

Molecular Analysis of *RASSF1* Gene Methylation and mRNA Expression in Sporadic Breast Cancer

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Abstract

Introduction: The aim of this study was to validate innovative and reliable methods for studying DNA methylation of the first promoter of *RASSF1* gene (where its primary transcript *RASSF1A* in breast tissue is transcribed from), its mRNA expression and their evaluation in sporadic breast cancer.

Materials and methods: DNA and RNA from 81 frozen breast cancer tissues with known histopathological data as well as 4 normal breast tissues were analyzed. DNA methylation levels were assessed by Pyrosequencing, analyzing 9 CpG dinucleotides in the CpG island of the first *RASSF1* promoter. For mRNA expression, a real-time RT-qPCR method was used and validated with synthetic standards, with the SYBR Green PCR kit and a suitable set of standardized primers (Qiagen) for all *RASSF1* transcript variants (except G). For relative quantification of *RASSF1* expression, the $2^{-\Delta\Delta Ct}$ method was used with beta2-microglobulin as a reference gene.

Results: 59 samples were characterized as *RASSF1* normally-methylated (72.8%), while 22 samples as hyper-methylated (27.2%). Also, 40 samples were classified as *RASSF1* mRNA over-expressing (49.4%), while 41 (50.6%) as sub-expressing. No inverse correlation was found between methylation and *RASSF1* expression ($p=0.207$). Logistic regression showed that the probability of lymph node infiltration was increased by the presence of negative ER receptors ($p=0.008$, OR 0.09, CI 0.14-0.52), the percentage of methylation ($p=0.006$, OR 7.96, CI 1.82-34.86) and the level of *RASSF1* expression ($p=0.047$, OR 3.94, CI 1.02-15.29). Survival analysis showed that there is a marginal statistically significant correlation between metastasis and mRNA *RASSF1* over-expression (log rank test, $p=0.040$).

Conclusions: The evaluated assays appear to provide potentially useful information for the clinical management of breast carcinomas. A similar reliable assay for the methylation of the second *RASSF1* promoter should be evaluated in the future. Regarding the expression study, a new design strategy with probes specific for all different *RASSF1* transcripts, would provide additional power in the study and potentiate the use of new *RASSF1* biomarkers in the clinical evaluation of sporadic breast cancer.

Keywords: *RASSF1*; DNA methylation; Pyrosequencing; RT-qPCR; mRNA expression; Splice variants

Abbreviations: CI: Confidence Intervals; Cq: Quantification Cycle; CV: Coefficient of Variation; ER: Estrogen Receptor; IHC: Immunohistochemistry; LOH: Loss of Heterozygosity; OR: Odds Ratio; PCR: Polymerase Chain Reaction; PR: Progesterone Receptor; qMSP: Quantitative Methylation Specific PCR; RASSF: Ras-Association Domain Family; RT-qPCR: Reverse Transcriptase-Quantitative Polymerase Chain Reaction; SB: Sodium Bisulfite; SD: Standard Deviation; Tm: Melting Point.

Introduction

According to the World Health Organization, breast cancer is the second most common form of cancer in the world and by far the most common cancer in women, with estimated 1.67 million new cases of cancer diagnosed in 2012 (25% of all cancers). Regarding mortality, it is considered as the fifth leading cause of death from cancer in both sexes (522,000 deaths) and the most common cause of cancer death in women, after lung cancer [1].

For many years there was a suspicion that one or more tumor suppressors are localized in the 3p21.3 locus, because this area frequently suffers loss of heterozygosity (LOH) in many types of cancer [2-4]. In 2000, a gene located in this region was cloned and termed as *RASSF1* (Ras association domain family 1), because the translated protein contains a putative Ras association (RA) domain [5].

RASSF1 is a member of the Ras-Association Domain Family (RASSF), which comprises ten members. *RASSF1* to *RASSF6* harbor a C-terminal RA domain while *RASSF7* to *RASSF10* contain an N-terminal one. *RASSF1* gene contains eight exons (1 α , 1 β , 2 $\alpha\beta$, 2 γ , 3, 4, 5 and 6) and by alternative splicing transcribes at least 8 different variants (*RASSF1A* – *RASSF1H*) from two promoters (Figure 1). Both promoter regions are covered by CpG islands. The first one (737 bp, 85 CpGs, 71.5% GC) contains the first promoter region that transcribes the main transcript *RASSF1A*, but also *RASSF1D*, *RASSF1E*, *RASSF1F*, and *RASSF1G* variants. The second CpG island is intragenic (1365 bp, 139CpG, 67.9% GC) and contains the promoter region for the transcription of *RASSF1B* and *RASSF1C* variants [6].

