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A rapid and accurate closed-tube Methylation-Sensitive High Resolution Melting Analysis assay for the semi-quantitative determination of *SOX17* promoter methylation in clinical samples

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ABSTRACT

Introduction: *SOX17* promoter methylation can provide important prognostic information in cancer. We developed a novel semi-quantitative MS-HRMA assay for *SOX17* promoter methylation.
Methods: The assay was optimized by using synthetic control samples and validated by analyzing 165 clinical samples: a) 107 formalin fixed paraffin embedded (FFPEs) samples of patients with early breast cancer, b) 27 FFPE samples of patients with metastatic breast cancer, c) 15 reduction mammoplasty specimens obtained from healthy women and d) 16 genomic DNA samples isolated from healthy blood donors. Comparison with real time MSP was also performed.
Results: The assay is highly specific and sensitive and provides a semi-quantitative estimation of *SOX17* promoter methylation. *SOX17* promoter was found methylated in 96/134 (71.6%) breast cancer samples, while none of the 31 non-cancerous samples tested was positive (0%). *SOX17* promoter methylation levels varied significantly among samples. When 165 clinical samples were analyzed both by MS-HRMA and real time MSP results were significantly comparable (concordance: 146/165, 88.5%).
Conclusions: This novel MS-HRMA assay for *SOX17* promoter methylation is closed-tube, highly sensitive, specific, cost-effective, rapid and easy-to-perform. It gives comparable results to Real-Time MSP in less time, while it offers the advantage of additionally providing an estimation of *SOX17* promoter methylation levels.

1. Introduction

During the last 20 years, DNA methylation has been recognized as an epigenetic mechanism, which plays a major role during the development and progression of many types of cancer [1]. It is known that inactivation of certain tumor-suppressor genes occurs as a consequence of hyper-methylation within the promoter regions and numerous studies have demonstrated a broad range of genes silenced by DNA methylation in different types of cancer [2]. DNA methylation is considered to be an early event in the process of cancer development and progression since tumor suppressor genes are frequently inactivated at very early stages in human cancer. Thus, DNA methylation is considered as a promising tumor biomarker for early detection and prognosis and extremely interesting for therapy approaches [3,4]. Especially during the last 10 years, an impressive technological advancement allows for the highly sensitive and accurate quantification of DNA methylation biomarkers in challenging sample types [1].

SOX17, a member of the Sry-related high mobility group box (SOX) family of transcription factors, is conserved in many species and plays a critical role in the regulation of development and stem/precursor cell function [5,6]. Global analysis of CpG island hypermethylation and gene expression in colorectal cancer cell lines has revealed that *SOX17* gene silencing is associated with DNA hypermethylation [7] and that *SOX17* plays a tumor suppressor role through suppression of the canonical Wnt/β-catenin signaling pathway [8]. *SOX17* is frequently methylated in human papillary thyroid carcinoma while loss of *SOX17* expression was induced by promoter region hypermethylation and methylation of *SOX17* activated the Wnt signaling pathway in human thyroid cancer [9]. Our group has recently shown that *SOX17* promoter is highly

Abbreviations: cfDNA, cell free DNA; CTCs, Circulating Tumor Cells; FFPEs, formalin fixed paraffin embedded; MS-HRMA, Methylation-Sensitive High Resolution Melting Analysis; MSP, methylation Specific PCR; SB, sodium bisulfite; SOX, Sry-related high mobility group box.

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methylated in primary breast tumors, in Circulating Tumor Cells (CTCs) isolated both from patients with early and metastatic breast cancer, and in corresponding cell free DNA (cfDNA) samples [10,11].

Sodium bisulfite (SB) modification of DNA is necessary for DNA methylation assays that are based on PCR amplification, since DNA polymerase does not recognize methylated nucleotides, and as a result methylation information is lost during amplification. Through SB treatment this information is maintained, since non-methylated cytosines are transformed into uracils, while 5-methylcytosines remain unaffected. There are two different approaches, which allow DNA methylation analysis through PCR amplification of SB modified DNA. The first approach is based on design of primers that specifically amplify methylated or non-methylated templates, and is adopted by methylation specific PCR (MSP) and quantitative MSP. The second approach is based on primers that amplify a region of the desired template including CpG islands, no matter what its methylation status is. In this case, information on the methylation status of that region is obtained through post-PCR analysis techniques like bisulfite sequencing, restriction digestion, single-strand conformation analysis, and high-resolution melting [12,13].

