

## Prognostic significance of *RASSF1A* promoter methylation in operable breast cancer

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### Abstract

**Objectives:** The aim of our study was to evaluate the prognostic significance of *RASSF1A* promoter methylation status in operable breast cancer.

**Design and methods:** By using Methylation Specific PCR, we evaluated the specificity of *RASSF1A* promoter methylation in 10 breast tumors and matching normal tissues, 10 breast fibroadenomas and 11 normal breast tissues. The prognostic significance of *RASSF1A* methylation was validated in 93 formalin fixed paraffin-embedded (FFPE) tissues obtained from patients with operable breast cancer.

**Results:** Methylation of *RASSF1A* promoter was observed in 1/31 (3.2%) non-cancerous breast tissues and 53/93 (57.0%) early stage breast tumors. The only positive sample in the non-cancerous breast tissues group was found in a histological normal tissue surrounding the tumor. During the follow-up period, 24/93 (25.8%) patients relapsed and 19/93 (20.4%) died. Disease-Free-Interval (DFI) was significantly associated with *RASSF1A* methylation ( $p=0.028$ ).

**Conclusions:** *RASSF1A* promoter methylation provides important prognostic information in early stage breast cancer patients.

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**Keywords:** *RASSF1A*; DNA methylation; Operable breast cancer; Epigenetic markers; Prognostic biomarker; Methylation Specific PCR

### Introduction

DNA methylation is one of the most frequently occurring epigenetic events taking place in the mammalian genome at cytosine bases that are located 5' to a guanine, in a CpG dinucleotide. Short CpG rich regions ranging from 0.5 to 4 kb, known as CpG islands, are found in the proximal promoter regions of almost half of the human genes and are generally unmethylated in normal cells, while most CpGs outside CpG islands are methylated [1,2]. In cancer cells however, methylation pattern includes a loss of global methylation and a gain of methylation at selected promoter CpG islands [3].

Promoter methylation is found in virtually every type of human neoplasm and is associated with transcriptional silencing

of hundreds of genes, including several known tumor suppressor genes [4]. In recent years, the list of tumor suppressor genes that are inactivated by epigenetic events rather than classic mutation events has been growing. Unlike mutational inactivation, methylation is reversible and demethylating agents and inhibitors of histone deacetylases are being used in clinical trials. Furthermore, highly sensitive and quantitative assays have been developed to assess methylation in tumor samples, early lesions, and bodily fluids. Hence, gene silencing by promoter methylation has potential clinical benefits in early cancer diagnosis, prognosis and treatment.

The *RASSF1* gene locus spans about 11,151 bp of the human genome and is comprised of eight exons. Differential promoter usage and alternative splicing generates seven transcripts (*RASSF1A-G*). The *RASSF1A* (RAS-association domain 1 isoform A) gene has been quite recently cloned and characterized [5–7] acting as a tumor suppressor gene that obeys

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Knudson's two hit model [8]; it is likely that the first hit is epigenetic silencing through methylation of its CpG island promoter and the second hit is 3p loss [9]. Particularly, *RASSF1A* is frequently silenced by promoter methylation in many primary tumors, including breast, lung, kidney, bladder, and other cancers [10]. Furthermore, *RASSF1A* is a member of a new group of Ras effectors, and its functional analysis has revealed an involvement in apoptotic signaling [11], microtubule stabilization [12] and mitotic progression [13].

*RASSF1A* has been previously demonstrated to be inactivated by CpG island methylation in breast cancer cell lines and primary tumor tissues. Despite the fact that methylation of this gene has been shown to have a significant impact on biological characteristics of breast tumors, the relationship between CpG island methylation of this gene and prognosis in breast cancer has not been reported as yet.

In this work we evaluated the prognostic significance of *RASSF1A* promoter methylation in early stage breast cancer. By using Methylation Specific PCR, we first evaluated the specificity of *RASSF1A* promoter methylation in 10 breast tumors and their matching non-cancerous breast tissues, 10 breast fibroadenomas and 11 normal breast tissues. The prognostic significance of *RASSF1A* methylation was validated in 93 formalin fixed paraffin-embedded (FFPE) tissues obtained from early stage breast cancer patients.

