Breast Cancer Res Treat (2012) 135:705–713
DOI 10.1007/s10549-012-2197-z

PRECLINICAL STUDY

Diagnostic values of *GHSR* DNA methylation pattern in breast cancer

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Received: 26 March 2012 / Accepted: 3 August 2012 / Published online: 17 August 2012

Abstract DNA methylation patterns have been recognised as cancer-specific markers with high potential for clinical applications. We aimed at identifying methylation variations that differentiate between breast cancers and other breast tissue entities to establish a signature for diagnosis. Candidate genomic loci were analysed in 117 fresh-frozen breast specimens, which included cancer, benign and normal breast tissues from patients as well as material from healthy individuals. A cancer-specific DNA methylation signature was identified by microarray analysis in a test set of samples ($n = 52$, $p < 2.1 \times 10^{-4}$) and its performance was assessed through bisulphite pyrosequencing in an independent validation set ($n = 65$, $p < 1.9 \times 10^{-7}$). The signature is associated with *SFRP2* and *GHSR* genes, and exhibited significant hypermethylation in cancers. Normal-appearing breast tissues from cancer patients were also methylated at these loci but to a markedly lower extent. This occurrence of methylated DNA in normal breast tissue of cancer patients is indicative of an epigenetic field defect. Concerning diagnosis, receiver operating characteristic curves and the corresponding area under the curve (AUC) analysis demonstrated a very high sensitivity and specificity of 89.3 and 100 %, respectively, for the *GHSR* methylation pattern (AUC >0.99). To date, this represents the DNA methylation marker of the highest sensitivity and specificity for breast cancer diagnosis. Functionally, ectopic expression of GHSR in a cell line model reduced breast cancer cell invasion without affecting cell viability upon stimulation of

Electronic supplementary material The online version of this article (doi:10.1007/s10549-012-2197-z) contains supplementary material, which is available to authorized users.

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cells with ghrelin. Our data suggest a link between epigenetic down-regulation of GHSR and breast cancer cell invasion.

Keywords DNA methylation · Breast cancer · Diagnosis · GHSR · Epigenetics

Introduction

Earlier breast cancer diagnosis and prevention of the disease is being worked at worldwide. Despite these efforts, the outcome of breast cancer has not changed very substantially in recent years. Mortality is mostly due to metastasis, which in many cases may be prevented when the cancer is diagnosed early. Conventional clinical breast examination and imaging procedures are being used to this end, yet they are limited in their efficacy to reduce mortality and morbidity significantly [1]. Concomitantly, however, cellular and molecular markers exhibit an overall promising performance and will unquestionably improve current detection power [2, 3].

DNA methylation—methylation of cytosines in the context of d(CG) dinucleotides, called CpGs—is a prominent epigenetic factor that plays a key role in regulating mammalian gene expression [4]. DNA methylation patterns seem to be cell-type specific, implying that tumour cells might have a distinct configuration. Indeed, cancer-associated DNA methylation patterns typically show global hypomethylation along with localised hypermethylation of DNA in CpG-rich regions, called CpG islands (CGIs). Islands are often found in the promoter or the first exon of tumour suppressor genes [5], for example. Aberrant DNA methylation events are abundant in tumours and occur in the early stages of tumourigenesis in different cancers, including those of breast [6]. As this modification takes place in DNA, a molecule type that is more stable than RNA and protein molecules, and through a covalently bound substitution, it can be readily detected in body fluids. These properties have strengthened the candidature of DNA methylation marks for clinical applications in cancer management [7]. For instance, DNA hypermethylation of certain genes has been suggested as a potential clinical marker for early diagnosis (e.g. *GSTP1*; prostate cancer) and prognosis ($p16^{INK4a}$; poor outcome in lung and colorectal cancers) or as a predictor of the response to particular treatment regimes (*MGMT*; response to temozolomide treatment in glioma patients) [reviewed in [8]]. Recently, hypermethylation of a *BRCA1* CGI has been proposed as a marker to predict the response of breast cancers to poly(adenosine-diphosphate)-ribose polymerase (PARP) inhibitors [9]. Nonetheless, sensitive DNA methylation markers for an early detection of breast cancer are missing, which is an important weakness for screening procedures.