High-Resolution Melting Analysis (HRMA), firstly introduced in 2003 [13], has several advantages for clinical analysis, since it is a closed-tube, probe-free technique, rapid, simple, cost-effective and non-destructive. Initially developed for mutation scanning and genotyping studies [14–19], high-resolution melting technology can now be useful for the detection of methylation as well [20–27].

In the present study, we developed and validated a novel, closed tube, highly specific and sensitive, cost-effective, rapid and easy-to-perform assay for *SOX17* promoter methylation based on MS-HRMA. The melting curves or derived melting peaks provide a profile of the methylation status of the entire pool of DNA molecules, thus permitting a semi-quantitative estimation of the gene promoter methylation levels in our clinical samples. We compared the MS-HRMA assay with Real-Time MSP and evaluated the agreement between these two methods. We found that *SOX17* promoter methylation levels varied significantly among these FFPE samples.

2. Materials and methods

2.1. Clinical samples

Our study material consisted of a total of 165 clinical samples: a) 107 FFPE samples of patients with early breast cancer, b) 27 samples of patients with metastatic breast cancer, c) 15 histologically cancer-free (reduction mammoplasty) specimens obtained from healthy women and d) 16 genomic DNA samples isolated from healthy blood donors (DNA was isolated from peripheral blood mononuclear cells). All samples were obtained from the Oncology Unit and Pathology Department, Helena Venizelou Hospital, and the Department of Medical Oncology, University Hospital of Heraklion Crete. All patients gave their informed consent to participate in the study which has been approved by the Ethical and Scientific Committees of our Institution. Tissue sections of 10 μm containing >80% of tumor cells were used for genomic DNA (gDNA) extraction and for subsequent Real-Time MSP and MS-HRMA analysis. gDNA from paraffin tissues was isolated with the QIAamp DNA FFPE Tissue Kit 50 (Qiagen, Germany). DNA concentration was determined in the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA). The clinicopathological characteristics for all patients included in the study are shown in Suppl. Table 1.

2.2. Sodium bisulfite conversion

1 μg of extracted DNA was modified with SB, in order to convert only all non-methylated cytosines to uracil. SB conversion was carried out using the EZ DNA Methylation Gold Kit (ZYMO Research Co., Orange, CA), according to the manufacturer's instructions. The converted DNA was stored at −70 °C until used.

2.3. Quality control

In each SB reaction, dH_2O and gDNA from breast cancer cell lines MCF-7 and SKBR3 were included as negative and positive controls, respectively. Moreover, human placental genomic DNA (gDNA; Sigma-Aldrich) and Universal Methylated Human DNA Standard (ZYMO Research Co., Orange, CA), were used as fully non-methylated and fully methylated controls respectively. Both controls underwent SB conversion, and a series of synthetic controls containing 1%–100% of methylated DNA were prepared by spiking the fully methylated DNA control into the non-methylated. These synthetic methylated DNA controls were used for the evaluation of the sensitivity of the assay and the semi-quantitative estimation of *SOX17* methylation in our clinical samples.

2.4. Methylation Sensitive High Resolution Melting Analysis (MS-HRMA)

2.4.1. In silico primer design

Our MS-HRMA primer set was first designed *in silico*, using the Primer Premier 5 software (Premier Biosoft International, USA). Primers were synthesized by the FORTH (Heraklion, Greece). In MS-HRMA, both methylated and non-methylated target sequences have to be amplified equally so as the percentage of the methylated products reflects their percentage in the original sample. In low annealing temperatures bias favor the non-methylated template. Therefore, the annealing temperature is critical. In order to reverse those PCR bias, to improve the sensitivity of the assay and ensure that only SB converted DNA is amplified our primer set was designed according to the guidelines set by Wojdacz et al. [13,29–32]. Our PCR amplicon consists of 99 bp and the exact position of CGs in the *SOX17* gene and the MS-HRMA primer set used in this study as well as their sequences are given upon request.

