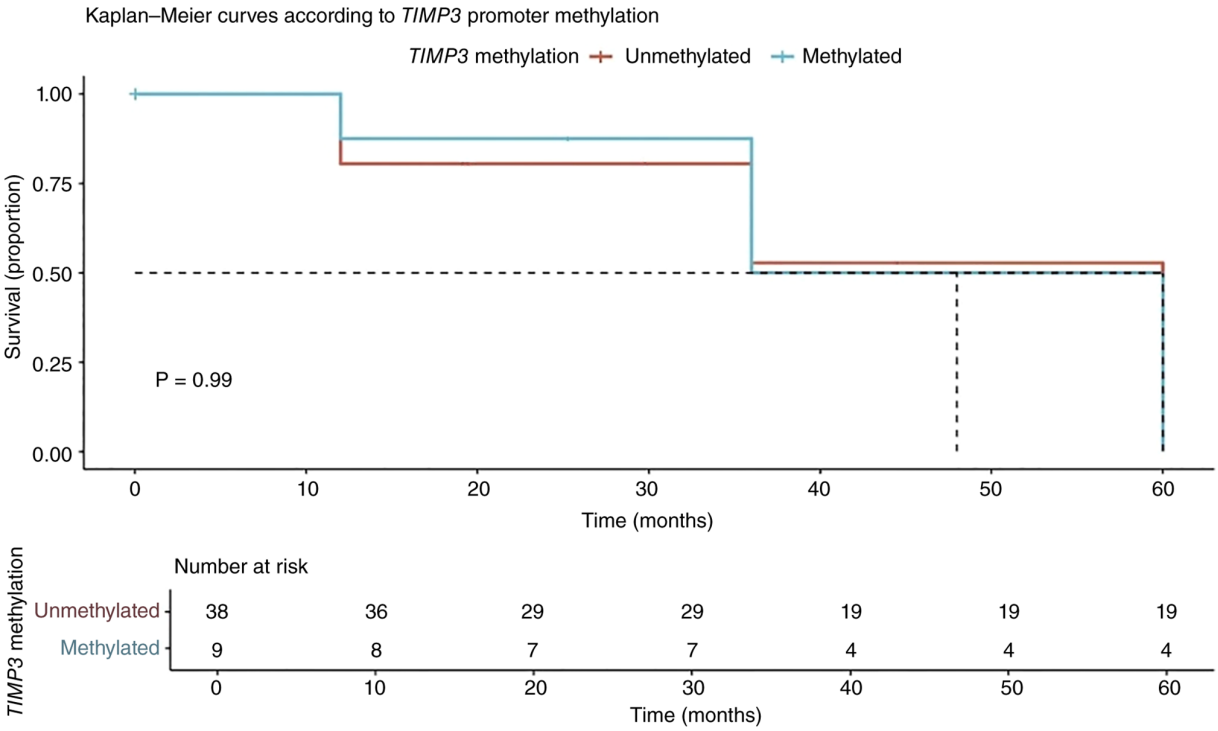

Figure 6. Kaplan-Meier curve according to *TIMP3* promoter methylation. Median PFS was estimated from time to progression and event status, that is, from the time of diagnosis confirmed by histopathology up to 5 years of follow-up. The probability of survival over the course of 12, 36 and 60 months was evaluated using the log-rank test (Mantel-Cox). The blue line represents patients with methylated *TIMP3* gene and the red line represents unmethylated *TIMP3* gene. *TIMP3*, tissue inhibitor of metalloproteinase 3; PFS, progression-free survival.

associated with an OR of 5.75 (14), which is similar to the value obtained in the present study (OR=5.44). This finding suggested that women >50 years of age may have a >5-fold increased risk of developing BC. These results were also consistent with data

from the U.S. National Cancer Institute (15), which reported that the average age of women diagnosed with BC is ~50 years. The increased risk of developing BC with advancing age can be explained by the accumulation of genetic mutations and the decline in DNA repair mechanisms over time (16).

