# High-Resolution Melting-Based Quantitative Analysis of *RASSF1* Methylation in Breast Cancer

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***Key Words**: DNA methylation; breast cancer; HRM; QMSP; RASSF1 gene.*

***Summary.** Background and Objective. Breast cancer is the leading cause of death from cancer among women worldwide. The aberrant promoter methylation of tumor suppressor genes is a typical epigenetic alteration for breast cancer and can be detected in early carcinogenesis. High-throughput and cost-effective methods are needed for the early and sensitive detection of epigenetic changes in clinical material. The main purpose of our study was to optimize a high-resolution melting (HRM) assay for the reliable and quantitative assessment of RASSF1 gene methylation, which is considered one of the earliest epigenetic alterations in breast cancer.*

*Material and Methods. A total of 76 breast carcinomas and 10 noncancerous breast tissues were studied by means of HRM and compared with the results obtained by means of quantitative methylation-specific polymerase chain reaction (QMSP) and methylation-specific polymerase chain reaction (MSP).*

*Results. Both quantitative methods, HRM and QMSP, showed a similar specificity and sensitivity for the detection of RASSF1 methylation in breast cancer (about 80% and 70%, respectively). In breast cancer, the mean methylation intensity of RASSF1 was 42.5% and 48.6% according to HRM and QMSP, respectively. Both methods detected low levels of methylation (less than 5%) in noncancerous breast tissues. In comparison with quantitative methods, MSP showed a lower sensitivity (70%), but a higher specificity (80%) for the detection of RASSF1 methylation in breast cancer.*

*Conclusions. HRM is as a simple, cost-effective method for the reliable high-throughput quantification of DNA methylation in clinical material.*

## Introduction

Breast cancer (BCa) is the most prevalent and leading cause of death from malignant diseases among women in Lithuania and worldwide. In Lithuania, about 1500 new cases and 600 deaths are registered every year (1). BCa is a heterogeneous disease, usually occurring in older women and is mainly related to natural factors, such as gender, hormone production, reproductive status, or family history. Nowadays, the status of only few molecular biomarkers, such as estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2), and the expression of cell proliferation marker Ki67 are used in diagnostics and prognostics of BCa (2). According to these biomarkers, BCa is divided into biological subtypes; however, it is insufficient to achieve successful individual therapy. The genome-wide profiling of gene expression of BCa can assist in more precise molecular subtyping of BCa, though such methods are too expensive for routine clinical application. The introduction of novel genetic/epigenetic biomarkers and modern methods for the sensitive detection of early molecular changes in the breast tissue could enable the detection of BCa at early stages and reduce the risk of disease progression and mortality rates.

Carcinogenesis overwhelms all cellular processes, including the regulation of gene expression by epigenetic mechanisms (3). Epigenetic alterations, especially silencing of tumor-suppressor genes by promoter DNA hypermethylation, are one of the leading causes of carcinogenesis of BCa. Modern techniques enable the sensitive detection of tumor-specific epigenetic alterations in tumor tissue and blood plasma or serum. Invention of the quantitative means of DNA methylation measurement enabled the identification of minor variations in the levels of methylation among tumors with different aggressiveness and provided a new tool for therapy individualization. Besides, quantitative methods are valuable for the studies of heterogeneous biological material with the different density of cancer cells. Quantitative methods for the detection of DNA methylation include combined bisulfite restriction analysis (COBRA) (4), matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) (5), pyrosequencing (6), quantitative methylation-specific polymerase chain reaction (QMSP), also known as MethyLight (7), and high-resolution melting (HRM) analysis (8). Of these methods, pyrosequencing and QMSP are the most widely used methods in cancer

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research; however, novel high-throughput instruments with HRM application currently provide a reliable and cheaper alternative for the quantification of DNA methylation in clinical biosamples.

The *RASSF1* gene (*Ras-association domain family 1*) promoter is frequently hypermethylated in BCa and is among the earliest epigenetic changes in carcinogenesis (9). The gene belongs to the Ras-association gene family, which encodes regulatory proteins with tumor-suppressor functions. *RASSF1* is encoded in 3p21.3 locus and produce a protein involved in the regulation of cell cycle, apoptosis, and microtubule formation (10). Despite extensive *RASSF1* involvement in the studies of epigenetic biomarkers of BCa, most of the investigations are done by using qualitative methods, particularly MSP (11). Few quantitative analyses of *RASSF1* methylation have been performed in BCa, and QMSP is the most frequently used method for this purpose (12–16). The usage of HRM for *RASSF1* methylation analysis in BCa was reported in only 3 studies (17–19).