2.4.2. Methylation Specific High Resolution Melting Analysis (MS-HRMA)

Our optimization experiments were performed both in the LightCycler® 1.5 instrument (Roche Applied Science, Germany) and LightScanner 32 (LS32™, Idaho Technology, USA) using glass capillary tubes, so that the method can be used in both these instruments that are widely used in clinical labs. Extensive optimization experiments were performed in order to maximize PCR amplification efficiency, including PCR program parameters, Mg^{2+}, primer and template concentrations. In addition, optimization for the annealing temperature in order to reverse PCR bias as described above was carried out. 1 μL (~100 ng) of SB converted DNA was added in the PCR reaction mix, which consisted of 1× PCR Buffer (Promega, USA), 0.2 mM for each dNTP (Invitrogen, USA), 0.05 U/μL GoTaq Hot Start Polymerase (Promega, USA), 0.25 μg/μL BSA (Sigma, Germany), 1× LC-Green Plus Dye (Idaho Technology, USA), 0.25 μM primers, and Mg^{2+} (2.5 mM). dH_2O was used to supplement up to 10 μL. The Real-Time PCR protocol began with one cycle at 95 °C for 2 min followed by 50 cycles of: 95 °C for 10 s, 63 °C for 15 s and 72 °C for 20 s. Immediately after amplification, a rapid cooling cycle to 40 °C for 30 s was introduced in order to prepare the melting curve acquisition step. Real-time fluorescence acquisition was set at the elongation step (72 °C). Samples whose amplification begun late or the relative fluorescence value on the raw melting-curve plot was low were not further processed. All HRMA reactions were performed in duplicate for each sample. HRMA assay optimization studies were performed in the HR-1 High Resolution Melter (Idaho Technology, USA). For this reason, glass capillary tubes were transferred after Real-Time PCR to the HR-1 High Resolution Melter. Melting data acquisition began at 69 °C and ended in 92 °C, using a ramp rate of 0.3 °C/s. Data processing included normalization, and resulted on the normalized melting curves and the respective negative derivative of

fluorescence over the temperature plots. The first step in analyzing the samples is to normalize the fluorescence data. In the instrument this opens a window that shows four vertical cursors numbered 1–4. Cursors 1 and 2 should be moved to identify a linear region of the melting curves prior to the major melting transition of the samples. Cursors 3 and 4 should be moved to identify a linear region of the melting curves following the melting transition of the samples. The cursors must be kept in the same numeric order, relative to each other, from left to right. There are no set rules for positioning the cursors, but as a general rule, it is recommended by the manufacturer to include a larger temperature range between each set of cursors as ramp rate increases. Since in our study the ramp rate was 0.3 °C/s, we set the temperature range between the cursors at 0.5 °C/s. Comparison of the melting curve or the peaks of an unknown sample with those of the controls gave the semi-quantitative estimation for the methylation level of that sample.

2.4.3. Real time MSP

Real time MSP for *SOX17* promoter methylation was used for comparison studies. In this assay that was developed and evaluated in our previous study we are using a specific primer set and a hydrolysis LNA probe for methylated DNA to distinguish the methylated sequence of *SOX17* promoter (11). For maximal discrimination between methylated and non-methylated alleles, both primers and probe contained several CpGs. The analytical sensitivity and specificity of this assay have been previously evaluated [11].

3. Results

3.1. MS-HRMA assay optimization

By using fully methylated and fully non-methylated DNA, as well as synthetic methylated DNA mixtures as controls, optimization of the assay conditions, and evaluation of the analytical sensitivity and specificity of the MS-HRMA assay, for *SOX17* promoter methylation was performed. More specifically:

1 Annealing temperature: three different annealing temperatures were tested (62 °C, 63 °C and 64 °C). The normalized melting curves and the respective derivative plots, as obtained for the synthetic methylated DNA mixtures in all these three temperatures, were best distinguishable from each other at 63 °C.
2 Analytical specificity: the developed MS-HRMA assay for *SOX17* promoter methylation is highly specific for SB treated DNA since under these experimental conditions only SB treated DNA is amplified. When genomic DNA isolated from the MCF7 cell line that was not SB modified was added, amplification under the same conditions was not observed. We could readily discriminate between SB treated methylated and SB treated non-methylated controls and no dimers or "non-specific" products were observed. The non-methylated and the fully methylated SB treated DNA controls gave only one peak at their expected Tm values respectively (Fig. 1).
3 Analytical sensitivity: to evaluate the analytical sensitivity of the assay, dilutions of fully methylated to fully non-methylated DNA (0%, 1%, 10%, 30%, 50% and 100%) were prepared and analyzed. Both peaks were detected as expected when synthetic mixtures containing both methylated and non-methylated *SOX17* promoter sequences were used (Fig. 2). Fluorescence difference plots were generated and the ability to discriminate melting transitions of methylated DNA samples from that of non-methylated DNA samples was assessed. As can be seen in Fig. 2 the presence of 1% of methylated *SOX17* sequence can be easily detected in the presence of 99% non-methylated *SOX17* sequence. When the analysis for the same control samples was repeated three times in three different days, melting curves were highly reproducible.