In the present study, the BMI in the BC and BD groups were 28.41 and 27.69 kg/m^2, respectively, with no significant difference (P=0.378), indicating an overweight BMI classification for both groups. According to the Ministry of Health of Mexico (17), ~70% of adults and 33% of children are overweight or obese, conditions that represent notable risk factors for the development of chronic non-communicable diseases, including diabetes mellitus type 2, hypertension, dyslipidemia and cardiovascular or cerebrovascular diseases such as ischemic heart disease or strokes. Since the 1980s, the prevalence of obesity in Mexico has demonstrated a notable increase, currently affecting >30% of the adult population. It has been estimated that by 2050, obesity rates will reach 54% in men and 37% in women in Mexico, indicating that the number of obese individuals will exceed those who are overweight. This increase has mainly been attributed to a higher consumption of high calorie foods and the adoption of more sedentary lifestyles. Obesity is associated with various comorbidities, including cardiovascular disease, type 2 diabetes mellitus, osteoarthritis, certain types of cancer (such as breast, colorectal, pancreatic and liver), obstructive sleep apnea and other health-related conditions (18). Complications can arise during BC disease progression when the patient is overweight or obese. For example, several studies reported that obesity is a major risk factor in the development and progression of BC (19-21). Women with a BMI >25 kg/m^2 have a higher risk of recurrence and lower survival rates compared with those of normal weight (19). Furthermore, obesity can decrease the effectiveness of chemotherapy even when the dose is correctly adjusted for body weight and is associated with higher surgical complications and a lower probability of breast reconstruction (20). Obesity has also been reported to increase the risk of developing BC after menopause and is associated with higher mortality (21).

Adipose tissue serves an active role in the pathophysiology of BC as it functions as an endocrine organ capable of producing estrogens, adipokines (such as leptin and IL-6) and proinflammatory factors (22). Aromatase, an enzyme expressed by adipocytes, converts androgens into estrogens, which promotes cell proliferation and tumor progression, particularly in estrogen receptor (ER)$^+$ tumors (22). Furthermore, adipose tissue-derived stem cells may participate in the formation of microcalcifications, which are associated with less favorable prognoses, while insulin resistance and leptin secretion contribute to tumor growth and metastasis (23). Although no significant differences in BMI were observed between the groups analyzed in the present study, these findings provide insight into how the interaction between metabolism, inflammation and hormonal signaling may influence the development and progression of BC. Lastly, it is key to consider not only the specific BMI value, but also the duration of obesity, to more fully understand its role in the clinical and biological evolution of BC.

The present study evaluated the promoter methylation status of the *BMP6*, *BRCA1*, *ESR2*, *GSTP1* and *TIMP3* genes in Mexican women with BC or BD. The findings highlighted *BRCA1* and *TIMP3* as the genes with the most notable clinical relevance. While *BMP6*, *ESR2* and *GSTP1* did not demonstrate statistically significant associations, these genes did demonstrate notable trends that may be further explored in future research.

In the present study, the *BRCA1* gene (a caretaker tumor suppressor gene) exhibited a higher frequency of methylation in the BC group (32.1%) compared with the BD group (16.6%), a statistically significant difference (P=0.040). Several studies have indicated that methylation in the promoter of the *BRCA1* gene decreases its expression (24,25). Within the *BRCA1* gene CpG sites region analyzed in the present study (from -315 to +97) there is a CCAAT box (position-173) (24); thus, methylation occurring near these sites could interfere with gene expression by interfering with the binding of transcription factors [transcription factor II (TFII)D, TFIIB, TFIIF, TFIIE and TFIIH] to the CCAAT box (25). In the present study, this epigenetic event was associated with increased susceptibility to BC (OR, 2.50; 95% CI, 1.02-6.42; P=0.0001). Similar to previous studies that identified *BRCA1* methylation was associated with risk, proliferation (Ki-67), metastasis and aggressive phenotypes (7,26-32), the present study identified a potential association with Ki-67 protein (P=0.057) and lymph node metastasis (P=0.059). The frequency of *BRCA1* methylation is reported to be between 2.7 and 65.2% in BC, although this percentage may vary depending on the selection criteria of the group and the techniques used (26-32). Lobanova *et al* (27) reported that methylation of the *BRCA1* gene promoter is significantly associated with late-stage metastatic cancer (OR, 4.04; 95% CI, 1.19-13.65; P=0.038). Furthermore, high levels of proliferation, as indicated by elevated Ki-67 protein, are reported to associate with greater tumor aggressiveness and a worse prognosis for patients with *BRCA1* methylated compared with unmethylated (32). Khan *et al* (7) reported that lymphatic vascular invasion, ductal carcinoma *in situ* and lymph node metastasis (≥3) are observed at a higher rate in tumors with *BRCA1* methylation compared with healthy tissue. This finding is particularly relevant, as *BRCA1* inactivation by methylation could have therapeutic implications in the selection of patients who are candidates for poly-ADP ribose polymerase inhibitors (33).

