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ORIGINAL PAPER

Validation of DNA promoter hypermethylation biomarkers in breast cancer — a short report

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Abstract
Purpose DNA promoter hypermethylation of tumor suppressor genes is known to occur early in cancer development, including breast cancer. To improve early breast cancer detection, we aimed to investigate whether the identification of DNA promoter hypermethylation might be of added value.
Methods The methylation status of a panel of 19 candidate genes (*AKR1B1, ALX1, ARHGEF7, FZD10, GHSR, GPX7, GREM1, GSTP1, HOXD1, KL, LHX2, MAL, MGMT, NDRG2, RASGRF2, SFRP1, SFRP2, TM6SF1* and *TMEFF2*) was determined in formalin-fixed paraffin-embedded normal breast and breast cancer tissue samples using gel-based methylation-specific PCR (MSP).
Results The promoters of the *AKR1B1, ALX1, GHSR, GREM1, RASGRF2, SFRP2, TM6SF1* and *TMEFF2* genes were found to be significantly differentially methylated in normal versus malignant breast tissues. Based on sensitivity, specificity and logistic regression analyses the best performing genes for detecting breast cancer were identified. Through multivariate analyses, we found that *AKR1B1* and *TM6SF1* could detect breast cancer with an area under the curve (AUC) of 0.986 in a receiver operating characteristic (ROC) assessment.
Conclusions Based on our data, we conclude that *AKR1B1* and *TM6SF1* may serve as candidate methylation biomarkers for early breast cancer detection. Further studies are underway to evaluate the methylation status of these genes in body fluids, including nipple aspirates and blood.

Keywords Breast cancer · Early detection · DNA promoter hypermethylation · Biomarkers · Validation

1 Introduction

Worldwide, breast cancer is the most common cancer in women [1]. Incidence and survival rates differ between countries with a lifetime risk of 1 in 9 women in The Netherlands. Due to increasing life expectancy and changes in lifestyle, the breast cancer burden is rising in developing countries. Survival rates range from more than 80 % in high developed countries to less than 40 % in developing areas [1]. Early detection is of essence to improve breast cancer survival. DNA promoter hypermethylation of tumor suppressor genes occurs early in carcinogenesis with a high frequency in many cancer types, including breast cancer [2–5]. Therefore, to improve early breast cancer detection, the identification of DNA promoter hypermethylation could be of added value. Previously, we reported that DNA promoter hypermethylation of 11 genes (i.e., *RARB, RASSF1, TWIST1, CCND2, ESR1, SCGB3A1, BRCA1, BRCA2, CDKN2A, APC* and *CDH1*) may be involved in the development of both sporadic and *BRCA1*-associated breast cancers [6]. Since then, genome-wide hypermethylation studies have revealed several additional methylation biomarkers (listed in supplementary Table S1). As yet, however, most of these biomarkers have not been validated in independent breast cancer cohort studies. In order to increase the sensitivity and specificity of our previously reported gene panel for the early detection of breast cancer, we selected 19 additional candidate genes (Table 1, see Table S1 for extended version) reported to be hypermethylated in breast cancer, based on genome-wide methylation screens and/or published literature data. These 19

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Table 1 Overview of the 19 investigated candidate methylation biomarkers for breast cancer. See Table S1 for more detailed information