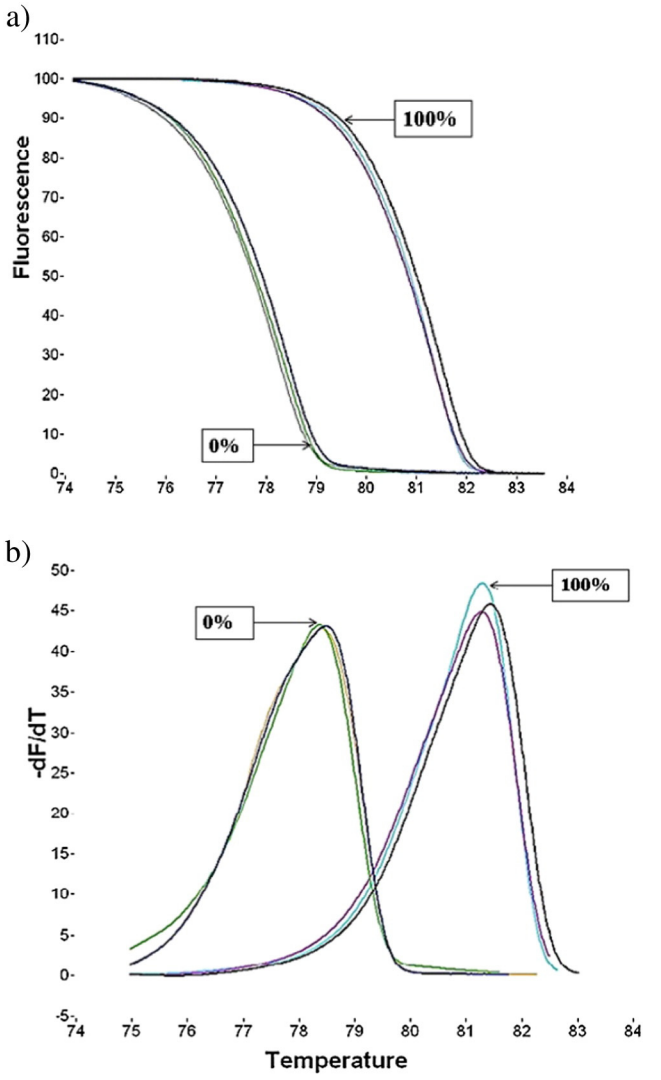

Fig. 1. Analytical specificity and reproducibility of the MS-HRMA assay for *SOX17* promoter methylation. a) Normalized melting curves of 0%: human placental genomic DNA, 100% methylated control: universal methylated human DNA standard, run three times at three different days, b) first derivative MS-HRMA plots of 0% and 100% methylated controls, run three times at three different days.

3.2. SOX17 promoter methylation in clinical samples by using the developed MS-HRMA assay

By using the developed MS-HRMA, we evaluated *SOX17* promoter methylation in a total of 165 DNA samples: a) 107 FFPE samples of patients with early breast cancer, b) 27 samples of patients with metastatic breast cancer, c) 15 non-cancerous breast tissue samples (mammoplasties) and d) 16 genomic DNA samples isolated from peripheral blood mononuclear cells (PBMC) of healthy donors.

SOX17 promoter was found to be highly methylated in 96/134 (71.6%) breast cancer samples; it was highly methylated both in early breast cancer 78/107 (72.9%) and metastatic disease 18/27 (66.7%). It is important to note that none of the 15 (0%) histologically cancer-free specimens from reduction mammoplasty (Fig. 3a) or the 16 non-cancerous DNA samples obtained from healthy blood donors (Fig. 3b) was found to be methylated for *SOX17* promoter.

3.2.1. Semi-quantification of SOX17 promoter methylation levels in clinical samples

The melting patterns of clinical samples when compared to that of the spiked control samples containing known percentages of *SOX17*

