

SOX17 and RASSAF1A Promoters Methylation in Circulation Tumor Cell and Cell Free Deoxyribonucleic Acid Isolated from Plasma in Breast Cancer

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ABSTRACT

Purpose: The study was to evaluate *SOX17*, and *RASSEF 1A* promoters methylation levels in patients with malignant breast tumors compared with healthy women.

Materials and methods: Fifty women with breast cancer (range 26 to 75 years) were included in this study. They were admitted at Al-Diwaniyah Teaching Hospital, Iraq, compared with 25 women as the control. We obtained plasma samples from 50 patients (breast cancer) stages II, III, IV and normal (healthy women). Cell-free deoxyribonucleic acid (DNA) (cfDNA) extracted and methylation analysis for *SOX17* and *RASSEF1A* gene.

Results: We found different methylation pattern of the *RASSEF1A* gene in plasma samples between patients with breast cancer and control ($p < 0.05$) ranging in all cases 30 (60%) and unmethylation in 20 (40%), hyper methylation frequency in all studied cases [13 (44.8%) for II stage, 12 (80%) III stage and 4 (66.6%) IV stage of breast cancer in this study association between the hypermethylation was significant with age ≥ 50 11.4 (71.2%) and < 50 26 (76%)] than normal cases, we found different methylation pattern of the *SOX17* gene in plasma samples between patients with breast cancer and control ($p < 0.05$) ranging in all cases 29 (58%) and unmethylation in 21 (42%), hyper methylation frequency in all studied cases 11 (37.9%) for II stage, 13 (86.6%) III stage and 5 (83.3%) IV stage of breast cancer in this study association between the hyper methylation was significant with age (≥ 50 13 (81.2%) and < 50 23 (67%) than normal cases also this study association between the hypermethylation was significant with menopause (premenopause 6 (66%), post menopause 31 (75.6%) than the control group (0%).

Conclusions: Our data indicate that methylation of the *RASSAF1A* and *SOX17* genes are the frequent event in primary breast cancer and that it plays a major role in the silencing of the expression of this gene during tumor development.

Keywords: Breast cancer, Cell-free deoxyribonucleic acid (DNA) Methylation, *RASSAF1A* gene, *SOX17* gene.

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INTRODUCTION

Breast cancer is the most common malignancy among women worldwide.^{1,2} The prognosis of this disease mainly depends on its early detection, which currently to a major part relies on mammography. Early detection of this disease can also be facilitated by new diagnostic biomarkers³ cell-free DNA (cfDNA) and circulating tumor cells (CTCs), released into the bloodstream by solid tumors, are considered real-time liquid biopsies in cancer patients reflecting the disease complexity and progression. The liquid biopsy represents a surrogate material for the molecular characterization of solid cancers.⁴ In fact, in the advanced stages when the tumor has acquired genetic, epigenetic and expression features which may be very different from those of the primary tissue, the liquid biopsy represents the actual molecular signature of the disease. Total cfDNA concentration, higher in cancer patients than healthy individuals,⁵ has been proposed as a cancer marker, but it has shown limited sensitivity and specificity.^{6,7} DNA methylation acts as a major epigenetic mechanism within the complex network of mechanisms regulating gene expression.⁸ The chemically stable addition of a methyl (CH₃) group at the carbon five positions of the cytosine ring by DNA methyltransferases (DNMTs) interferes with the capacity of several proteins to bind DNA.⁹ Methylation in CpG-rich gene promoters serves as recognition signals for proteins mediating transcriptional repression and constitutes a mechanism for gene regulation.^{10,11} Methylation in repetitive transposable elements plays an important role in their silencing and contributes to global genomic stability¹²⁻¹⁴ Physiologically methylation patterns are established during early embryogenesis.