For the assessment of suitability of the HRM method for the reliable quantification of DNA methylation in the *RASSF1* gene promoter, 76 early-stage breast carcinomas and 10 noncancerous tissue specimens were studied by two quantitative methods – QMSP and HRM. For comparison with the widely used MSP method, the cases were dichotomized into methylation-positive and methylation-negative and compared with our previously collected MSP data on *RASSF1* methylation in the same group of cases. Furthermore, the analysis of associations between *RASSF1* promoter methylation and demographic and clinicopathological characteristics was performed, and the results of quantitative methods were compared.

## Material ant Methods

*Patients and Samples*. A total of 76 tumor and 10 control specimens were collected from the patients treated at the Oncology Institute of Vilnius University during the 2007–2009 period, as a part of the BCa biomarker study approved by the Lithuanian Bioethics Committee. The mean age of the patients was 56 years (range, 27–83 years). All the patients were diagnosed with early invasive breast carcinoma of T1 or T2 stages (n=43 and n=33, respectively). Both ductal (n=70) and lobular (n=6) breast carcinomas were analyzed. Among the cases of BCa, 13 cases of relapse and 4 cases of death due to BCa were registered. Noncancerous breast tissues were collected from the patients with fibroadenoma, and the mean age of the group was 46 years. The main immunohistochemical (IHC) biomarkers of BCa were analyzed (both visually and digitally) at the National Centre of Pathology as published previously (20). The intrinsic subtypes of BCa were identified based on the IHC status of molecular markers ER, PR, HER2 and Ki67, and the results of HER2 fluorescence in situ hybridization (FISH) according to the highlights of the St Gallen International Expert Consensus (2).

*DNA Extraction and Bisulfite Modification*. DNA was extracted by the standard phenol-chloroform purification and ethanol precipitation method; the deparaffinization step was used for formalin-fixed paraffin-embedded (FFPE) tissues (n=10). DNA (400 ng) was modified with bisulfite using an EZ DNA Methylation™ Kit (Zymo Research, USA) according to the manufacturer's recommendations. The efficiency of bisulfite modification was verified with a Cells-to-CpG™ Methylated and Unmethylated gDNA Control Kit (Life Technologies, USA), and only completely modified DNA was used in the analysis.

*Methylation Analysis by High-Resolution Melting*. Several sets of primers with CpG dinucleotides (CpGs) or without CpGs in the sequence were tested for the robust discrimination of methylated and unmethylated DNA in the promoter region of *RASSF1* (Fig. 1A). Based on the optimal resolution of melting profiles, a pair of CpG-free primers (forward, 5′-AGTTTGGATTTTGGGGGAGG-3′; reverse, 5′-CAACTCAATAAACTCAAACTCCCC-3′) that amplify a 136-bp fragment containing 12 CpGs was selected for the further optimization of the assay. The thermocycling parameters and the concentrations of reaction components were adjusted for the optimal discrimination of methylated and unmethylated sequences. The optimized HRM reaction included 1× MeltDoctor™ HRM Master Mix (Life Technologies, USA), 200 nM of each primer, and 1 $\mu$L of bisulfite-modified DNA in a final reaction volume of 20 $\mu$L. DNA-intercalating dye SYTO® 9 was used to record DNA amplification. Reactions were prepared in 96-well plates (MicroAmp® Fast, Life Technologies, USA) sealed with an adhesive film (Life Technologies, USA) and carried out on the ViiA™ 7 Real-Time PCR system (Life Technologies, USA). Each sample was run in duplicate, and several no-template controls were included in each plate. A standard curve consisting of 2× serial dilutions of in vitro methylated DNA (SssI methylase, Zymo Research, USA) with unmethylated DNA from leukocytes of healthy donors was included in each run. Standards with the known levels of methylation (0%, 6.25%, 12.5%, 25%, 50%, and 100%) were used for the preparation of a standard curve (Fig. 1B, C). Enzyme activation at 95°C for 10 minutes was followed by 40 amplification cycles that were performed under the following conditions: 95°C for 15 seconds and 62.5°C for 1 minute. The melting procedure was performed for 10 seconds at 95°C, 1 minute at 60°C and a temperature heating gradient of 0.025°C per 1 second for every step of fluorescence measurement, from 60°C to 95°C. Melting profiles were analyzed by the ViiA™ 7 Software v1.1 (Life Technologies, USA).