In the present study, the *TIMP3* gene (classified as a gatekeeper gene) demonstrated no significant differences in methylation frequency between the BC (18.7%) and BD (20%) groups. However, an association was observed between *TIMP3* gene methylation and the development of distant metastasis (OR, 8.65; P=0.028), suggesting that epigenetic loss of *TIMP3* may promote metastasis through matrix dysregulation. This may be due to methylation inhibiting gene function and suppressing the regulation of angiogenesis and metastasis, which is generally associated with greater tumor aggressiveness compared with normal tissue (9). Although the frequency of *TIMP3* methylation was similar between the BC and BD groups, the relationship with distant metastasis suggested that this epigenetic event could be more relevant in advanced stages of the disease.

Regarding the other genes analyzed in the present study, although *BMP6* demonstrated higher methylation in the BC

group (14.3%) compared with that in the BD group (3.6%), the difference was not statistically significant (P=0.093), nor were there any significant differences regarding *BMP6* methylation and the clinical and histopathological characteristics. In another study conducted by our research group, *BMP6* promoter methylation was analyzed in women with BC (n=97) and BD (n=25) and the study demonstrated that the percentage of methylation was higher in the BC group (9.3%) compared with the BD group (4%) (34). However, these differences were not statistically significant either (P=0.365). The results of the present study and that of García (34) are similar since no statistically significant differences were reported in both studies examining this same population of Mexican women; however, they differ from European studies where notable methylation was reported in tumor tissue (5,6).

Various environmental, dietary and lifestyle factors can modify DNA methylation patterns and contribute to the development of BC. Factors such as diet, alcohol and tobacco consumption, exposure to pollutants, endocrine disruptors and medication use can alter the activity of DNA methyltransferases, the availability of methyl group donors and cellular redox balance (35,36). Furthermore, ethnicity influences methylation profiles, which may explain the differences in cancer incidence and mortality among different population groups (35,37-39). According to GLOBOCAN 2022 estimates, breast cancer was the most commonly diagnosed cancer among women worldwide, with approximately 2.3 million new cases and 666,000 mortalities. The distribution of the disease burden showed significant differences across continents. Asia accounted for the highest number of new cases, 985,817 and mortalities, 315,309, followed by Europe, with 557,532 new cases and 144,439 mortalities. North America recorded 306,307 new cases and 49,744 mortalities, while Latin America and the Caribbean reported 220,124 cases and 59,700 mortalities. Africa, meanwhile, reported 198,553 new cases and 91,252 mortalities, standing out for having the highest mortality-to-incidence ratio among the regions evaluated, suggesting significant challenges in accessing timely diagnosis and treatment. Finally, Oceania recorded 28,507 cases and 5,483 mortalities (35,37-40).

Diet serves a key role as nutrients such as folate, vitamin B12, methionine and omega-3 fatty acids promote methylation, while polyphenols and vitamin D can inhibit methylation (36). Additionally, comorbidities such as diabetes mellitus type 2 alter DNA methylation by generating insulin resistance, chronic inflammation and metabolic dysregulation, favoring the activation of oncogenes and the inactivation of tumor suppressor genes (36).

The differences between the results of the present study and those of García (34) compared with those previously reported by Radpour *et al* (5) and Barekati *et al* (6) may be explained by methodological factors. For example, Radpour *et al* (5) used matrix-assisted laser desorption/ionization-time of flight mass spectrometry, Barekati *et al* (6) used the T-cleavage assay and mass spectrometry and García (34) used Sanger sequencing. Furthermore, in the present study, BC tissue samples were compared with those from women with BD, whereas Radpour and Barekati *et al* (6) analyzed adjacent tissue. The present study considered that adjacent tissue is not an adequate control since, according to the tumor microenvironment theory, there may be cancer cells involved in the development, invasion and metastasis in this type of tissue (41).

In the present study, the *ESR2* gene exhibited frequent methylation (38.2% in the BC group vs. 28.3% in the BD group) without reaching statistical significance (P=0.378) or statistical significance with clinical and histopathological characteristics. Silencing of *ESR2* by methylation resulted in decreased expression, not only in the early stages of BC (42), but also in premalignant stages, suggesting that *ESR2* promoter methylation is a focal event rather than a phenomenon that generally occurs in the breast due to aging processes (43). Although no statistically significant differences were observed between the BC and BD groups in the present study, the high frequency of *ESR2* methylation suggested that this epigenetic event occurs early during the transformation of the mammary epithelium. The gatekeeper nature of *ESR2* implies that it could facilitate the proliferative imbalance that precedes invasive cancer.

