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Original contribution

Clinicopathologic significance of DNA methyltransferase 1, 3a, and 3b overexpression in Tunisian breast cancers☆

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Summary DNA methyltransferase 1, 3a, and 3b affect DNA methylation, and it is thought that they play an important role in the malignant transformation of various cancers. The current study was designed to analyze DNA methyltransferase expression by immunohistochemistry in a series of 94 Tunisian sporadic breast carcinomas. Results were correlated to clinicopathologic parameters and promoter methylation status of 8 tumor suppressor genes (*BRCA1*, *BRCA2*, *RASSFA1*, *TIMP3*, *CDH1*, *P16*, *RARβ2*, and *DAPK*). Overexpression of DNA methyltransferase 1, 3a, and 3b was detected in 46.8%, 32%, and 44.7% of cases, respectively. A significant correlation was found between DNA methyltransferase 1 overexpression and Scarff-Bloom-Richardson histologic grade III (P = .01). DNA methyltransferase 3a overexpression was significantly associated with menopausal status (P = .01), Scarff-Bloom-Richardson histologic grade III (P = .0001), estrogen (P = .04) and progesterone (P = .007) receptor negativity, and HER2 overexpression (P = .004). However, DNA methyltransferase 3a overexpression was found less frequently in the luminal A intrinsic breast cancer subtype (9.7%) than in luminal B (53%), HER2 (41%), and triple-negative (50%) subtypes (P = .001). DNA methyltransferase 3b overexpression shows significant correlation with promoter hypermethylation of *BRCA1* (P = .03) and *RASSFA1* (P = .04) and with the hypermethylator phenotype (more than 4 methylated genes, P = .01). These data suggest that overexpression of various DNA methyltransferases might represent a critical event responsible for the epigenetic inactivation of multiple tumor suppressor genes, leading to the development of aggressive forms of sporadic breast cancer.

1. Introduction

Breast cancer is the most common type of cancer in several parts of the world and the second leading cause of cancer mortality among all women [1]. Each woman's breast cancer risk may be higher or lower, depending on several factors including family history, genetics, age of menstruation, and other factors that have not yet been identified [2]. The lack of somatic mutations in sporadic breast carcinomas suggests that gene inactivation might be achieved by mechanisms other than coding region mutations, such as epigenetic or regulatory changes [3]. Hypermethylation of the cytosine-phospho-guanine (CpG) islands of gene promoters is an important epigenetic mechanism for gene silencing, which may confer a growth advantage to tumor cells [4]. Many cellular pathways are inactivated by this epigenetic event, including DNA repair, cell cycle, apoptosis, cell adherence, and detoxification [3].

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Methylation of cytosine bases in CpG dinucleotides in gene promoters is coordinated by a family of DNA methyltransferases (DNMTs) comprising DNMT1, DNMT3a, and DNMT3b [5]. DNMT1 is the "maintenance" methyltransferase that ensures faithful transmission of the methylation profile from maternal to daughter cells during cell division, whereas DNMT3a and DNMT3b are mainly involved in de novo establishment of methylation patterns during embryogenesis [6,7]. Disruption of DNMT1 in mice results in early embryonic lethality and severe genomic hypomethylation [8]. Mice disrupted for DNMT3a or DNMT3b undergo abnormal mammalian development and have a loss of genomic DNA methylation in pericentromeric repeats [6].

Overexpression of DNMTs has been reported in various types of cancers [7,9,10,11,12]. The degree of overexpression varies between reports depending on the tumor type and the method of analysis. Many studies have reported that DNMT expression may have important therapeutic implications. Indeed, reexpression of promoter-methylated genes has been obtained with DNMT inhibitor treatment such as 5-aza-2′deoxycytidine treatment [13-15].

However, few studies have examined the expression of DNMTs in breast tissues [10,16], and none has evaluated all 3 enzymes simultaneously by immunohistochemistry in breast tissues. Moreover, no clear relationship between DNMT overexpression and clinicopathologic parameters has been established.

Breast cancer rates and median age of onset differ between Western Europe and North Africa [17]. Compared with Western populations, Tunisian breast cancer is characterized by a younger age at onset and a more aggressive tumor phenotype, especially large tumor size, high histologic grade, and hormone receptor negativity [18]. These differences may be caused by the discrepancy in environmental risk factors and/or genetic susceptibilities.

