Impact of *RASSF1A* gene methylation on the metastatic axillary nodal status in breast cancer patients

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Abstract. Hypermethylation of CpG islands is a hallmark of cancer and occurs at an early stage in breast tumorigenesis. To gain insight into the epigenetic switches that may promote and/or contribute to the initial neoplastic events during breast carcinogenesis, the present study focused on the DNA methylation profile of invasive breast carcinoma. The aim of the study was to evaluate the prognostic significance of Ras association domain family 1 isoform A (*RASSF1A*) promoter methylation status in operable breast cancer, and to analyze the utility of this biomarker regarding its association with metastatic and nonmetastatic axillary nodal status. For this purpose, formalin-fixed, paraffin-embedded tissue specimens from 116 breast cancer patients with known axillary nodal status were subjected to assessment of *RASSF1A* promoter methylation status by methylation-specific polymerase chain reaction (MSP) and methylation-sensitive high-resolution melting assay, and the results were subsequently validated by bisulfite sequencing. A multinomial logistic regression model was used to model the dependence of distinct levels of methylation status of the *RASSF1A* promoter on the nodal status. Promoter region CpG hypermethylation was identified by MSP in 97 (83.6%) of 116 primary breast tumors, while hypermethylation of *RASSF1A* was confirmed by MS-HRM in 107 (92.2%) of 116 cases of breast cancer. Based on the results of the multinomial logistic regression model, there was no significant difference between the frequency of *RASSF1A* promoter methylation and axillary lymph node status of patients in general. However, upon adjustment of pN stage, an association was identified between pN0 lymph node-negative status (without axillary metastases) and percentage of *RASSF1A* methylation in two groups of heterogeneous methylated alleles with ≤50% methylated ($P<0.05$) and >50% methylated alleles ($P<0.0001$). If a patients' nodal status changes from pN- to pN+ then the risk of having >50% methylated alleles increases by 7%. The present study revealed a specific phenomenon, suggesting that the presence of heterogeneous methylated alleles in the *RASSF1A* gene is significantly associated with lymph node-negative status in breast cancer patients. Furthermore, greater significance with negative axillary nodal status was observed with a higher level of heterogeneous methylated alleles in the *RASSF1A* gene.

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Introduction

Malignant tumors belong to priority areas of concern of any responsible society and its scientific community. For decades, the most common malignant disease in female patients has been breast cancer (1). The pathological evaluation of benign and malignant breast lesions underwent remarkable changes with the introduction of molecular diagnostic methods, and thus, increased knowledge about the biological nature of individual lesions (2).

Mammary carcinoma is a cancer that most commonly affects women, and dissemination of tumor cells by the lymph-vascular pathway at an early stage of development is considered a decisive factor of mortality (3). An early diagnosis of breast carcinoma favors a better prognosis. Breast cancer, at a very early stage of its development, has already cell clones with such a severe genetic defect that can result in metastatic potential and formation of metastases in secondary sites (4,5). In ≤30% of breast cancer patients diagnosed with distant metastasis, conventional treatment methods fail to stop the disease progressing, which suggests an early event of lymph node invasion (5,6). Thus, the detection of occult invasion and lymph node metastasis prediction requires novel, and preferentially more sensitive, methods such as molecular genetics.

By using these methods, structural changes in different genes associated with alterations in the function of proteins can be observed. Such changes may alter or reduce the levels of certain gene protein products, including tumor-suppressor

or metastatic-suppressor genes, thus leading to neoplasia or metastasis formation in secondary sites (7). Currently, part of the standard diagnostic tools monitoring breast carcinoma are biological markers with good informative value, including estrogen receptor (ER), progesterone receptor (PR) and lymph nodes status, which are important prognostic and predictive biomarkers (8). However, although the presence or absence of metastases in axillary lymph nodes is the strongest prognostic factor for patients with primary breast cancer, it only indirectly reflects the tendency of the cancer to spread (9).

Histological examination of axillary lymph nodes, including lymphatic mapping of metastatic spread of the disease, is essential for the detection or exclusion of any tumor cells in the node (10). False-negative examinations of lymph nodes may have severe clinical consequences for the patient. Based on false-negative results, regional lymphadenectomy or systematic chemotherapy is not indicated, and thus, lymph nodes affected by metastatic turnover change may result in untreated disease progression (11). For this reason, nodal status must be investigated more thoroughly than only by using conventional staining techniques of lymph nodes with hematoxylin and eosin, which could be inadequate (12). Currently, there are four standard methodological approaches for surgical identification of sentinel lymph nodes (SLNs): i) ^{99m}Tc-nanocolloid lymphoscintigraphy; ii) blue color tracking methods such as Patent Blue V; iii) a combined method involving the use of both the above substances at the same time; and iv) a paramagnetic method using iron oxide nanoparticles (13).