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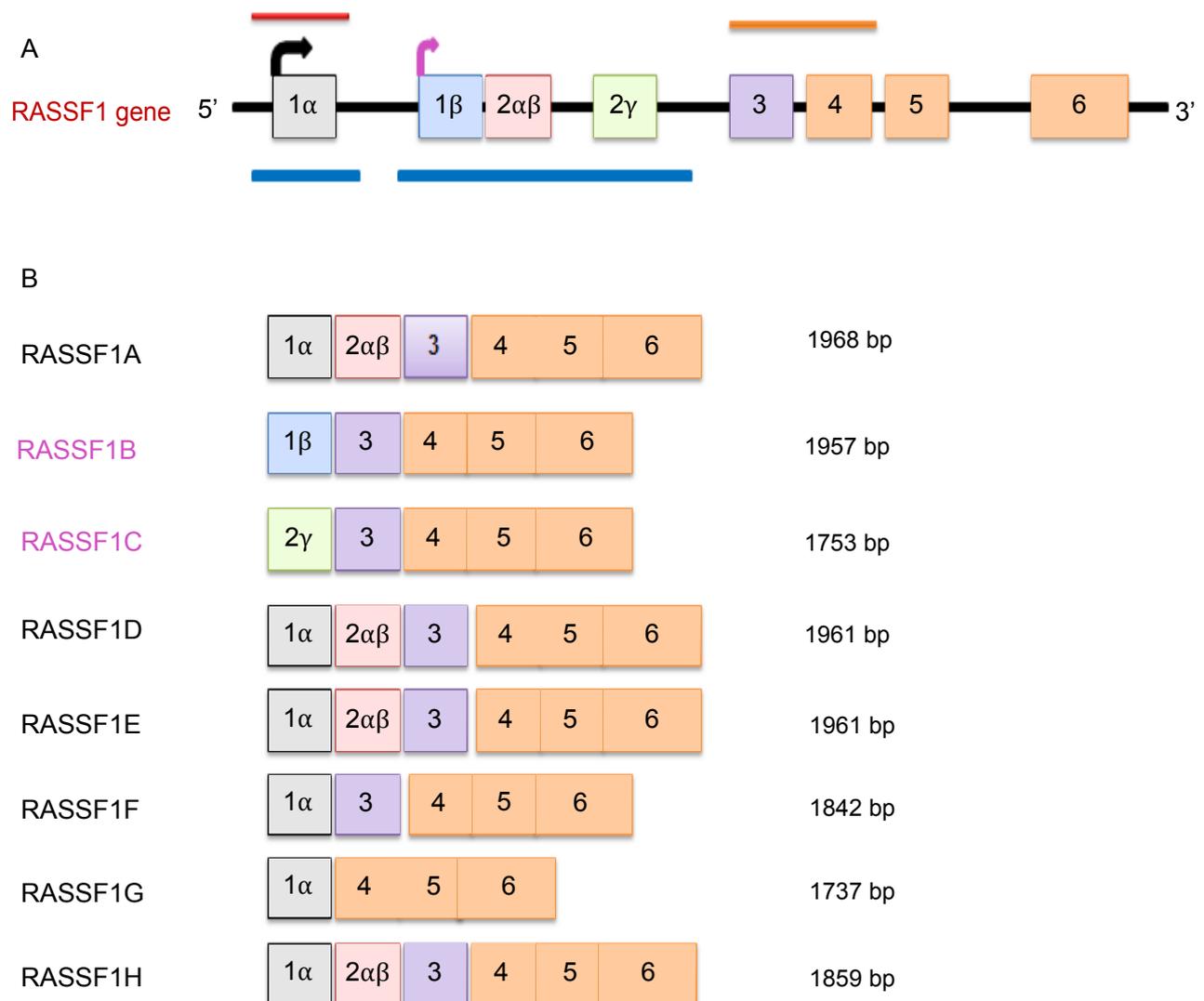


Figure 1: A) *RASSF1* genomic area in 3p21.3 contains eight exons (colored boxes) and two promoters (black and purple arrows). Two CpG islands (blue lines underneath) are covering both *RASSF1* promoter regions. The areas that are amplified before methylation analysis (red line, 117bp) and during RT-qPCR (orange line, 121 bp) are also shown. B) By alternative splicing, eight different variants have been identified (*RASSF1A* – *RASSF1H*) and their exon composition and transcript sizes are depicted.

The major splice variant of *RASSF1* is *RASSF1A* and its expression is variably down-regulated in many types of tumors and the main cause appears to be its promoter CpG island hypermethylation that has been proposed to be a valuable biomarker in many studies [7-14]. *RASSF1A* is characterized as a tumor suppressor gene [7], in contrast to the *RASSF1C* variant, that is considered a potential oncogene, since it was shown that it has opposing effects on cancer cells [15,16].

Most studies at the protein level have also focused on the *RASSF1A* isoforms role. It has been showed that *RASSF1A* can regulate microtubule dynamics, cell cycle progression and apoptosis, through interactions with various factors and their signaling pathways, e.g., with MST1 for inducing apoptosis or through participation in the complex KRas-*RASSF1A*-MOAP-1 that leads to the Bax activation pathway and cell death, also with Cdc20 for interaction with microtubules [2,5,17-25]. It was also recently shown that *RASSF1A* forms a DNA damage-regulated complex with the key DNA repair protein XPA; the latter requires *RASSF1A* in order to have full repair activity [26]. In addition,

the absence of a functional *RASSF1A*/*MST2*/*LATS1*/*CDK2* pathway leads to lack of phosphorylation of the BRCA2 C-terminal RAD51 binding site by *CDK2* and as a result of it, to chromosomal instability, impaired homologous recombination and malignant transformation [27].

Therefore, we started dissecting *RASSF1* expression by analyzing the methylation status of the first promoter of *RASSF1* gene in tissue DNA from sporadic breast cancer patients. We utilized a robust and thorough Pyrosequencing method previously developed by a member of our research team (TL) [28]. In order to add an additional prognostic biomarker for our patients, we then validated a reverse-transcriptase quantitative Polymerase Chain Reaction method (RT-qPCR) for *RASSF1* mRNA expression based on the SYBR Green detection format in the real-time Rotor-Gene Q MDX platform. Both differential methylation status of the first promoter of *RASSF1* gene and expression of *RASSF1* mRNA were investigated for their potential utility as biomarkers associated with particular clinic pathological parameters.

Materials and Methods

Patients

Eighty one tissues from breast cancer patients and four normal breast tissues (from mammoplasty and benign breast hyperplasia) were collected from the Pathology Laboratory of Evgenidio Hospital from consecutive cases of residents of the Metropolitan Athens area during 2007-2011. The inclusion criteria were the availability of the material, the presence of >70% cancer cells in the frozen section and the written informed consent of the patients (family history was not used as a criterion for the selection of patients in this study). The study was approved by the Ethics and Conduct Committee and the Scientific Council of Evgenidio Hospital. No patient had received any adjuvant therapy prior to surgery. The samples were stored in RNA Later stabilizing material (Ambion, USA) for 1-2 days at 4°C and then stored at -80°C until the isolation of total RNA and DNA.