## Design and Methods

### Cell lines and tissues samples

The study material consisted of a total of 134 breast formalin fixed paraffin-embedded tissues (FFPEs): 93 paraffin-embedded breast carcinomas, obtained from patients with operable breast cancer with a known clinical outcome and a long follow-up period, 10 paraffin-embedded breast carcinomas together with their 10 paired adjacent normal tissues, 10 breast fibroadenomas and 11 breast tissue specimens obtained from reduction mammoplasty (histologically cancer-free). In total, the 31 non-cancerous paraffin-embedded breast tissues were used as controls for the evaluation of the specificity of *RASSF1A* promoter methylation in tumors. All patients were enrolled to adjuvant chemotherapy research protocols of the Hellenic Oncology Research Group (HORG) [i.e. FEC regimen or sequential docetaxel followed by epirubicin in combination with cyclophosphamide (D/EC regimen) or docetaxel in combination with epirubicin (DE regimen)]; patients with breast conservative surgery also received radiation treatment and those with hormone receptor-positive tumors received adjuvant tamoxifen for 5 years. All patients gave their informed consent and the study has been approved by the Ethical and Scientific Committees of our Institution. Tumor sections of 10  $\mu\text{m}$  containing more than 80% of tumor cells were cut and were used for DNA extraction and Methylation Specific PCR (MSP) analysis. The breast cancer cell line MCF-7 was used as positive control in MSP reactions for the detection of *RASSF1A* methylation.

### Isolation of genomic DNA

Genomic DNA was isolated from both paraffin tissues and breast cancer cell lines with the High Pure PCR Template Preparation kit (Roche, Germany), according to the manufacturer's protocol. DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA). Before proceeding to the sodium bisulfite conversion and MSP reaction steps, the genomic DNA integrity of all samples was assessed by amplifying BRCA1 exon 20 for mutation analysis by using the same primers as previously described [14].

### Sodium bisulfite conversion

1  $\mu\text{g}$  of extracted DNA was modified with sodium bisulfite (SB), in order to convert all unmethylated, but not methylated-cytosines to uracil. Bisulfite conversion was carried out using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), according to the manufacturer's instructions. Briefly, 1  $\mu\text{g}$  of denatured genomic DNA (gDNA) was treated with sodium bisulfite for 20 min. Subsequently, SB-treated samples were applied to supplied columns, columns were washed, desulfonation was conducted, samples were washed again and DNA was subsequently eluted with 10  $\mu\text{L}$  elution buffer. The converted DNA was stored at  $-70^\circ\text{C}$  until used. In each sodium bisulfite conversion reaction, dH<sub>2</sub>O and MCF-7 were included as a negative and positive control, respectively.

### Methylation Specific PCR (MSP)

MSP reaction was performed in a total volume of 25  $\mu\text{L}$ . 1  $\mu\text{L}$  of sodium bisulfite converted DNA was added into a 24  $\mu\text{L}$  reaction mixture that contained 0.1  $\mu\text{L}$  of Taq DNA polymerase (5U/ $\mu\text{L}$ , Platinum DNA polymerase; Invitrogen), 2.5  $\mu\text{L}$  of the supplied 10 $\times$ PCR buffer, 1.0  $\mu\text{L}$  of MgCl<sub>2</sub> (50 mmol/L), 0.5  $\mu\text{L}$  of dNTPs (10 mmol/L; Fermentas) and 1  $\mu\text{L}$  of the corresponding forward and reverse primers (10  $\mu\text{mol/L}$ ); finally dH<sub>2</sub>O was added to a final volume of 25  $\mu\text{L}$ . Thermocycling conditions used were 1 cycle at 95  $^\circ\text{C}$  for 5 min, followed by 40 cycles of 95  $^\circ\text{C}$  for 30 s, 56  $^\circ\text{C}$  for 45 s and 72  $^\circ\text{C}$  for 45 s, with a final extension cycle of 72  $^\circ\text{C}$  for 5 min. MSP products were fractionated on 2% agarose gels containing 40 mM Tris-acetate/1.0 mM EDTA (pH=8.0) and visualized by ethidium bromide staining. The sequences of primers used in this study were previously described [15]. Human placental genomic DNA (gDNA; Sigma-Aldrich) methylated *in vitro* with *SssI* methylase (NEB, Ipswich, MA) was used, after sodium bisulfite conversion, as a fully methylated (100%) MSP positive control; the same unmethylated placental gDNA, was used, after sodium bisulfite conversion, as a negative MSP control. All MSP reactions were performed in a blinded fashion with regard to the patient's clinical outcome.