However, there is still uncertainty and no optimal method for precise metastatic event detection, particularly at the level of micrometastases and isolated tumor cells. This assessment has been recently made by using immunohistochemistry and multilevel serial incision of lymph nodes (14). Axillary lymph node dissection (ALND) has traditionally been a routine component of the management of early breast cancer. The benefits of ALND include its impact on disease control (axillary recurrence and survival), its prognostic value and its role in treatment selection. However, the anatomic disruption caused by ALND may result in lymphedema, nerve injury and shoulder dysfunction, which compromise functionality and quality of life (15). ALND is the typical approach for women who have clinically palpable axillary nodes or positive nodes confirmed by methods such as ultrasound-guided fine needle aspiration. For patients who have clinically negative axillary lymph nodes, SLN biopsy is a method of staging the axilla with less morbidity than that of ALND (16). Logically, sentinel lymph node biopsy (SLNB) without ALND has been recommended as the standard procedure for the management of SLN-negative patients with early breast cancer; however, the efficiency of SLNB for SLN-positive patients remains unclear (16). Pathologists have limited time and ability to perform a precise node examination under surgery. However, the performance of nodal assessment several days prior to the main surgery is now available, and allows pathologists to have a detailed assessment of the lymphatic tissue, either performed as a fine needle aspiration biopsy or as a complete SNB (17-20).

Isolated tumor cells are clusters usually diagnosed by immunohistochemistry and molecular biology methods. It is considered that these cells have no metastatic activity, and the histopathological staging is designated as pN0mi in the regional lymph nodes (21). Currently, micrometastases and isolated tumor cells are undergoing renewed scientific focus in order to identify their prognostic value and clinical outcomes. In general, an urgent requirement to define their prognostic value by promoter methylation status assessment of the Ras association domain family 1 isoform A (*RASSF1A*) gene in the affected nodes both at the level of micrometastases and macrometastases exists (22). Furthermore, these micrometastases can remain dormant for years prior to re emerging as incurable secondary tumors that are insensitive to adjuvant chemotherapies that were previously effective against the primary tumor (21). Further experimental analyses are required to investigate the precise function of *RASSF1A* methylation in breast cancer invasion and metastasis.

As hypermethylation of tumor-suppressor genes is consi dered an early event of breast carcinogenesis, the present study detected aberrant methylation of the tumor-suppressor and cancer-associated gene *RASSF1A* in order to identify its potential correlation with an early stage of axillary nodal affection, since hypermethylation of the *RASSF1A* gene promoter has been reported to be an early event of carcinogenesis and to participate in various gynecological neoplasia (23,24), such as breast cancer (25,26).

As hypermethylation is a transient and markedly sensitive event, the present study used rapid assays for the detection of small levels of heterogeneous methylated alleles in breast cancer patients based on a methylation-sensitive high-resolution melting (MS-HRM) technology and a methylation-specific polymerase chain reaction (PCR) (MSP) approach. These methods were applied to assess the possible role of *RASSF1A* gene hypermethylation in early axillary nodal affection in women with breast cancer.

Materials and methods

Clinical specimens. Formalin-fixed, paraffin-embedded tissue sections from 116 breast cancer patients operated on between June 2013 and June 2016 at the Department of Obstetrics and Gynaecology, Jessenius Faculty of Comenius University and University Hospital Martin (Martin, Slovakia) were evaluated. The histopathological data reflecting cancer biology, including lymph node involvement, were obtained from the medical database at the Department of Pathology, Jessenius Faculty of Comenius University and University Hospital Martin. All participants were of Caucasian origin and residents in the geographic area of Slovakia (Table I). The Regional Ethics Committee of Jessenius Faculty of Medicine (registered under IRB00005636 at the Office for Human Research Protection, USA Department of Health and Human Services) approved the present study protocol (code no. EK 1269/2013). Written informed consent was obtained from all patients.

Histopathological analysis. Tumor and lymph node specimens were fixed in formalin and embedded in paraffin; basic histological examination was performed on 4-5-μm-thick slides stained with hematoxylin and eosin. In selected cases, lymph nodes were stained immunohistochemically (cytokeratin 19) to detect potential isolated tumor cells or micrometastases. Classical morphological indicators such as tumor type and histological grade, were evaluated according

Table I. Clinicopathological characteristics of the cohort (n=116 patients).