Recent reports have shown that substantial differences can exist in the degree of methylation of different parts of an individual CGI [10]. Consequently, a high-resolution analysis of multiple CpGs along the given CGI needs to be applied in a screen for robust markers. Microarrays and more recently deep sequencing provide the means for an analysis of methylation in multiple CGIs with high resolution [11]. Furthermore, although patient-matched control samples are necessary for the exclusion of methylation patterns that are induced by factors such as environment, nutrition and ageing, they may not be sufficient on their own for the identification of early cancer-associated methylation alterations of high specificity but should be supplemented with tissue material from entirely healthy individuals [12]. Employing custom arrays that analysed some genomic regions at high resolution, we profiled a panel of different sample types for the identification of DNA methylation signatures for breast cancer diagnosis. The specimens studied included cancers and benign lesions of the breast as well as normal tissue samples from cancer patients and healthy individuals. Identified markers were validated in an independent set of samples by sequencing, yielding markers of high robustness and accuracy.

Materials and methods

We used a strategy of screening and validation to develop the breast cancer DNA methylation signature. In the screening analysis, specialised microarrays were applied to screen DNA methylation patterns in the first series of 52 samples to identify the methylation signature. The second series of 65 samples, as an independent validation set, were analysed for the methylation signature by bisulphite pyrosequencing.

Breast tissue samples

We included 117 samples in this study. Fresh-frozen breast tissue samples were obtained, with written informed consent from female individuals from the Hospitals of Tehran University of Medical Sciences, Shahid Beheshti University of Medical Sciences and Odessa State Medical University after approval by the Institutional Review Boards at

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the corresponding universities in Iran and Ukraine. They included cancers, normal-appearing tissues from cancer patients, benign samples, normal samples from patients with benign disease and normal breast tissue specimens from individuals undergoing aesthetic surgery. The last group had no known personal or family history of breast cancer (Table 1). Samples were inspected pathologically, and cancer samples were classified according to the origin of the tumour, cell shape, patterns and staining. Samples that did not have one of the main hallmarks of malignant/benign proliferation were considered normal. DNA isolated from tissue samples was used for either microarray-based methylation analysis of 44 candidate CGIs in the screening set or bisulphite pyrosequencing in the validation set. Detailed experimental procedures are available in supplementary information.

Microarray experiments and data analysis

Fifty-two samples (31 cancers, 9 normal-appearing breast specimens from cancer patients, 5 normal samples from healthy people and 7 benign lesions) were screened on microarrays as described in supplementary information. Microarray data are submitted to ArrayExpress (accession number; E-TABM-1171).

In order to develop the breast cancer DNA methylation signature, the methylation profiles were analysed with R/Bioconductor packages [13]. The normalised methylation indices were used to compute *p* values via the limma package [14]. In limma, a systematic methylation effect for each CpG was described by a linear model using a design matrix and a CpG-specific vector of regression coefficients. A complete pairwise contrast matrix, which defines the comparisons of interest between samples in the experiment, was set up to test differential methylation patterns across all samples. The regression coefficients represent comparisons of interest between samples in the experiment. These coefficients were estimated using a least squares linear model fitting procedure and were tested for differential DNA methylation with moderated Student's *t* statistic via the empirical Bayesian statistics described in the limma package. *P* values computed for the *F* statistic, 'multi-group' [14] were adjusted for multiple testing to control the false discovery rate (FDR) at 5 % [15]. The adjusted *p* values can serve to accept or reject the null hypothesis based on the significance level. CpGs that exhibited adjusted *p* values smaller than 0.05 were selected as differentially methylated CpGs. In order to visualise the results, correspondence analysis (CA) was used [16].

