DNA methylation of polycomb group target genes in cores taken from breast cancer centre and periphery

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Breast Cancer Res Treat (2010) 120:345–355
DOI 10.1007/s10549-009-0384-3

PRECLINICAL STUDY

DNA methylation of polycomb group target genes in cores taken from breast cancer centre and periphery

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Received: 14 February 2009 / Accepted: 19 March 2009 / Published online: 8 April 2009

Abstract We previously demonstrated that methylation of neugogenic differentiation 1 (*NEUROD1*) gene, a polycomb group target (PCGT) gene is a predictor of response to neoadjuvant chemotherapy in breast cancer. Here, we address the question whether *NEUROD1* methylation provides clinical information independent from its expression level, and whether PCGT methylation is homogeneous in breast cancer. We examined: (1) *NEUROD1* methylation and mRNA expression in 9 breast cancer cell lines and 63 tumour specimens, (2) DNA methylation in a training set of 55 PCGT genes taken from the centre (TUC) and periphery (TUP) of 15 breast cancer specimens, and compared this with 22 non neoplastic controls, and finally, (3) validated statistically significant genes in an independent set of 20 cases versus 18 controls. 8/9 cell lines demonstrated *NEUROD1* methylation, whereas, there was only one cell-line that showed *NEUROD1* expression. There was no association between methylation and expression in breast tumour specimens, with only 14% exhibiting *NEUROD1* expression. Of the 55 PCGT genes analysed, 24% (13/55) were shown to be cancer specific ($p < 0.05$) with a receiver-operating-characteristic (ROC) area-under-the-curve (AUC) of >0.7 (range 0.71–0.95). DNA methylation accurately predicted the presence of cancer in both TUC and TUP. DNA methylation of PCGT genes predicts the presence of breast cancer and is not subject to tumour heterogeneity. Further work will reveal if methylation of PCGT genes will serve as a robust means for the clinical detection and assessment of breast cancer.

Keywords DNA methylation · Breast cancer · Intra-tumour heterogeneity

The authors Evangelia-Ourania Fourkala and Cornelia Hauser-Kronberger are equally contributed this work.

Electronic supplementary material The online version of this article (doi:10.1007/s10549-009-0384-3) contains supplementary material, which is available to authorized users.

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Introduction

Breast cancer is the most common female cancer. The overall lifetime risk of developing an invasive breast cancer is 10%. Despite progress in early detection and treatment, breast cancer still accounts for the second largest number of cancer related deaths in the western world [1]. Therefore, identification of novel molecular markers with the potential for optimal disease management and improved survival rates is essential.

During the last decade, a huge emphasis has been given to the identification of genetic changes and expression profiles that correlate with clinical characteristics of the

disease, in an attempt to discover genetic markers predicting prognosis and response to treatment [2–6]. Many of these studies have been based on a single sample from within the tumour, assuming that this single region reflects the genetic signature of the whole cancer. However, there are increasing reports of the presence of intra-tumour heterogeneity and its effect on expression profiling in several cancer types [7–13], including breast cancer [14, 15].

Intra-tumour heterogeneity is the result of a multifactorial microenvironment which exhibits a zonal heterogeneity from central to peripheral regions [16]. Studies comparing the central with the peripheral zone have identified expression of different molecules within these regions. In the centre, which is characterised by hypoxic conditions, genes such as vascular endothelial growth factor (*VEGF*) have been shown to be regulated and molecules such as the matrix metalloproteinases (MMPs) are overexpressed. In the tumour periphery, which is localised at the stromal border, forming the biologically active invasion front and cancer stem cell reservoir, molecules such as E-cadherin have been shown to be down-regulated [17, 18]. Even, for oestrogen (ER) and progesterone receptors (PR), which are the most significant markers in treatment strategies, there is conflicting data with studies also reporting to be differently expressed in different regions within the tumour [19–21]. This has led to an increased emphasis for the need to study more than one part of the tumour to ensure the generation of accurate and reproducible data, especially as these data are used to guide patient management. Based on these observations we suggest that the problem of intra-tumour heterogeneity may be overcome by studying DNA based alterations, such as epigenetic changes, specifically DNA methylation, which may not be affected by the zonal microenvironment of the tumour.