The aims of the current study were to analyze the 3 catalytically active human DNMTs (DNMT1, DNMT3a, and DNMT3b) for overexpression by immunohistochemistry in a series of 94 Tunisian breast cancer cases and to seek relationships between the overexpression of these enzymes, the clinicopathologic parameters, and hypermethylation promoters of several tumor suppressor genes.

2. Materials and methods

2.1. Patients and tissue samples

Our analysis includes 94 invasive ductal carcinomas selected from the breast cancer samples collected in the Department of Pathology, Farhat Hached Hospital of Sousse, Tunisia. Cases were selected based on the availability of sufficient paraffin-embedded sections and their corresponding frozen tumor biopsy specimens. Familial breast cancer cases were not included in this study. Patients' age at diagnosis ranged from 31 to 75 years, with a mean of 48.7 years and a median of 47 years. Tumors were graded according to the modified Scarff-Bloom-Richardson (SBR) system. Clinicopathologic collected information included patient age, menopausal status, tumor size, SBR histologic grade, lymph node metastasis, hormonal receptors (estrogen [ER] and progesterone [PR]), and HER2 status.

Tumors were grouped according to their ER, PR, and HER2 status into 4 intrinsic subtypes according to Cheang et al [19]: luminal A (ER+ and/or PR+, and HER2−), luminal B (ER+ and/or PR+, and HER2+), HER2 overexpressing (HER2+, and ER− PR−), and triple negative (ER− PR− HER2−).

2.2. Immunohistochemical analysis of DNMTs expression

The immunohistochemical staining procedure was performed on formalin-fixed, paraffin-embedded breast cancer sections using a rabbit polyclonal anti-DNMT1 antibody (clone H-300, dilution 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), a mouse monoclonal anti-DNMT3a antibody (clone 64B1446, dilution 1:200; Imgenex, San Diego, CA), and a mouse monoclonal anti-DNMT3b antibody (clone 52A1018, dilution 1:200; Imgenex). Briefly, 5-μm thick tissue sections were cut, dried overnight at 56°C, deparaffinized in toluene, rehydrated through a series of alcohol, and washed in Tris-buffered saline (TBS) (0.05 mmol/L Tris–HCl; 1.15 mmol/L NaCl, pH 7.6). For antigen retrieval, sections were boiled in a water bath with EDTA buffer (10 mmol/L, pH 8.0) for 40 minutes until the temperature reached 98°C. Sections were then allowed to cool at room temperature for 20 minutes, rinsed thoroughly with water, and placed in TBS. Endogenous peroxidase activity was blocked with hydrogen peroxide/methanol for 5 minutes. Subsequently, sections were rinsed gently with TBS and incubated at 4°C overnight with the appropriate primary antibody. Immunostainings were performed using the high-sensitive polymer-based EnVision system (DakoCytomation, Glostrup, Denmark) according to the manufacturer's instructions. Immunoreactivity was visualized with 3,3′-diaminobenzidine tetrahydrochloride. Sections were counterstained with Mayer hematoxylin, permanently mounted, and viewed with a standard light microscope.

The normal staining pattern for DNMTs is nuclear, and a case was considered positive only in the presence of nuclear staining cells. A case was considered negative for expression of DNMTs only when there was a complete absence of nuclear staining cells in the presence of an unquestionable internal positive control represented by normal epithelial cells or stromal lymphocytes. For negative control preparations, primary antibodies were omitted and replaced by TBS. In all cases, immunostaining results were evaluated independently by 2 pathologists (M. T. and S. Z.) using a semiquantitative scoring system [20] taking into consideration the staining

intensity obtained (0, negative; 1, mild; 2, moderate; 3, high) and the proportion of positive cells observed (0, negative; 1, ≤10%; 2, 11% to ≤33%; 3, 34% to ≤66%; 4, ≥66%). The 2 scores were then added for each slide, and *overexpression* was defined as grade 6 or greater.