Characteristic	Patients, n (%)
Age, years	
<50	20 (17.3)
≥50	96 (82.7)
Stage	
1	65 (56.0)
2	34 (29.3)
3	7 (6.0)
4	6 (5.2)
X	4 (3.5)
Histological grade	
1	21 (18.1)
2	42 (36.2)
3	48 (41.4)
X	5 (4.3)
Lymph node metastases	
Positive	52 (44.8)
Negative	64 (55.2)
ER status	
Positive	95 (81.9)
Negative	21 (18.1)
PR status	
Positive	89 (76.7)
Negative	27 (23.3)
HER-2 amplification	
Positive	23 (19.8)
Negative	93 (80.2)
Molecular subtype	
Luminal A (ER^+ and/or PR^+, $HER\text{-}2^-$)	86 (74.1)
Luminal B (ER^+ and/or PR^+, $HER\text{-}2^+$)	10 (8.6)
Basal-like (ER^-, PR^-, $HER\text{-}2^-$)	8 (6.9)
HER-2 type (ER^-, PR^-, $HER\text{-}2^+$)	12 (10.4)
Tumor type	
Ductal	104 (89.7)
Lobular	7 (6.0)
Other	5 (4.3)

ER, estrogen receptor; PR, progesterone receptor; HER, human epidermal growth factor receptor; X, not determined/not available.

to the World Health Organization criteria and Nottingham grading modification (27,28). The pathologic (p) stage of breast cancer takes into consideration the characteristics of the tumor (T) and the presence of any lymph nodes metastases (N) or distant organ metastases (M). These major tumor characteristics were assessed according to the criteria of the latest tumor-node-metastasis classification (29). Biological parameters, including estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor (HER)-2, were detected immunohistochemically, and their interpretation was based on the American Society of Clinical Oncology/College of American Pathologists criteria from 2010 and 2013 (30-32). Briefly, immunohistochemistry. For ER, PR and HER-2 was performed concurrently on serial sections with ready-to-use (RTU) reagents using an automated immunostainer Autosteiner Link 48 (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA).

Primary ER (Flex Monoclonal Rabbit ERα; clone EP1, RTU; cat. no. IR08461) and PR antibody (Flex Monoclonal Mouse; clone PgR636, RTU; cat. no. IS0683) were supplied by Dako; Agilent Technologies, Inc. Antigen retrieval was performed using EnVision™ Flex Target Retrieval High pH solution (pH 9.0) for 20 min at 97-98˚C in a PT Link instrument (both Dako; Agilent Technologies, Inc.). Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide for 10 min, followed by primary antibody incubation for 20 min at room temperature. The EnVision Flex/HRP High pH kit (cat. no. K8000; Dako; Agilent Technologies, Inc.) was used to visualize staining according to the manufacturer's protocol.

The immunohistochemistry for HER-2 was performed using a HercepTest™ Breast+Gastric kit (cat. no. SK001; Dako; Agilent Technologies, Inc.). Antigens were retrieved in HercepTest Epitope Retrieval Solution (pH 6.0), using PT Link for 40 min at 97-98˚C. Sections were blocked for endogenous peroxidase in 3% hydrogen peroxide for 10 min, and then incubated with the primary antibody for 30 min at room temperature. HercepTest Visualization reagents from the kit were used 30 min at room temperature according to the manufacturer's protocol.

Tumors were considered as ER and PR positive if ≥1% of neoplastic cells stained positively. HER-2-expressing tumors had to exhibit a 3+ reaction in ≥10% of neoplastic cells to be considered positive. Cases with 2+ reaction of HER-2 staining were considered as equivocal and were analyzed by fluorescent *in situ* hybridization to confirm or exclude HER-2 gene amplification. Briefly, slides were hybridized with probes to locus-specific identifier, HER2/neu and centromere 17 using the PathVysion HER-2 DNA Probe kit (Abbott Pharmaceutical Co. Ltd., Lake Bluff, IL, USA) according to manufacturer's protocol.

Definite positivity of HER-2 status in tumors was defined as a HER-2/chromosome enumeration probe 17 ratio of ≥2.0 or an average HER-2 copy number of ≥6.0 signals per cell (31,32).

