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SCGB3A1 gene DNA methylation status is associated with breast cancer in Egyptian female patients

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Abstract

Background: In recent years, hypermethylation of gene promoters has emerged as one of the fundamental mechanisms for the inactivation of tumor suppressor genes and has a potential role in the early detection of breast cancer. The present study is a case-control study aimed to quantify the methylation levels in the promoters of secretoglobin 3A1 (*SCGB3A1*), and ataxia-telangiectasia mutated (*ATM*) genes and evaluate their relation to clinicopathological features of the tumor in a cohort of Egyptian female patients with breast cancer.

Methods: Genomic deoxyribonucleic acid (DNA) was extracted from 100 tissue samples, 50 breast cancer tissues and 50 adjacent non-cancerous breast tissues, then, it was subjected to bisulfite conversion. The converted DNA was amplified by real-time PCR; then, pyrosequencing was performed to quantify DNA methylation levels in four CpG sites in *ATM* and *SCGB3A1* gene promoters. The methylation data were presented as the percentage of average methylation of all the observed CpG sites and were calculated for each sample and each gene.

Results: The percentage of DNA methylation of the *SCGB3A1* promoter was significantly higher in the tumor group than in the normal group ($P = 0.001$). However, a non-statistical significance difference was found in the DNA methylation percentage of the *ATM* promoter in the tumor group compared to the normal group ($P = 0.315$). The *SCGB3A1* promoter methylation frequency was significantly associated with estrogen receptors (ER) and progesterone receptors (PR) positive tumors, lymph node metastasis, and lymphovascular invasion. However, no association was found between *ATM* methylation status and the different clinicopathological features of the tumor.

Conclusions: The findings of this work showed that the *SCGB3A1* promoter methylation was significantly higher in the tumor group and was significantly associated with different clinicopathologic features in breast cancer. It may be considered as a suitable biomarker for diagnosis and prognosis. However, the promoter methylation levels of the *ATM* gene in breast cancer cases were unable to distinguish between breast cancer tissues and adjacent normal tissues, and there is no evidence that epigenetic silencing by *ATM* methylation has a role in breast cancer pathogenesis.

Keywords: Breast cancer, DNA methylation, Pyrosequencing, *SCGB3A1*, *ATM*

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Background

Epigenetic changes are inherited DNA alterations that affect the function and expression of genes without changing the DNA sequence. The mechanisms of epigenetic alterations include DNA methylation, alterations in chromatin condensation, histone modifications, and RNA interference [1].

DNA methylation comprises the addition of a methyl group by the DNA methyltransferase enzyme in the 5′ position of cytosine in a cytosine-guanine (CpG) dinucleotide, which aggregates in the promoter region leading to a densely packed chromatin and transcriptional gene silencing. It affects regulator genes that cover a wide range of cellular pathways. Abnormal DNA methylation patterns have been associated with almost all types of cancer, including breast cancer [2].

Breast cancer is the most common neoplasm among women all over the world, and there are more than 1, 300,000 newly diagnosed cases every year [3]. Despite early detection and improvement in its management, its mortality increases annually, accounting for 14% of the total cancer deaths [4]. It is considered a complex multifactorial disease. It has been widely established to be caused by an interaction between environmental and genetic factors [5]. There is increasing evidence that hypermethylation of tumor-related genes may have added to the pathogenesis of breast cancer in recent years [6].

Secretoglobin 3A1 (*SCGB3A1*) gene, also called high in normal-1 (*HIN-1*) is a gene located at chromosome 5q35.3 and encodes 104 amino acids protein, which is a member of the secretoglobin family that consists of small secretory proteins [7]. The *SCGB3A1* gene is highly expressed in the epithelial cells of normal lung, uterus, prostate, and breast [8]. It is involved in regulating epithelial cell proliferation, differentiation, and morphogenesis. Thus, its abnormal expression may result in the development of malignant phenotype in human tumors [9]. The aberrant methylation of the *SCGB3A1* gene was displayed in multiple human cancer types at an early stage of malignant transformation, including breast cancer [10].

The ataxia-telangiectasia mutated (*ATM*) gene which is located at 11q22.3 codes for a protein kinase; a member of the 3-kinase phosphatidylinositol family, that shows a significant role in the activation of cellular responses to double-stranded DNA breaks through downstream phosphorylation of principal players in the response pathways of DNA damage which include BRCA1, p53, and Chk2 [11]. It plays a central role in maintaining the integrity of the genome by activating the checkpoints of the cell cycle and promoting the repair of double-strand breaks of DNA. It is involved in recognizing the damaged DNA, recruitment of repair proteins, signaling to cell cycle checkpoints, transcriptional regulation, and apoptosis activation [12]. *ATM* promoter hypermethylation has been observed in a variety of cancers, including cancer colon, head and neck squamous cell carcinoma, glioma, and gastric lymphoma. However, data on the methylation status in breast tumors showed contradictory results [13–16].