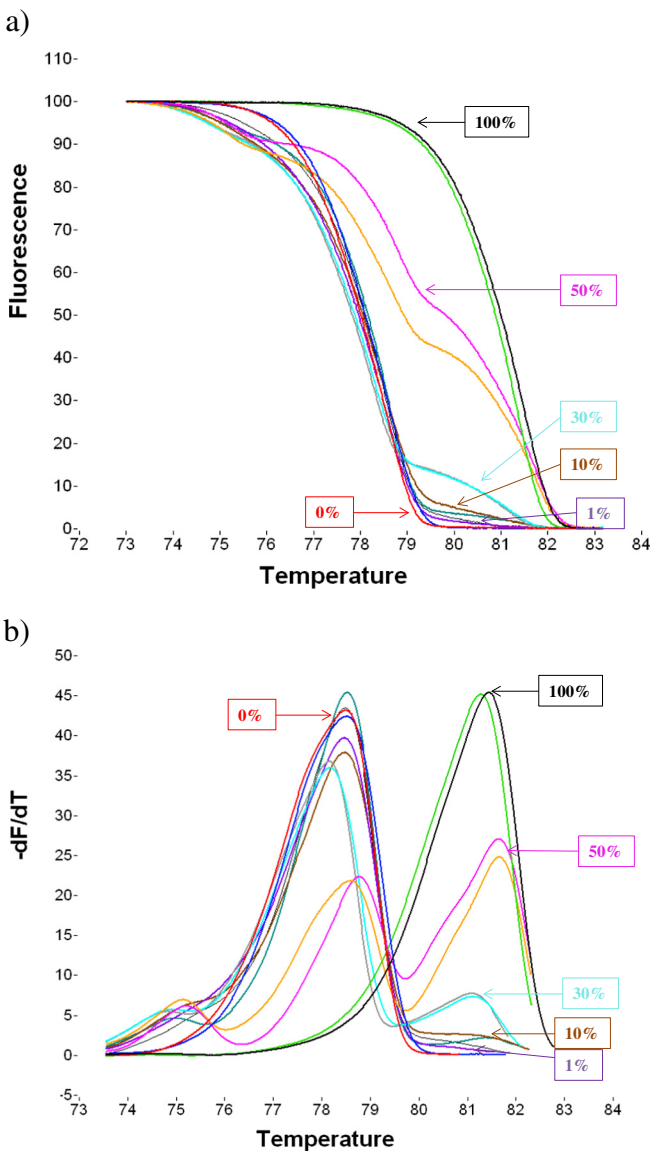

Fig. 2. Analytical sensitivity of the MS-HRMA assay by using synthetic mixtures of non-methylated to fully methylated DNA. a) normalized melting curves of red: 0%, purple: 1%, brown-dark green: 10%, turqoise-grey: 30%, margenta-orange: 50%, black-green: 100% methylated DNA, b) first derivative MS-HRMA plots of synthetic mixtures: Red: 0%, green: 1%, orange: 10%, pink: 30%, blue: 50%, black: 100% methylated DNA.

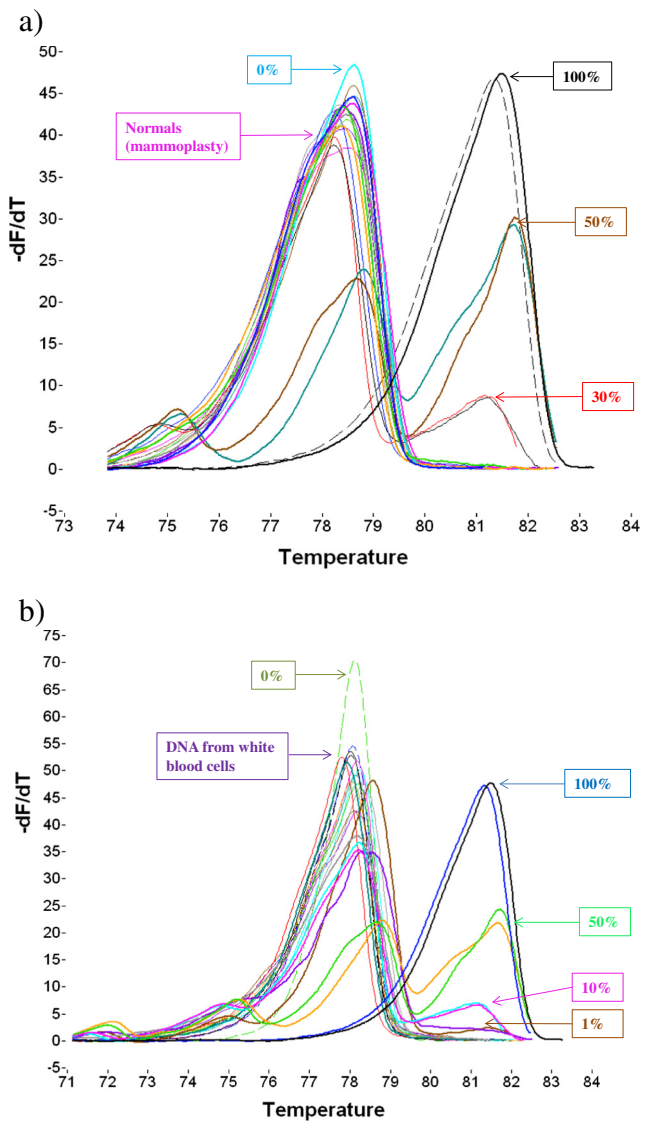

Fig. 3. Specificity of the MS-HRMA assay: *SOX17* promoter methylation of non-cancerous samples. a) First derivative plots of histologically cancer-free specimens obtained from healthy women during reduction mammoplasty (n = 15) combined with the control levels of methylation, b) DNA isolated from PBMC obtained from healthy blood donors (n = 16).