Gene	Full name	Reference	Material used in methylation analysis (N)	Hypermethylation validated in BC patients (N)	If not validated in BC patients, validated in other cancer patients	Hypermethylation validated in nipple/ductal fluid of BC patients
ALX1	ALX homeobox protein1	[11]	BC patient tissue (103), normal breast tissue (21)	No	Yes	No
AKR1B1	Aldo-keto reductase family 1, member B1 (aldose reductase)	[11, 12]	BC patient tissue (103), normal breast tissue (21); sera recurrent stage 4 BC (24) and healthy women (28)	Primary tumors (82); sera recurrent stage 4 BC (33) and healthy women (27)	NA	No
ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7	[18, 12]	BC tissue (39); sera recurrent stage 4 BC (24) and healthy women (28)	BC tissue (132); sera recurrent stage 4 BC (33) and healthy women (27)	NA	No
FZD10	Frizzled family receptor 10	[19]	Random fine needle aspiration healthy women (136)	No	No	No
GHSR	Growth hormone secretagogue receptor	[20]	BC samples (31)	BC samples (26)	NA	No
GPX7	Glutathione peroxidase 7	[11, 12]	BC patient tissue (103), normal breast tissue (21); sera recurrent stage 4 BC (24) and healthy women (28)	Sera recurrent stage 4 BC (33) and healthy women (27)	NA	No
GREM1	Gremlin 1	[21]	BC cell lines (4) different metastatic potential	BC samples (127)	NA	No
GSTP1	Glutathione S-transferase pi 1	[22]	Invasive BC tissue (20)	Invasive BC tissue (30), DCIS (35)	NA	[23]
HOXD1	Homeobox D1	[21, 24]	BC cell lines (4) different metastatic potential; low grade in situ and invasive BC (10) vs. healthy (10)	BC samples (120); invasive (75) and in situ (13) BC samples	NA	No
KL	Klotho	[21]	BC cell lines (4) of different metastatic potential	BC samples (30)	NA	No
LHX2	LIM homeobox 2	[25]	Stage 1 BC samples (3)	BC samples (39)	NA	No
MAL	Mal, T cell differentiation protein	[26]	BC cell lines (14)	BC samples (36)	NA	No
MGMT	O-6-methylguanine-DNA methyltransferase	[27–29]	Tripe negative BC (92); BC tissue ranging from DCIS to invasive tumors stage I to IV (238); basal-like BC (26)	[30-32]	NA	No
NDRG2	NMYC downstrean-regulated gene (NDRG)family member 2	[21]	BC cell lines (4) of different metastatic potential	BC samples (127)	NA	No
RASGRF2	Ras protein-specific guanine nucleotide-releasing factor 2	[18, 12]	BC tissue (39); sera recurrent stage 4 BC (24) and healthy women (28)	BC tissue (132); sera recurrent stage 4 BC (33) and healthy women (27)	NA	No
SFRP1	Secreted frizzled-related protein 1	[21, 22]	BC cell lines (4) of different metastatic potential; invasive BC tissue (20)	BC samples (30); IDC tissue (30), DCIS (35)	NA	No
SFRP2	Secreted frizzled-related protein 2	[20]	BC samples (31)	BC samples (26)	NA	No
TM6SF1	Transmembrane 6 superfamily member 1	[12]	Sera recurrent stage 4 BC (24) and healthy women (28)	Sera recurrent stage 4 BC (33) and healthy women (27)	NA	No
TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains	[22, 11, 12]	Invasive BC tissue (20); BC patient tissue (103), healthy breast tissue (21); sera recurrent stage 4 BC (24) and healthy women (28)	IDC tissue (30), DCIS (35); primary tumors (82); sera recurrent stage 4 BC (33) and healthy women (27)	NA	No

candidate genes were specifically chosen on the basis of (i) the frequency by which they were identified as potential biomarkers and (ii) their presumed hypermethylation status in low-grade breast cancers. We performed a validation study using methylation-specific PCR (MSP) to assess DNA promoter hypermethylation of the selected genes in 21 cancerous and 10 normal breast tissue samples. MSP is a sodium bisulfite-based, qualitative method for DNA promoter methylation analysis [7]. PCR primers were designed to be complementary to completely methylated or completely unmethylated target DNA [8]. The breast cancer samples included in this study consisted of high and low grade invasive breast cancers, combined with ductal carcinomas in situ (DCIS) samples to represent different stages of breast cancer progression.

2 Materials and methods

2.1 Patients and tissue samples

Formalin-fixed paraffin-embedded (FFPE) tissue samples were obtained from the Pathology Department of the University Medical Center Utrecht (UMC Utrecht). Tissue samples were selected based on their tumor status: 4 from patients diagnosed with high grade breast cancer, 2 from patients with intermediate grade breast cancer, 10 from patients with low grade breast cancer, 5 from patients with DCIS, and 10 normal breast tissue samples obtained from mammoplasty specimens as controls. Hematoxylin and eosin (H&E) stained slides were reviewed by an experienced pathologist (PvD) to confirm the presence or absence of malignancy. The percentage of carcinoma cells, if applicable, was estimated to be at least 50 %. The clinical characteristics of the study samples are listed in Table 2. The mean ages of the women in the control group (normal breast tissue) and the breast cancer group were 33.6 and 55.9 years, respectively. This study was performed in accordance with the institutional ethical guidelines. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in the UMC Utrecht [9].