Pyrosequencing is a quantitative sequencing-by-synthesis approach that provides a rapid, high-throughput means of quantifying methylation levels globally and at regulatory elements of methylation-sensitive genes [17]. It is a fast, reproducible, and easy-to-use method to analyze not only CpG-rich but also CpG-poor regions, making it possible to define a threshold discriminating hypomethylation or hypermethylation and differences in DNA methylation among various tissue types [18].

To better understand the role of DNA methylation and its relation to clinicopathological features in breast cancer, we used the pyrosequencing technique to assess the methylation status in two candidate genes, *SCGB3A1* and *ATM*, which are known to have significance in the regulation of cellular processes and to evaluate their association with the clinicopathological features in breast cancer cases. This could lead to a better understanding of the tumorigenesis process and possibly improve the diagnosis and treatment of this cancer.

Methods

Study population and sample collection

A total of 50 female patients with primary breast cancer who underwent surgery at the Department of Surgery at the Medical Research Institute Hospital, from December 2018 to June 2019 were consecutively enrolled in this study. The surgical intervention was conservative breast surgery (96%) or modified radical mastectomy (4%).

All female patients were recruited after their diagnosis with primary breast cancer based on the standard clinical, radiological, and histological parameters. We did not specify certain age groups or tumor characteristics. Patients with a previous history of neo-adjuvant chemotherapy, radiotherapy, or hormonal therapy were excluded from the study. Also, patients with a history of other cancers, acute medical or surgical conditions, were excluded. Informed consent was collected from all patients. The study was approved by the ethics committee of the Medical Research Institute (Ref. IORG 0008812), which is following the Declaration of Helsinki code of ethics for research involving humans.

Before surgery, all patients underwent full history taking, thorough physical examination, fine-needle aspiration cytology, or ultrasound-guided core biopsy from the breast mass for preoperative pathological examination. Also, mammography, breast ultrasound, pelvi-

abdominal ultrasound, chest X-ray, and bone scan were done.

Total 100 fresh tissue specimens, 50 from the excised breast cancer tissues and 50 from the adjacent normal tissues after leaving a clearly defined safety margin, were collected during the operation. Serial 5 μm thick paraffin sections from all cases were subjected to routine hematoxylin and eosin staining to assess the histologic tumor type, tumor grade, lymphovascular invasion, and lymph node metastasis. The collected fresh samples were stored immediately at −80 °C until use.

Breast cancer hormonal assay in the form of ER, PR, and human epidermal growth factor receptor 2 (HER2/neu) were evaluated using immunohistochemistry. The clinicopathological parameters were assessed, including the histopathology of the tumor, pathological grade, and the clinical staging, which was performed according to the tumor-node-metastasis (TNM) classification system.

Detection of methylation status of *SCGB3A1* and *ATM* genes

Genomic DNA extraction and bisulfite treatment

DNA extraction was performed from 25 mg tissue samples using QIAamp DNA Mini Kit (Qiagen, Germany. Cat No: 51304) following manufacture instructions. The concentration and purity of the extracted DNA were assessed using the Thermo Scientific NanoDrop™ 1000 spectrophotometer.

Five microliters of the extracted DNA samples was bisulfite-treated to convert all unmethylated cytosine residues to uracil, while 5-methylcytosine residues stay unaffected, using EpiTect Fast Bisulfite Conversion Kit (Qiagen, Germany Cat No. 59824). The modified DNA was then stored at −20 °C for further analysis.

DNA amplification

The converted DNA was amplified by PCR using the PyroMark PCR Kit (Qiagen, Cat No. 978703) according to the manual instructions. The final volume of each PCR reaction mix was 25 μL. Each reaction consisted of 12.5 μL PyroMark PCR master mix (2×), 2.5 μL CoralLoad concentrate (10×), 2.5 μL sodium bisulfite-treated DNA, 1.25 μL (200 nM) final concentrations of each primer, and 5.0 μL RNase-free water. The primers for PCR amplification and pyrosequencing, one of them being biotinylated, were purchased from Qiagen, Germany; PyroMark CpG Assays (Cat No. 978746). The PCR conditions consisted of initial denaturation at 95 °C for 15 min, 45 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s, then a final extension at 72 °C for 10 min. The PCR products were separated on agarose gel electrophoresis to confirm successful amplification.