2.3. Methylation-specific polymerase chain reaction

The methylation status of the promoters on 8 tumor suppressor genes was determinate by methylation-specific polymerase chain reaction (PCR) arrays as previously described [18]. Briefly, genomic DNA was isolated from fresh-frozen tumor samples by phenol/chloroform preceding proteinase K treatment. The extracted nucleic acid was examined by electrophoresis, and the yield was measured spectrophotometrically on a Biophotometer (Eppendorf, Hamburg, Germany) before use and stored at −20°C. Extracted DNA was assessed for its suitability for PCR analysis by a control reaction designed to amplify a fragment of 407 bp of the *β-globin* gene as described previously [21].

Bisulfite conversion of genomic DNA was performed as described previously by Herman et al [22]. This process converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged. Bisulfite-modified DNA was used as a template, and methylation-specific PCR was performed to determine the methylation status for 8 tumor suppressor genes involved in cell-cycle regulation (*P16* and *RASSF1A*) [23,24], cell adhesion and invasion (*CDH1* and *TIMP3*) [25,24], DNA repair (*BRCA1*and *BRCA2*) [26,27], apoptosis (*DAPK*) [28], and hormone receptor–mediated cell signaling (*RARβ2*) [24].

The PCR amplification was performed with treated DNA as template in a total volume of 25 μL containing 0.25 μmol/L of each primer pair, 200 μmol/L of each dNTP, Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 5% dimethylsulfonide (DMSO), and 1 unit of *Taq* DNA polymerase (Promega, Madison, WI, USA). Amplification was performed in a PTC 200 DNA engine thermal cycler (MJ Research, Watertown, MA). Amplified products were electrophoresed, using 2% agarose gel stained with ethidium bromide, against a 50-bp DNA ladder (Promega), and the images were captured under ultraviolet light using the GelDoc2000 System (Bio-Rad, Marnes-la-Coquette, France). All experiments were performed at least 2 times.

Tumors were categorized according to the number of methylated genes into 2 groups: high-methylated group (tumors showing >4 methylated genes) and low-methylated group (tumors showing ≤4 methylated genes).

2.4. Statistical analysis

Statistical analysis was carried out with the SPSS software package (version 17.0; SPSS, Chicago, IL). Pairwise correlations between DNMTs overexpression and clinicopathologic parameters and methylation status were investigated by χ^2 test or Fisher exact test, where appropriate. Probability values of $P < .05$ were regarded as statistically significant.

3. Results

3.1. DNMT overexpression and clinicopathologic parameters

By immunohistochemical analysis, overexpression of DNMT1, DNMT3a, and DNMT3b was observed in 44 (46.8%), 30 (32%), and 42 (44.7%) of the 94 breast carcinoma cases respectively investigated. Representative results for DNMT1, DNMT3a, and DNMT3b staining are shown in Fig. 1.

Fig. 2 summarizes the differential expression of DNMTs in breast carcinomas. Overall, no significant correlations were found between the overexpression of these enzymes. However, the 3 enzymes were simultaneously overexpressed in 11.7% of cases. Coexpression of DNMT1 and DNMT3b was found in 12.7% of cases. Coexpression of DNMT1 and DNMT3a was noted in 6.3% of cases; the same frequency was found for DNMT3a and DNMT3b coexpression.

With regard to clinicopathologic parameters (Table 1), DNMT1 overexpression was significantly associated with SBR histologic grade III ($P = .01$), whereas DNMT3a overexpression correlated significantly with menopausal status ($P = .01$), SBR histologic grade III ($P = .0001$), ER ($P = .04$) and PR ($P = .007$) negativity, and HER2 overexpression ($P = .004$). However, no significant correlation was observed between DNMT3b overexpression and any of the clinicopathologic parameters analyzed.

Table 2 shows the relationship between DNMT overexpression and intrinsic tumor subtypes. No significant differences in DNMT1 and DNMT3b overexpression were detected between the different intrinsic subtypes. However, we found significantly more frequent DNMT3a overexpression in luminal B (53%), HER2 (41%), and triple negative (50%) groups than in luminal A (9.7%) ($P = .001$).

3.2. DNMT overexpression and DNA hypermethylation

Promoter hypermethylation of *BRCA1*, *BRCA2*, *RASSF1A*, *TIMP3*, *CDH1*, *P16*, *RARβ2*, and *DAPK* genes were detected in 61 (65%), 64 (68%), 74 (79%), 18 (19%), 42 (45%), 35 (37%), and 35 (37%) of the 94 breast tumors cases, respectively.