Ras association domain family one isoform (*ARASSF1A*) is a tumor suppressor gene, whose inactivation, mainly achieved by promoter hypermethylation, is involved in the development of many cancers.¹⁵ So far the role

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of *RASSF1A* methylation as a biomarker in cfDNA has been investigated, mostly in combination with other parameters, in a variety of tumor such as breast cancer.¹⁶ Methylation of *RASSF1A* increases significantly with advancing clinical stage, suggesting that the inactivation of this gene is associated with tumor progression.¹⁷ *RASSF1A* promoter hypermethylation has been detected in cfDNA from melanoma patients¹⁸ in association to a worse response to therapy and reduced overall survival.¹⁹ In melanoma²⁰ *SOX17*, a member of the Sry-related high-mobility group box gene family is a high-mobility group box transcription factor that is known to function as a key regulator in various developmental and disease contexts, including endoderm organ development,^{21,22} vascular development,²³ and stem cell function regulation.²⁴ Besides, *SOX17* also plays an essential role in human carcinogenesis. Hypermethylation of the *SOX17* promoter is correlated with poor prognosis in several cancers.²⁵ In a previous study, we demonstrated that *SOX17* is often hypermethylated and provides important prognostic information in breast cancer patients. Recent studies have shown that *SOX17* is also epigenetically silenced in circulating tumor cells isolated from the peripheral blood of patients with breast or gastric cancer²⁶ and that such silencing can be used as a molecular diagnostic marker in stage cancer. Therefore, this study aimed to evaluate the prognostic significance of *SOX17* promoter methylation in breast cancer cancer (Fig. 1).^{27,28}

MATERIALS AND METHODS

Study Patients

A total of 50 women with breast cancer (range 26 to 75 years) were included in this study. They were admitted at Al-Diwaniyah Teaching Hospital, Iraq, compared with 25 women as the control. We obtained plasma samples from 50 patients (breast cancer) stage II, III, IV and normal (healthy women). This study was conducted during the period from September 2016 to April 2017, were analyzed and compared to determine the tumor.

Cell-free DNA was extracted blood (5 mL) was collected in K3 EDTA-containing tubes. The plasma was isolated by low-speed centrifugation: 500 × g for 10 minutes using centrifuge (Gottingen, Germany). It was (cfDNA) was isolated from 2 mL plasma using was synthesized using M-MLV cfDNA synthesis kit (Quick-cfDNATM serum and plasma DNA miniprep kit, USA) Transcript expression was evaluated using gene-specific primers unmethylation (*RASSF1A*)TM 57° C with the following cycling parameters: followed by 40 cycles and methylation (*RASSF1A*)TM 58° C with the following cycling parameters: followed by 40 cycles the PCR products were examined by electropho-

resis polymerase chain reaction (PCR) conditions were as follows: 95° C for 4 min and then 40 cycles of 95° C for 40seconds, 58° C for 20seconds, and 72° C for 30seconds, with a final extension for 5 minutes at 72° C.

Also, unmethylation (*SOX17*) Tm (58)° C with the following cycling parameters: followed by 40 cycles and methylation (*SOX17*)TM (59)° C with the following cycling parameters: followed by 40 cycles the PCR products were examined by electrophoresis PCR conditions were as follows: 95° C for 4 minutes and then 40 cycles of 95° C for 40seconds, 59° C for 20seconds, and 72° C for 30seconds, with a final extension for 5 minutes at 72° C.

Methylation Analysis

Genomic DNA was extracted and treated with bisulfite by using the EZ MethylationTM Kit (USA). Methylation specific PCR (MSP) primers of four selected candidate genes were prepared as described previously.²⁶ MSP was performed using 5 µL of modified cfDNA (USA) PCR system Furthermore, the methylation was calculated from threshold cycles (CT) values. Lastly, MSPs were analytically validated using methylated DNA by using gene-specific primers methylation (*RASSF1A*) (MR) (5'-CCC TCC CAA ACG CCG A-3') MF (5'-GCT AAC AAA CGA GAA CCG-3')TM 58° C and unmethylated (57° C with the following cycling parameters: followed by 40 cycles, also MSPs were analytically validated using methylated DNA by using gene-specific primers methylation (*SOX17*)TM 59° C and methylated 58° C MR (5'-TTG CGT TAG TCG TTT GCG TTC-3') MF (5'-CAA AAA CGA ATC CCG TAT CCG ACG-3') with the following cycling parameters: followed by 40 cycles.

Statistical Analysis

By using Statistical Package for the Social Sciences (SPSS version 23), student test (t-test) was used for the quantitative data. The lowest level of significance was when the probability (p < 0.05).