### Statistical analysis

Correlation between methylation status and clinicopathological features of the patients was assessed by the Chi-square test. The prognostic significance of *RASSF1A* promoter methylation was assessed according to our results of the FFPEs samples from the 93 breast cancer patients. Disease free interval (DFI) and overall survival (OS) curves were calculated by using the Kaplan–Meier method and comparisons were performed using the log rank test. A Cox-regression analysis was performed in order to determine the relative contribution of various variables to the assessment of DFI and OS. *p* values  $\leq 0.05$  were considered statistically significant. Statistical analysis was performed by using the SPSS Windows version 11.0 (SPSS Inc., Chicago, IL).

## Results

### Sensitivity of *RASSF1A* promoter Methylation Specific PCR

The sensitivity of the developed MSP assay for *RASSF1A* was evaluated by using 1  $\mu\text{g}$  of a fully methylated DNA sample as positive control (100% methylation) serially diluted (10-fold dilutions) in 1  $\mu\text{g}$  of sodium bisulfite converted unmethylated DNA. The MSP assay for *RASSF1A* was performed in duplicate and it was sensitive enough to detect 0.5 ng of the methylated *RASSF1A* sequence in the presence of 1  $\mu\text{g}$  of unmethylated *RASSF1A* sequence. The sensitivity was 1:2000, corresponding to 0.05% *RASSF1A* methylated gDNA (Fig. 1A).

### Specificity of *RASSF1A* promoter methylation

To validate the specificity of the primers used for the methylated sequence of *RASSF1A*, we first tested these primers in silico and then in PCR reactions, using as a template bisulfite modified human placental gDNA that is not methylated: no amplification of *RASSF1A* was observed, while the same primers clearly recognized the methylated sequence when we used our fully methylated (100%) positive control (Fig. 1B, lane#12). The specificity of *RASSF1A* promoter methylation in tumor tissues was further confirmed by the absence of any detectable methylation of the *RASSF1A* promoter in all normal breast tissues from reduction mammaplasty (0/11), nor in any breast fibroadenoma (0/10); conversely, *RASSF1A* promoter was methylated in 4 (40%) out of 10 breast tumors and in only 1 (10%) out of 10 matching normal tissues. In total only 1/31 (3.2%) non-cancerous paraffin-embedded breast tissues were positive for *RASSF1A* promoter methylation (Fig. 1B).

### Clinical relevance of *RASSF1A* methylation status in operable breast cancer patients

The methylation status of *RASSF1A* was evaluated in paraffin-embedded breast carcinomas from 93 patients diagnosed with early breast cancer in double-blinded experiments. Patients' clinicopathological characteristics and clinical outcome data became available upon completion of analysis and were compared to the methylation status of *RASSF1A* (Table 1). *RASSF1A* promoter was found methylated in 53 (57.0%) out of