DNA extraction and bisulfite modification. Paraffin sections of tissue were subjected to deparaffinization by incubation with an organic solvent (xylene) and a decreasing series of 96, 80 and 70% ethanol solutions. Subsequently, the ethanol was removed from the sections by drying the samples at room temperature until the ethanol had evaporated completely. Tissues were suspended in 200 μl of lysis buffer (Buffer AL) and digested using proteinase K (both Qiagen GmbH, Hilden, Germany) for 3 days, or longer if necessary, at 56˚C. Subsequent genomic DNA extraction was performed using DNeasy Blood & Tissue kit (Qiagen GmbH) according to the manufacturer's recommendations. Bisulfite modification of 116 target DNA samples (≤3-5 μg) was performed with the EpiTect Bisulfite kit (Qiagen GmbH) according to the manufacturer's protocol with minor changes. Instead of incubating the columns for 5 min at 56˚C

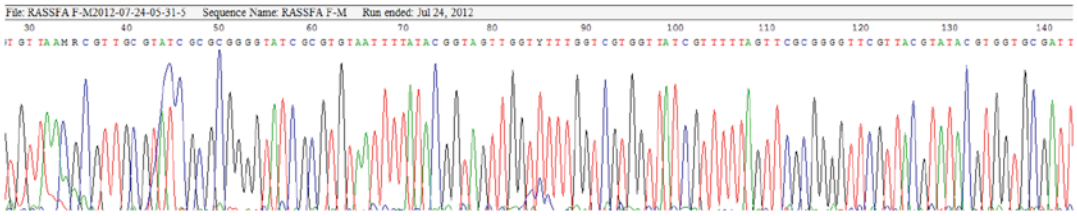

Figure 1. Electropherogram showing a region of the DNA sequence reamplified by methylation-specific polymerase chain reaction with a forward primer for the *RASSF1A* gene promoter region. Sequences were subjected to comparison with the AF132675.1 sequence in the GenBank database. The blue/black CG peaks in the gene represents the presence of methylation in the CpG islands *RASSF1A*, Ras association domain family 1 isoform A.

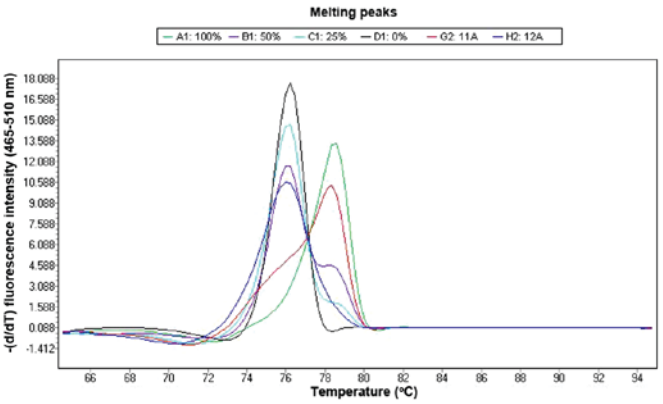

Figure 2. Melting peaks for hypermethylated *RASSF1A* gene promoter showing standards as 100% methylated alleles (green), 50% methylated alleles in a background of unmethylated DNA (purple), 25% methylated alleles in a background of unmethylated DNA (turquoise) and unmethylated control (black). Representative methylation profile of samples with >50% methylated alleles, 11A (red) and with unmethylated alleles as 12A (dark blue) is also shown.

in a heating block, the columns were incubated for 15 min at 56˚C in a thermostat in the present study.

As positive (methylated) and negative (unmethylated) controls, commercially available EpiTect methylated and unmethylated controls (Qiagen GmbH) were used, which contained 0.1 μg/μl methylated and fully unmethylated DNA, respectively.