Pyrosequencing methylation analyses

Quantitative DNA methylation analysis of the two studied genes was performed using the Pyromark Q24 Instrument (Qiagen, Germany). PyroMark Gold Q24 Reagents Kit (Qiagen, Cat No. 971802) which contained all enzymes, substrates, and nucleotides needed in the pyrosequencing cascade was used. A pyrosequencing reaction mix consisted of 40 μL PyroMark Binding buffer (Cat No. 979306), 1 μL streptavidin-coated Sepharose beads, and 29 μL nuclease-free water was prepared for each reaction before adding 10 μL biotinylated PCR products. The beads were captured with the vacuum tool on the PyroMark Vacuum Workstation. They were thoroughly washed and subsequently denatured generating single-stranded DNA suitable for pyrosequencing. This template DNA was released into the pyrosequencing reaction plate containing 22.5 μL annealing buffer and 2.5 μL sequencing primer (0.3 μM) for each reaction. The primers used in pyrosequencing are summarized in Table 1. After primer annealing, the plate was placed into the PyroMark instrument, and the sequencing reaction was started. The plates were prepared for pyrosequencing according to the manufacturer's protocol. Only data that passed appropriate quality control thresholds were included for analysis. Pyrosequencing results were evaluated using the PyroMark Q24 version: 2.0.8 software (Qiagen, Germany). The methylation percentage at each CpG site was calculated from the ratio of heights of a cytosine peak (methylated signal) divided by the sum of cytosine and thymine peaks (methylated and unmethylated signals) and displayed on a pyrogram. An example of the pyrogram produced by pyrosequencing of the promoters of the two genes was shown in Figs. 1 and 2. Methylation levels were presented as the percentage of average methylation of all the measured CpG sites and then, were calculated for each sample and each gene [19].

Statistical methods

All statistical calculations were done using the IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Wilcoxon signed-rank test was used for abnormally distributed quantitative variables to compare between two related samples. Comparisons between groups for categorical variables were evaluated using the chi-square test (Fisher's exact or Monte Carlo correction). The significance of the obtained results was judged at $P \leq 0.05$.

The median value of the percentage of DNA methylation levels of normal tissue samples of each gene was used as a cut-off point. Therefore, the frequencies of DNA hypermethylation were represented as the percentage of samples with methylation levels above or equal to

Table 1 The primers used in pyrosequencing

Gene symbol	Entrez Gene ID	Qiagen product code	Pyrosequencing primers*	Chromosomal location	Number of CpG sites	PCR product size
ATM	4863/472	Hs_NPAT/ATM_01_PM (PM00153622)	CGCGGACGCGGGAWGGAGGGTTAT TGGACCCGGC	Chromosome 11, BP 1080931X-1080932XX	4	203 bp
SCGB3A1/ HIN-1	92304	Hs_SCGB3A1_01_PM (PM0022687)	GGGGCGTTTTTCTGTCTTCGAAACTA GCCTCGACGC	Chromosome 5, BP 1800190XX-1800190XX	4	90 bp