Validation of the signature

In order to validate the DNA methylation signature developed from microarray data, we used bisulphite pyrosequencing. Methylation patterns of the signature loci were interrogated in an independent set of 65 samples (26 cancers, 14 normal-appearing specimens from cancer patients, 12 benign lesions, 10 normal samples from the tissue adjacent to the benign lesions and three normal samples from healthy individuals) (Table 1). Pyrosequencing reactions were performed using the Pyro Gold Reagent Kit (Qiagen, Hilden, Germany) in a PSQ HS 96 Pyrosequencing System (Biotage, Uppsala, Sweden) according to the manufacturer's instructions. Methylation levels were quantified with the methylation Software PyroQ-CpG v.1.0.9 (Biotage). PCR conditions and pyrosequencing primers are listed in the supplementary Table 1. In order to control potential PCR amplification bias, we used control DNA samples of 0, 25, 50, 75 and 100 % methylation degree prepared from completely methylated and unmethylated control human DNA (EpiTect PCR control DNA set; Qiagen) and calibration curves were used for the correction, when necessary [17].

Receiver operating characteristic (ROC) curves

In order to characterise the accuracy of DNA methylation signature, ROC analysis was performed. The most popular summary measure of accuracy is the area under the ROC curve, often denoted as AUC. It ranges in value from 0.5 (chance) to 1.0 (perfect discrimination or accuracy). ROC and AUC analysis were performed using Stata statistical package, version 8. Adjusted ROC curves were produced with STATA 'lroc' command after logistic regression analyses.

Cell viability and invasion assays

Highly invasive MDA-MB-231 breast cancer cells were obtained from ATCC (Manassas, VA, USA) and cultured in DMEM medium supplemented with 10 % (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37 °C and 5 % (v/v) CO_2. For viability assays, 4×10^4 cells were transfected with 60 ng of either GHSR1a cDNA-expressing or control empty vectors (both from Origene, Rockville, USA) in 24-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Twenty-four hour thereafter, cells were treated with Ghrelin (25 ng/ml) for 48 h, and cell viability was assessed by Sulforhodamine B (SRB) colorimetric assay [18]. For invasion assay, 2.5×10^5 cells were transfected with 150 ng of control or GHSR1a vector using Lipofectamine 2000 in 6-well plates. After 24 h of starvation, cells were seeded in BioCoat Matrigel™ invasion plates (Becton Dickinson Bioscience, Franklin Lakes, NJ, USA). Ghrelin (25 ng/ml) was added

Table 1 Characteristics of samples

	Screening set ($n = 52$)				Validation set ($n = 65$)				Overall ($n = 117$)				
	Cancer	Normal from Cancer	Benign	Healthy	Cancer	Normal from cancer	Benign	Normal from Benign	Healthy	Cancer	Normal from Cancer	Benign	Normal from benign	Healthy
Number	31	9	7	5	26	14	12	10	3	57	23	19	10	8
Mean age (years; range)	50 (33–75)	48 (37–74)	43 (35–58)	32 (27–40)	47 (32–83)	48 (35–83)	41 (18–66)	43 (18–66)	26.6 (22–31)	49 (32–83)	48 (35–83)	42 (18–66)	44 (18–66)	30 (22–40)
Pathological subtype														
Ductal carcinoma in situ	–	–	–	–	8 (31 %)	1 (7 %)	–	–	–	8 (14 %)	1 (4 %)	–	–	–
Invasive ductal carcinoma	29 (93 %)	8 (89 %)	–	–	16 (61 %)	11 (79 %)	–	–	–	45 (79 %)	19 (83 %)	–	–	–
Invasive lobular carcinoma	2 (7 %)	1 (11 %)	–	–	1 (4 %)	1 (7 %)	–	–	–	3 (5 %)	2 (9 %)	–	–	–
Lobular ductal carcinoma	–	–	–	–	1 (4 %)	1 (7 %)	–	–	–	1 (2 %)	1 (4 %)	–	–	–
Fibroadenoma	–	–	7 (100 %)	–	–	–	11 (92 %)	9 (90 %)	–	–	–	18 (95 %)	9 (90 %)	–
Fibrocystic changes	–	–	–	–	–	–	1 (8 %)	1 (10 %)	–	–	–	1 (5 %)	1 (10 %)	–

to cells in the upper chamber and after 24 h, the number of invaded cells was determined by flow cytometry.