RESULTS

We found different methylation pattern of the *RASSEF1A* gene in plasma samples between patients with breast cancer and control (p < 0.05) ranging in all cases 30 (60%) and unmethylation in 20(40%), hypermethylation frequency in all studied cases [13 (44.8%) for II stage, 12 (80%) III stage and 4 (66.6%) IV stage] of breast cancer in this study association between the hypermethylation was significant with age [≥ 50 11.4 (71.2%) and 50 26 (76%)] than normal cases also this study association between the hypermethylation was significant with Menopause [premenopause 5 (55%), postmenopause 27.3 (65.8%)]

than control group (0%) as shown in Figures 2A and B and Table 1.

We found different methylation pattern of the *SOX17* gene in plasma samples between patients with breast cancer and control ($p \leq 0.05$) ranging in all cases 29 (58%) and unmethylation in 21 (42%), hypermethylation frequency in all studied cases [11 (37.9%) for II stage, 13 (86.6%)

III stage and 5 (83.3%) IV stage] of breast cancer in this study association between the hypermethylation was significantly with age [$\geq 50^{13}$ (81.2%) and 50 23 (67%)] than normal cases also this study association between the hypermethylation was significant with Menopause (premenopause 6 (66%), postmenopause 31 (75.6%) than control group (0%) as shown in Figures 3A and B and Table 1.