*Primer sequences were adopted from PyroMark Assay Database

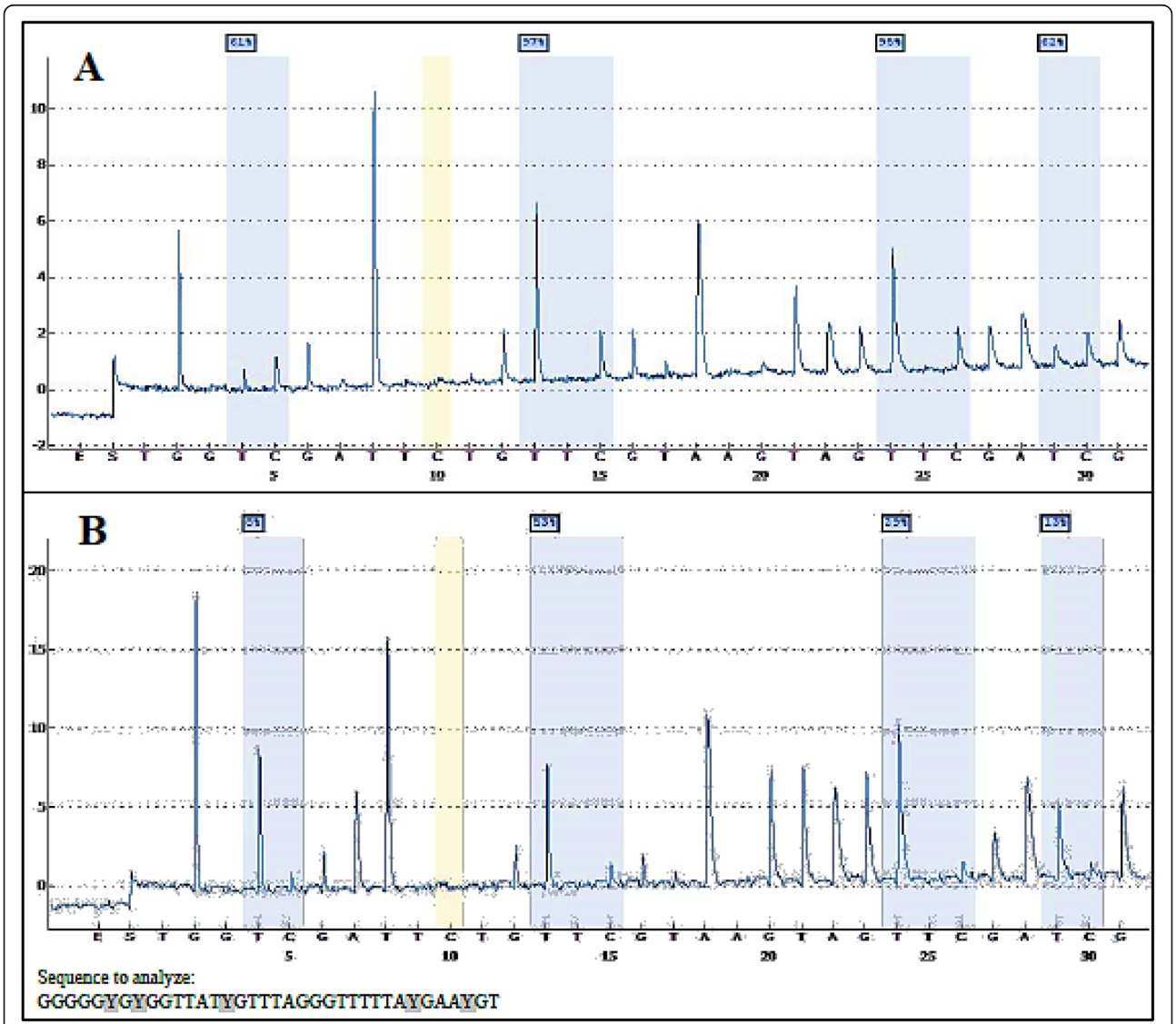

Fig. 1 An example of a pyrogram produced by pyrosequencing of the *SCGB3A1* gene promoter. **A** Tumor group. **B** Normal group. The gray highlights mark the tested CpG sites and the percentage methylation readings. X-axis, dispensation order; Y-axis, relative light intensity