Results

The DNA methylation of 609 CpG sites was analysed in a screening set of 52 breast tissue specimens on a custom-made oligonucleotide microarray that could discriminate between methylated and unmethylated DNA subsequent to a bisulphite treatment of the DNA. Initially, separate pairwise comparisons were performed between the methylation profiles in DNA from the cancer samples and the profiles of the other breast tissue entities. This analysis resulted in three sets of CpG sites, which discriminated on the basis of methylation variations between (i) cancers and healthy samples (215 CpGs), (ii) cancers and normal-appearing tissues of cancer patients (100 CpGs) and (iii) cancers and benign lesions (107 CpGs) ($p < 0.05$, FDR = 5 % in all comparisons) (Supplementary Figure 1; Tables 2–4). Subsequently, we concentrated on the 55 CpGs that were present in all three identified sets (Supplementary Table 5) and tested their differential methylation across all samples. This identified a core set of 12 CpGs with the highest differentiating power across all breast tissue entities.

In order to visualise the result, CA was used. In the projection plot (Fig. 1), each sample is depicted as a coloured square and the CpG sites that are exhibiting the most significant differential methylation levels (adjusted p values $<2 \times 10^{-4}$; Table 2) are represented as black dots. Based on the methylation of only 12 CpG dinucleotides (hereafter called signature CpGs) which are associated with two genes—*GHSR* and *SFRP2*—samples clustered into four distinct groups that are representing the four sample types used in the analysis. In CA, similarity among tissue samples is depicted in terms of proximity; the closer their location, the more similar is the methylation pattern. A CpG that is particularly methylated in a certain sample group will be located in the direction of this group from the centroid. The further the distance from the centroid in this direction, the stronger is the association [16]. In Fig. 1, all CpG sites co-localise with the cancer samples at the right side of the plot, indicating that the highest methylation level is found in cancer. In contrast, the healthy samples are located to the left, in the opposite direction off the centroid, indicating that the CpGs are at the lowest level of methylation in these samples. Likewise, based on the localisation of normal-appearing tissues of cancer patients and benign samples along the horizontal axis (first principal component; i.e. the direction along which the samples show the largest variation), it can be seen that an intermediate methylation load existed in these samples.

The analysis showed that hypermethylation at 12 CGIs of *GHSR* (growth hormone secretagogue receptor) and *SFRP2* (secreted frizzled-related protein 2) can distinguish between cancer and non-cancer tissue.

Subsequently, the discriminative power of the methylation signature was validated in an independent set of 65 samples by interrogating the related *GHSR* and *SFRP2* loci using bisulphite pyrosequencing. In addition to the four samples types used in the microarray analysis, we included normal breast samples from persons with benign lesions in the validation set (Table 1) as an age-matched control group. Pyrosequencing data (Supplementary Table 6) confirmed that methylation at the *GHSR* and *SFRP2* CGIs is a determinant for breast cancers. Figure 2 shows the methylation levels of these two CGIs in all samples analysed in our study. Regardless of pathological subtypes, cancers were markedly hypermethylated in both loci as compared to the other samples. Moreover, although not statistically significant, normal breast tissue samples from healthy individuals held the minimum load of DNA methylation among the control samples.

We also assessed the sensitivity and specificity of the signature for detection of breast cancers. Due to the limited number of samples, we combined the data of the screening and validation sets and performed the analysis on this dataset. ROC curves (Fig. 3) for both *GHSR* (AUC = 0.9866, 95 % CI 0.9715–1.0000) and *SFRP2* (AUC = 0.9585, 95 % CI 0.9172–0.9998) genes exhibited a high degree of both sensitivity and specificity for detecting breast cancers from other tissue entities (Table 3). The cancer detection power of both genes was slightly improved following adjustment for age and