MSP. The first step of MSP was performed with 2.0 μl of bisulfite-modified DNA template in 25 μl of reaction mixture containing 2.5 mmol/l $MgCl_2$, 10 pmol/l of each forward and reverse external primers, 0.5 mmol/l of each of the four deoxynucleotides and 2.5 mmol/l of 10X ReddyMix PCR buffer (ABgene; Thermo Fisher Scientific, Inc.). Negative-control samples without DNA target were included. The external primers used in first MSP step were forward, 5'-TTGAGT TGYGGGAGTTGGTAT-3' and reverse, 5'-CCCAAATAA AATCRCCACAAAAAT-3'. The amplification reaction was performed with a hot start at 95˚C for 8 min, followed by 45 cycles of denaturation at 95˚C for 30 sec, annealing temperature for external primers of 60˚C for 30 sec, extension at 72˚C for 30 sec and a final step of 8 min at 72˚C. In total, 9 μl of each reaction were loaded onto a 1.5% agarose gel stained with Gel Red™ (Biotium, Inc., Hayward, CA, USA) and visua lized under ultraviolet light. The PCR product for the external primer had a length of 198 bp. The second step of MSP was performed using 1 μl of the PCR product (10-50 ng cDNA) obtained in the first step of the reaction with internal primers, diluted in a 25 μl reaction volume. For methylated DNA targets, the following primers were used: Forward, 5'-GTG TTAACGCGTTGCGTATC-3' and reverse, 5'-AACCCCGCG AACTAAAAACGA-3'. For unmethylated DNA targets, the following primers were used: Forward, 5'-TTTGGTTGGAGT GTGTTAATGTG-3' and reverse, 5'-CAAACCCCACAAACT AAAAACAA-3'. The reamplification products were analyzed on a Gel Red™-stained agarose gel and subsequently validated by bisulfite sequencing.

Bisulfite sequencing. To validate the results from MSP, DNA sequencing was performed on PCR-reamplified MSP products. The PCR products were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherery-Nagel, GmbH, Düren, Germany) according to the manufacturer's recommendations. The purified PCR products were amplified in a sequencing reaction with BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and subsequently purified on a Sepharose™ SigmaSpin Post-Reaction Clean-Up Column (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The products were denatured and then analyzed by capillary electrophoresis in a 3500 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific, Inc.). The resulting sequences were analyzed by using Chromas software 2.0 (Technelysium Pty Ltd., South Brisbane, Australia) and compared with the sequence of the gene *RASSF1A* (AF132675.1) in the GenBank database (https://www.ncbi.nlm.nih.gov/gene?Cmd=DetailsSearch&Term=11186) (Fig. 1).

MS-HRM. MS-HRM detected ≤1% methylated DNA in a background of unmethylated DNA. The technology is sensitive, inexpensive and thus likely to become an appropriate technique for a diagnosis of methylation of the *RASSF1A* gene in breast cancer patients as a predictor of bilateral nodal spread (33). To compare the sensitivity of detection of methylation by various methods, MS-HRM was used, since this method is able to define more precisely the extent of methylation in the sample than MSP (34). MS-HRM was conducted on a LightCycler® 480 System (Roche Diagnostics, Basel, Switzerland). PCR products (5-10 ng cDNA) of the first step of MSP were diluted to the desired concentration in a 10 μl reaction volume, and the standards were prepared by mixing methylated DNA (0.1 μg/μl) with unmethylated DNA (0.1 μg/μl) (both Qiagen GmbH) to obtain 100, 50, 25 and 0% methylated/unmethylated DNA dilutions (Fig. 2). MS-HRM was performed with the same internal primers as described earlier. MS-HRM was performed in a total volume of 10 μl

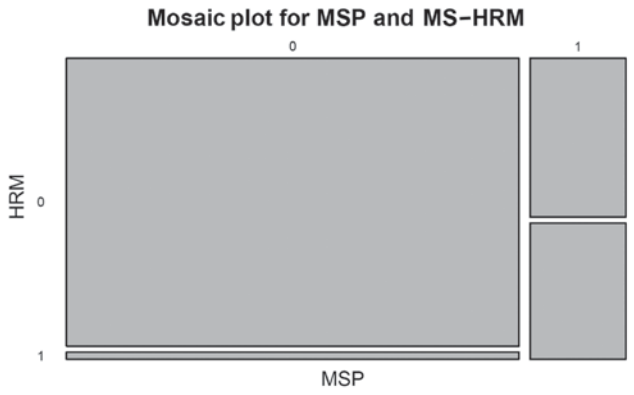

Figure 3. Compliance of the two methods used for detection of the methylation status of the Ras association domain family 1 isoform A gene: MSP and MS-HRM. To compare the two methods, the 4 levels of methylation obtained by MS-HRM were combined into two categories: 1, ≤50% methylated and unmethylated alleles; 0, >50 and 100% methylation statuses. In 3 cases, the samples considered to be 0 (methylated) by MSP were considered to be 1 (unmethylated) by MS-HRM. In 14 cases, the samples considered to be 0 (methylated) by MS-HRM were considered to be 1 (unmethylated) by MSP. The rectangular area represents the number of samples in the particular category. MSP, methylation-specific polymerase chain reaction; MS-HRM, methylation-sensitive high-resolution melting.