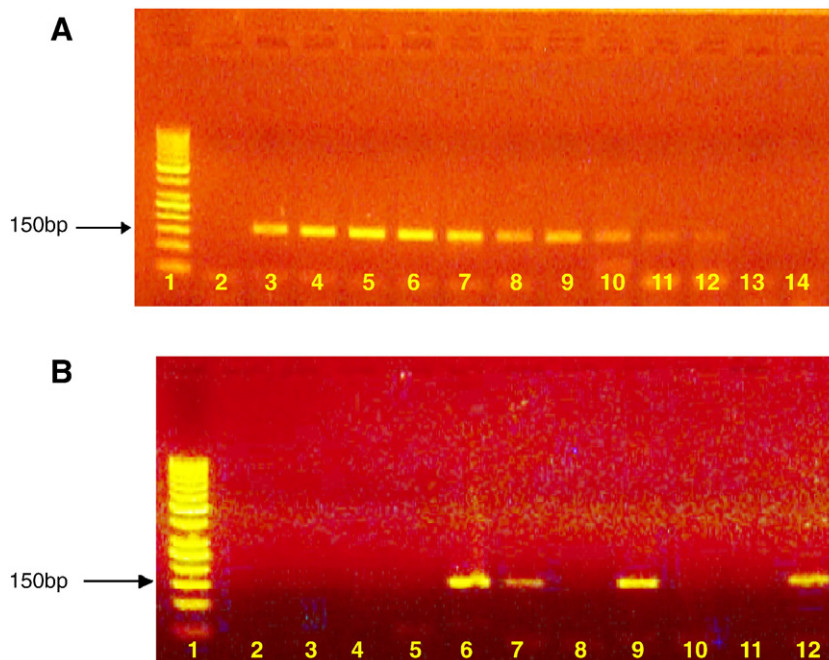


Fig. 1. (A) Sensitivity of Methylation Specific PCR for *RASSF1A*: #1. DNA marker 50 bp, #2. Negative Control, #3. 100% methylated DNA, #4. 50% methylated DNA, #5, 6. 5% methylated DNA, #7, 8. 0.5% methylated DNA, #9, 10. 0.1% methylated DNA, #11, 12. 0.05% methylated DNA, #13, 14. 0.005% methylated DNA. (B) Agarose gel electrophoresis of methylation specific PCR products for *RASSF1A* promoter methylation in breast tumors: # 1. DNA marker 50 bp, # 2. Negative Control, #3. Normal breast tissue, # 4. Breast fibroadenoma, # 5, 6. Pair of breast tumor and adjacent normal tissue, #7, 9. Breast tumors (methylated), #8, 10, 11 Breast tumors (unmethylated), #12. Positive control (MCF-7).

Table 1  
Correlation of *RASSF1A* methylation status with clinicopathological features of the patients.

Patient's characteristics	<i>RASSF1A</i>		<i>p</i> <sup>b</sup>
	<i>n</i> <sup>a</sup>	Methylation (%)	
<i>Menopausal status</i>			
Pre	38	21 (55.3)	0.780
Post	55	32 (58.2)	
<i>Tumor size (cm)</i>			
0–2.0	26	12 (46.1)	0.346
2.1–5.0	56	35 (62.5)	
>5.0	10	5 (50.0)	
<i>Axillary lymph node</i>			
0	27	13 (48.1)	0.514
1–3	30	19 (63.3)	
≥4	34	19 (55.9)	
<i>Tumor grade</i>			
I, II	42	26 (61.9)	0.906
III	42	24 (57.1)	
Lobular	5	3 (60.0)	
<i>Tumor stage</i>			
I	24	12 (50.0)	0.422
II	69	41 (59.4)	
<i>Estrogen receptor</i>			
Positive	56	35 (62.5)	0.187
Negative	37	18 (48.6)	
<i>Progesterone receptor</i>			
Positive	30	17 (56.7)	0.965
Negative	63	36 (57.1)	
<i>HER2 score</i>			
0–2+	72	40 (55.5)	0.283
3+	11	8 (72.7)	

<sup>a</sup> In cases that the total number of patients is less than 93, this is due to non available clinical information.

<sup>b</sup> Chi-square test.

93 breast tumor samples. As can be seen in Table 1, there was no correlation between the *RASSF1A* promoter methylation and the major characteristics of the patients.

After a median follow-up period of 76 months (range 12–116 months), 24 (25.8%) out of 93 patients relapsed. The incidence of relapses was significantly higher in patients with methylated (35.8%) than in those with unmethylated *RASSF1A* promoter (12.5%; Table 2;  $p=0.011$ ). The Kaplan–Meier estimates of the cumulative DFI for patients with methylated and non-methylated *RASSF1A* promoter were significantly

different in favor of patients with non-methylated *RASSF1A* promoter ( $p=0.028$ ; Fig. 2A).

During the follow-up period, 19 (20.4%) patients died as a consequence of disease progression. The incidence of deaths was higher in patients with methylated (28.3%) than in patients with unmethylated *RASSF1A* promoter (10.0%; Table 2;  $p=0.030$ ). Nevertheless, there was no difference in survival for patients with methylated or unmethylated *RASSF1A* promoter ( $p=0.128$ ; Fig. 2B).