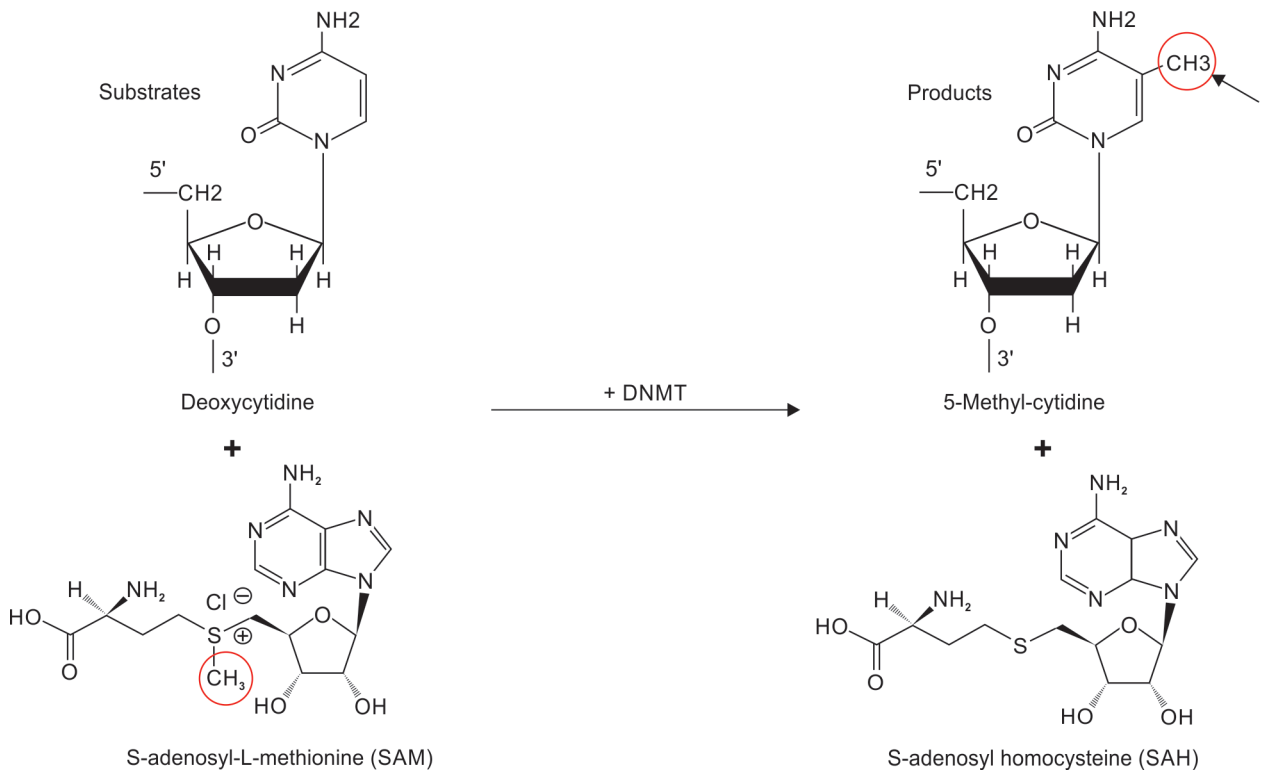
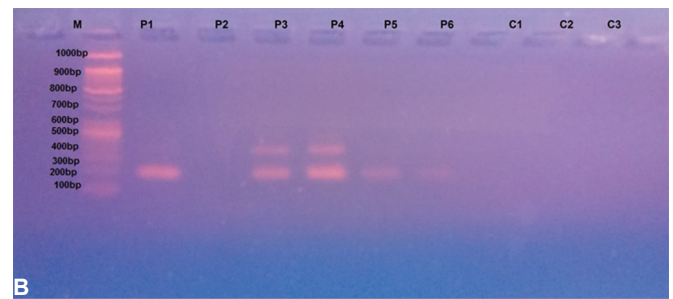
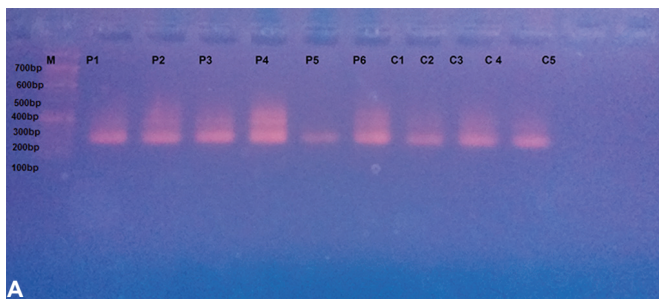
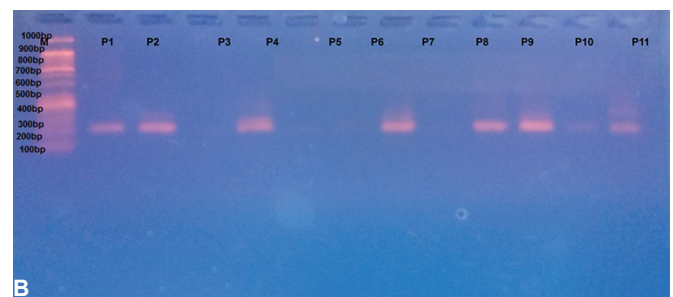
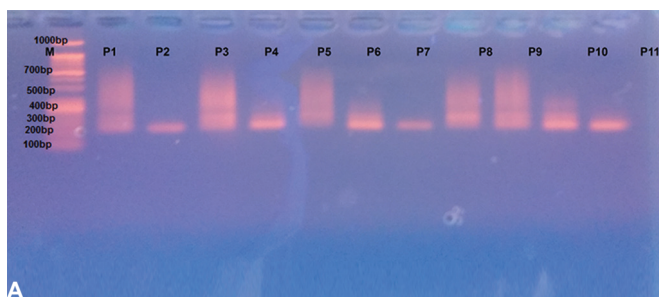


Fig. 1: Methylation of cytosine catalyzed by DNMTs²⁸



Figs 2A and B: (A) Unmethylated GEL picture of *RASSF1A* gene; (B) Methylated GEL picture of *RASSF1A* gene (p: patient, c: control) in breast cancer



Figs 3A and B: (A) Unmethylated GEL picture of *SOX17* gene (p: patient) in breast cancer; (B) Methylated GEL picture of *SOX17* gene (p: patient) in breast cancer

Table 1: Correlation analysis of RASSF1A gene and SOX17 methylation in breast cancer plasma

Clinical information	N	RASSF1A M %	RASSF1A UM %	SOX17 M %	SOX17 UM %	t-test
All cases	50	30 (60%)	20 (40%)	29 (58%)	21 (42%)	p ≤ 0.05
Age ≥ 50	16	11.4 (71.2%)	5 (31.2%)	13 (81.2%)	3 (18.7%)	p ≤ 0.05
< 50	34	26 (76%)	8 (23.5%)	23 (67%)	11 (32.3%)	
Stage						
II	29	13 (44.8%)	16 (55%)	11(37.9%)	18 (62%)	p ≤ 0.05
III	15	12 (80%)	3 (20%)	13 (86.6%)	2 (13.3%)	
IV	6	4 (66.6%)	2 (33.3%)	5 (83.3%)	1 (16.6%)	
Menopause						
Pre menopause	9	5 (55%)	4 (44%)	6 (66%)	3 (33%)	p ≤ 0.05
Post menopause	41	27.3 (65.8%)	14 (43%)	31 (75.6%)	10 (24.3%)	