Table 3 summarizes the relationships between the methylation status of these genes and DNMT overexpression. No significant correlation was found between promoter hypermethylation of these tumor suppressor genes and

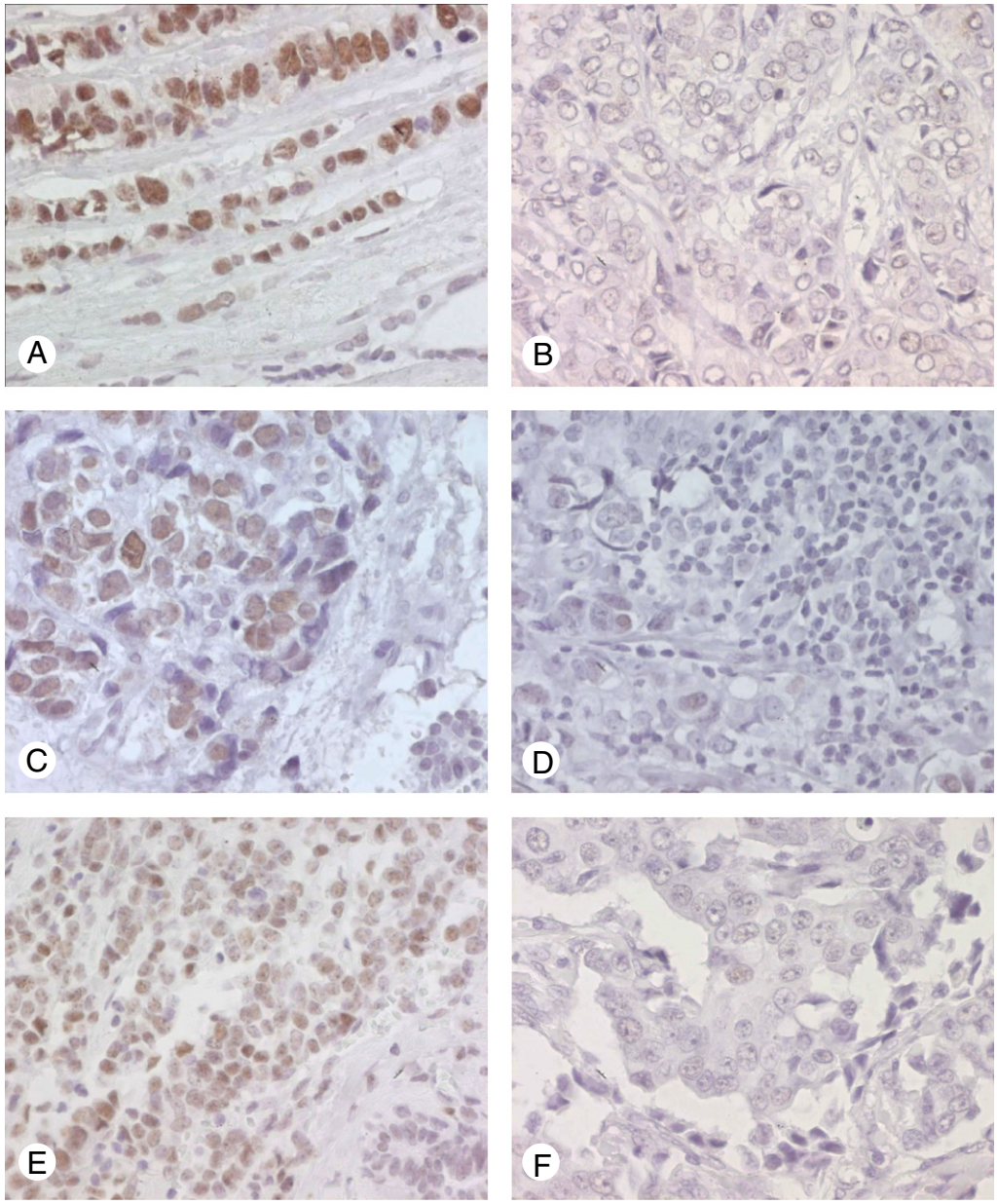

Fig. 1 Immunohistochemical staining patterns of DNMT1, DNMT3a, and DNMT3b protein expression in breast carcinomas (original magnification ×400). A-C, Representative examples of positive immunostaining for DNMT1 (A), DNMT3a (B), and DNMT3b (C). Note the intense and diffuse positivity in the nuclei of tumor cells compared with normal acini (lower right corner of figures). D-F, Representative examples of negative immunostaining for DNMT1 (D), DNMT3a (E), and DNMT3b (F).

overexpression of DNMT1 or DNMT3a. However, DNMT3b overexpression correlated significantly with the hypermethylation of *BRCA1* ($P = .03$) and *RASSF1A* ($P = .04$).