Epigenetic modifications, and in particular DNA methylation, is known to be an early event in carcinogenesis and to precede major genetic changes leading to cancer. The main differences between epigenetic and genetic alterations are that the former occuring at a higher frequency, are reversible upon treatment with pharmacological agents, and arise at defined regions within a gene [22, 23]. These characteristics make them an attractive alternative for cancer detection and assessment [24, 25]. Several studies have shown multiple genes to be differently methylated in normal versus tumour tissue [26–29]. Recently, we and others demonstrated that stem cell polycomb group-target (PCGT) genes are more likely to have cancer specific promoter DNA hypermethylation than non-PCGT genes [30–32]. Furthermore, we showed that hypermethylation of *NEUROD1* within pretreatment core biopsies preferentially discriminated between neoplastic and non-neoplastic breast tissue samples, and was associated with a favourable response to treatment [27]. Given the data showing that expression profiles are affected by intra-tumour heterogeneity, it is essential to establish that the methylation profile of the core biopsy is representative of the entire tumour.

In this study, we initially analysed the expression and DNA methylation profile of *NEUROD1* in 9 breast cancer cell lines and 63 frozen breast cancer tissues. Based on the results we postulated that a DNA methylation signature may carry information independently from the expression profile of the tumour. In order to further investigate both the predictive role and homogeneity of PCGT gene methylation in breast cancer, we compared methylation levels using MethyLight, a highly sensitive real-time PCR methylation assay [33], of PCGT genes in paraffin embedded breast cancer tissue biopsies taken from the central (TUC) and peripheral (TUP) parts of the tumour and compared this with non-neoplastic breast tissue.

Materials and methods

Subjects

The project was jointly approved by the UCL/UCLH Research Ethics Committee, University London College and by the University of Salzburg. The samples were collected at the Department of Pathology, Paracelsus Private Medical University Salzburg (Salzburg, Austria). Clinical and pathologic data were stored in a database in accordance with hospital privacy regulations. For the mRNA analysis, frozen breast tissue samples were collected from 63 patients with breast cancer. The breast cancer specimens were obtained immediately after resection of the breast or lumpectomy, and therefore prior to treatment. The specimens were processed by the pathologist and part of the tissue was pulverized under liquid nitrogen and stored at −70°C. Clinicopathological features of these samples have previously been reported [27]. For the PCGT gene methylation analysis, tissue samples were collected from postmenopausal women undergoing surgery for ER+ve breast cancer or benign breast changes. Core biopsies were dissected from the centre of pT1 and pT2 invasive ductal carcinoma and from the peripheral cancer stromal border, as shown in Supplementary S1. Relevant tissue areas from tumours at least 1 cm in diameter were selected on Haematoxylin and Eosin (H&E) slides and used to guide dissection from the paraffin block. For DNA extraction, 3 mm diameter core punches were used. The samples were a priori separated into two sets: training and validation. Although grading ($p = 0.073$) and sentinel status ($p = 0.072$) p values almost reached statistical significance, no other clinicopathological feature demonstrated significant differences (p values < 0.05) between the two sets as shown in Table 1.

Table 1 Clinicopathological features are shown for the breast cancer cases that tissue was taken from the centre and periphery for both training and validation set

Clinicopathological features		Training set	Validation set	*p* value
Age		60	59.55	0.298
Histological type	IDC[a]	5	8	0.227
	IDC + DCIS[b]	10	12	
Grading	I	1	0	0.073
	II	10	17	
	III	4	3	
Staging	1	11	11	0.096
	2	4	9	
PR	Positive	12	15	0.237
	Negative	3	5	
HER2+	1	7	5	0.121
	2	1	3	
	0	7	12	
Sentinel	Positive	5	9	0.072
	Negative	15	11	

[a] Invasive ductal carcinoma

[b] Ductal carcinoma in situ

Cell-lines, culture conditions and reagents

Human breast cancer cell lines BT-20, ZR-75-1, MCF7, MDA-MB-231, T-47D, and SK-BR-3 were obtained from the American type culture collection (ATCC) and cultured according to the recommended guidelines. The following cell lines were generously provided: HBL-100 from NE Hynes, F Miescher Institute, Basle, Switzerland and Hs 578T from GC Buehring, School of Public Health, Berkley, CA, USA and were cultured in Dulbecco's modified eagle medium (DMEM; Gibco Invitrogen Corporation, Lofer, Austria) containing 10% fetal bovine serum (Biochrom AG, Berlin, Germany).