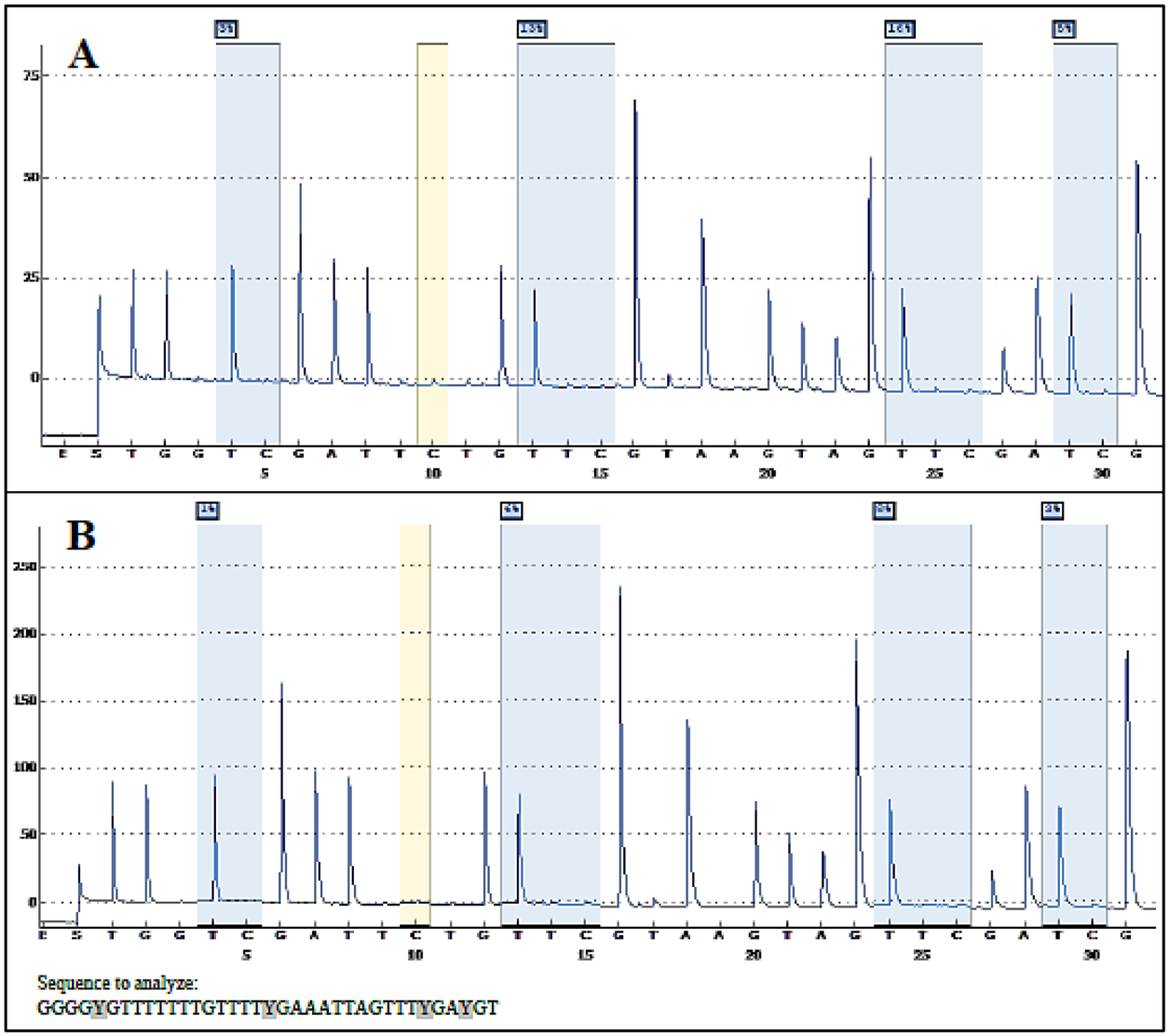

Fig. 2 An example of a pyrogram produced by pyrosequencing of the *ATM* gene promoter. **A** Tumor group. **B** Normal group. The gray highlights mark the tested CpG sites and the percentage methylation readings. X-axis, dispensation order; Y-axis, relative light intensity

the cut-off value for each gene, which was 43.4% for the *SCGB3A1* gene, and 8.4% for the *ATM* gene.

Results

Bisulfite pyrosequencing was used to quantify DNA methylation levels of *SCGB3A1* and *ATM* genes in 50 samples of breast cancer tissues (tumor group) and in the corresponding 50 samples of adjacent normal tissues (normal group).

The clinical data of the patients and the tumor characteristics are summarized in Table 2. The age of the involved subjects was above 50 years in 50% of cases. Sixty-four percent were in the post-menopausal state. Positive ER was encountered in 72% of patients, and positive PR was 64%, while HER2/neu receptors were found positive in 78% of cases. Regarding the molecular type, 22% were luminal A, 50% were luminal B, and 28% were HER2-enriched (Table 2).

SCGB3A1 and *ATM* promoter methylation were assessed in the two studied groups. The DNA methylation percentage of *SCGB3A1* promoter was significantly higher in the tumor group compared to the normal group (P = 0.001) (Table 3) (Fig. 3). However, a non-statistical significance difference was found in the DNA methylation percentage of the *ATM* promoter in the tumor group compared to the normal group (P = 0.315). The frequency of *SCGB3A1* promoter methylation was significantly associated with ER, and PR positive tumors

Table 2 Patients and tumor characteristics (N = 50)

Variables	No.	%
Age (years)		
≤ 50	25	50
> 50	25	50
Family history		
Yes	23	46
No	27	54
Menstrual history		
Post menopause	32	64
Pre menopause	18	36
Focality		
Unifocal	44	88
Multifocal	6	12
Tumor size (cm)		
≤ 2	25	50
> 2	25	50
Histopathological type		
IDC	44	88
ILC	6	12
Pathological grade		
Grade I	7	14
Grade II	29	58
Grade III	14	28
Lymph vascular invasion		
Positive	43	86
Negative	7	14
LN metastasis		
Positive	28	56
Negative	22	44
TNM stage		
IA	9	18
IIA+IIB	32	64
III	9	18
ER		
Positive	36	72
Negative	14	28
PR		
Positive	32	64
Negative	18	36
HER2/neu		
Positive	39	78
Negative	11	22
Molecular type		
Luminal A	11	22
Luminal B	25	50
HER2 enriched	14	28

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *LN* lymph node, *TNM* tumor-node-metastasis classification system, *ER* estrogen receptors, *PR* progesterone receptors, *HER2/neu* human epidermal growth factor receptor 2

(P = 0.005, and 0.022 respectively), lymph node metastasis (P = 0.015), and lymphovascular invasion (P = 0.012) (Table 4). However, no association was found between *ATM* methylation frequency and the different clinicopathological features of the tumor.

Discussion

Breast cancer is the most common cancer associated with high mortality rates among women worldwide [3]. The silencing of tumor suppressor genes by hypermethylation is an essential epigenetic mechanism in breast tumorigenesis [20]. Epigenetic alterations differ from genetic ones primarily in their greater frequency, incomplete reversibility, and their location in fixed genome regions. They affect the early stages of the carcinogenic process, nominating them as promising markers in early diagnosis [21]. In the present study, we assessed the promoter methylation of two tumor suppressor genes, *SCGB3A1* and *ATM*, in breast cancer tissues and adjacent normal tissues, and we evaluated their association with the clinicopathological features of the tumor.

The current findings demonstrated that DNA methylation levels of the *SCGB3A1* promoter were significantly higher in the tumor group compared to the normal group (P = 0.001). In accordance with previous results, Cho et al. [22] showed significant hypermethylation of the *SCGB3A1* gene in the tumor tissues compared to the adjacent normal tissues. Vavoulidis et al. [23] found that the *SCGB3A1* promoter was hypermethylated in 82.1% of all malignant cases, in contrast with the low frequencies in patients negative for neoplasia or with benign breast lesions. In addition, Feng et al. [9] found that *SCGB3A1* was densely methylated in cancer cell lines and was frequently methylated in breast cancer tissues with a 49% positive rate. Park et al. [24] concluded that the CpG island methylation of the *SCGB3A1* gene is an early event that predominantly occurs during the preinvasive stage of breast cancer and accumulates with breast cancer progression. Moreover, Gurioli et al. [25] reported that the *SCGB3A1* methylation levels gradually increased from low and intermediate in healthy noncancerous tissue to high in cancerous tissue. Another study demonstrated that a three-gene panel including the *SCGB3A1* gene could distinguish between breast ductal carcinoma in situ and normal breast tissues with 96% sensitivity and 87% specificity [26].