We also examined the relationship between the methylation of multiple genes and DNMT overexpression. We found that 34% of our cases belong to the high-methylated group (more than 4 genes methylated) and 66% belong to the low-methylated group. No correlation was found between the number of methylated genes and the overexpression of DNMT1 and DNMT3a, whereas a significant association was found between DNMT3b overexpression and the high-methylated group ($P = .01$) (Table 3).

4. Discussion

Aberrations in DNA methylation are common in cancers and have important roles in breast tumor initiation and progression [3,18]. Dysregulated expression of the 3 functional DNMTs, which catalyze cytosine methylation, may therefore be important in dysregulating gene expression, especially of tumor suppressor genes during breast tumorigenesis. The aim of our current study was to investigate the overexpression of DNMT1, DNMT3a, and DNMT3b using immunohistochemistry in a series of 94 sporadic breast cancers, and second, we evaluated the relationship of DNMT

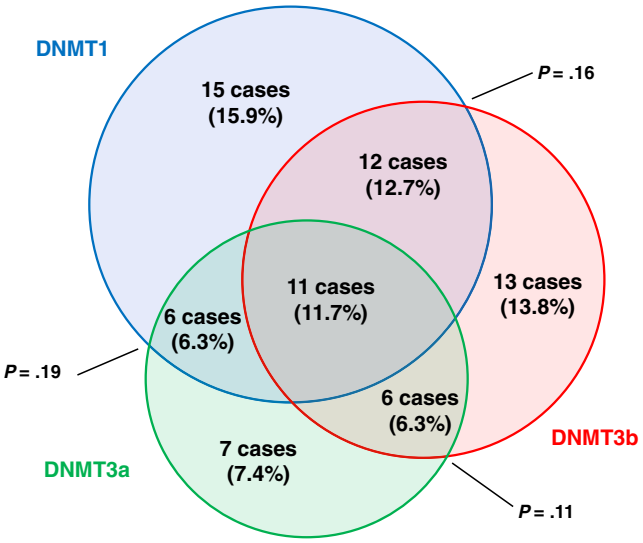

Fig. 2 Venn diagram of DNMT1, DNMT3a, and DNMT3b differential overexpression in breast carcinoma.

overexpression with clinicopathologic parameters and hypermethylation of 8 tumor suppressor genes.

We found that DNMT1, DNMT3a, and DNMT3b are frequently overexpressed in Tunisian breast cancer (46.8%, 32%, and 44.7% of cases, respectively). To date, only a small number of studies have investigated DNMT expression in breast cancers. These studies have shown variable rates of expression for DNMTs in breast cancers. Overall, the levels of DNMT1, DNMT3a, and DNMT3b messenger RNA (mRNA)/protein in breast carcinomas are significantly higher than in noncancerous breast tissues, and the highest expression range was observed with DNMT3b [10,16]. Indeed, Girault et al [10] have reported a predominant role of DNMT3b in breast tumorigenesis, relative to DNMT1 and DNMT3a. DNMT3b overexpression was observed in 30%; in contrast, only 5.4% and 3.1% of the patients' tumors overexpressed DNMT1 and DNMT3a, respectively. However, Butcher and Rodenhiser [16] have found that DNMT3b was overexpressed in 80% of the tumor cases. The different levels of DNMT expression could be largely caused by breast tumor heterogeneity and the diversity of research methodologies.

In fact, all DNMTs have homology and different functions [10]. DNMT1 maintains established methylation patterns in hemimethylated genes by copying methylation patterns from the parent strand to the daughter and is expressed during the S-phase [29]. DNMT3a and DNMT3b, referred to as de novo methyltransferases, methylate unmethylated DNA. They initiate normal DNA methylation during embryonic development [10].