#### Univariate and multivariate analysis

*RASSF1A* promoter methylation, menopausal and axillary lymph node status, tumor size, stage and grade, estrogen and progesterone receptor status and HER2 score were tested in univariate analysis for association with DFI. Detection of *RASSF1A* promoter was significantly associated with decreased DFI ( $p=0.028$ ); in addition, tumor stage and grade, number of involved axillary lymph nodes and HER2 score were significantly associated with decreased DFI ( $p=0.038$ ,  $p=0.025$ ,  $p=0.002$  and  $p=0.038$ , respectively). The same clinical and epigenetic variables were also tested in univariate analysis for OS. Estrogen receptor-negative tumors, number of involved axillary lymph nodes and tumor grade were significantly associated with a decreased OS ( $p=0.013$ ,  $p=0.037$  and  $p=0.001$ , respectively).

Multivariate analysis demonstrated that the number of involved axillary lymph nodes was independently associated with a high risk of relapse (HR = 5.982; 95% CI: 1.338 to 26.739  $p=0.019$ ). Similarly, the absence of estrogen receptor positivity (HR = 3.406; 95% CI: 1.215 to 9.547  $p=0.020$ ) was independently associated with an increased risk of death due to the disease.

#### Discussion

Epigenetic silencing due to DNA hypermethylation often leads to inactivation of the wild-type allele at sites of LOH that introduces one hit in the well-known Knudson's model for tumorigenesis that accounts for loss-of-function of tumor suppressor genes [8]. The potential of DNA methylation as a novel area of new biomarkers discovery is very promising. A number of interesting tumor suppressor genes, whose silencing through DNA methylation occurs has been evaluated in many types of cancer and especially in breast cancer as novel prognostic and predictive biomarkers [9,16,17].

In the present study the methylation status of *RASSF1A* promoter was evaluated as prognostic biomarker in operable

Table 2  
Incidence of disease-relapse and disease-related death according to the methylation status of *RASSF1A* promoter.

Gene	Methylation status	Relapses (%)	<i>p</i> <sup>c</sup>	Median DFI (range)	Deaths (%)	<i>p</i> <sup>c</sup>	Median OS (range)
<i>RASSF1A</i>	M <sup>a</sup> ( <i>n</i> =53)	19 (35.8)	<b>0.011</b>	90 (80–101)	15 (28.3)	<b>0.030</b>	102 (93–110)
	U <sup>b</sup> ( <i>n</i> =40)	5 (12.5)		97 (90–104)	4 (10)		114 (106–122)

<sup>a</sup> Methylated.

<sup>b</sup> Unmethylated.

<sup>c</sup> Chi square test.

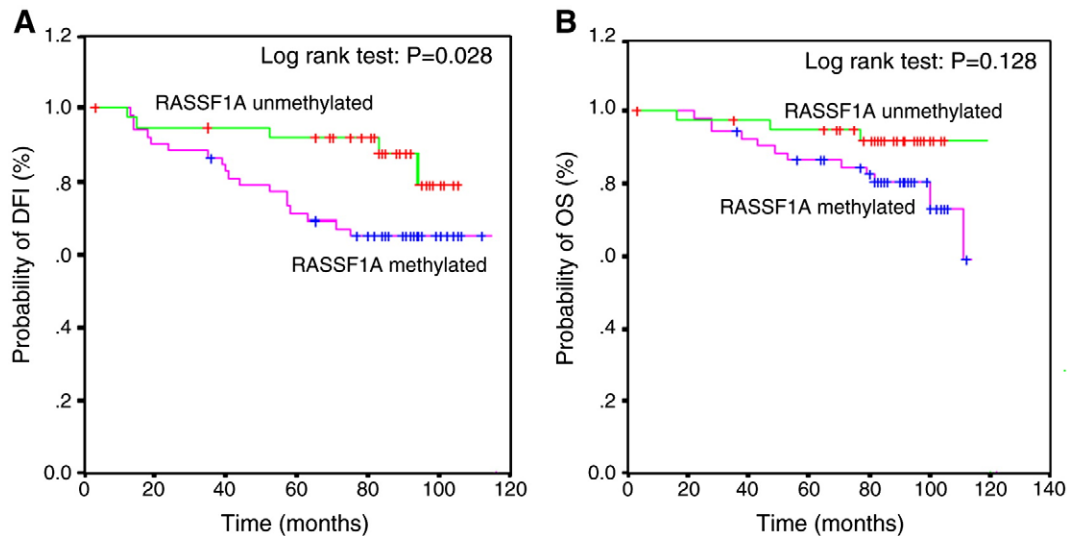


Fig. 2. (A) Kaplan–Meier estimates of DFI for operable breast cancer patients with or without *RASSF1A* methylation ( $p=0.028$ ). (B) Kaplan–Meier estimates of OS for operable breast cancer patients with or without *RASSF1A* methylation ( $p=0.128$ ).

breast cancer by MSP. MSP can reliably detect, as verified by our own experiments in serial dilutions, 1 methylated allele in a total of 2,000 unmethylated alleles [18]. The presented data show for the first time that the detection of methylation of this gene in FFPEs has significant prognostic implications in operable breast cancer patients.