SCGB3A1 is expressed with an increased amount in the normal terminal duct lobular unit in the mammary gland, and it has a role in regulating the proliferation, differentiation, and morphogenesis of epithelial cells [27]. It was reported that reintroduction of SCGB3A1 into breast cancer cell line inhibited cell growth. This indicated that *SCGB3A1* is a tumor suppressor gene that is deactivated with high frequency in the early stages of breast tumorigenesis. Its promoter hypermethylation may be associated with transcriptional gene suppression, possibly leading to uncontrolled cell growth [28]. The results of the current study agreed with these findings and supported the role of *SCGB3A1* promoter methylation in cancer pathogenesis. We suggest that it could be an important characteristic as a diagnostic biomarker.

The status of the hormone receptors is an important prognostic factor in breast cancer patients. It is also considered a predictive marker for the response to hormonal therapy [9]. In this study, we demonstrated a significant association between *SCGB3A1* methylation

Table 3 DNA methylation percentage of *ATM* and *SCGB3A1* promoters in the studied groups

	Tumor group (n = 50)		Normal group (n = 50)		*P*
	Mean ± SD	Median (IQR)	Mean ± SD	Median (IQR)	
***ATM* (%)**					
CpG_1	2.9 ± 1.8	2 (3)	3.4 ± 2.2	3 (3)	0.194
CpG_2	8.3 ± 4	7.5 (7)	9.6 ± 4.3	10 (6)	0.356
CpG_3	11.4 ± 5.2	10 (9)	12 ± 4.8	12.5 (8)	0.550
CpG_4	4.6 ± 2.2	5 (3)	5.1 ± 2.5	4.5 (4)	0.495
Average CpG sites	6.8 ± 2.8	6.3 (4.8)	7.5 ± 3	8.4 (5.3)	0.315
***SCGB3A1* (%)**					
CpG_1	32.3 ± 20.7	22.5 (37)	23.9 ± 17.1	22.5 (22)	0.001*
CpG_2	78.6 ± 23.5	89.5 (47)	69.9 ± 24.6	72.5 (39)	0.010*
CpG_3	55.1 ± 24.8	49.5 (49)	48 ± 22.8	45 (33)	0.012*
CpG_4	29.1 ± 14.9	24 (29)	20 ± 15.3	20 (23)	< 0.001*
Average CpG sites	48.8 ± 16.6	49.3 (26.5)	40.4 ± 16.3	43.4 (27.8)	0.001*

P value, compares the studied groups using Wilcoxon signed ranks; *, Statistical significance at $P \leq 0.05$; *SD* standard deviation, *IQR* interquartile range. CpG_1, CpG_2, CpG_3, and CpG_4 stand for the four CpG sites studied in each assay

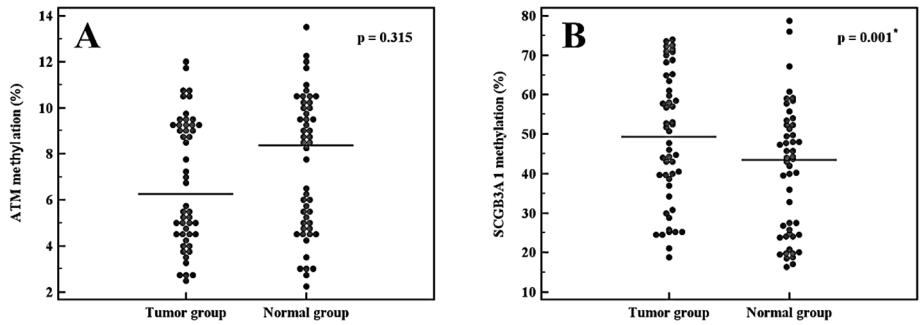

Fig. 3 Comparison of the methylation levels of *ATM* (**A**) and *SCGB3A1* (**B**) genes in tumor samples and paired normal samples. Horizontal lines provided median methylation levels of each group

frequency and ER, and PR positive tumors (P = 0.005, and P = 0.022 respectively). This study and others [29–31] provided an evidence for the association between epigenetic changes and hormonal receptor regulation in breast cancer. In addition, Conway et al. [30] reported that *SCGB3A1* promoter was highly methylated in the luminal A type of breast tumor and linked the aberrant methylation events with exposures that modulate the risk of tumor subtypes. On the other hand, previous studies showed no consistent associations with hormonal status [23, 24]. This disagreement could be related to differences in laboratory or statistical methodology, the sample size or clinical status of the subjects.