The histological type and grade were assigned according to the World Health Organization and the modified criteria of Bloom-Richardson. Also, the clinical features of patients (age, tumor size, node filtration, and metastasis) were recorded. The paraffin-embedded tissues were examined by routine Immunohistochemistry (IHC) for the status of Estrogen Receptors (ER), Progesterone Receptors (PR) and overexpression of HER2 protein. If the IHC result for HER2 was intermediate (i.e., 2+) for a patient, the sample was then further examined by a chromogenic in situ hybridization method for HER2 gene amplification in order to reclassify into either a positive or a negative result.

Most of the specimens originated from lumpectomy surgery and the mean size was 2.0 cm (range: 1.0-5.5 cm). The majority of the tumors (76.5%) were ductal infiltrating carcinomas (the rest were lobular mostly, papillary and mucinous) and were classified as grade I (3 samples), grade II (45 samples) and grade III (14 samples). Grades I and II were grouped together as the low grade group because of the small number of grade I tumors. Lymph node involvement was examined in most of the samples and the presence of any recurrences or metastasis was also recorded for those patients with sufficient follow-up data. The characteristics of the 81 tissues and patients with sporadic breast cancer are summarized in the left part (Table 1).

Tissue DNA extractions

DNA was extracted by using the NucleoSpin Tissue Kit (Macherey-Nagel, Germany) according to the manufacturer's instructions, after crushing and homogenizing the tumor tissues under liquid N₂. DNA concentration was determined by the Quant-iT dsDNA Broad range Assay kit in the Qubit 1.0 Fluorometer (Invitrogen/Thermo Fisher, USA).

Pyrosequencing methylation analysis of the first *RASSF1* promoter

Tumor DNA and appropriate negative and positive controls were modified after treatment with sodium bisulfite (SB) that converts all unmethylated cytosine to uracil while leaving methylated cytosines untouched by using the EZ-DNA Methylation Gold Kit (Zymo, USA) according to manufacturer's instructions. Converted DNAs were stored up to 3 months at -20°C. The fully-methylated positive control was a SB-converted placental DNA sample treated by *SssI* methylase in the presence of S-adenosyl methionine (both from NEB, USA) while the negative unmethylated control was the same placental sample that was SB-converted but *SssI* untreated. Serial mixtures of the aforementioned positive and negative controls were constructed in order to validate the *RASSF1* Pyro sequencing assay (range of the constructed controls: 0-80% positive).

Detection of methylation of the first promoter of *RASSF1* gene was performed by Pyrosequencing, where a total of 9 CpG dinucleotides were analyzed in 117 bp of the first CpG island of *RASSF1* gene. The primers that amplify the region that would be further analyzed by Pyrosequencing have been previously described [28]. Polymerase Chain Reaction (PCR) is performed, by using both forward (5'-AGTATAGTAAAGTTGGTTTTAGAAA-3') and reverse (5'-CCCTTCCTCCCTCCTT-3') primers in a 0.2 μM final concentration, 1 μg of the SB-modified genomic DNA as the template, 200 μM dNTPs, 0.15 μL Hot Star Polymerase (Qiagen, Germany) and 3 μL of its corresponding Coral Load 10X Buffer in a 30 μL total volume. The cycling conditions consisted of an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 48°C for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. The quality and quantity of the 117 bp PCR products were confirmed by electrophoresis of a 10 μL aliquot in a 2% w/v agarose gel. The remaining PCR product was mixed with 50 μL PyroMark Binding Buffer (Qiagen, Germany), 2 μL Streptavidin-Sepharose High Performance Beads and 18 μL ddH₂O and incubated at room temperature for 10 min. Following immobilization and clean-up of the product on the Q96 Vacuum Workstation (Qiagen, Germany) the single stranded product was released in 45 μL annealing mix containing 330nM sequencing primer (5'-AAGTTGGTTTTAGAAATA-3'). Pyrosequencing analysis proceeded on a Pyro-mark Q96 instrument (Qiagen, Germany).

Additionally, in order to check the quality of our tumor samples and to assist in the selection of normal samples, a Pyrosequencing assay for the LINE-1 retrotransposable element was undertaken as previously described [29,30]. This assay evaluates global DNA methylation status.

Total RNA isolation

Total RNA was extracted by the NucleoSpin RNA kit (Macherey-Nagel, Germany). DNA was removed by an in-column recombinant DNase treatment. Total RNA was eluted in RNase-free water and stored at -80°C until further use. RNA concentration was determined by the Quant-iT RNA Assay kit in the Qubit 1.0 Fluorometer (Invitrogen/Thermo Fisher, USA) that employs a dye specific for RNA and not for DNA. All RNAs were of adequate quantity.

Complementary DNA synthesis

cDNA was synthesized from 1 μg of total RNA and random hexamers in a 20 μL total volume, according to the Transcriptor First strand cDNA synthesis kit instructions (Roche Applied Science, Switzerland). Appropriate controls were added: a RNA negative control (blank), a Reverse Transcriptase-negative (RT-) control and a RNA-positive (RT+) control that contained 100 ng of the control RNA provided by the kit. In order to test the quality and purity of cDNA samples, were subjected to a control PCR reaction for the actin reference gene as previously described [31]. cDNA samples that are free of genomic DNA produce a unique fragment of 587 bp (and not an additional fragment of 1122 bp, if genomic DNA exists). The cDNA samples were then stored at -20°C.