Table 2 Clinicopathological characteristics of breast cancer and DCIS tissue samples

		High grade BC N=6	Low grade BC N=10	DCIS N=5
Age (years)	Mean	50.8	60.5	54.0
Histological type	Ductal	5	7	NA
	Ductulolobular	1	1	
	Lobular	0	1	
	Other	0	1	
ER status	+	4	9	NA
	–	2	1	
PR status	+	3	10	NA
	–	3	0	
HER2 IHC score	0–1+	3	9	NA
	2+	0	1	
	3+	3	0	
Grade	1	0	10	1
	2	2	0	2
	3	4	0	2
Mitotic activity index	<13	3	10	NA
	≥13	3	0	
Tumor size (cm)	Mean	6.0	1.8	NA
Lymph node metastases	Yes	5	6	NA
	No	0	4	
	Unknown	1	0	

2.2 DNA extraction

For the isolation of DNA from the FFPE tissues, one to five 10 μm unstained sections were deparaffinized in xylene and rehydrated through a graded series of alcohol. Relevant tissues were scraped from the slides and 100 μl lysis buffer (0.5 % Tween-20, 50 mM Tris pH 8) containing 20–40 μg proteinase K (Sigma, P6556) was added. After incubation at 56 °C overnight, the reactions were heat inactivated for 10 min at 95 °C and the mixtures were centrifuged at 14,000 rpm for 3 min. The supernatants were transferred to new tubes and the DNA concentrations, as well as 260/280 absorbance ratios, were determined using a spectrophotometer (NanoDrop ND-2000, Thermo Scientific). The samples were stored at 4 °C until further analysis.

2.3 Sodium bisulfite conversion

Sodium bisulfite conversion was performed using the Epitect bisulfite kit (Qiagen, 59104) according to the manufacturer's protocol (Sodium Bisulfite Conversion of Unmethylated Cytosines in Small Amounts of Fragmented DNA; protocol version 09/2009) with an input of 1.5 μg DNA. Human sperm DNA was used as a negative control and *Sss*I methylase-treated MDA-231 gDNA was used as a positive control. In addition, a non-template control was included in each bisulfite conversion reaction.

2.4 External multiplex PCR

External PCR was performed with a primer mix to co-amplify the DNA promoters of the following 19 genes, regardless of their methylation status: *AKR1B1, ALX1, ARHGEF7, FZD10, GHSR, GPX7, GREM1, GSTP1, HOXD1, KL, LHX2, MAL, MGMT, NDRG2, RASGRF2, SFRP1, SFRP2, TM6SF1* and *TMEFF2*. The respective primer sequences are listed in Table S2. For each PCR reaction 10 μl Epitect-treated DNA was added to 15 μl reaction mix consisting of 1x MSP buffer (67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 10 mM β-

mercaptoethanol, 0.1 % DMSO), 1.25 mM dNTPs, 2.5 units of Platinum Taq (Life Technologies, 10966–083) and 2.2 μM of each of the forward and reverse primers (dissolved in distilled water containing 50 μg/ml salmon sperm DNA). The PCR conditions used were: 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 45 s and 72 °C for 45 s, with a final extension cycle of 72 °C for 7 min. In each multiplex PCR run no template controls were included. The PCR products were 5000x diluted in dilution buffer (distilled water containing 1x MSP buffer and 100 μg/ml salmon sperm DNA) for further analysis.