In line with previous studies [32, 33], the frequency of *SCGB3A1* promoter methylation was significantly associated with LN metastasis (P = 0.015) and lymphovascular invasion (P = 0.012). This finding indicates that *SCGB3A1* methylation can be used as a prognostic marker for breast cancer.

In the present work, we found no association between *SCGB3A1* methylation frequency and the age (P = 0.564), tumor size (P = 0.083), histopathological type (P = 0.672), pathological grade (P = 0.123), or TNM staging (P = 0.061). Similar results were detected by Feng et al. [9] and Callahan et al. [31]. On the other hand, previous studies reported significant differences in tumor size [22, 29, 32], the grade [32], and stage of the tumor [26].

ATM phosphorylate a large number of downstream proteins after its activation by double-stranded DNA breaks. These proteins include those involved in the cell-cycle checkpoint arrest, DNA repair like BRCA1 and RAD51, and apoptosis such as p53. It also modulates networks not immediately engaged in DNA repair like insulin-like growth factors and other metabolic and stress-response pathways. Subsequently, silencing of the *ATM* gene expression may hinder the repair of the damaged DNA and augment cancer development [34]. Previous studies reported that diminished *ATM* expression occurs in a significant portion of breast tumors [35, 36]. These findings led us to hypothesize the link between epigenetic events and the reduced ATM function in sporadic breast cancer. However, the reported results about the role of DNA hypermethylation as a mechanism for ATM protein expression deregulation in breast tumors are conflicting.

In this work, a non-significant difference was found in *ATM* methylation levels in the tumor group compared to the normal group (P = 0.315). Similarly, Cao et al. [13] used two different assays and reported the same results. Krasteva et al. [21] demonstrated a normal methylation profile of the *ATM* promoter in all studied cases. Other studies [37–39] come to the same conclusion, implying that *ATM* promoter hypermethylation is not involved in the neoplastic pathways of sporadic breast cancer.

On the other hand, previous studies have demonstrated *ATM* hypermethylation in breast cancer patients. Flanagan et al. [16] showed significant hypermethylation of one intragenic repetitive element in breast cancer cases when compared to matched controls, and Brennan et al. [40] found a strong association of *ATM* methylation levels in the family group, but the CpG sites they investigated were in the gene body or intragenic region not in the promoter. Other studies [2, 41] showed promoter hypermethylation for the *ATM* gene in cancer tissue samples compared to normal tissues. The differences in sample size or methylation methodology could explain this distinct methylation pattern.

In our study, we found no correlation between *ATM* promoter DNA methylation and the various clinicopathological features of the tumor. This result agreed with Cao et al. [13] who displayed the same finding. On the other hand, Begam et al. [2] has detected an association between *ATM* promoter methylation and the age, tumor size, and advanced stage of the disease.

The current study results did not support the contribution of the *ATM* promoter hypermethylation in the neoplastic pathways of breast cancer. However, such an

Table 4 The associations between *ATM* and *SCGB3A1* methylation frequency and the clinicopathological characteristics of the studied patients

Variables	*ATM*		*P*	*SCGB3A1*		*P*
	< 8.4 (n = 29)	≥ 8.4 (n = 21)		< 43.4 (n = 20)	≥ 43.4 (n = 30)	
	n (%)	n (%)		n (%)	n (%)	
Age (years)						
≤ 50	12 (48)	13 (52)	0.152	9 (36)	16 (64)	0.564
> 50	17 (68)	8 (32)		11 (44)	14 (56)	
Family history						
Yes	14 (60.9)	9 (39.1)	0.704	11 (47.8)	12 (52.2)	0.297
No	15 (55.6)	12 (44.4)		9 (33.3)	18 (66.7)	
Menstrual history						
Post menopause	17 (53.1)	15 (46.9)	0.352	12 (37.5)	20 (62.5)	0.630
Pre menopause	12 (66.7)	6 (33.3)		8 (44.4)	10 (55.6)	
Focality						
Unifocal	24 (54.5)	20 (45.5)	0.380^{FE}	20 (45.5)	24 (54.5)	0.069^{FE}
Multifocal	5 (83.3)	1 (16.7)		0 (0)	6 (100)	
Tumor size (cm)						
< 2	17 (68)	8 (32)	0.152	7 (28)	18 (72)	0.083
≥ 2	12 (48)	13 (52)		13 (52)	12 (48)	
Histopathological type						
IDC	25 (56.8)	19 (43.2)	1.000^{FE}	17 (38.6)	27 (61.4)	0.672^{FE}
ILC	4 (66.7)	2 (33.3)		3 (50)	3 (50)	
Pathological grade						
Early (GI + GII)	21 (58.3)	15 (41.7)	0.939	12 (33.3)	24 (66.7)	0.123
Late (GIII)	8 (57.1)	6 (42.9)		8 (57.1)	6 (42.9)	
Lymph vascular invasion	27 (62.8)	16 (37.2)	0.115^{FE}	14 (32.6)	29 (67.4)	0.012*^{FE}
LN metastasis	19 (67.9)	9 (32.1)	0.111	7 (25)	21 (75)	0.015*
TNM stage						
IA	4 (44.4)	5 (55.6)	0.709^{MC}	6 (66.7)	3 (33.3)	0.061^{MC}
IIA+IIB	19 (59.4)	13 (40.6)		13 (40.6)	19 (59.4)	
III	6 (66.7)	3 (33.3)		1 (11.1)	8 (88.9)	
ER positive	21 (58.3)	15 (41.7)	0.939	10 (27.8)	26 (72.2)	0.005*
PR positive	18 (56.3)	14 (43.8)	0.738	9 (28.1)	23 (71.9)	0.022*
HER2/neu positive	22 (56.4)	17 (43.6)	0.741^{FE}	18 (46.2)	21 (53.8)	0.163^{FE}
Molecular type						
Luminal A	7 (63.6)	4 (36.4)	0.741^{FE}	2 (18.2)	9 (81.8)	0.163^{FE}
Luminal B	14 (56)	11 (44)	0.774	8 (32)	17 (68)	0.248
HER2 enriched	8 (57.1)	6 (42.9)	0.939	10 (71.4)	4 (28.6)	0.005*