With regard to clinicopathologic parameters, we found a significant relationship between DNMT1 overexpression and high SBR histologic grade (P = .01). The above evidence indicates that increased DNMT1 expression may play a role in the malignant progression. To our knowledge, only 1 previous study has examined the expression of DNMT1 in breast carcinomas. Using real-time reverse transcription–PCR, Girault et al [10] have found that DNMT1 was overexpressed in only a few tumors (5.6%), but the authors did not check their result by immunohistochemistry. Using real-time PCR and Western blot techniques, Xiong et al [30] have found increased DNMT1 expression in grade III endometrioid cancers. In addition, and by immunohistochemistry, DNMT1 expression was found to be increased significantly in high-grade hepatocellular carcinomas, suggesting that DNMT1

Table 1 Association between DNMTs overexpression and clinicopathologic parameters

Parameters	Total no.	DNMT1, n (%)	DNMT3a, n (%)	DNMT3b, n (%)
Age (y)				
≤45	44	23 (52)	18 (40)	17 (38)
>45	50	21 (42)	12 (25)	25 (50)
P value		.31	.07	.26
Menopausal status				
Pre-	51	23 (45)	21 (41)	21 (41)
Post-	43	21 (49)	8 (18)	22 (51)
P value		.71	.01*	.33
Tumor grade [a]				
Grade I	17	3 (17)	2 (11)	8 (47)
Grade II	36	17 (47)	5 (13)	13 (36)
Grade III	41	24 (58)	23 (56)	21 (51)
P value		.01*	.0001*	.40
Tumor size (mm)				
≤20	19	8 (42)	7 (36)	9 (47)
>20	75	36 (48)	23 (30)	33 (44)
P value		.64	.60	.79
Lymph node involvement [b]				
Negative	34	20 (59)	12 (35)	18 (53)
Positive	42	16 (38)	12 (28)	18 (43)
P value		.07	.53	.38
ER status [c]				
Negative	55	24 (43)	22 (40)	23 (41)
Positive	39	20 (51)	8 (20)	19 (48)
P value		.46	.04*	.50
PR status [c]				
Negative	50	24 (48)	22 (44)	26 (52)
Positive	44	20 (45)	8 (18)	16 (36)
P value		.80	.007*	.14
HER2 status [d]				
Negative	69	30 (43)	18 (26)	32 (46)
Positive	25	14 (56)	12 (48)	10 (40)
P value		.28	.04*	.58

NOTE. *P* value for χ^2 or Fisher exact test; asterisks indicate significant correlations.

[a] SBR classification.

[b] Pathologic nodal status was missing in 18 cases.

[c] ER and PR receptors status was evaluated by immunohistochemistry and considered positive if 10% or greater of tumor cells showed nuclear staining.

[d] Evaluated by immunohistochemistry and considered positive if scored 2+ or 3.

Table 2 Correlation between DNMTs overexpression and intrinsic subtypes in breast carcinomas

Intrinsic histologic subtypes	Total	DNMT1, n (%)	DNMT3a, n (%)	DNMT3b, n (%)
Luminal A (ER/PR+, HER2−)	41	17 (41)	4 (9.7)	18 (43)
Luminal B (ER/PR+, HER2+)	13	8 (61)	7 (53)	5 (38)
HER2 overexpressing (ER−, PR−, HER2+)	12	6 (50)	5 (41)	5 (41)
Triple negative (ER−, PR−, HER2−)	28	13 (46)	14 (50)	14 (50)
P value		.64	.001∗	.90

NOTE. *P* value for χ^2 or Fisher exact test; asterisks indicate significant correlations.

overexpression plays a role in the early stage of hepatocarcinogenesis [20]. Using semiquantitative reverse transcription–PCR, Chen et al [9] have demonstrated that DNMT1 overexpression in ovarian epithelial cancers was correlated with low tumor differentiation, advanced clinical stage, and lymphatic metastasis. By using immunohistochemistry, a significant correlation was reported between DNMT1 overexpression and higher-stage tumors in 63 gastroenteropancreatic neuroendocrine tumors, suggesting that the overexpression of DNMT1 was related to tumorigenesis and progression of these tumors [11]. However, given the limited studies exploring the DNMT1 expression in breast cancer, further research is required to better elucidate the precise clinical significance of DNMT1 expression in this cancer.