2.5 Gene-specific internal PCR

To investigate a specific target gene promoter of interest, a nested methylation-specific PCR (MSP) was performed for each candidate gene separately. The respective primer sequences are listed in Table S3. In the internal PCR reaction, 4 μl of the 5000x diluted multiplex PCR product was added to 16 μl of reaction mix consisting of 1x MSP buffer, 200 μM dNTPs, 1.25 units of Platinum Taq (Life Technologies, 10966–083), 50 μg/ml salmon sperm DNA, and 800 nM of each primer. The PCR conditions were: 95 °C for 7 min, followed by 20 to 35 cycles (see Table S3) of 98 °C for 15 s and 65 °C for 1 min. The PCR products (10 μl) were analyzed on 2 % agarose gels and visualized through UV illumination. The intensity of the bands was quantified using the ImageQuant software package (1D analysis; TL Control Centre, Amersham Biosciences, v2003.02). The methylation percentages were calculated by dividing the intensity of the methylated band (M) by the sum of the intensities of the methylated and unmethylated (UM) bands of each gene, and corrected for the methylation percentage in the positive control (if not 100 %). The cumulative methylation index (CMI) was calculated per sample as the sum of the methylation percentages of all investigated genes.

2.6 Statistical analyses

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated based on the presence or absence of a methylation signal. For this analysis, high grade, low grade and DCIS samples were taken as malignant samples. The Pearson Chi-Square test was used to compare binary values, and the One-Sample Kolmogorov-Smirnov test was used to determine the distribution of continuous values. An independent sample *T*-test was performed if the distribution was normal, and an independent-Samples Mann–Whitney *U* Test if the distribution was not normal. Two-sided p values < 0.05 were considered to be statistically significant.

Logistic regression was performed, including age and genes having a specificity > 50 %, using a backward selection procedure, retaining covariates with p values ≤ 0.10. For the independent predictors, a combined receiver operating characteristic (ROC) graph was made and the area under the curve (AUC) with 95 % confidence interval (CI) was determined. For all statistical analyses IBM SPSS Statistics version 20 was used.

3 Results

To determine the DNA promoter methylation status of 19 a priori selected genes (see materials and methods), a semi-quantitative methylation-specific PCR (MSP) was carried out. By doing so, we found that the promoters of the *AKR1B1, ALX1, GHSR, GREM1, RASGRF2, SFRP2, TM6SF1* and *TMEFF2* genes were significantly differentially methylated between normal and malignant tissues (Table 3), i.e., the absolute methylation levels of these genes were higher in breast cancer tissues compared to normal breast tissues. In case of the *GHSR* gene, the breast cancer samples were generally hypomethylated compared to the normal breast tissues. Figure 1 depicts the methylation signals in the breast cancer, DCIS and normal breast tissue samples of the *TM6SF1* and *GHSR* gene promoters.

Table 3 shows the sensitivity, specificity, and positive and negative predictive values based on methylation being present or absent (see also supplementary Figure S1). The *AKR1B1, ALX1, GPX7, RASSGRF2, SFRP2, TM6SF1* and *TMEFF2* gene promoter methylation significantly differed between the normal and breast cancer tissues based on dichotomized methylation values. Since normal breast specimens were obtained from breast reduction surgeries that usually take place at a relatively young age, the women in the control group were significantly younger (p <0.000) than those in the breast cancer group. However, using logistic regression analysis, *AKR1B1*, *TM6SF1* methylation signals and age appeared to be significant independent predictors of breast cancer (AUC 0.986; CI95 0.949–1.000). By using the cumulative methylation index (CMI) of *AKR1B1* and *TM6SF1*, the sensitivity of detecting breast cancer was found to be 76.2 % and its specificity 100 %. For *AKR1B1*, methylation was detected in 60 % of the DCIS samples, 56 % of the low grade and 83 % of the high grade breast cancer samples. For *TM6SF1* these numbers were 60, 67 and 80 %, respectively.

4 Discussion

Through this study, we aimed to independently validate the diagnostic value of a panel of 19 candidate genes previously reported to be differentially methylated in breast cancer. Based on a semi-quantitative assessment of their methylation levels

Table 3 Predictive value of promoter hypermethylation of 19 tumor suppressor genes for detecting breast cancer