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *LN* lymph node, *TNM* tumor-node-metastasis classification system, *ER* estrogen receptors, *PR* progesterone receptors, *HER2/neu* human epidermal growth factor receptor 2

P value, compares the studied groups using χ^2 Chi-square test, *MC* Monte Carlo, *FE* Fisher's exact tests; *, statistical significance at $P \leq 0.05$

association could not be excluded. So, more studies to validate these results are needed. Other epigenetic mechanisms may have a role in the functional activity of *ATM* in the tumorigenesis process. Moreover, the molecular pattern of the *ATM* gene, and its protein expression, which are lacking in our study, are complementary, and both should be considered to be done in further research.

Various environmental factors such as nutrition, stress, working habits, smoking, alcohol consumption, and

lifestyle trigger epigenetic mechanisms, including DNA methylation, so that genetic methylation profile is different among populations [42, 43]. This study focused on the Egyptian population with their geographic, environmental, and lifestyle characteristics.

Other factors should also be considered when interpreting our results, the relatively small sample size, the different CpG sites, and the technique used for quantification, although we used the sensitive pyrosequencing assay for methylation analysis.

Larger sample sizes from different populations and ethnicities, as well as more studied CpG sites, are recommended to shed more light on the role of *SCGB3A1* and *ATM* promoters' methylation in breast cancer development and the potential use of these genes as biomarkers for breast cancer detection. The current study is also limited by the lack of correlation of our results with the molecular pattern of both genes and their protein expression, so additional research involving more insight on these parameters is recommended in the future, which may clarify more details and help to get a clearer picture on these molecular processes and breast cancer development.

Conclusions

In conclusion, *SCGB3A1* promoter methylation was significantly higher in the tumor group and was significantly associated with different clinicopathologic features of the tumor. It was associated with ER and PR positive tumors, LN metastasis, and lymphovascular invasion. These findings provided an empirical evidence that aberrant *SCGB3A1* promoter methylation may contribute to the pathogenesis of breast cancer and may have a role in distinguishing between malignant disease and normal. Thus, it may be considered as a suitable biomarker for diagnosis and prognosis. However, the promoter methylation levels of the *ATM* gene in breast cancer cases were unable to differentiate between breast cancer tissues and adjacent normal tissues. No association was found between the DNA methylation status of the *ATM* gene promoter and the clinicopathological features of the tumor. More investigations are recommended to validate these results and give more insight into the role of both genes in breast cancer development, diagnosis, and prognosis.

Abbreviations

SCGB3A1: Secretoglobin 3A1; *ATM*: Ataxia-telangiectasia mutated; CpG: Cytosine-guanine; HIN-1: High in normal-1; IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; LN: Lymph node; TNM: Tumor-node-metastasis classification system; ER: Estrogen receptors; PR: Progesterone receptors; HER2/neu: Human epidermal growth factor receptor 2; TNM: Tumor-node-metastasis classification system

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Authors' contributions

AMN performed the design of the study, made the literature review, carried out the molecular genetic studies, analyzed and interpreted the patient data, and was a major contributor in writing the manuscript. SSA performed the immunohistochemistry and pathological examination of the samples and revised the manuscript. AFM provided the surgical specimens and revised the manuscript. HEM participated in writing and revision of the manuscript. HMN participated in the design of the study, carried out the molecular genetic studies, and revised the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and analyzed during the current study are available on request.

Declarations

Ethics approval and consent to participate

The study was approved by the local ethics committee of the Medical Research Institute, Alexandria University (reference number: IORG 0008812), in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for research involving humans, and written informed consents were obtained from all included patients before the acquisition of the tissue specimens.

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

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