RNA isolation and RT-PCR

Total cellular RNA was extracted from the tumour specimens using the acid guanidium thiocyanate-phenol-chloroform method, and reverse transcription was performed as previously described [34, 35]. Primers and probes for *NEUROD1* RT-PCR analysis were purchased from Applied Biosystems (Applied Biosystems Assay ID: Hs00159598_m1). Primers and probes for the TATA box-binding protein (TBP; a component of the DNA-binding protein complex TFIID as endogenous RNA control) were used according to Bieche et al. [36]. Real-time PCR was performed using an ABI Prism 7900HT Detection System (Applied Biosystems, Foster City, CA). The standard curves were generated using serial dilutions of standard cDNA derived from the HBL-100 breast carcinoma cell-line.

DNA isolation, bisulphite modification and analysis of DNA methylation

DNA was extracted using the QIAGEN/QIA-amp Tissue kit as previously described [27]. Specific criteria were set up to select the eligible samples to perform for the analysis as follows: The quality of the genomic DNA was checked by two methods: (1) quantification and (2) real-time PCR using three housekeeping genes collagen 2A1 (*COL2A1*), β-actin (*ACTB*) and glyceraldehydes 3-phosphate dehydrogenase (*GAPDH*) (primer sequences are provided in Supplemental Table S1). The samples that had sufficient DNA for the purpose of analysis and a mid-exponential cycle threshold (Ct) value of <36, were included in the study (data not shown). The eligible samples were bisulphite modified based on the manufacturer's instructions (Zymo Research, Orange, CA) and MethyLight analysis was performed as described previously [27]. The specificity of the reactions for methylated DNA was confirmed using *SssI* (New England Biolabs, UK) treated human white blood cell DNA (heavily methylated). The percentage of fully methylated molecules at a specific locus, as defined by percentage of methylated reference (PMR), was calculated by dividing the *GENE*:*COL2A1* ratio of a sample by the *GENE*:*COL2A1* ratio of the *SssI*-treated human white blood cell DNA, and multiplying by 100. The analysis was performed blinded, and cases and controls were randomly mixed during bisulphite treatment and real-time PCR. The concentration of bisulphite modified DNA (assessed by the level of the reference gene *COL2A1*) was the same between cases and controls (data not shown). A detailed list of the nucleotide sequences for MethyLight primers and probes in the promoter or 5′ end region for all analysed loci is provided in supplemental Table S1.

Statistics

Statistical analysis was carried out using a computer assisted program-SPSS version 12.0.1, Chicago, IL. For both training and validation sets, for each gene the percent of non-zero results, the median and the *p* values from the Mann–Whitney test, were calculated. The genes in both training and validation sets were also assessed using receiver-operating-characteristic (ROC) curves and the area-under-the curve (AUC) value. When a gene was denoted with a 'reverse decision rule' this meant that the higher the methylation value the more likely the subject would be a control rather than a case. In order to assess if there is a difference between the TUC and TUP groups, a non-parametric paired test (Wilcoxon rank test) was carried out comparing the rank order of values for TUC versus

TUP. Spearman correlation analysis was performed in order to examine any association between the two zones, tumour taken from the centre and periphery.

Results

NEUROD1 methylation does not correlate with *NEUROD1* mRNA expression

Based on two of our recent studies, we showed that *NEUROD1* was one of the best discriminators between breast cancer and non neoplastic tissue samples [30] and that methylated *NEUROD1* promoter could be a good predictive marker for chemosensitivity in breast cancer [27]. Since cancer specific methylation of some of the PCGT genes, such as myogenic differentiation 1 (*MYOD1*) and neugogenin 1 (*NEUROG1*) has been shown not to be associated with expression in epithelial cancers [30], we wanted to investigate the association of the methylation and expression profile of *NEUROD1* in breast cancer. Therefore, we analysed and compared *NEUROD1* methylation and *NEUROD1* mRNA expression in a panel of nine human breast cancer cell lines. As it is illustrated in Figure 1, from the 9 tested cell lines only 1 expressed *NEUROD1* whereas, *NEUROD1* methylation was observed in 8 out of 9 cell lines. To further examine this observation, we analysed *NEUROD1* expression and methylation of 63 frozen breast cancer tissue samples. The experiments were performed in triplicate and, when at least two signals of the analysis had given a negative value, the expression was considered as zero. As is seen in supplemental Table S3, the majority of the cases, 54/63 (85.7%) did not express *NEUROD1* in contrast to *NEUROD1* methylation, which was detected in all cases with a PMR value ranging from 0.05 to 633.