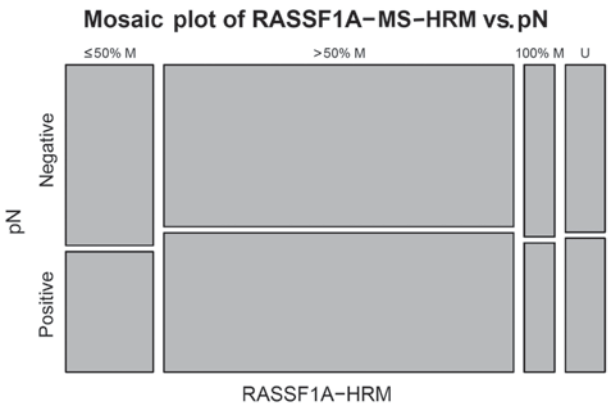

Figure 4. *RASSF1A* methylation profiles by MS-HRM in positive- or negative-pN status. The percentage of *RASSF1A* methylation had a range of 0 (unmethylated allele), ≤50, >50 or 100%. The rectangular area represents the number of samples in the particular category. pN, pathological node; MS-HRM, methylation-sensitive high-resolution melting; M, methylated; U, unmethylated; *RASSF1A*, Ras association domain family 1 isoform A.

of reaction mixture containing 2X EpiTect HRM Master Mix (Qiagen GmbH), 10 μM of each primer, 1 μl of the diluted PCR product from the first step of MSP (5-10 ng per reaction) and RNase-free water to a final volume of 10 μl. Amplification consisted of an initial denaturation step at 95˚C for 5 min, followed by 45 cycles of the following steps: Denaturation for 10 sec at 95˚C, annealing for 30 sec at 61˚C and extension for 10 sec at 72˚C. To perform high-resolution melting analysis, the temperature was increased from 65 to 95˚C. The fluorescence of the binding fluorescent dye was measured continuously as the temperature was increased at a speed of 0.02˚C/sec and was plotted against the temperature.

Statistical analysis. All statistical tests were performed using R software (version 3.2.3) (35). Pearson's χ^2 test with Yates continuity correction was used to test the compliance of the

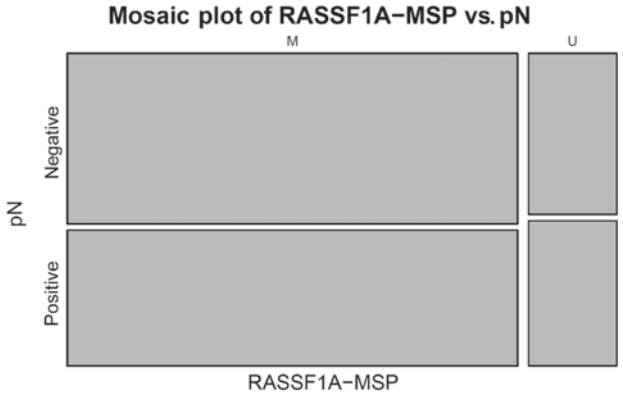

Figure 5. *RASSF1A* methylation profiles by MSP vs. positive or negative nodal status. The method allows the detection of methylated and unmethylated alleles where the extent of methylation can not be assessed. The rectangular area represents the number of samples in the particular category. MSP, methylation-specific polymerase chain reaction; pN, pathological node; M, methylated; U, unmethylated; *RASSF1A*, Ras association domain family 1 isoform A.

two methods used (MSP vs. MS-HRM) (Fig. 3). A multinomial logistic regression model was used to model the dependence of the methylation status levels derived from MS-HRM on the patients' clinicopathological characteristics. P<0.05 was considered to indicate a statistically significant difference.

Results

Overall methylation results. The present study used MSP and MS-HRM to examine the methylation status of the promoter region of the *RASSF1A* gene in paraffin sections of 116 patients with breast cancer (Table I). Promoter region CpG hypermethylation was identified by MSP in 97 of 116 (83.6%) primary tumors, while hypermethylation of *RASSF1A* was confirmed by MS-HRM in 107 (92.2%) of cases.