methylation, always run in parallel, allowed for their classification as non-methylated or methylated, while the percentage of methylation in these samples could also be estimated (Fig. 4). According to our findings, the methylation levels in our clinical samples ranged from slightly lower than 1% up to approximately 100%. A graph presenting in a semi-quantitative way *SOX17* promoter methylation percentage for each sample across all sample groups tested is shown in Fig. 5. Mann–Whitney and Kolmogorov–Smirnov non-parametric tests were performed to evaluate whether a significant difference in methylation levels between those groups exists. Fig. 5 demonstrates that the methylation levels for tumor FFPE samples from operable breast cancer patients (n = 107) were significantly different ($p < 0.001$) than corresponding methylation levels for DNA samples isolated from PBMC cells obtained from healthy blood donors (n = 16), and FFPE non-cancerous DNA samples belonging to healthy individuals that underwent mammoplasty surgery (n = 15) ($p < 0.001$). However, there was not a significant difference between operable breast cancer patients samples and samples from 27 patients with metastatic disease ($p = 0.385$ for the Mann–Whitney test and $p = 0.342$ for the Kolmogorov–Smirnov test).

3.3. Comparison between MS-HRMA and real time MSP

Furthermore, we compared the newly developed semi-quantitative MS-HRMA assay with our previously reported Real-Time MSP assay for *SOX17* promoter methylation [11]. When all samples were analyzed by both assays, results were comparable (Table 1); in total, for 146/165 (88.5%) samples these two assays gave comparable results (Table 1). More specifically, 55 samples were found negative and 91 samples were found positive by both assays, while 14 samples were positive for Real-Time MSP and negative for MS-HRMA and 5 samples were positive by MS-HRMA and negative by Real-Time MSP. We evaluated the agreement between these two assays by calculating the kappa index adjusted for a 2-way comparison that has been developed as a measure of agreement that is corrected for chance [33]. According to the Guidelines for Strength of Agreement Indicated with K Values,