DISCUSSION

Solid tumor release into the circulation cfDNA and CTCs which represent promising biomarkers for cancer diagnosis. Circulating tumor DNA may be studied in plasma from cancer patients by detecting tumor-specific alterations, such as genetic or epigenetic modifications association *RASSEF1A*. *RASSEF1A* promoter methylation is variably present in breast carcinoma. In this study, we investigated the diagnostic performance of *RASSF1A* promoter methylation in cfDNA as a non-invasive marker of tumor DNA in patients. We focused our attention on the methylated form of the marker since it is supposed to represent the fraction of circulating DNA deriving from the tumor by any of the hypothesized mechanisms of necrosis, apoptosis and active release.²⁹ We considered patients at different stages of breast cancer disease to test the reliability of the epigenetic marker under study independently from the stage of the tumor. The absolute levels of the epigenetic marker are significantly higher in breast cancer compared to controls and increases during tumor progression (from *in situ* to invasive and metastatic disease). To evidence, both the diagnostic and prognostic. We found no significant correlation among the two biomarkers analogously to what recently reported in a study on breast cancer patients.³⁰ The univariate analysis performed allowed us to assess the performance of methylated *RASSF1A* as a marker of tumor-related ctDNA in breast cancer for diagnostic purposes being able to discriminate the *in situ* and invasive breast cancer from control and as an indicator of patients prognosis.⁷

The *RASSF1A* family members were found to be frequently inactivated in many cancers, and such inactivation was correlated with promoter region methylation.^{31,32} The CpG island methylation of *RASSF1A* was reported in previous studies,³³ detected a correlation between age and methylation status.³⁴

On the other hand, our study found a correlation between tumor stage and differential with *RASSF1A*. It is well accepted that DNA promoter region methylation could result in loss and alteration of gene expression gene expression in a dose-dependent manner. Therefore, all the combined data from our study confirmed the epigenetic effect on gene expression. These study similar to the studies.³⁵ *RASSF1A* is a tumor suppressor gene silenced by promoter hypermethylation in a variety of human cancers including breast cancer.⁷

SOX17 promoter methylation is variably present in breast carcinoma *SOX17*, one of the tumor suppressor genes shown to be epigenetically silenced in CTCs of patients with breast cancer.³⁶ For this study, we report our findings that *SOX17* was epigenetically inactivated and there are differences in levels of methylation between healthy individuals and patients with malignant conditions. We have shown that the *SOX17* promoter is highly methylated in primary breast tumors, in cfDNA isolated both from patients with breast cancer and those with metastasis-verified breast cancer. These results to other studies³⁷ methylation status of cancer-related genes is considered to be a promising biomarker for the early diagnosis and prognosis of tumors. This study investigated the methylation status of the *SOX17* gene in breast cancer. *SOX17* methylation correlated with different stage and lymph node metastasis suggested that epigenetic silencing of *SOX17* may also accelerate the spread of cancer through influencing the development of an invasive and biologically aggressive phenotype and thus expedite the progression of breast cancer suggesting that *SOX17* methylation in peripheral blood samples may be a good tumor marker for the diagnosis of breast cancer. Thus, *SOX17* promoter methylation in plasma DNA is highly specific and can provide important prognostic information for patients with breast cancer. *SOX17* gene silencing due to promoter methylation may deactivate its tumor suppressor

role. The tumor-suppressing function of no methylated *SOX17* may be partially maintained in patients without *SOX17* methylation, and this may slow the progress of tumor development.³⁸

CONCLUSION

Our data indicate that methylation of the *RASSAF1A* and *SOX17* genes are a frequent event in primary breast cancer and that it plays a major role in the silencing of the expression of this gene during tumor development.

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