RASSF1 RT-qPCR assay

In order to study the *RASSF1* mRNA expression, a real-time RT-qPCR method was validated in the Rotor-Gene Q MDX (Qiagen) real-time thermal cycler by using the SYBR Green PCR kit (Qiagen) and a suitable set of standardized primers from Qiagen covering exons 3-4 wherein almost all *RASSF1* transcript variants are detectable (except *RASSF1G*). As a reference gene, the beta2-microglobulin was used, with a standardized set of Qiagen primers as well. Manufacturer's

Clinical features	N (%)	RASSF1 DNA methylation			RASSF1 mRNA expression		
		Low n (%)	High n (%)	Pearson χ^2 or Fisher's exact p-value	Low n (%)	High n (%)	Pearson χ^2 or Fisher's exact p-value
Age Group				0.126			0.323
≤ 50 years	24 (35.3)	20 (83.3)	4 (16.7)		15 (62.5)	9 (37.5)	
>50 years	44 (64.7)	29 (65.9)	15 (34.1)		22 (50.0)	25 (50.0)	
Tumor Size				0.328			0.339
≤ 2.0 cm	52 (65.8)	40 (76.9)	12 (23.1)		29 (55.8)	23 (44.2)	
>2.0 cm	27 (34.2)	18 (66.7)	9 (33.3)		12 (44.4)	15 (55.6)	
Histopathological Type				0.925			0.211
Intraductal infiltrating	62 (76.5)	45 (72.6)	17 (27.4)		29 (46.8)	33 (53.2)	
Lobular & rest	19 (23.5)	14 (73.7)	5 (26.3)		12 (63.2)	7 (36.8)	
Lymph-node Involvement				0.073			0.120
Negative	49 (66.2)	39 (79.6)	10 (20.4)		27 (55.1)	22 (44.9)	
Positive	25 (33.8)	15 (60.0)	10 (40.0)		9 (36.0)	16 (64.0)	
Metastasis				0.117			0.275
Negative	45 (83.3)	36 (80.0)	9 (20.0)		19 (42.2)	26 (57.8)	
Positive	9 (16.7)	5 (55.6)	4 (44.4)		6 (66.7)	3 (33.3)	
Grade				0.683			0.305
Low (I-II)	56 (76.7)	40 (71.4)	16 (28.6)		25 (44.6)	31 (55.4)	
High (III)	17 (23.3)	13 (76.5)	4 (23.5)		10 (58.8)	7 (41.2)	
ER Status				0.016*			0.744
Negative	19 (24.1)	18 (94.7)	1 (5.3)		9 (47.4)	10 (52.6)	
Positive	60 (75.9)	40 (66.7)	20 (33.3)		31 (51.7)	29 (48.3)	
PR Status				0.064			0.909
Negative	40 (50.6)	33 (82.5)	7 (17.5)		20 (50.0)	20 (50.0)	
Positive	39 (49.4)	25 (64.1)	14 (35.9)		20 (51.3)	19 (48.7)	
HER2 Status				1.000			1.000
Negative	73 (94.8)	53 (72.6)	20 (27.4)		38 (52.1)	35 (47.9)	
Positive	4 (5.2)	3 (75.0)	1 (25.0)		2 (50.0)	2 (50.0)	

Table 1: Clinicopathological characteristics of the 81 tissue samples from patients with sporadic breast cancer and their association with DNA methylation and mRNA expression of the *RASSF1* gene (* denotes statistical significance).

instructions were followed for the RT-qPCR protocol for both genes and the only change was the number of cycles (45 cycles). The expected product of *RASSF1* gene amplification is 121 bp long, while the beta2-microglobulin gene is 98 bp long. RT-qPCR products were additionally checked for size and purity by electrophoresis of the products on 2% w/v agarose gels. Within every run, each cDNA sample was amplified for both genes. Also for our lab internal quality control, a positive tumor cDNA sample was chosen to be included in all runs.

In order to prepare calibrators (standards) for the beta2-microglobulin and *RASSF1* gene assays, several PCR products were united and then purified by the PureLink PCR Purification Kit (Invitrogen/Thermo Fisher) followed by measuring the concentration by the Quant-iT dsDNA Broad range Assay kit in the Qubit 1.0 Fluorometer (Invitrogen/Thermo Fisher, USA). The copies/ μ L was

calculated as described previously [32]. The highly concentrated calibrator was serially diluted and standard curves were obtained for both genes.

The analytical validation of the RT-qPCR assay was performed following the established MIQE guidelines [31-33]. From various runs of the calibrators and the positive lab internal control, quantification cycle (C_q), curve characteristics and melting points (Tms) of the amplicons were recorded. Their averages, SD (standard deviation) and CV% (coefficient of variation) were calculated in order to estimate the within and between run precision. The efficiency (E) of each standard curve was calculated by using the equation: $E=10^{-1/Slope}$ and the average were reported. As method of relative quantification, the $2^{-\Delta Ct}$ of Livak and Schmittgen was used ($RQ=2^{-\Delta Ct}$, wherein RQ is the mRNA expression) [34,35].

Statistical Methods

Statistical analysis was performed with IBM SPSS v.21 in order to evaluate any associations between the differential methylation of the first promoter of *RASSF1* gene, *RASSF1* mRNA expression and the clinicopathological characteristics of the patients. Initially, descriptive statistical analysis of the clinicopathological features of tissues and patients was performed followed by normality check of the quantitative variables with the Kolmogorov-Smirnov test. Methylation of the first promoter of *RASSF1* gene and mRNA *RASSF1* expression (RQ results were multiplied by 1000 to have an easier oversight of the numbers with an integral and two decimal places) were divided in two categories (high or low). Then, the data were evaluated by two-dimensional contingency tables (cross tabulation or contingency table). The existence of independence or not between categorical variables was tested by Pearson χ^2 test or Fisher's exact test. For continuous variables following the normal distribution, parametric test t-test was applied for comparing averages, while for those that do not follow the normal distribution, the non-parametric Mann-Whitney test was applied for the comparison of median values between two variables, respectively. The Pearson and Spearman correlation coefficients were used to study the strength of the correlation between continuous variables (with parametric or non-parametric way, depending on the normality). Furthermore, Linear Regression was used for the correlation between the two continuous measured variables, i.e., the degree of methylation and mRNA expression. Binary Logistic Regression was used in order to find the model that relates either the lymphatic infiltration or metastasis with clinicopathological features of patients and the measured parameters of our study. Finally, in patients where follow-up data on metastasis was available, Kaplan-Meier survival analysis was performed in order to examine whether metastasis was associated with the degree of methylation and mRNA expression of *RASSF1*. All the observed statistical significance (p-value) was two-sided and the confidence level was set at 0.05.