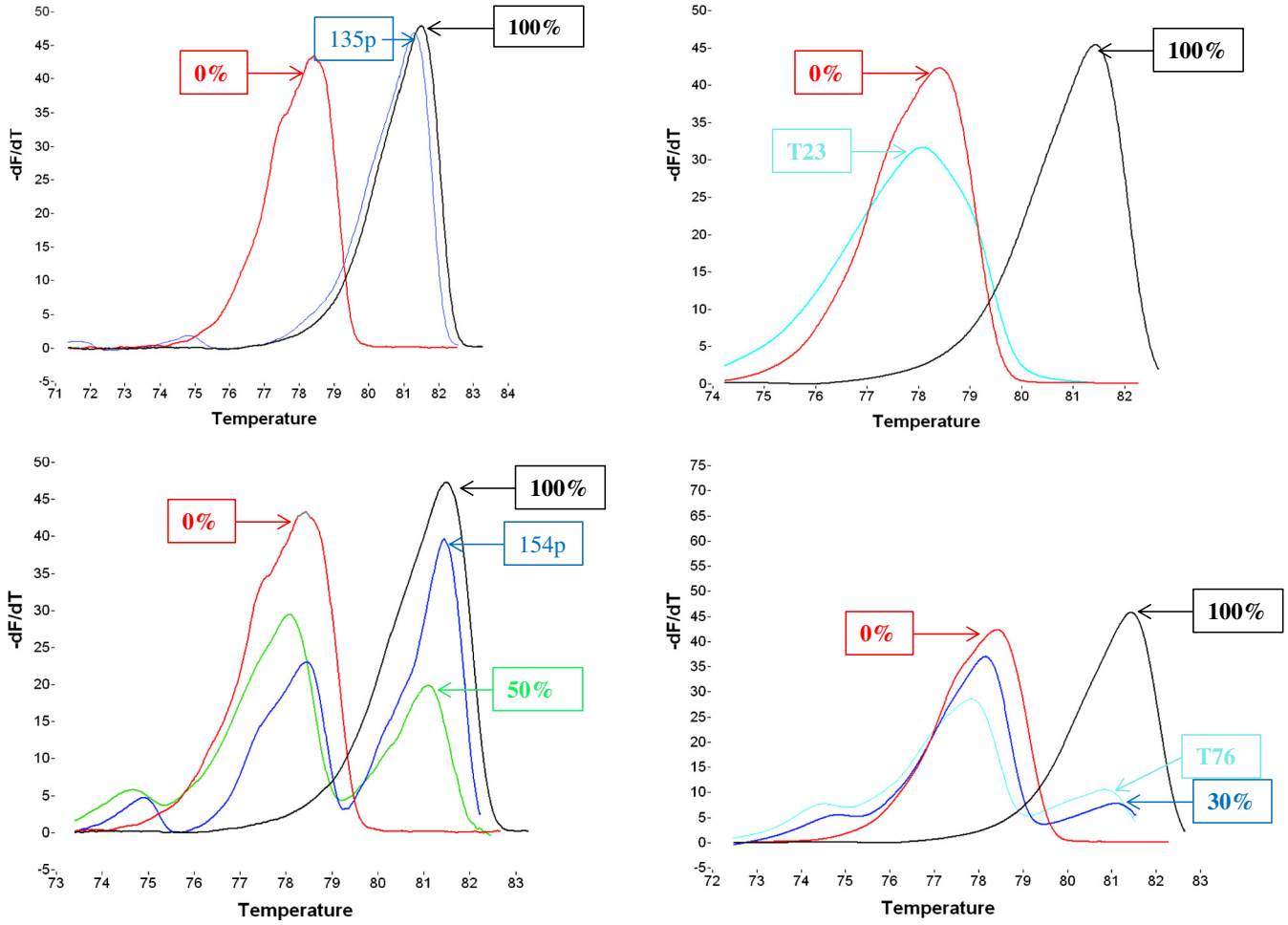

Fig. 4. Characteristic first derivative plots for the semi-quantitative estimation of % methylation for *SOX17* promoter methylation by MS-HRMA in four tumor FFPE samples: Sample#135p: 100%, Sample#T23: 0%, Sample#154p: >50%, and Sample#T76: 30%–50%.

the resulting kappa value of 0.7589 is indicative of a substantial agreement between these two methods. Kappa index was calculated according to a program that is available online (http://vassarstats.net/kappa.html) while statistical analysis was performed using the SPSS Windows version 22.0 (SPSS Inc., Chicago, IL).

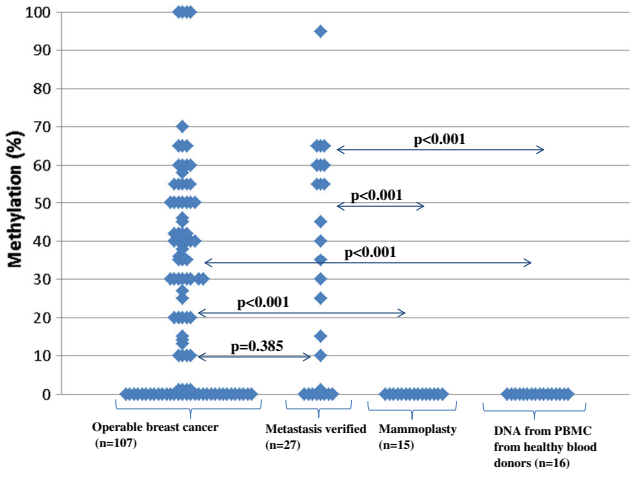

Fig. 5. *SOX17* methylation levels for individual clinical samples as estimated by MS-HRMA (P values were estimated by the Mann–Whitney test).

4. Discussion

SOX17 plays a critical role in the regulation of development and stem/precursor cell function [5,6]. Recently our group has shown for the first time by using real time MSP that *SOX17* promoter is methylated

Table 1
Contingency table which tabulates the outcomes of MS-HRMA and real time MSP for *SOX17* promoter methylation for all samples tested and kappa index values (n = 165).

Method		Real-Time MSP		Total
		Pos	Neg	
MS-HRMA	Pos	91	5	96
	Neg	14	55	69
Total		105	60	165

Concordance: 146/165 = 88.5%

Indices of agreement for MS-HRMA and real time MSP for *SOX17* methylation

Agreement index	Type of agreement	Calculated values	Standard error	CI (95%)
p_o	Overall	0.8848		
p_{pos}	Positive	0.9055		
p_{neg}	Negative	0.8527		
p_e	Chance	0.5223		
Kappa index	Chance corrected	0.7589	0.0516	0.6577–0.8601

in CTCs and cell free DNA isolated from peripheral blood of breast cancer patients [10,11]. In our previous studies [10,11] we reported our findings in a qualitative matter as positive or negative for the presence of *SOX17* methylated sequences in our samples. Based on this real time MSP, even if our data indicated which patients were considered to arbitrarily be positive or negative, we had no quantitative indication of the percentage of *SOX17* promoter methylation that exists in each individual patient and in different patient groups as compared to normal.