MS-HRM for RASSF1A vs. lymph node status. The examined methylation status of *RASSF1A* included 64 breast tumor samples with stage N0 (negative lymph nodes) and 52 samples with stages N1, N2 and N3 (positive lymph nodes). The percentage of *RASSF1A* promoter methylation had a distinct range in tissues from patients with different lymph node metastatic stage. Based on the result of the multinomial logistic regression model, there was no significant difference between the frequency of *RASSF1A* promoter methylation among lymph node-positive and node-negative patients in general. However, unexpectedly, an association between pN0 lymph node-negative status (without axillary metastases) and percentage of methylation was detected by MS-HRM in two groups of methylated alleles for *RASSF1A*: ≤50% methy lated group (P<0.05) and >50% heterogeneous methylated group, where a stronger significant association was observed (P<0.0001) (Fig. 4). The MSP method did not identify any significant association (Fig. 5).

MS-HRM vs. MSP results. In the breast cancer samples, comparable results were obtained with the two assays used. More specifically, 48 samples were observed to be methylated and 4 unmethylated in lymph node-positive cases by

MS-HRM, while 59 samples were observed to be methylated and 5 unmethylated in lymph node-negative cases. There were only small differences in the second method used. Only 43 samples were identified as methylated and 9 as unmethylated in lymph node-positive samples by MSP, while 54 samples were methylated and 10 samples were unmethylated in the lymph node-negative cases of breast cancer by MSP.

Statistical outputs. In the pN-negative group, the risk of having >50% methylated alleles was identified as 8.6. In the pN-positive group the risk was 9.25. The comparison of the risks, as estimated by the odds ratio, indicates that if a patient's nodal status changes from pN- to pN+ then the risk of possessing >50% methylated alleles increases by 7%. This is in contrast with the results from the MSP, where moving from pN-negative to pN-positive decreases the risk of possessing >50% methylated alleles by 6%.

Discussion

The tumor-suppressor *RASSF1A* gene is the first identified RASSF family member that is frequently epigenetically inactivated in a wide range of cancer types (36). *RASSF1A* has been reported to be epigenetically inactivated in lung, ovary, bladder, kidney, endometrium and breast tumor tissue (37), and is methylated in ~60-70% of breast cancers (38,39). As a tumor-suppressor gene, *RASSF1A* regulates the activation of cell death (40), cell cycle (41) and microtubule formation (42). The methylation signature of *RASSF1A* is considered to be one among the earliest cellular changes in tumorigenesis (38,39).

At present, DNA methylation is a widely studied epigenetic event (43). A previous study indicated that formalin-fixed, paraffin-embedded tissue is a valuable source for breast cancer biomarkers, for its biologic profiling or validation of certain signaling pathways (44,45). Blocs can be also used for the detection of promoter hypermethylation as a diagnostic and prognostic biomarker in various cancers (46,47). The methylation status of particular tumor-suppressor genes identified in paraffin-tissue samples displayed higher sensitivity for breast cancer origin than conventional biomarkers (43). Similarly, methylation occurs at the early stages of breast cancer development (48), and it may potentially reflect its metastatic potential into lymph nodes (49). Therefore, the present study used MSP and MS-HRM assays to identify a potential association between methylation of *RASSF1A* in breast cancer tissue and bilateral axillary nodal involvement. Bisulfite sequencing was used to validate the results.

Previous studies have identified *RASSF1A* promoter methylation as a potentially useful breast cancer biomarker for the presence of invasiveness of disease (44,50). In addition, other studies have reported that methylation of the *RASSF1A* promoter provides an important prognostic information in operative breast tumors, and that methylation serves an important role in the clinical behavior of breast cancer (26). Although there is a significant effect of *RASSF1A* methylation on the biological characteristics of breast tumors, the association between methylation of CpG islands of this gene in breast tumor tissue obtained from paraffin sections and prognosis prediction by assessment of nodal affection has not been fully established yet. The prognostic value of aberrant methylation of *RASSF1A* in breast tumors has been demonstrated in cell-free DNA circulating in serum prior to therapy, and *RASSF1A* has been reported to be one among the 39 genes with prognostic significance in association with unfavorable development of the disease (45). According to that study, the results on *RASSF1A* methylation from paraffin sections also provide important prognostic information, since patients with *RASSF1A* methylation in the promoter region had a shorter disease-free interval than those with absence of methylation in this gene (45). It is likely that *RASSF1A* gene silencing due to promoter methylation causes deactivation of its tumor-suppressor role, and is therefore a possible contributor to short survival in patients with breast cancer.