Results

RASSF1 DNA methylation analysis

LINE-1 Pyro-sequencing assay was performed in order to measure the global methylation status for the selected samples. LINE-1 sequences are autonomous retrotransposons comprising 21% of the human genome that are kept suppressed by methylation in normal cells [29]. Therefore, LINE-1 hypomethylation is common in cancer cells but not in normal cells. 42% of our tumor samples were LINE-1 hypomethylated while the four selected normal samples were methylated (69.22 ± 6.35 and 72.26 ± 1.66 , respectively). This test was an important selection criterion since initially, seven normal samples had been collected but because of significant LINE-1 hypomethylation, three of these samples were rejected [30].

The PCR amplification of the first promoter region of *RASSF1* gene was specific, as it was confirmed by a single band at the expected size of 117 bp during agarose gel electrophoresis (Supplementary Figure 1). The *RASSF1* Pyrosequencing assay previously developed by a member of our research team (TL) was further validated successfully since all synthetically derived positive controls that we have constructed yielded the expected results: e.g., the 40% positive control showed a 46.75% average methylation across the 9 examined CpGs sites (Figure 2) while the negative control sample provided a zero result (data not shown) [28].

A background methylation was observed in the normal breast samples. In order to set the cut-off threshold for *RASSF1* methylation, the average and the standard deviation of methylation of normal

samples were calculated and then the sum of average+2 SD was used as the upper limit of normal background methylation (with 95% confidence). By this way, the cut-off of methylation was set as 57.09%. In Figures 3 and 4, two Pyrograms are shown that concern a normally methylated and a hypermethylated sample, respectively. Of the total 81 samples, 59 (72.8%) were classified as normally methylated while 22 (27.2%) as hypermethylated (Table 2).

RASSF1 RT-qPCR assay

Amplifications of the *RASSF1* gene and beta2-microglobulin were specific, as confirmed by the presence of single bands at their expected sizes of 121 bp and 98 bp respectively during electrophoresis of the products on 2% w/v agarose gels (Supplementary Figure 2). As an additional element of specificity, melting curves were performed. The average $T_m \pm SD$ of the beta2-microglobulin amplicon was $80.5^\circ\text{C} \pm 0.2$ (CV=0.2%) and of the *RASSF1* amplicon was $87.4^\circ\text{C} \pm 0.2$ (CV=0.3%). For each sample, both genes were amplified at the same time. In Supplementary Figures 3 and 4, the standard curves are shown for each gene. For the beta2 microglobulin standard curve the slope was -3.39 ± 0.24 (CV=7.14%), the efficiency 0.98 ± 0.10 (CV=10.10%) and the intercept 35.57 ± 3.00 (CV=5.97%). For the *RASSF1* gene standard curve the slope was -3.24 ± 0.13 (CV=4.08%), the efficiency 1.04 ± 0.06 (CV=6.15%) and the intercept 34.79 ± 0.51 (CV=1.48%). Repeatability and reproducibility from the Cqs of all calibrators were less than 3% and 5% for both genes. Reproducibility of the internal quality control sample in copies/ μL was less than 12% for both genes.

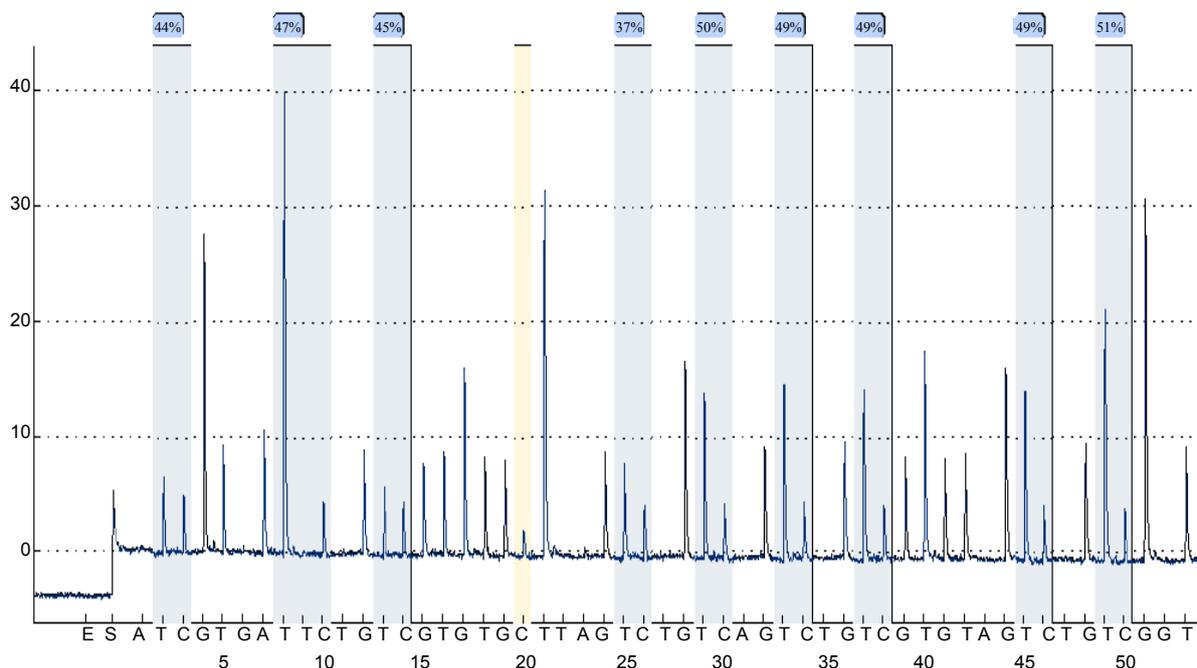
The average of the relative expression values (RQ*1000) from the tumor samples was 4.45 (range 0.03-63.37). Due to their non-normal distribution, their median (2.27) was used in order to separate them either as sub or over-expressed. Therefore, from the 81 samples, 40 (49.4%) were classified as over-expressed, while 41 (50.6%) as sub-expressed (Table 2).