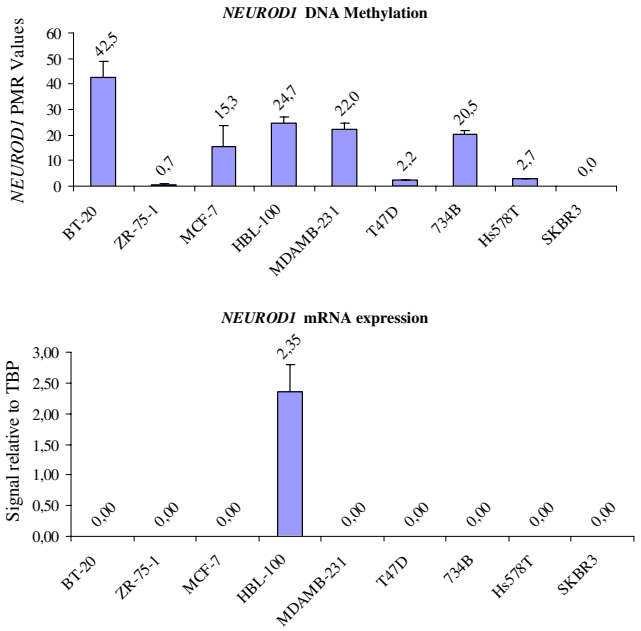

Fig. 1 *NEUROD1* methylation and mRNA expression in breast cancer cell lines. Nine different breast cancer cell lines were tested for their DNA methylation and mRNA status for *NEUROD1*

PCGT genes can predict the presence of breast cancer

In order to further investigate the methylation level changes of the PCGT genes in breast cancer we analysed a sample set consisting of TUC and TUP tissues from 35 postmenopausal women with ER+ve breast cancer and compared these with non neoplastic breast tissue from 40 postmenopausal women who had undergone surgery for benign breast changes. The samples were divided into a training set consisting of 15 cases and 22 controls, and a validation set of 20 cases and 18 controls.

In the training set, we examined methylation of 55 PCGT genes in 14 TUC and 15 TUP samples and 22 controls. 24% (13 out of 55) of the genes analysed were cancer specific ($p < 0.05$), being more frequently methylated in tumour samples when compared with non neoplastic tissues as illustrated in Table 2. To test the hypothesis that the selected genes are cancer predictors, we assessed them by ROC analysis as seen in Table 3. In this table, for both TUC and TUP groups, the p values given reflect whether the AUCs are significantly different from 0.5 (a straight line from bottom left to top right corners, implying a decision rule no better than chance). The predictive value of the 13 genes was statistically significant showing an AUC range of 0.667–0.930 for the TUC samples and 0.714–0.955 for the TUP samples.

To further validate these findings, we analysed in an independent validation set consisting of 19 TUC, 20 TUP samples and 18 controls, the 13 PCGT genes from the training set that had a $p < 0.05$ in the Mann–Whitney analysis for both TUC and TUP. All 13 genes (Table 4) were confirmed and were shown to be statistically significant in the validation set ($p < 0.05$). We calculated the predictive potential of these 13 genes by ROC analysis as it is illustrated in Table 5. Interestingly, even though the sample size is low, the results were consistent and the same panel of genes that were shown to be statistically significant with the Mann–Whitney test also had a statistically significant AUC value ($p < 0.05$).

DNA methylation of PCGT genes is not affected by intra-tumour heterogeneity

Even though we show methylation levels of specific genes to be comparable between selected TUC and TUP tissue samples in both the training and validation sets, there were also

Table 2 Summary statistics of controls versus tumour taken from the centre (TUC) and tumour taken from the periphery (TUP) from the breast cancer cases analysed in the training set