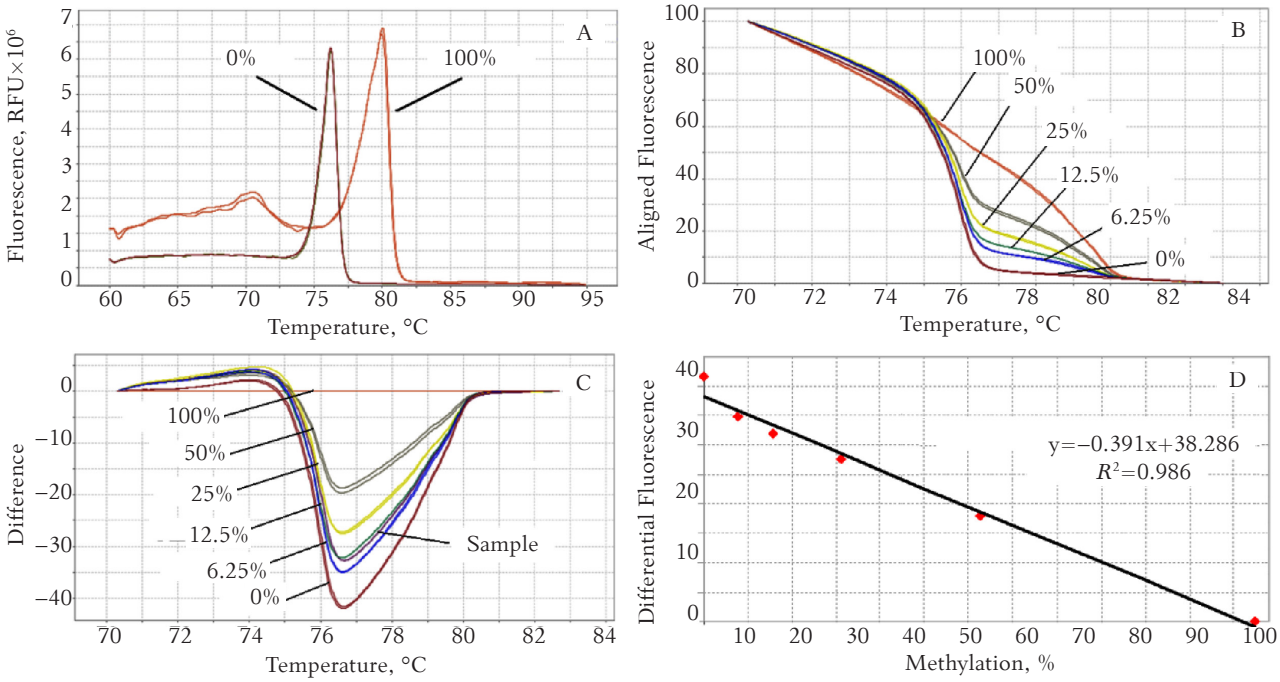


*Fig. 1.* Example of the flow-chart of HRM method for *RASSF1* analysis

A, melting curve peaks of 100% methylated and unmethylated DNA; B, a standard curve made by 2× dilutions of 0%, 6.25%, 12.5%, 25%, 50%, and 100% methylated DNA; C, normalization of sample methylation level against unmethylated control (violet); D, linear regression equation derived from the standard curve used for the evaluation of each sample fluorescence.

For the quantification of the methylation level of the analyzed clinical samples, a linear regression equation was derived based on the fluorescence of each standard normalized against an unmethylated control (0%) and plotted against the percentage of methylation (21) (Fig. 1D). Before the calculation, the value of the fluorescence of each sample was also normalized against an unmethylated control. For the qualitative evaluation of *RASSF1* methylation status, a cut-off value derived from the analysis of noncancerous breast tissues was used.