By examining the relationship between DNMT3a overexpression and clinicopathologic parameters, we found significant correlations with menopausal status ($P = .01$), SBR histologic grade III ($P = .0001$), ER ($P = .04$) and PR ($P = .007$) negativity, and HER2 overexpression ($P = .004$); this observation suggests that DNMT3a overexpression may be a specific marker of tumor aggressiveness and proliferation and also that it may play an important role in breast carcinogenesis. Unfortunately, no previous study has examined the relationship between DNMT3a overexpression and clinicopathologic parameters in breast carcinomas.

Table 3 Association between DNMTs overexpression and DNA promoter hypermethylation

Genes	Total	DNMT1		DNMT3a		DNMT3b	
		n (%)	*P*	n (%)	*P*	n (%)	*P*
BRCA1			.28		.82		.03∗
Unmethylated	33	13 (39)		11 (33)		10 (30)	
Methylated	61	31 (50)		19 (31)		32 (52)	
BRCA2			.19		.22		.28
Unmethylated	30	17 (56)		7 (23)		11 (36)	
Methylated	64	27 (42)		23 (35)		31 (48)	
RASSFA1			.23		.15		.04∗
Unmethylated	20	7 (35)		9 (45)		5 (25)	
Methylated	74	37 (50)		21 (28)		37 (50)	
TIMP3			.76		.67		.11
Unmethylated	76	35 (46)		25 (32)		31 (40)	
Methylated	18	9 (50)		5 (27)		11 (61)	
CDH1			.16		.28		.74
Unmethylated	52	21 (40)		19 (36)		24 (46)	
Methylated	42	23 (54)		11 (26)		18 (42)	
P16			.86		.64		.57
Unmethylated	50	23 (46)		17 (34)		21 (42)	
Methylated	44	21 (47)		13 (29)		21 (47)	
RARβ2			.79		.59		.55
Unmethylated	59	27 (45)		20 (33)		25 (42)	
Methylated	35	17 (48)		10 (28)		17 (48)	
DAPK			.87		.32		.55
Unmethylated	59	28 (47)		21 (35)		25 (42)	
Methylated	35	16 (45)		9 (25)		17 (48)	
Methylated genes			.12		.13		.01∗
≤4 genes	62	25 (40)		23 (37)		22 (35)	
>4 genes	32	19 (59)		7 (21)		20 (62)	

NOTE. *P* value for χ^2 or Fisher exact test; asterisks indicate significant correlations.

However, DNMT3a overexpression was also found to be associated with tumor aggressively in other types of cancers. Indeed, a significant correlation between DNMT3a protein expression and higher tumor stage was reported in gastroenteropancreatic neuroendocrine tumors [11]. Moreover, a poorer recurrence-free survival was noted in hepatocarcinomas with increased DNMT3a mRNA [12].

In our current study, we found that DNMT3a overexpression is significantly correlated with triple-negative breast carcinomas and tumors overexpressing HER2 (P = .001). Triple-negative carcinomas are themselves a subgroup of "basal-type" breast tumors and account for 10% to 17% of all breast cancers. These carcinomas are characterized by a poor prognosis compared with tumors that are positive for hormone receptors. Despite being sensitive to chemotherapy, many women with triple-negative breast carcinomas relapse quickly and commonly develop visceral metastases, including lung, liver, and brain metastases. Combinations of epigenetic drug treatments, especially DNMT inhibitors, could potentially work synergistically in increasing therapeutic effects and may have important therapeutic implications in this subtype of cancer [13-15,31].

We found no link between DNMT3b overexpression and any of the clinicopathologic parameters analyzed in our present study. Few trials have been performed on solid tumors to investigate DNMT3b expression in breast carcinomas. Butcher and Rodenhiser [16] have examined DNMT3b expression by immunohistochemistry in a series of 54 sporadic breast carcinomas from Canada and reported a high frequency of DNMT3b overexpression (80% of cases), but the authors did not seek any relationship of their results with clinicopathologic parameters. The only previous study that examined the relationship between DNMT3b expression and clinicopathologic parameters in breast carcinomas was that described by Girault et al [10]. Using real-time PCR, the authors found that DNMT3b mRNA overexpression is significantly associated with high SBR histologic grade, ER negativity, and strong expression of the proliferation marker Ki-67, but not with patients' age, menopausal status, lymph node involvement, or macroscopic tumor size. They also found an association between DNMT3b overexpression and poor relapse-free survival in patients who received adjuvant hormone therapy in univariate analysis. The authors of this study suggested that DNMT3b may be a marker of both tumor aggressiveness and proliferation.