Statistical Analysis

No significant negative correlation was found between *RASSF1* methylation and mRNA expression levels (p=0.207), data that was further supported by the inability to find a linear regression model, under which *RASSF1* mRNA expression could be calculated from methylation levels. Furthermore, a statistically significant difference of the average methylation levels depending on the condition of the ER receptors was detected (t-test, p=0.001) that was also confirmed by the division into groups and χ^2 -test (p=0.016) (Table 1). The *RASSF1* hyper-methylation classification was also marginally correlated with positive PR receptors (χ^2 , p=0.06) and lymph node infiltration (χ^2 , p=0.07), (Table 1). Binary logistic regression (Table 3) showed that the probability of lymph node infiltration was increased by the presence of negative ER receptors (p=0.008, OR 0.09, CI 0.14-0.52), the percentage of *RASSF1* methylation (p=0.006, OR 7.96, CI 1.82-34.86) and marginally with the level of *RASSF1* expression (p=0.047, OR 3.94, CI 1.02-15.29). Survival analysis (Kaplan-Meier) showed that there is marginally statistically significant correlation between metastasis and *RASSF1* gene mRNA over-expression (log rank test, p=0.040), curve in (Supplementary Figure 5).

Discussion

The tumor suppressor *RASSF1* gene from 2000 till now has been the subject of research in many cancer types. The hyper-methylation of CpG island of the promoter of a tumor suppressor gene, such as *RASSF1*, may cause silencing and termination of its operation [5,6,36]. *RASSF1* methylation has shown its value as a diagnostic biomarker in



Sequence to analyze:

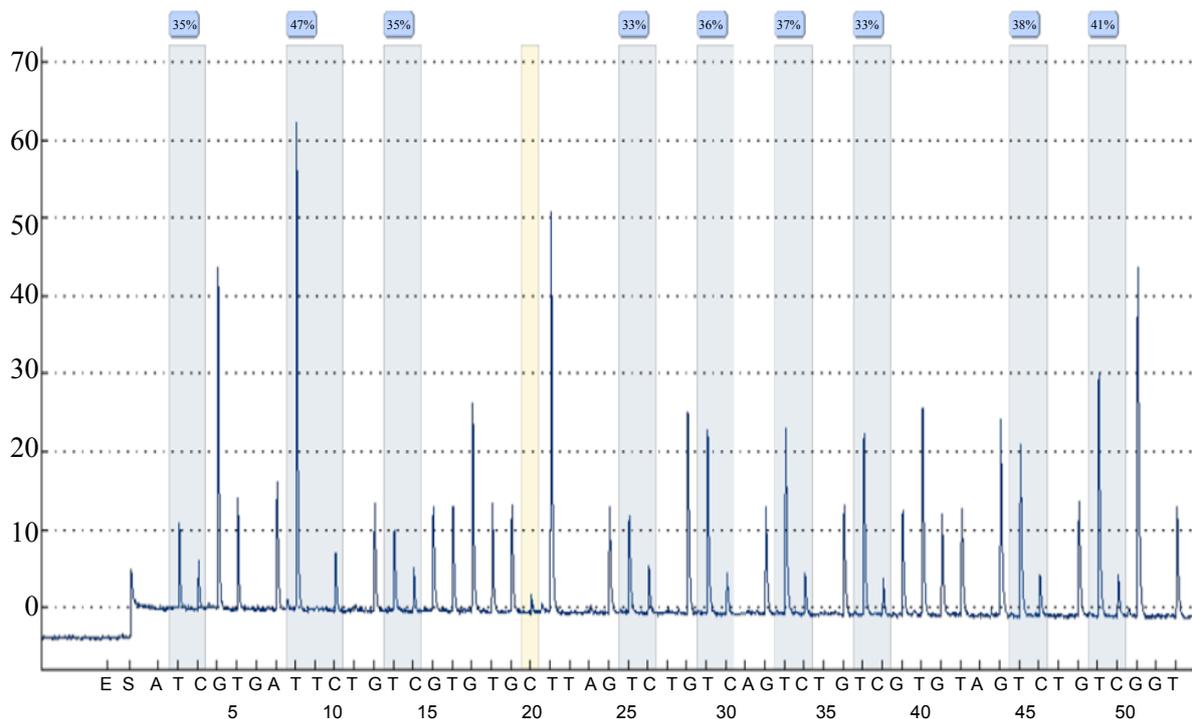
YGGGTATTTTYGYGTGGTGTTTTGYGGTYGTYGTYGTGTTGTGGTYGTTYGGGGTGGGGTGTGAGGAGGGGA

Position	1	2	3	4	5	6	7	8	9
Quality	Passed								
Meth (%)	44	47	45	37	50	49	49	49	51

Figure 2: Pyrogram of the 40% methylated standard for the *RASSF1* DNA methylation assay. The blue ribbons represent the 9 CpGs sites, while the yellow ribbon the cytosine that was chosen in order to access the efficiency of SB-DNA conversion. The methylation percentage in every single CpG is derived from the T/C+T ratio and the total percentage is the average of the examined 9 CpGs (the average of methylation measured for this standard was 47%).