*Methylation Analysis by Quantitative Methylation-Specific Polymerase Chain Reaction.* The primers and hydrolysis probe of QMSP analysis were designed for the selective amplification of bisulfite-converted fully methylated DNA in the *RASSF1* promoter (22). For normalization of the fluorescence signal, 6-carboxy-X-rhodamine (ROX), which is a passive reference dye, was used. The amplification of endogenous control gene beta-actin (*ACTB*) was included in each assay to normalize for DNA input. For QMSP, the final reaction volume of 25 $\mu$L included 300 nM of each primer, 50 nM of probe, 10 nM of ROX, 1× Maxima® Probe qPCR Master Mix (Fermentas, Thermo Fisher Scientific, Lithuania), and 1 $\mu$L of bisulfite-converted DNA. QMSP was carried in 96-well plates (MicroAmp® Fast, Life Technologies) sealed with an adhesive film and run on ViiA™ 7. Thermocycling consisted of 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Fluorescence was recorded at the end of each cycle. Each sample was run in duplicate; several no-template controls were added into each run.

The results were generated using the ViiA™ 7 Software v1.1 (Life Technologies, USA). Exact methylation intensity was calculated by using the comparative ΔΔCq method for relative quantification, where in vitro methylated leukocyte DNA was used as a reference. Values were expressed in percentages. Due to an incomplete in vitro methylation reaction, some of the cases showed the intensity of methylation exceeding 100%, but in further calculations were equated to 100%. For the qualitative assessment, the *RASSF1* gene was considered methylated when the methylation level was higher than the mean methylation level in noncancerous tissue samples.

*Statistical Analysis.* Correlations between hypermethylation and clinicopathological characteristics of BCa were analyzed using the Spearman rank-order correlation. The 2-sided Fisher exact test was used to analyze categorical data in qualitative analysis, whereas the Mann-Whitney rank-sum test was used to indicate quantitative data connections between groups. Differences were considered statistically significant when a $P$ value was $<0.05$. The level of agreement among data obtained using different methods was verified by calculating the Kappa coefficient ($\kappa$). Calculations were performed with the GraphPad InStat 3, STATISTICA 7, and Microsoft Excel programs.

## Results

*Methylation Analysis by High-Resolution Melting and Quantitative Methylation-Specific Polymerase Chain Reaction.* Standard curves generated from standards with the known methylation levels were used for the validation of both quantitative methods – HRM and QMSP. Good linearity and a high goodness of fit were observed for both methods. Fig. 1D shows an example of the standard curve derived by the HRM method.

A total of 76 malignant and 10 nonmalignant breast samples were analyzed by means of the HRM method. The mean methylation level was 42.5% in BCa tissues and 4.7% in noncancerous breast tissues ($P<0.001$). Of the 76 BCa cases, 62 were considered methylation-positive in the qualitative assessment, based on the cut-off value. The results are presented in Table 1.

A total of 72 malignant and 10 nonmalignant breast samples were analyzed by the QMSP method. The mean methylation level of *RASSF1* was 48.6% in BCa tissues and 3.3% in noncancerous tissues ($P<0.001$). The comparison of methylation levels identified by HRM and QMSP is presented in Fig. 2. According to the cut-off value, BCa cases were divided into methylated (n=58) and unmethylated (n=14).

*Comparison of Results of Quantitative Methylation-Specific Polymerase Chain Reaction and High-Resolution Melting With Those of Methylation-Specific Polymerase Chain Reaction.* According to the identified cut-off values, the quantitative results of HRM and QMSP were dichotomized for comparison with the MSP results collected for the same clinical samples in our previous study (Table 1). The main discrepancies between MSP and one or both quantitative methods were observed in the evaluation of the methylation status of FFPE specimens. Thirteen cases were evaluated as unmethylated by the MSP method; of these 13, 5 were from FFPE tissues. Because of the wide intervals in the levels of methylation (from 0% to 194.2%), the quantification of methylation in FFPE tissues was also complicated when using the QMSP method. Despite some fluctuation of the fluorescence signals among replicates, the HRM method generated satisfactory results for FFPE tissues and showed the methylation levels from 21.1% to 46.6%. In addition, 1 noncancerous case was classified as unmethylated by MSP, but showed a significant methylation level by QMSP and HRM (4.1% and 10.1% respectively). Moreover, 2 cases were classified as methylated in the MSP assay, but showed low methylation levels (below the cut-off value) by QMSP and HRM.

Kappa statistic was used to measure the agreement among the tests. The data on FFPE tissues were excluded from this analysis. The best agreement ($\kappa=0.86$) was detected between the QMSP and HRM tests, while the concordance with the results obtained by MSP was only moderate ($\kappa=0.61$ and $\kappa=0.59$ for QMSP and HRM, respectively).