Genes	Control ($n = 22$)		TUC ($n = 15$)			TUP ($n = 14$)			
	Positive (%)	Median PMR	Positive (%)	Median PMR	p value	Positive (%)	Median PMR	p value	p value (rank)
HOXD9	77.3	0.01	100	1.42	**0.000**	100	2.48	**0.000**	1
HOXA7	90.9	0.28	100	5.39	**0.000**	100	3.96	**0.000**	3
PENK	9.1	0.00	73.3	0.09	**0.000**	78.6	4.22	**0.000**	2
TMEFF2	50	0.00	93.3	8.96	**0.000**	92.9	7.06	**0.000**	5
HOXA1	59.1	0.03	80	11.74	**0.002**	85.7	11.31	**0.000**	6
MT1A	95.5	69.92	100	28.72	**0.003**	100	19.20	**0.003**	15
CRABP1	0	–	33.3	0.00	**0.004**	42.9	0.00	**0.001**	9
GATA4	22.7	0.00	60	0.11	0.007	28.6	0.00	0.609	35
HOXD11	63.6	0.75	80	17.53	**0.007**	92.9	35.48	**0.000**	8
HOXD12	54.5	0.03	73.3	10.12	**0.010**	85.7	7.75	**0.002**	12
NEUROD1	54.5	0.00	73.3	0.39	**0.020**	78.6	5.19	**0.002**	13
GAD1	100	0.72	100	2.57	**0.020**	100	4.33	**0.000**	7
HOXA13	54.5	1.14	66.7	177.49	**0.021**	100	181.40	**0.000**	4
PITX2 (II)	72.7	0.26	73.3	4.33	**0.029**	85.7	10.64	**0.001**	10
HIC1	63.6	28.33	100	42.58	0.043	100	31.32	0.054	22
PGR	23.8	0.00	0	–	0.045	21.4	0.00	0.680	39
HOXD8	63.6	0.18	80	4.57	0.052	64.3	7.04	0.111	28
ITGA4	0	–	13.3	0.00	0.083	28.6	0.00	0.009	17
PITX2 (I)	0	–	13.3	0.00	0.083	28.6	0.00	0.009	18
CACNA1G	0	–	13.3	0.00	0.083	14.3	0.00	0.072	24
TWIST	13.6	0.00	33.3	0.00	0.086	14.3	0.00	0.829	44
MT3	77.3	0.07	46.7	0.00	0.107	57.1	0.00	0.041	21
EBF3 (DKFZ)	4.5	0.00	20	0.00	0.121	42.9	0.00	0.004	16
GABRA2	4.5	0.00	20	0.00	0.136	21.4	0.00	0.115	29
DLC1	13.6	0.00	0	–	0.142	14.3	0.00	0.957	50
GATA5	13.6	0.00	33.3	0.00	0.144	50	0.02	0.010	19
HOXA11	100	26.31	100	19.15	0.146	100	19.77	0.092	27
CDH13	13.6	0.00	33.3	0.00	0.156	14.3	0.00	1.000	55
SFRP4	50	0.00	33.3	0.00	0.171	21.4	0.00	0.057	23
NEUROD2	59.1	0.02	60	2.34	0.189	85.7	7.14	0.001	11
ESR1	50	0.00	20	0.00	0.201	14.3	0.00	0.024	20
HOXB7	77.3	0.02	53.3	0.00	0.202	78.6	0.02	0.636	37
NEUROG1	0	–	6.7	0.00	0.226	14.3	0.00	0.072	26
HOXA6	59.1	2.21	40	0.00	0.230	42.9	0.00	0.410	33
SLC6A20	9.1	0.00	0	–	0.236	7.1	0.00	0.892	47
SFRP1	45.5	0.00	26.7	0.00	0.242	35.7	0.00	0.885	46
ZBTB16	54.5	0.00	33.3	0.00	0.266	28.6	0.00	0.269	31
HOXA10	95.5	2.91	80	7.13	0.300	85.7	5.03	0.626	36
DCC	4.5	0.00	13.3	0.00	0.311	7.1	0.00	0.713	41
SLIT2	63.6	0.12	46.7	0.00	0.317	50	0.02	0.637	38
IGF2	4.5	0.00	13.3	0.00	0.343	7.1	0.00	0.713	40
HOXC9	18.2	0.00	6.7	0.00	0.376	7.1	0.00	0.331	32
GDNF	18.2	0.00	6.7	0.00	0.376	14.3	0.00	0.764	43
TITF1	18.2	0.00	6.7	0.00	0.376	14.3	0.00	0.920	48
HOXA9	100	11.88	86.7	7.74	0.404	100	11.52	1.000	54
CYP27B1	100	4.86	100	4.65	0.458	0	6.34	0.183	30