Lastly, in the present study, *GSTP1* was the gene with the highest overall methylation frequency (>90% in both groups, BC and BD), with no statistically significant differences (P=0.83). Although certain studies have proposed *GSTP1* methylation as a prognostic biomarker (44,45), its clinical value was limited in the present study cohort. In the present study, no relationship was observed between *GSTP1* gene methylation and prognostic variables such as age, BMI, progesterone receptor (PR), ER, clinical stage, Ki-67 protein proliferation index or metastasis and progression.

Overall, the results of the present study highlighted that *BRCA1* and *TIMP3* methylation had a more evident clinical impact since *BRCA1* was associated with tumor susceptibility and aggressiveness, while *TIMP3* to metastatic risk. In the present cohort *BMP6, ESR2* and *GSTP1* appear to be involved in specific stages or contexts of tumor progression; however, without clear diagnostic or prognostic value. These findings underscored the importance of validating epigenetic biomarkers in specific populations and suggested that the integration of *BRCA1* and *TIMP3* into predictive models could improve risk stratification and therapy selection in BC.

A limitation of the present study is that methylation status was assessed qualitatively using methylation-specific PCR, based on the presence or absence of bands on agarose gels, and the lack of confirmatory tests on all samples, such as DNA sequencing or bisulfite pyrosequencing. Gel interpretation was not conducted under blinded conditions, which may introduce potential observer bias. However, this approach is extensively used for the detection of methylation patterns, although it does not provide quantitative information on methylation levels.

Another limitation of the present study is the lack of availability of relevant gynecological and obstetric variables in the present study cohort, including reproductive history, parity and use of hormonal therapy. These factors may act as confounders and could influence DNA methylation patterns as well as clinical outcomes. The absence of these variables may have affected the assessment of the association between methylation status and the analyzed outcomes. Despite this, key clinical variables such as age, BMI, molecular subtype, ER and PR status, clinical stage, Ki-67 proliferation index and the presence of distant and lymph node metastasis were included as covariates in the

binary logistic regression models to partially control for confounding.

In conclusion, the methylation status of the *BMP6*, *BRCA1*, *ESR2*, *GSTP1* and *TIMP3* genes was evaluated in women with BC or BD in the present study to determine the association with poor prognosis. Only the *BRCA1* gene exhibited a significantly higher frequency of methylation in women with BC (32.1%) compared with BD (16.6%) (P=0.040), particularly in the triple-negative subtype and methylation demonstrated a potential association with cell proliferation Ki-67 (P=0.057) and lymph node metastasis (P=0.059). Methylation of the *TIMP3* gene was associated with up to an eight-fold increased risk of distant metastasis, indicating that both the *BRCA1* and *TIMP3* genes could be prognostic biomarkers. Methylation of the *BMP6*, *ESR2* and *GSTP1* genes did not exhibit statistically significant differences between the BC and BD groups, nor with the examined clinical and pathological characteristics, which may be due to interindividual variability, cell selection, technique sensitivity or confounding factors such as reproductive history. These results highlighted the potential utility of *BRCA1* and *TIMP3* methylation as prognostic biomarkers and the need for further research into DNA methylation as a diagnostic and therapeutic tool in BC.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

SRDLTG contributed to conception and design, data acquisition, data analysis and interpretation, manuscript drafting and reviewing of key intellectual content. AMGM contributed to sampling, clinical data collection and analysis. BPC contributed to sampling and clinical data collection. CVG contributed to data analysis, manuscript drafting and interpretation. MPGA contributed to data interpretation and statistical analysis. ESR contributed to data analysis and interpretation. SOMC contributed to analysis and interpretation of clinical and experimental data and sample collection. JYSL contributed to conception and design and reviewing of key intellectual content. SRDLTG and JYSL confirm the authenticity of all the raw data. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and was approved by the Institutional Review Board (Ethics Committee 1305) of Western Biomedical Research Center (approval no. R-2023-1305-024; Guadalajara, Mexico) for studies involving humans. All participants were invited to take part in the study and signed informed consent forms at the Breast Clinic of the Mexican Social Security Institute in Guadalajara (Mexico).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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