Concerning the specificity, *RASSF1A* promoter was not found methylated in histologically normal breast tissue obtained by reduction mammoplasty, nor in breast fibroadenomas. Conversely, it was found methylated in 4 (40%) out of 10 breast tumors and in only 1 (10%) sample of matching normal breast tissues. In total, methylation of *RASSF1A* promoter was observed in 1/31 (3.2%) non-cancerous breast tissues while in 53/93 (57.0%) operable breast tumors. The only positive sample in the non-cancerous breast tissues group was found in a histological normal tissue surrounding the tumor. This result is in accordance to a previous report by Dammann et al. [16] who detected *RASSF1A* methylation in 7.5% of samples that were classified as normal tissue removed during the tumor excision; this finding was attributed to methylation which may occur as part of the aging process [19], or to the contamination of the normal samples by tumor cells.

In our study *RASSF1A* was identified as a target of methylation and silencing in 57% of tumors of early breast cancer patients, suggesting that inactivation of this gene is a frequent event in the process of mammary tumorigenesis. It is also interesting to note that according to our data, presented in Table 1 there was no difference in the methylation status of *RASSF1A* between groups of premenopausal and postmenopausal women. In breast cancer, the incidence of *RASSF1A* methylation, among independent studies, is around 60% [10] and our results are in agreement with such a high incidence of *RASSF1A* methylation. Particularly, Dammann et al. [16] detected *RASSF1A* promoter methylation in 62% of primary mammary carcinomas, Honorio et al. [20] demonstrated that *RASSF1A* promoter was methylated in 65% of invasive breast

carcinomas and in 42% of corresponding ductal carcinoma in situ (DCIS) and Burbee et al. [21] reported that 49% of primary breast tumors exhibited the methylated *RASSF1A* allele. Furthermore, Fackler et al. [15] demonstrated that *RASSF1A* was methylated in 62% of lobular carcinoma in situ (LCIS), in 84% of invasive lobular carcinoma (ILC), in 88%, 58% and 78% of DCIS grade 1, 2 and 3, respectively, and in 70% of invasive ductal carcinoma (IDC), while Yeo et al. [22] reported one of the highest frequencies of *RASSF1A* methylation (95%) in breast tumor tissues. It was very recently shown that ER-positive and ER-negative status relates to epigenetic changes in breast cancer-related genes including *RASSF1A* [23]. The importance of epigenetic silencing of *RASSF1A* is investigated in many types of cancer [24–26].

However, despite the fact that *RASSF1A* methylation has been shown to have a significant impact on the biological characteristics of breast tumors, the relationship between CpG island methylation of this gene and prognosis in breast cancer FFPE tissues has not been reported as yet. The prognostic value of *RASSF1A* aberrant methylation in breast cancer has only been shown in cell-free DNA circulating in pretherapeutic serum and was found among 39 genes to be of prognostic significance and independently associated with poor outcome [27]. In accordance to that study, our results demonstrate that *RASSF1A* methylation in FFPEs provides also important prognostic information, since patients with *RASSF1A* promoter methylation had shorter DFI than those without. It is probable that *RASSF1A* gene silencing due to promoter methylation deactivates its tumor suppressor role and can thus possibly contribute to a shorter survival in breast cancer patients.

In conclusion, our data demonstrate that *RASSF1A* promoter methylation provides important prognostic information in operable breast cancer patients and that this methylation plays an important role in the clinical behavior of breast tumors. Nevertheless, the methylation status of this gene should be

prospectively evaluated as a promising prognostic biomarker in a large cohort of patients with operable breast cancer.

#### Conflict of interest

None declared.

#### Disclaimers

None declared.

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