Table 2 continued

Genes	Control ($n = 22$)		TUC ($n = 15$)			TUP ($n = 14$)			
	Positive (%)	Median PMR	Positive (%)	Median PMR	p value	Positive (%)	Median PMR	p value	p value (rank)
MYOD1	22.7	0.00	26.7	0.00	0.498	64.3	0.21	0.003	14
SFRP5	81.8	2.43	60	0.63	0.708	71.4	1.54	0.744	42
CYP1B1	4.5	0.00	6.7	0.00	0.752	0	–	0.425	34
CALCA	45.5	0.00	40	0.00	0.784	35.7	0.00	0.942	49
FLJ39739	50	0.00	33.3	0.00	0.973	35.7	0.00	0.873	45
GATA3	0	–	0	–	1.000	14.3	0.00	0.072	25
PYCARD	0	–	0	–	1.000	0	–	1.000	51
TP73	0	–	0	–	1.000	0	–	1.000	52
BCL22	0	–	0	–	1.000	0	–	1.000	53

p value from the Mann–Whitney test for each gene is provided (significant p value less than 0.05). The genes were ordered according to the rank of the p value for the test of TUC versus control, and a further column for the TUP group gave the rank order value for the TUP versus control group to facilitate comparison between tests. Values in bold are the p values of the 13 cancer specific genes that were further tested

PMR Percentage of methylated reference

Table 3 Receiver operating characteristic (ROC) analysis for both TUC and TUP in training set

Genes	TUC				TUP				
	AUC	95% CI lower	95% CI upper	p value	AUC	95% CI lower	95% CI upper	p value	Rank
HOXD9	0.930	0.852	1.008	0.000	0.955	0.894	1.015	0.000	5
HOXA7	0.891	0.788	0.993	0.000	0.935	0.859	1.011	0.000	11
TMEFF2	0.853	0.719	0.987	0.000	0.888	0.767	1.009	0.000	9
PENK	0.830	0.682	0.979	0.001	0.883	0.745	1.021	0.000	10
HOXA1	0.792	0.619	0.966	0.003	0.867	0.715	1.019	0.000	1
*MT1A**	0.788	0.051	0.373	0.003	0.795	0.048	0.361	0.003	13
HOXD11	0.761	0.584	0.937	0.008	0.860	0.726	0.994	0.000	6
HOXD12	0.745	0.564	0.927	0.012	0.808	0.650	0.967	0.002	12
GAD1	0.727	0.557	0.897	0.020	0.870	0.733	1.007	0.000	2
NEUROD1	0.721	0.540	0.902	0.024	0.805	0.633	0.977	0.002	4
HOXA13	0.718	0.526	0.910	0.026	0.922	0.835	1.009	0.000	3
PITX2 (II)	0.712	0.513	0.911	0.030	0.825	0.656	0.993	0.001	7
CRABP1	0.667	0.477	0.856	0.089	0.714	0.526	0.903	0.032	8

TUC Tumour taken from the centre, *TUP* Tumour taken from the periphery, *AUC* Area under the curve, *CI* Confidence interval

The performance of each gene as a predictor of breast cancer was assessed using ROC curves and the AUC value. The genes were ordered according to the rank of the p value for the test of TUC and TUP versus control. Significance required a p value of less than 0.05

* 'Reverse decision rule' applied means that the higher the methylation value, the more likely the subject is a control rather than TUC or TUP breast cancer case

some genes that demonstrated differential methylation levels between the two tumour zones. This was true not only for both cancer specific genes (genes that were shown to be specifically methylated in the breast tumour tissue when they were compared with the controls) but also for the genes that were shown to be non-specifically methylated in cancer. In order to verify these results we performed non-parametric paired test for the genes one by one. The p values for each gene, suggested that there was no difference in the methylation level between the two zones within the tumour for cancer specific genes (Table 6) and non-cancer specific genes (supplemental Table S4). In order to investigate whether there is any correlation between the two different zones, we performed Spearman correlation analysis. The analysis showed 4 out of the 13 genes to be positively correlated in both the training and validation for the two different zones of the tumour (Table 6).