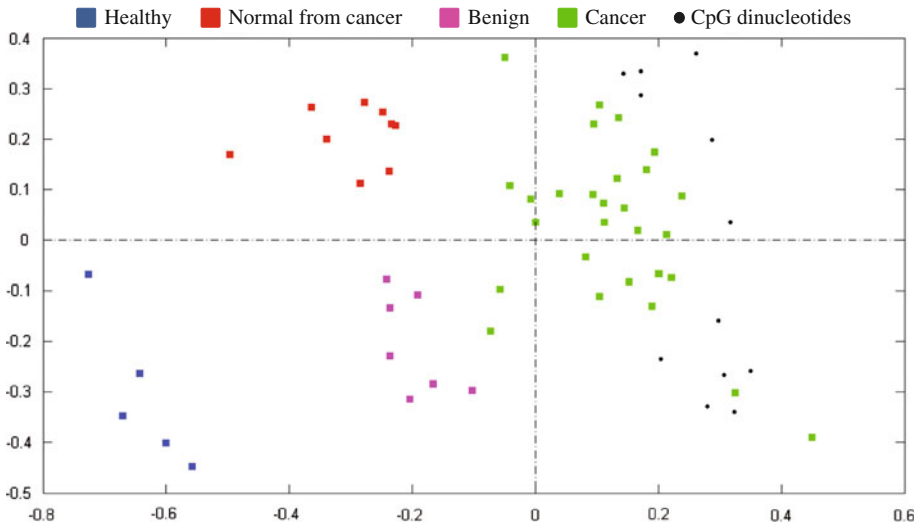

Fig. 1 Sample profiling. Correspondence analysis resulted in a biplot of the samples and differentially methylated CpG sites. Samples are depicted as *squares* that are coloured according to disease state; *black spots* represent the 12 signature CpGs. The smaller the distance between two samples, the higher is the concordance of their methylation profile. Measurements located in the same direction off the centroid of the plot, which is indicated by the crossing of the *dotted lines*, exhibit similar methylation

Table 2 CpGs with the most differential methylation patterns between breast cancers and the other tissue specimens

Probe ID	Gene symbol	Entrez gene ID	Chromosome	Distance to TTS	Adjusted *p* value[a]
GHSR_251	*GHSR*	2693	3	251	1.20×10^{-12}
GHSR_426	*GHSR*	2693	3	426	5.60×10^{-12}
GHSR_118	*GHSR*	2693	3	118	9.72×10^{-9}
SFRP2_−201	*SFRP2*	6423	4	−201	2.15×10^{-8}
GHSR_245	*GHSR*	2693	3	245	4.22×10^{-7}
GHSR_130	*GHSR*	2693	3	130	8.27×10^{-7}
GHSR_133	*GHSR*	2693	3	133	9.63×10^{-7}
GHSR_168	*GHSR*	2693	3	168	2.01×10^{-6}
SFRP2_−313	*SFRP2*	6423	4	−313	2.70×10^{-6}
SFRP2_−321	*SFRP2*	6423	4	−321	3.40×10^{-6}
GHSR_159	*GHSR*	2693	3	159	1.00×10^{-4}
SFRP2_−343	*SFRP2*	6423	4	−343	2.00×10^{-4}

TTS transcription start site

[a] Cancers were compared with other entities (healthy, normal from cancer, benign)

country of origin of samples, such that adjusted AUC values reached to 0.9906 and 0.9601 for *GHSR* and *SFRP2*, respectively (Table 3). Accordingly, using a minimum of 20.9 % *GHSR* DNA methylation as cut-off value, we obtained 100 % specificity and 89.3 % sensitivity for breast cancer detection. Further reduction of this cut-off value to 15.49 % resulted in 83.3 and 100 % specificity and sensitivity, respectively.

It has been reported that hypermethylation of *GHSR* is associated with down-regulation of this gene in breast cancer [19]; however, the potential outcome of this down-regulation of expression has not been studied in the context of breast cancer. Therefore, we sought to examine whether GHSR has an anti-tumour activity. To this end, we studied the effect of ectopic expression of GHSR variant 1a (GHSR1a encodes the functional protein which acts as receptor for the Ghrelin hormone) on viability and invasion potential of breast cancer cells upon Ghrelin induction. While over expression of GHSR1a in the presence of Ghrelin did not affect the viability of cancer cells, the invasion of cells was reduced as compared to control transfection (Fig. 4) suggesting the potential involvement of GHSR down-regulation in the pathology of breast cancer.