In the present study, we also investigated the relationship between DNMT overexpression and DNA methylation. We found no correlation between overexpression of DNMT1 or DNMT3a and promoter hypermethylation of the tumor suppressor genes investigated. Although more CpG islands still need to be examined to draw conclusions, the lack of correlation between expression of these enzymes and DNA methylation in this study implies that increased DNMT1 and DNMT3a expression in breast cancer may have other roles during breast carcinogenesis than merely altering DNA methylation of tested genes. In the absence of previous studies exploring the expression of DNMT1 and DNMT3a in breast cancer and their relation to DNA methylation, we cannot draw consistent conclusions about the role of these 2 enzymes in DNA methylation. Other studies analyzing larger series of breast cancer and taking into account the methylation status of a larger number of tumor-related genes are required to clarify the role of these enzymes in breast carcinogenesis.

With regard to DNMT3b, few significant relationships were found in our study. Indeed, DNMT3b overexpression correlates significantly with hypermethylation of *BRCA1* (P = .03) and *RASSF1A* (P = .04) in our series of cases. This suggests that DNMT3b may be the primary DNMT responsible for methylation of these 2 critical genes. The subsequent epigenetic alteration of BRCA1 activity would predispose a cell to tumorigenesis, because of defects in BRCA1-dependent DNA repair, and cycle regulation and proliferation [16]. RASSF1A regulates several biological processes including cell cycle progression, apoptosis, and microtubule stability. It is currently thought that RASSF1A functions as a scaffold for the assembly of multiple tumor suppressor complexes and may relay proapoptotic signaling by K-RAS. Several reports indicate the presence of aberrant methylation in *RASSF1A* in various types of human tumors [32-34]. Promoter hypermethylation of *BRCA1* and *RASSF1A* is common in sporadic ovarian cancer [33]. Furthermore, Gao et al [34] confirmed that the presence of a RASSF1A functional polymorphism was associated with breast cancer pathogenesis in general and modified breast cancer age of onset in *BRCA1/2* mutation carriers.

Data on the existence of a hypermethylator phenotype in breast cancer are controversial [35]. There is no standard definition of highly methylated tumors that most likely represent the hypermethylator phenotype. In the present study, we examined the relationship between multigene methylation and DNMT overexpression. We found that 34% of our cases belong to the high-methylated group (more than 4 genes methylated). Moreover, we found a significant association between DNMT3b overexpression and the high-methylated group (P = .01). These findings support the existence of a hypermethylator phenotype in breast cancer and suggest a role for DNMT3b in this phenotype.

Epigenetic changes are reversible, raising the possibility that inhibition of specific enzymes that regulate epigenetic marks would have antitumor effects. Promoter methylation can be reversed by using DNMT inhibitors, such as 5-aza-2′-deoxycytidine-decitabine or 5-aza-cytidine [13-15]. These molecules are metabolized intracellularly to 5-aza-2′-deoxycytidine-triphosphate, which is incorporated into DNA in place of 2′-deoxycytidine-triphosphate. This results in the covalent binding of DNMT1, DNMT3a, and DNMT3b to modified DNA; inhibition of DNMT activity; and DNMT degradation. The use of these epigenetic drugs allows restoration of the expression of genes that are silenced by hypermethylation in breast cancer and thus restores normal cell growth control or induces apoptosis in tumor cells. These

agents will be effective especially in breast cancer overexpressing DNMTs with multiple methylated genes and may help patients to live longer with fewer side effects than is possible with standard chemotherapy.

In conclusion, we found that DNMTs are frequently overexpressed in sporadic breast carcinomas and correlate significantly with promoter hypermethylation of multiple tumor suppressor genes and aggressive forms of Tunisian breast cancer. These data suggest that overexpression of various DNMTs might represent a critical event responsible for the epigenetic inactivation of several tumor suppressor genes and lead to the development of breast cancers, especially those with aggressive phenotype.

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