Gene	Mean methylation % normal samples (number of available samples)	Mean methylation % BC samples (number of available samples)	Methylation signal DCIS samples (%)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	*P*-value Pearson Chi-Square	*P*-value Logistic regression[a]	AUC (CI 95)
AKR1B1	0.0 (7)	32.8 (20)	60	65	100	100	50	0.003	0.046	0.825 (0.672–0.978)
ALX1	0.0 (7)	22.5 (21)	80	67	100	100	50	0.002	0.255	NA
ARHGEF7	0.0 (7)	19.2 (20)	25	25	100	100	32	0.143	NA	NA
FZD10	16.8 (10)	26.5 (21)	60	67	30	67	30	0.853	NA	NA
GHSR	66.7 (10)	43.8 (21)	100	100	0	68	0	NA[b]	NA	NA
GPX7	0.0 (9)	13.0 (20)	50	35	100	100	41	0.042	0.255	NA
GREM1	19.5 (10)	32.4 (21)	80	91	20	70	50	0.416	NA	NA
GSTP1	0.0 (10)	4.5 (21)	20	10	100	100	35	0.313	NA	NA
HOXD1	28.3 (9)	19.7 (17)	40	29	56	56	29	0.443	NA	NA
KL	50.5 (7)	89.0 (17)	100	100	13	74	100	0.111	NA	NA
LHX2	74.5 (10)	70.8 (21)	100	100	0	68	0	NA[b]	NA	NA
MAL	0.0 (9)	5.5 (21)	40	29	100	100	38	0.073	NA	NA
MGMT	11.0 (10)	12.1 (21)	40	33	70	70	33	0.853	NA	NA
NDRG2	0.0 (8)	3.4 (14)	20	7	100	100	38	0.439	NA	NA
RASGRF2	7.6 (10)	31.7 (21)	80	86	50	78	63	0.038	NA	NA
SFRP1	17.4 (10)	24.1 (19)	80	79	30	68	43	0.593	NA	NA
SFRP2	19.0 (10)	59.1 (19)	100	100	30	73	100	0.012	NA	NA
TM6SF1	0.0 (10)	22.7 (21)	60	57	100	100	53	0.002	0.012	0.786 (0.629–0.943)
TMEFF2	17.3 (10)	47.7 (21)	100	95	60	83	86	0.001	0.882	NA