Discussion

In this study, we have established a molecular classifier that has the highest accuracy for DNA methylation-based diagnosis of breast cancer. This was achieved by combining high-resolution DNA methylation scanning of multiple genomic loci and a utilisation of different types of control samples. All cancer samples were distinguishable from the other breast tissue samples. Our data show that this was due to substantial DNA hypermethylation at the signature CpGs in malignant tissues. Much less but still significantly increased methylation of these sites was detected in benign samples, which might be of clinical importance. This observation is consistent with a previous study by Huang and colleagues, who reported that genes frequently hypermethylated in breast tumours are also methylated in benign breast lesions, albeit at a lower level [20]. Furthermore, the presence of abnormal DNA methylation has also been reported in benign breast lesions obtained from women who were at risk to develop breast cancer [21]. This abnormal change occurs more frequently in benign breast epithelium of women who are at high risk for breast cancer than in people at low risk [22]. These findings point to the

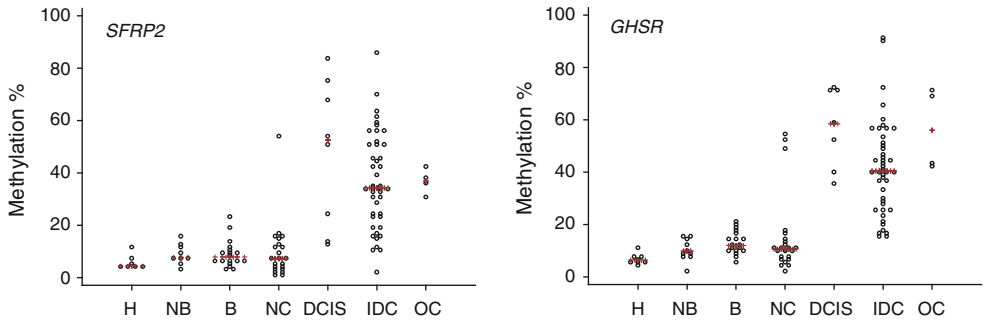

Fig. 2 Methylation levels of the signature genes *SFRP2* and *GHSR* in all samples analysed. Percentage of methylation on the *Y* axis is plotted against the sample type on the *X* axis. The *red crosses* represent the median value. *H* healthy, *NB* normal from benign, *B* benign, *NC* normal from cancer, *DCIS* ductal carcinoma in situ, *IDC* invasive ductal carcinoma, *OC* other cancer (invasive lobular carcinoma and lobular ductal carcinoma)

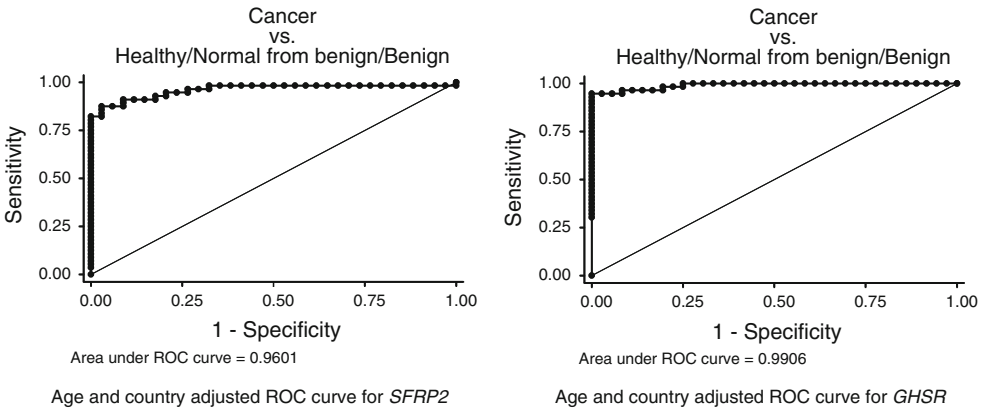

Fig. 3 ROC curves using *SFRP2* and *GHSR* methylation. Cancers were compared with healthy, benign and normal tissue samples of benign patients