Further studies demonstrated that *RASSF1A* methylation confers poor prognosis (44,45,51) and significantly higher methylation with increasing tumor stage (from *in situ* to stage III) was observed, with a trend towards HER-2$^+$ tumors, in women who were lymph node-positive at the time of diagnosis (52). Another study supported these results, since it observed that *RASSF1A* was frequently methylated in metastatic lymph nodes (53). Involvement of axillary lymph node metastasis is one of the single most important prognostic factors in the management of patients with primary breast cancer, and is considered to be a predictor of disease-free survival and overall survival in breast cancer (54,55). Only 20-30% of node-negative patients will develop recurrence within 10 years, compared with ~70% of patients with axillary nodal involvement (55,56). In general, patients with ≥4 involved nodes at initial diagnosis have a worse outcome upon relapse than patients with negative lymph nodes (56). Furthermore, nodal metastasis is not only a marker of diagnosis at a later point in the natural history of breast cancer, but also a marker of an aggressive phenotype (57). Similarly, micrometastases have been associated with decreased survival in the early stage of breast cancer (9). Despite negative SLN-findings, metastases were detected in 7% of patients (58). In another study, 6 patients were identified with lymph node-negative, ER$^+$/HER-2$^-$ breast cancer, and low 21-gene expression assay results (recurrence score of 0-17) were able to determine the risk of distant recurrence within 5 years of their breast cancer diagnosis (59).

The present study is in agreement with the above studies, since hypermethylation of *RASSF1A* was mostly observed in lymph node-positive cases. In addition, methylation occurred even in lymph node-negative cases, which suggests the onset of an epigenetic process in early breast carcinogenesis. Using the MS-HRM method, the results of the current study revealed that *RASSF1A* methylation correlated with SLN metastasis, while no significant association with SNL metastasis was observed using the MSP method. These findings suggest that the silencing of the *RASSF1A* gene is consistent with its role as a tumor suppressor in breast carcinogenesis. The present study provides methylation data in correlation with lymph node status in breast cancer, suggesting that promoter hypermethylation of the *RASSF1A* gene is a molecular predictor of early disease progression.

A great advantage of using MS-HRM is its ability to detect a methylated template in an unmethylated background, with a sensitivity similar to that of MSP. Furthermore,

MS-HRM-based methylation screening is cost-, labor- and time-efficient, in contrast to direct bisulfite sequencing, which therefore, is unsuitable as a screening method. However, it still requires to determine the methylation status of individual CpG sites (60,61).

Compared with MSP, the MS-HRM method provides comparable but not consistent results. The differences between MS-HRM and MSP can be explained by the different principles on which these methods are based (62). In MSP, a positive signal is obtained only in cases where the specific designed methylated primers bind a specific CpG island site in the sequence. However, it is known that different specimens may have different methylation sites in a specific sequence of the promoter region. For instance, if a sample is methylated in positions 2, 5 and 8, and the MSP primers are designed to discern methylation of CpG sites in positions 3, 4 and 7, MSP will provide a negative result, while MS-HRM will provide a positive result, since it is affected by the presence of any methylated CpG island that is located between the primers. On the other side, if the methylation sites that are recognized by the MSP primers are not included in the region amplified by the MS-HRM primers, a sample detected as positive by MSP will be detected as negative by MS-HRM (62). Furthermore, these differences were demonstrated in the results of the methylation analyses in the present study. In addition, the methylation status in the promoter region of the *RASSF1A* gene could be detected by MS-HRM with higher precision than by MSP.

In conclusion, *RASSF1A* is one of the most frequently hypermethylated tumor-suppressor genes detected in breast cancer, and the present results are consistent with those from previous studies (25,26,36). These findings suggest the importance of *RASSF1A* methylation in breast cancer. Furthermore, the association of *RASSF1A* hypermethylation with known clinicopathological features, including lymph node metastasis, provides a better understanding of breast aggressiveness, and it could serve as an important prognostic marker during the treatment of breast cancer patients. Based on the current results, it can be assumed that heterogeneous methylation of the *RASSF1A* gene in breast carcinoma may indicate a potential connection with early-stage metastasis and invasion in ipsilateral axillary lymph nodes, even at a low level. However, this should be demonstrated by using detailed analytical methods, thus increasing the accuracy of this assumption. Such studies must focus mainly on geno-proteomic comparisons between node-positive and node-negative cases in order to examine the same events in metastatic tissue from the affected lymph nodes and the primary tumor. Particularly, based on the results from the IBCSG 23-01 and Z0011 studies, this biological activity and extension assessment is relevant for patient management, and thus axillary dissection could be avoided for patients with limited SN involvement (63,64). Additionally, the results derived from such molecularly focused studies may lead to an improvement in the early detection of axillary metastatic spread of breast cancer in women compared with that of current diagnostic procedures.

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