Recently, the development of a new generation of melting instrumentation and the introduction of highly sensitive fluorescent dye chemistries, allowed the development of MS-HRMA. This technique is based on the different melting profiles of non-methylated and methylated PCR products, due to their different sequence composition in respect to the CG content [13]. MS-HRMA is characterized by high sensitivity, reproducibility and accuracy, and is very appropriate for molecular diagnostic applications, since it is a closed tube technique less prone to contamination problems [28]. We have recently developed a MS-HRMA assay for the investigation of *CST6* promoter methylation that is highly methylated in cancer, and we have shown that its detection can provide important prognostic information in breast cancer patients [20].

In this study we present a novel closed tube MS-HRMA assay for *SOX17* promoter methylation that is highly sensitive, specific, cost-effective, rapid and easy-to-perform. This method gives comparable results to Real-Time MSP in less time, while it offers the' advantage of additionally providing an estimation of the gene promoter methylation levels in clinical samples. The newly developed MS-HRMA assay that we present here enables us to visualize the difference in methylation levels between normal and malignant samples. A key note of our findings is that by using this assay we are showing for the first time that *SOX17* promoter methylation levels differ significantly between individual samples. The clinical importance of this finding has to be evaluated later, when the clinical outcome of these operable breast cancer patients is known.

Through several recent studies it has been shown that besides breast cancer, *SOX17* promoter methylation is of clinical importance in many other types of cancer as well. We have recently shown that *SOX17* promoter methylation in cell free DNA of patients with operable gastric cancer is a frequent event and may provide important information regarding prognosis in this group of patients [34]. In another study, Kuo et al. identified *SOX17* among a panel of CpG methylation biomarkers that are important for prognosis prediction of esophageal squamous cell carcinoma (ESCC) patients [35] by using Illumina's GoldenGate methylation arrays. In addition, to the prognosis related important findings, they also detected an inverse correlation between CpG hypermethylation and the mRNA expression level of *SOX17* gene in ESCC patients, indicating that DNA hypermethylation was responsible for decreased expression of SOX17 [35]. In primary high-risk human papillomavirus (hrHPV)-DNA testing in a cervical cancer screening setting, a methylation signature comprising the 5′ regions of five genes including *SOX17* that is specific for CIN3 and cervical cancer (termed CIN3 +) was identified and validated. According to the findings reported by Hansel et al. [36], a high detection rate of CIN3 + was obtained if at least 2 of these five gene markers were methylated, indicating that clinical validation studies are required to determine the usefulness of these novel markers. Another recent study in cervical cancer identified 14 hypermethylated genes including *SOX17* that were significantly hypermethylated in CIN3 + lesions. The concurrent methylation of these genes in precancerous lesions suggests the presence of a driver of methylation phenotype in cervical carcinogenesis [37]. A recent study investigated promoter methylation of several Wnt-pathway antagonists including *SOX17* in non-polypoid adenomas that are a subgroup of colorectal adenomas that have been associated with a more aggressive clinical behavior compared to their polypoid counterparts [38]. Goeppert et al. [39] investigated the molecular mechanisms underlying the genesis of cholangiocarcinomas (CCs) by performing a genome-wide analysis for aberrant promoter methylation in human CCs and found that in CC cell lines, silencing of genes involved in Wnt signaling, such as *SOX17*, was reversed after 5-aza-2′ deoxycytidine administration. Several candidate genes of cancer-relevant signaling pathways were identified, and closer analysis of selected Wnt pathway genes confirmed the relevance of this pathway in CC. Based on all this recent information, further validation of *SOX17* methylation as a biomarker in many types of cancer in large population-based studies is needed.

The described closed tube MS-HRMA assay for *SOX17* promoter methylation is highly sensitive, cost-effective, rapid and easy-to-perform. It gives comparable results to Real-Time MSP in less time, while it offers the advantage of additionally providing an estimation of *SOX17* promoter methylation levels. By using this assay we are showing for the first time that *SOX17* promoter methylation levels differ significantly between individual clinical samples. This assay can be used in a variety of cancers where *SOX17* methylation is frequent, so that the clinical importance of different *SOX17* methylation levels can be evaluated. We strongly believe that many very interesting questions can be answered in the near future, by applying our semi-quantitative assay for *SOX17* promoter methylation in a variety of clinical samples.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2015.02.035.

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