Table 3 The AUC values

Methylated gene[a]	Area under the ROC curve (AUC)								
	Cancer vs. healthy				Cancer vs. 'healthy/normal from benign/benign'				
	n	Crude	(95 % CI)	Age-adjusted	*n*	Crude	(95 % CI)	Age-adjusted	Age- and country-adjusted
SFRP2									
Screening	36	0.9935	(0.9757–1.0000)	1.0000	43	0.9651	(0.9181–1.0000)	0.9651	1.0000
Validation	27	0.96	(0.8816–1.0000)	1.0000	47	0.9418	(0.8604–1.0000)	0.9418	NC
All	63	0.9745	(0.9357–1.0000)	0.9923	90	0.9585	(0.9172–0.9998)	0.9569	0.9601
GHSR									
Screening	36	1.0000	(1.0000–1.0000)	1.0000	43	0.9731	(0.9347–1.0000)	0.9704	1.0000
Validation	28	1.0000	(1.0000–1.0000)	1.0000	49	0.9933	(0.9805–1.0000)	0.9933	NC
All	64	1.0000	(1.0000–1.0000)	1.0000	92	0.9866	(0.9715–1.0000)	0.9866	0.9906

NC not calculable

[a] Average of methylation percentage of different CpGs in the gene CGI

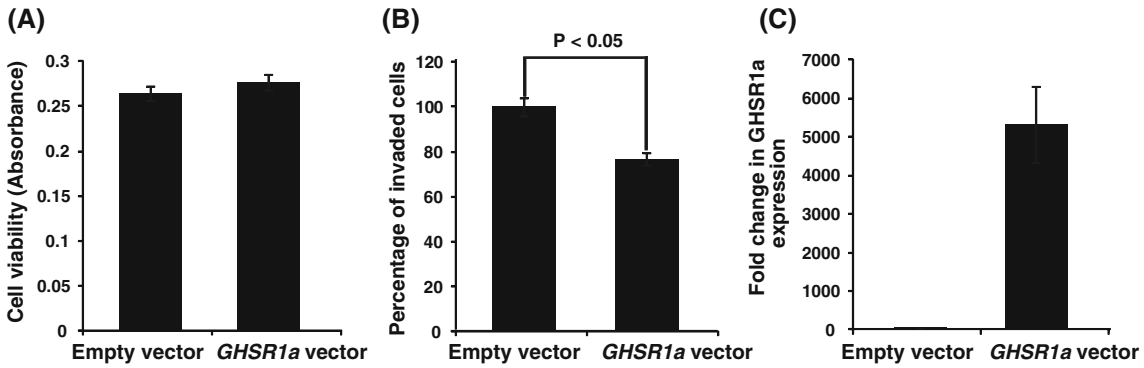

Fig. 4 Effects of GHSR on viability and invasion of breast cancer cells in the presence of Ghrelin. GHSR over expression does not affect the viability (**a**) but reduces the invasion of MDA-MB-231 breast cancer cells ($p < 0.05$) (**b**). **c** Over expression of GHSR following transfection was confirmed by quantitative real-time PCR analysis of the *GHSR1a* transcript

notion that abnormal DNA methylation may entail a risk for breast cancer development.

Our analysis documented that increased methylation of the signature CpGs is also present in the normal-appearing breast tissues collected from cancer patients but not in samples obtained from healthy donors. The lower DNA methylation levels of healthy samples may be partially explained by the age of the donors as they were, on average, younger than the patients. Age-dependent DNA methylation of many genes has been reported as a hallmark of cancer [23]. Notably, similar to our observation, Yan et al. have reported on genes being hypermethylated in breast tumours and normal tissues adjacent to the tumours but not in normal breast cells of cancer-free individuals [24]. These data suggest the involvement of DNA methylation in a 'field defect' in breast cancer. Field defect (or field cancerization) refers clinically to the existence of pre-neoplastic alterations in the cells of a tissue that are associated with local recurrences. From a molecular point of view, this phenomenon has been explained by genetic abnormalities in patients with familial cancers [25]. Our data together with other recent reports propose a contribution of epigenetic alterations to the field defect in sporadic cancers [26–28].