ID	Methylation	Expression									
1	49.57	7.04	22	62.52	2.23	43	36.26	0.28	64	17.85	0.05
2	23.77	3.67	23	84.44	7.70	44	49.18	14.08	65	20.43	0.73
3	56.86	5.37	24	70.24	0.55	45	34.65	1.45	66	32.22	1.67
4	30.08	8.73	25	16.92	5.26	46	22.05	1.46	67	61.58	2.04
5	30.17	11.28	26	69.43	1.89	47	68.89	2.47	68	39.96	0.62
6	56.66	12.87	27	47.59	3.24	48	20.33	3.40	69	74.69	63.37
7	26.94	5.76	28	53.64	7.14	49	41.96	6.85	70	47.55	0.44
8	31.99	4.30	29	72.40	3.33	50	57.65	4.46	71	82.24	1.64
9	27.82	7.04	30	34.23	0.76	51	54.12	2.88	72	67.19	0.28
10	52.98	5.84	31	13.98	0.53	52	19.61	6.09	73	73.97	0.92
11	48.49	11.44	32	26.06	0.68	53	46.18	9.69	74	43.53	0.23
12	62.85	4.68	33	23.64	0.03	54	21.74	13.14	75	46.17	0.65
13	47.34	11.60	34	44.46	12.17	55	37.05	1.40	76	72.41	11.92
14	19.62	3.11	35	55.05	0.27	56	43.95	3.67	77	55.78	0.55
15	15.76	0.11	36	55.88	1.19	57	21.08	1.48	78	59.18	0.36
16	76.75	1.48	37	30.77	0.13	58	39.67	8.20	79	81.13	0.64
17	19.92	2.06	38	17.39	2.21	59	26.59	0.89	80	59.51	0.50
18	42.01	3.42	39	45.40	8.73	60	16.31	2.71	81	90.05	0.50
19	19.97	1.71	40	86.95	1.65	61	62.43	0.80			
20	46.20	1.29	41	48.23	5.84	62	68.78	0.83			
21	49.49	2.29	42	42.62	2.24	63	13.29	2.42			

Table 2: Average methylation and mRNA expression levels of the *RASSF1* gene, for each tumor sample; the unbold values represent the normally-methylated and under-expressed samples while the bold values the hyper-methylated and over-expressed samples.



Sequence to analyze:

YGGGTATTTTGYGTGGTGTTTTGYGGTYGTYGTYGTGTTGTGGTYGTTYGGGGTGGGGTGTGAGGAGGGGA

Position	1	2	3	4	5	6	7	8	9
Quality	Passed								
Meth (%)	35	47	35	33	36	37	33	38	41

Figure 3: Pyrogram of a normally-methylated sample (according to our classification) with a 37% average of methylation in the 9 CpGs analyzed in the *RASSF1* first promoter area.

		Variables in the Equation						95% C.I. for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step	Size group	0.140	0.689	0.041	1	0.839	1.150	0.298	4.440
	GRADE	1.242	0.793	2.451	1	0.117	3.462	0.731	16.394
	ER group	-2.469	0.926	7.116	1	0.008*	0.085	0.014	0.519
	PR group	0.538	0.807	0.444	1	0.505	1.712	0.352	8.333
	HER2 group	0.055	1.532	0.001	1	0.971	1.057	0.053	21.271
	<i>RASSF1</i> meth group	2.074	0.754	70.575	1	0.006*	7.959	1.817	34.863
	RQ group	1.371	0.692	3.930	1	0.047*	3.941	1.016	15.290
	Constant	-2.155	1.301	2.742	1	0.098	0.116		

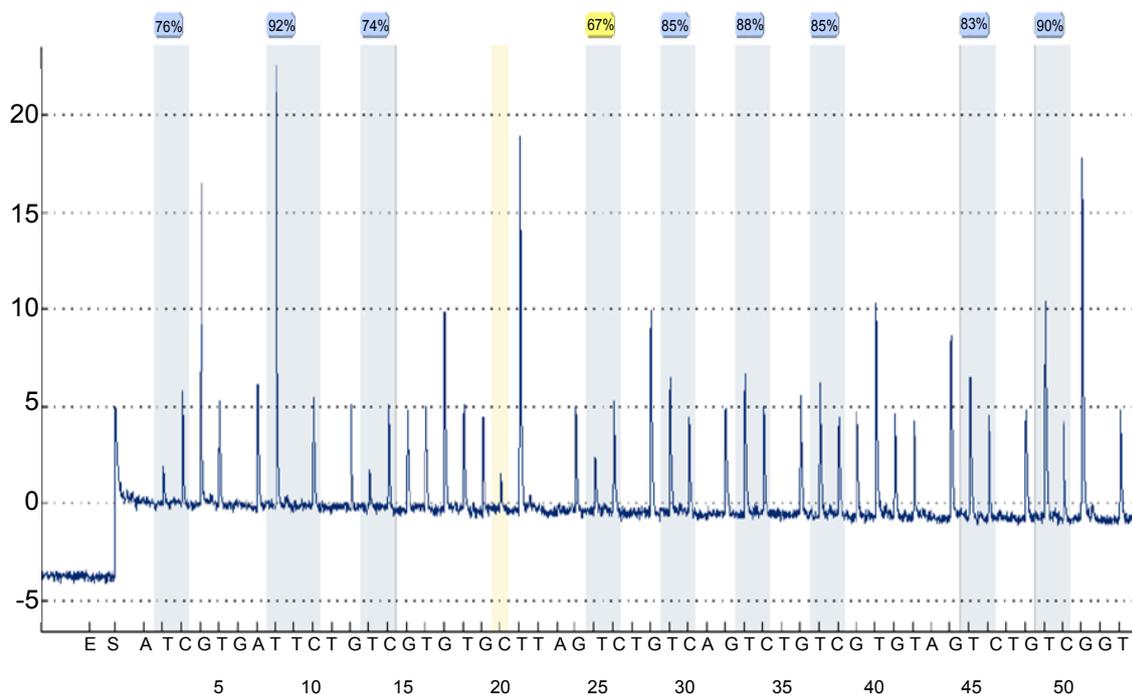
Table 3: Binary logistic regression for the prediction of lymph node infiltration from clinical variants and our measured parameters [*RASSF1* meth and RQ group] (* denotes statistical significance).

biological fluids assessed for lung cancer [37] and is being examined as a prognostic tissue biomarker in many other cancer types [7-14].

The purpose of this study was to develop and validate innovative and reliable methods for the analysis of all aspects of *RASSF1* expression: a) DNA methylation of the first promoter of *RASSF1* gene (from which the primary transcript *RASSF1A* is transcribed) and b) the mRNA expression. Then correlation of *RASSF1* methylation with its mRNA

expression and correlations both of the aforementioned measurements with clinicopathological data and patient survival were investigated.