Finally, the sensitivity and specificity of these 3 tests were evaluated. The MSP method showed a sensitivity of 69.7% and a specificity of 80.0%; QMSP, 80.6% and 70.0%; and HRM, 81.6% and 70.0%, respectively.

*Associations With Clinicopathological Variables.* The results of the quantitative tests in overlapping samples (n=72) were included in this analysis. Based on the results of HRM test, the methylation frequency and intensity in the *RASSF1* promoter decreased with an increasing tumor grade. The G1

*Table 1.* Methylation Characteristics of Samples Analyzed by HRM, QMSP, and MSP Methods

| | HRM | QMSP | MSP |
|---|---|---|---|
| Samples, N | | | |
| Malignant | 76 | 72 | 76 |
| Nonmalignant | 10 | 10 | 10 |
| Cut-off value, % | 4.72 | 3.32 | – |
| Methylation level, % | 42.48 | 48.56 | – |
| Frequency of methylation (sensitivity), % (n/N) | 81.6 (65/76) | 80.6 (58/72) | 69.7 (53/76) |
| Specificity, % (n/N) | 70.0 (7/10) | 70.0 (7/10) | 80.0 (8/10) |

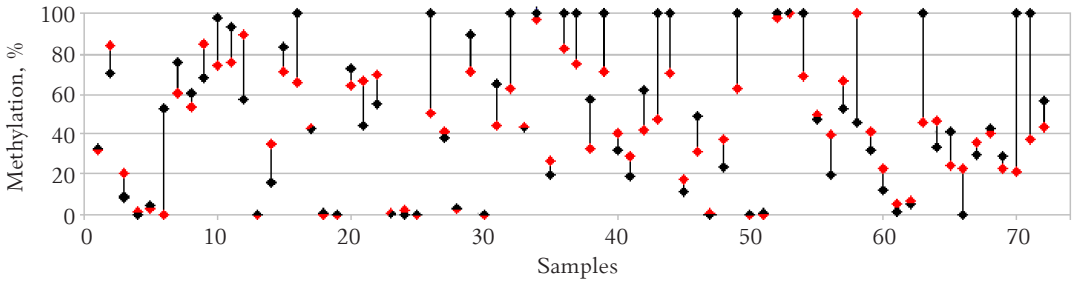


*Fig. 2.* Mean methylation levels of each sample identified by QMSP and HRM methods (black and red, respectively)
A black line indicates a difference in methylation detected by the two methods in the same BCa sample.

tumors were significantly more frequently methylated than the G3 tumors ($P$=0.01), and the level of methylation in the G1 tumors was higher than in the G2 tumors ($P$=0.02). *RASSF1* methylation was significantly more common in the ER-positive (ER+; $P$<0.001), PR-positive (PR+; $P$=0.03), and Ki67-negative ($P$=0.01) BCa cases as compared with the ER–, PR–, and Ki67-positive cases, respectively. A significant difference in methylation levels was observed between the ER+ and ER– tumors (48% vs. 18%, $P$<0.001), with the ER+ tumors showing more intense methylation of the gene. All these associations show the predominance of *RASSF1* methylation in less aggressive BCa of low grade with low proliferative potential, and positive response to hormonal signals. In support to this observation, the HRM test showed a low frequency and intensity of *RASSF1* methylation in highly aggressive triple negative (ER–, PR–, HER2–) BCa cases, and both parameters were significantly lower than in other intrinsic subtypes of BCa ($P$<0.05).

The analysis with the QMSP test revealed similar associations (except for some intrinsic subtypes, PR and Ki67 status), but the strength of associations was greater in the HRM analysis. All the mentioned associations are summarized in Table 2.

Meanwhile, the qualitative DNA methylation data obtained by the MSP test showed sparse associations with clinical variables. A greater frequency of RASSF1 methylation in the PR+ than PR– cases was the only statistically significant difference detected ($P$=0.02).