[a] Corrected for age and using dichotomous outcomes

[b] No *p* value is available since all outcomes are 1

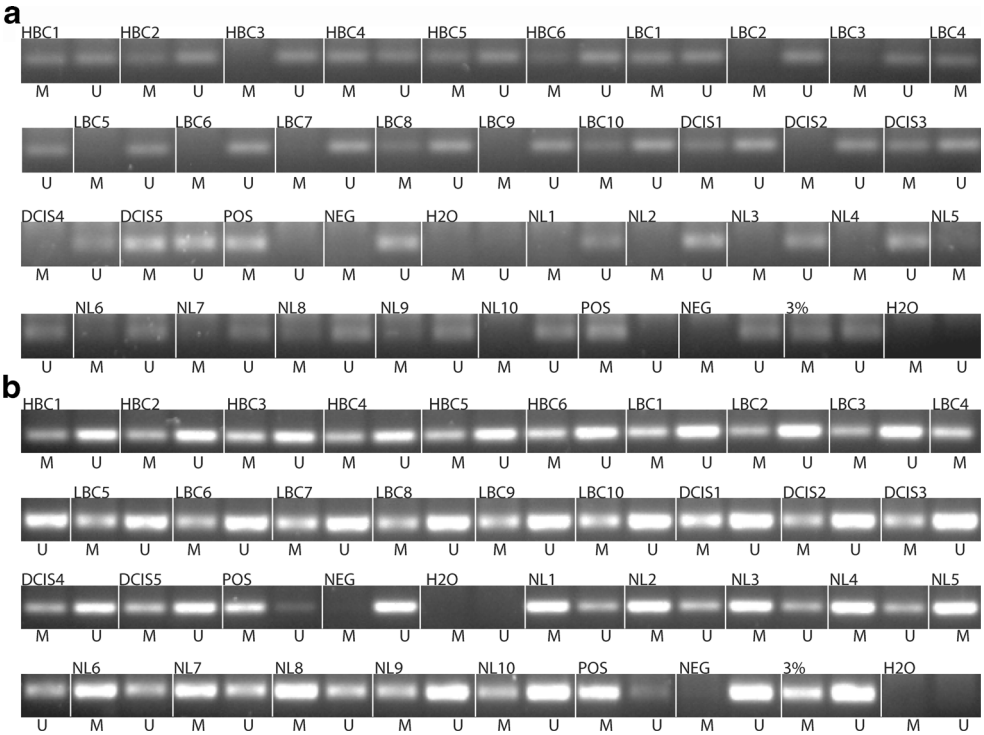

Fig. 1 Example of breast cancer (grade 2 and 3 = HBC; grade 1 = LBC), DCIS and normal breast (NL) samples ran on a 2 % agarose gel for methylation status analysis of *TM6SF1* (**a**) and *GHSR* (**b**). *M*=methylated signal; *U*=unmethylated signal. "Pos" refers to a 100 % methylated control, "Neg" to a 100 % unmethylated control. The "3 %" refers to a sample with 3 % methylated DNA

by MSP, we found that *AKR1B1, ALX1, GHSR, GREM1, RASGRF2, SFRP2, TM6SF1* and *TMEFF2* were significantly differentially methylated between normal and malignant tissues. In a multivariate analysis, including age, we found that a combination of *AKR1B1* and *TM6SF1* could detect (low grade, high grade and in situ) breast cancer with an AUC of 0.986 in ROC analysis, despite a lower sensitivity of the individual genes. Although there was an age difference between the normal and breast cancer tissue groups, both biomarkers turned out to act as independent predictors. In addition, previous studies have shown that age does not necessarily affect methylation status [10]. Interestingly, both *AKR1B1* and *TM6SF1* were previously found to be hypermethylated in a genome-wide methylation screen of sera from metastatic breast cancer patients [11, 12].

The *AKR1B1* gene encodes a member of the aldo/keto reductase super family. AKR1B1 catalyzes the reduction of certain aldehydes, including the aldehyde form of glucose, and plays a role in diabetes mellitus by catalyzing the reduction of glucose to sorbitol [13]. AKR1B1 has also been implicated in carcinogenesis by mediating inflammatory signals induced by growth factors, cytokines, chemokines and carcinogens. Furthermore, products of lipid-derived aldehydes and metabolites produced after reduction by AKR1B1 have been shown to be involved in the activation of transcription factors such as NF-κB and AP-1, which affect inflammatory cytokines. Increased levels of inflammatory cytokines and growth factors promote cell proliferation, and inhibition of AKR1B1 has been reported to prevent cancer cell growth, both in vitro and in vivo [14]. Moreover, AKR1B1 has been reported to be involved in the development of resistance to various chemotherapeutics, such as daunorubicin and cisplatin [14]. In endometrial cancers, the expression of AKR1B1 was found to be reduced compared to adjacent normal tissues [15]. In another study in which AKR1B1 expression was assessed using the Oncomine gene expression database in major human cancer types, no overall significant differences were found in ductal and lobular breast cancers compared to normal tissues. [16] The expression of AKR1B1 has also been found to be elevated in cancers of the bladder, brain, cervix, esophagus, head and neck, kidney, and in leukemias, lymphomas and melanomas. In prostate cancers AKR1B1 expression was found to be significantly lower than in normal prostate tissues [16]. Hypermethylation of *AKR1B1* was for the first time found to occur in breast cancer tissue [11] and, more recently, in sera from patients with recurrent metastatic breast cancer [12]. The present study provides an independent validation of these findings in a series of breast cancer samples from the Netherlands.

As yet, the function of TM6SF1 is largely unknown. Hypermethylation of the *TM6SF1* gene promoter in both breast cancer tissues and sera was shown recently [12]. Another study showed that single hepatocytes isolated from liver tissues of hepatitis B virus-related hepatocellular carcinomas exhibited higher *TM6SF1* methylation levels than adjacent and normal hepatocytes and, together with 19 other genes, *TM6SF1* was found to be hypermethylated in cancerous hepatocytes [17].

In conclusion, we found that *AKR1B1, ALX1, GHSR, GREM1, RASGRF2, SFRP2, TM6SF1* and *TMEFF2* are significantly differentially methylated in normal versus malignant breast tissues, and that these genes, in particular *AKR1B1* and *TM6SF1,* may serve as candidate methylation biomarkers in body fluids such as nipple aspirates, ductal lavages or blood, especially when combined. The next step, which is currently in progress, will be to evaluate whether the addition of these genes to our previously described gene methylation panel [6] will facilitate the (early) detection of breast cancer.

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