The signature identified from the screening set was composed of CpGs that belong to CGIs associated with the *SFRP2* and *GHSR* genes. In breast cancer, *SFRP2* hypermethylation is associated with poor patient survival [29]. Corroborating previous reports [19], our results emphasise *GHSR* methylation as the molecular classifier with the highest sensitivity and specificity for DNA methylation-based breast cancer detection. Discrimination of breast cancers from healthy tissue samples exhibited 100 % specificity and sensitivity in our analysis. Moreover, differential methylation was observed between ductal carcinoma in situ (DCIS), which is considered an early stage breast

cancer that may progress to invasive cancer, and healthy as well as benign breast lesions. With regard to this, pending further multicenter validation, *GHSR* gene methylation could be particularly attractive for early detection, which is the key factor in breast cancer control. The relevance of this molecular predictor for clinical practice needs to be clarified by addressing several issues such as the feasibility of a molecular diagnostic approach based on DNA methylation and potential medical interest for this. Owing to the recent technical advancements, the whole process of DNA methylation analysis by PCR can be completed in less than 9 h in serum [30]. The same procedure can be optimised for the analysis of luminal fluids. In their seminal study, Evron et al. [31] showed that it is possible to detect breast cancer in ductal lavage fluid using methylation-specific PCR. Subsequently, Fackler et al. [32] reported that analysing DNA methylation in ductal lavage cells doubled the detection rate of breast cancer as compared to conventional cytologic analysis. Moreover, recent data have demonstrated that by oxytocin-supported nipple aspiration, sufficient DNA for methylation analysis using quantitative multiplex-MSP was obtained in more than 90 % of women [33]. These findings suggest that an assessment of DNA methylation at the *GHSR* locus could be readily implemented into the intraductal approach to breast cancer [1]. Thus, in combination with cytological evaluation, *GHSR* methylation could provide an adjunct to imaging modalities in early diagnosis of breast cancer and could be particularly helpful for accurate assessment of breast cancer risk in carriers of *BRCA1* mutations, for instance. Such an approach might help to reduce the rate of interval malignancies in women with high breast density in whom the sensitivity of mammography is decreased [34].

The power of DNA methylation-based detection of breast cancer has also been confirmed in cells collected by fine-needle aspiration (FNA) [35]. Established in the preoperative diagnosis of breast cancer, FNA-cytology shows a high rate of inconclusive results and, thus, has been largely replaced by core-needle biopsy [36]. Core-needle biopsy is reliable in breast cancer diagnosis. However, it is accompanied by a significant range of side effects [37]. Therefore, cytology analysis could be complemented by assaying *GHSR* methylation for the diagnosis of breast cancer in FNA, thereby possibly avoiding the non-essential biopsies. As the cost of core-needle biopsy is nearly five times more than that of ultrasound-guided FNA [38], combination of cytology and PCR-based DNA methylation assays in FNA would not be more expensive than core-needle biopsy, possibly even cheaper.

Our study has some limitations. Although we used two independent sets of clinical samples from two different populations (screening set from Iranian population and validation set from Ukrainians), further multicenter studies are warranted to validate the potential of this signature for diagnosis of breast cancer. Furthermore, the diagnostic value added by the methylation signature to that of cytological analysis needs to be evaluated in future studies with a larger sample size. Moreover, whether hypermethylation of *GHSR* can be detected in serum samples of cancer patients could not be verified in this study, due to the lack of appropriate samples.

The current study shows that DNA methylation patterns at a single gene locus represent highly specific and sensitive cancer-associated marks that are promising for early detection of breast cancer. Further in depth evaluation of more samples is needed, however, to ensure the usefulness of this marker also for other tumour types. The high frequency of *GHSR* methylation in breast cancer patients necessitates functional studies towards identification of mechanisms by which GHSR is involved in breast cancer. Our in vitro data show that over expression of GHSR leads to lower invasion of breast cancer cell line MDA-MB-231 without affecting the viability of the cells. These data indicate that down-regulation of GHSR by DNA hypermethylation can contribute to the pathogenesis of breast cancer. However, further detailed studies are required to identify the molecular pathways mediating GHSR effect on cancer cell invasion. Such data may also provide a rational to develop novel therapeutic strategies in future.

Acknowledgments This study was supported by Bundesministerium für Bildung und Forschung as part of the NGFN programme (Grant Number 01GS08117). The authors thank Verena Beier and Neeme Tõnisson for discussions, and Achim Stephan for technical support.

Conflict of interest The authors declare that they have no conflict of interest.

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