## Discussion

The HRM method is based on the analysis of the melting profiles of a PCR product by monitoring the fluorescence of DNA-intercalating dye, like SYTO 9. After bisulfite exposure, unmethylated cytosine in a DNA strand is converted to uracil, while methylated cytosine remains intact. Due to the differences in melting temperature between methylated and unmethylated DNA, the HRM method enables the sensitive identification and quantification of methylated DNA in clinical biosamples (8). CpG-free primers used in the HRM assay enable the reliable amplification of methylated and unmethylated amplicons, and even DNA with heterogeneous methylation status in particular CpGs is amplified during the HRM reaction. A standard curve generated by a serial dilution of standard methylated and unmethylated DNA serves the reliable quantification of methylation levels, while the normalization of results according to an unmethylated control does not allow the overestimation of methylation levels.

*Table 2.* Significant Differences in Frequency and Intensity of Methylation According to Clinical-Pathological Variables Identified by HRM Method

| Comparison | Methylation | |
|---|---|---|
| | Frequency, % | Intensity, % |
| Triple negative vs. other intrinsic subtypes | 20.0 vs. 94.5 | 8.8 vs. 47.1 |
| Tumor differentiation grades | 100.0 vs. 65.2 (G1 vs. G3) | 55.7 vs. 34.5 (G1 vs. G2) |
| ER+ vs. ER– tumors | 89.7 vs. 42.9 | 47.5 vs. 18.4 |
| PR+ vs. PR– tumors | 87.0 vs. 61.1 | 45.1 vs. 32 |
| Ki-67+ vs. Ki-67– tumors | 65.5 vs. 90.5 | Nonsignificant |

QMSP is also a real-time fluorescence-based PCR method used for the quantification of DNA methylation. The method relies on the binding of primers and probe specifically designed to detect completely methylated DNA. Promoters with the heterogeneous methylation of target CpGs will not be amplified by this method. Besides, in vitro methylated DNA is used as a reference of 100% methylation level, and the incomplete methylation of control DNA sometimes generates the methylation level of biosample exceeding 100%. The high sensitivity of this method is specifically useful for the detection of DNA-methylation biomarkers in noninvasive clinical specimens such as urine or blood, but is less informative for the assessment of tumor tissues with high and heterogeneous methylation levels.

For many years, MSP has been the most popular method for the detection of DNA methylation in clinical material (23). Although MSP is a very sensitive and cost-effective method, it does not allow the quantification of DNA methylation and shows insufficient efficiency for the detection of DNA methylation in freely circulating tumor DNA. The MSP method relies on the methylation status of few CpGs included in a primer sequence and does not amplify DNA with heterogeneous methylation status. In our study, due to this limitation, MSP identified some of the cases with the low levels of methylation as methylation-positive, while some methylation-positive cases were missed in the MSP assay possibly due to the heterogeneous methylation status of target CpGs.

Several studies were done to evaluate the strengths and weaknesses of HRM in comparison with other methods for the detection of DNA methylation, including MSP, QMSP, or pyrosequencing (8, 24–25). In all cases, a good agreement between results generated by quantitative methods was reported indicating HRM as a simple, reproducible, and cost-effective method for the quantification of DNA methylation in clinical material. In some studies, HRM was chosen as the main method for the detection of methylation in the *RASSF1* promoter in BCa samples. Avraham et al. (19) detected the *RASSF1* gene being methylated in more than 80% of BCa and 40% of serum samples, which shows a good applicability of HRM for the detection of methylation in circulating cell-free tumor DNA. Huang et al. (17) also used the HRM method for the assessment of *RASSF1* methylation and detected an 83.3% frequency and a 10%–50% intensity of methylation, which is in line

with our results. In contrast, the study by Wojdacz et al. (18) showed a higher frequency (97%) of *RASSF1* methylation in BCa. None of these studies analyzed associations between *RASSF1* methylation and the clinical-pathological parameters of BCa.

QMSP studies (12–16) found methylated *RASSF1* in 65% up to 86% of BCa cases, with a mean methylation frequency of almost 80% that corresponds well to our results. Significant associations between *RASSF1* methylation and clinical-pathological features, including tumor differentiation grade, and ER and Ki67 status have been identified in QMSP studies. In agreement with these results, our study revealed the predominance of *RASSF1* methylation in less aggressive BCa cases, while in highly aggressive triple negative BCa, the frequency and intensity of the gene methylation was low.

## Conclusions

In summary, *RASSF1* methylation is a biomarker of low-grade, low-stage BCa. These early BCa-specific epigenetic changes can be quantified by the HRM method – the reliable and cost-effective mean for high-throughput analysis of clinical material.

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## Statement of Conflict of Interest

The authors state no conflict of interest.

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