The material analyzed was 81 breast cancer tissues and 4 breast tissues from women without any malignancy that were all impregnated in suitable stabilizing material for isolating DNA/RNA (RNA Later). Patient sampling was representative of the patients with breast cancer in Athens and it was not based on family history, but on the availability



Sequence to analyze:

YGGGTATTTT¹YGY²GTGGTGT³TTTGYGGTY⁴GTY⁵GTY⁶GTTGTGGTY⁷GTTY⁸GGGGTGGGGTGTGAGGAGGGGA

Position	1	2	3	4	5	6	7	8	9
Quality	Passed								
Meth (%)	76	92	74	67	85	88	85	83	90

Figure 4: Pyrogram of a hyper-methylated sample (according to our classification) with an 82% average of methylation in the 9 CpGs analyzed in the *RASSF1* first promoter area.

of the material and the existence of >70% of tumor cells in the frozen section. It should be noted that the quality of the clinical samples, in terms of malignancy was assessed in a previous study of our group that performed LINE-1 Pyrosequencing methylation assay [30].

The majority of the female breast cancer patients were over 50 years (64.7%), had a relatively small tumor size (65.8%), were mostly diagnosed with the invasive ductal histological type (76.5%), were grade I and II (76.7%), had negative lymph nodes (66.2%), showed no metastasis (83.3%), were ER positive (75.9%) and HER2 negative (94.8%). Therefore, our tissue sample set was appropriate for further analysis.

Most studies assessing *RASSF1* methylation have targeted the first promoter and used as a detection method MSP or variants like qMSP and Methyl Light, i.e., mostly detecting methylation in a qualitative way [7-14]. Xu et al. in 2012 used Pyrosequencing as a method of analysis of methylation, but they examined only four CpG di-nucleotides in the *RASSF1* first promoter [38].

The assessment of methylation in the first promoter of *RASSF1* gene in our study was performed with the Pyrosequencing method developed by a member of our research team (TL) [28]. This method is reliable, innovative, rapid and sensitive and provides quantitative and not only qualitative data. A total of 9 CpG di-nucleotides were

analyzed in a 117 bp stretch of the first CpG island of *RASSF1* gene. Methylation was given as a percentage in each CpG dinucleotide and finally, the average methylation of all 9 CpG di-nucleotides is provided. The method was further validated by using synthetic standard samples with different methylation percentages. The availability of only 4 normal samples (that were well-ascertained by LINE1 analysis, as aforementioned), certainly limited our statistical power in order to define the methylation threshold. It was defined by the average+2 SD of the normal samples and therefore, 59 tumor samples (72.8%) were classified as *RASSF1* normally-methylated while 22 (27.2%) as hyper-methylated.

Then, for studying the mRNA expression of *RASSF1* gene, real-time RT-qPCR was performed with the Rotor-Gene SYBR Green PCR kit and a suitable Qiagen Primer kit Assay (standardized primers). The primers of this assay are located in exons 3 and 4 and therefore, all transcript variants of *RASSF1* gene are detected (except G). As a reference gene, beta2-microglobulin was used. The method was validated by obtaining the correct size of the expected bands in gels, the reproducibility data from calibrators of the standard curves for each gene and the internal quality control sample and finally, the Tm of the amplicons of each gene. Both RT-qPCR assays performed adequately according to their validation data. Based on the median of their RQ values for total *RASSF1* mRNA expression, 40 (49.4%) tumor samples

were classified as *RASSF1* over-expressed, while 41 (50.6%) as sub-expressed.

An important finding of our study was that the methylation level of the first promoter of *RASSF1* gene and the level of its mRNA expression are not correlated ($p=0.207$), in contrast to the expected or bibliographic data [36]. On this basis, it is possible that not only the hyper-methylation of the first, but also the methylation of the second promoter of the *RASSF1* gene and/or other epigenetic modifications (e.g., acetylation, miRNA regulation) are required in order to decrease significantly or even shut down *RASSF1* expression and function. Therefore, the methylation analysis of the second promoter that affects transcription of *RASSF1B* and *RASSF1C* mRNA is also needed in order to draw safer conclusions. Additionally, lymph node infiltration was found to be increased by both the percentage of methylation *RASSF1* ($p=0.006$) and the level of total *RASSF1* expression ($p=0.047$). This last contradictory and unexpected finding could lead to the speculation that the expression of another transcript has increased -besides the main *RASSF1A* variant- that increases the overall *RASSF1* expression and leads to the poor prognostic factor of lymphatic infiltration. This is further reinforced by the fact that in several samples there is a combination of high *RASSF1* mRNA expression and high *RASSF1* DNA methylation (Table 2).

A working hypothesis is that variant *RASSF1C* could be overexpressed; a potential oncogene [15,16] that the RT-qPCR assay we used also detects. When *RASSF1A* is downregulated in tumors, the *RASSF1C* is overexpressed [16]. Unfortunately, the transcripts of *RASSF1* gene are showing high homology and contain palindromic sequences and high GC rich areas that complicate design and accurate amplification. As examined by bioinformatics, there is no suitable pair of primers that can be detect solely a single *RASSF1* transcript with the SYBR Green detection system format.

In conclusion, a robust methylation detection method of the first promoter of *RASSF1* gene was validated, which could in the future be extended in an analogous fashion in the second promoter of *RASSF1* gene in a reliable way to allow for a better estimation of the overall activity of the gene promoters. As for the expression study, a new design method using primers and/or probes (Taqman, dual hybridization etc.) specific for each alone of the *RASSF1* transcripts should be used, as we have already designed and performed in another gene [39]. In conjunction with specific antibodies for the detection of different *RASSF1* protein isoforms (by IHC and/or Western Blot), it would enable specificity and power in this study, overcoming the existing problems and offer additional useful predictive and prognostic tools for monitoring and treatment of patients with breast cancer. If this turns out to be true, *RASSF1* transcript variants could be included in multi-parametric expression biomarker panels (such as e.g., Oncotype, Mammaprint etc.) analyzing expression on the tissue and probably in the future, this assay could be expanded in a non-invasive material such as in peripheral blood.

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