Discussion

Over the last few years, the role of DNA methylation in cancer has been the subject of many studies and it is

Table 4 Percentage of positive cases and distribution of methylation levels of the 13 genes tested in validation set

Genes	Controls ($n = 18$)		TUC ($n = 19$)			TUP ($n = 20$)			
	Positive (%)	Median PMR	Positive (%)	Median PMR	p value	Positive (%)	Median PMR	p value	p value (rank)
HOXA1	44.40	0.00	94.70	26.15	0.000	89.50	25.54	0.000	3
GAD1	100	0.78	100	15.00	0.000	94.70	14.72	0.000	2
HOXA13	38.90	0.00	94.70	152.61	0.000	84.20	126.08	0.000	5
CRABP1	0	–	73.70	5.16	0.000	57.90	3.12	0.000	7
NEUROD1	44.40	0.00	89.50	9.73	0.000	89.50	6.51	0.000	4
HOXD9	100	0.24	100	8.02	0.000	100	1.84	0.002	10
PITX2 (II)	38.90	0.00	84.20	5.69	0.000	84.20	4.25	0.000	6
HOXD11	66.70	0.10	94.70	39.07	0.000	84.20	32.94	0.001	9
TMEFF2	83.30	0.15	89.50	22.34	0.000	100	16.57	0.000	1
PENK	22.20	0.00	68.40	0.29	0.001	73.70	0.85	0.000	8
HOXA7	100	1.86	94.70	6.22	0.004	100	4.31	0.025	12
HOXD12	66.70	0.17	78.90	4.98	0.006	89	6.97	0.002	11
MT1A	100	73.06	100	37.55	0.023	100	46.07	0.027	13

TUP Tumour taken from the centre, *TUP* Tumour taken from the periphery, *PMR* Percentage of methylated reference

Controls and breast cancer cases (TUC and TUP) showing the percentage of positive cases and the median PMR values. p values are provided from Mann–Whitney test for each gene. Significance required a p value of less than 0.05 after Mann–Whitney test

Table 5 ROC analysis for both TUC and TUP in validation set

Genes	TUC				TUP				
	AUC	95% CI lower	95% CI upper	p value	AUC	95% CI lower	95% CI upper	p value	Rank
HOXD9	0.889	0.787	0.991	0.000	0.801	0.659	0.944	0.002	9
HOXA7	0.778	0.626	0.930	0.004	0.716	0.551	0.881	0.025	12
TMEFF2	0.860	0.722	0.997	0.000	0.977	0.936	1.017	0.000	1
PENK	0.792	0.642	0.943	0.002	0.822	0.68	0.963	0.001	8
HOXA1	0.950	0.872	1.029	0.000	0.901	0.788	1.014	0.000	3
*MT1A**	0.719	0.111	0.450	0.020	0.713	0.637	0.942	0.027	13
HOXD11	0.883	0.767	0.999	0.000	0.825	0.679	0.971	0.001	7
HOXD12	0.760	0.598	0.923	0.007	0.789	0.638	0.941	0.003	11
GAD1	0.936	0.853	1.018	0.000	0.918	0.81	1.026	0.000	2
NEUROD1	0.904	0.795	1.012	0.000	0.892	0.78	1.004	0.000	4
HOXA13	0.925	0.837	1.014	0.000	0.858	0.729	0.987	0.000	5
PITX2 (II)	0.876	0.752	0.999	0.000	0.852	0.723	0.981	0.000	6
CRABP1	0.868	0.742	0.994	0.000	0.789	0.637	0.942	0.003	10

ROC Receiver operating characteristic, *AUC* Area under the curve, *TUP* Tumour taken from the periphery, *TUC* Tumour taken from the centre, *CI* Confidence interval

The performance of each gene as a predictor of cancer was assessed using ROC curves and the AUC value. The significant assessment required a p value of less than 0.05. The genes were ordered according to the rank of the p value for the test of TUC and TUP versus control

* 'Reverse decision rule' applied means that the higher the methylation value, the more likely the subject is a control rather than TUC or TUP cancer case

generally accepted that methylation of gene promoters is associated with gene silencing. However, as accumulating evidence suggests that DNA methylation can occur at loci without an effect on gene expression, we wanted to investigate the correlation between *NEUROD1* methylation and expression. Based on this analysis no association between DNA methylation and gene expression was found. These data are in agreement with previous reports suggesting that PCGT genes with tumour-specific promoter DNA methylation are not normally expressed in the

Table 6 Comparison of DNA methylation changes between TUC and TUP with non-parametric paired test and Spearman correlation analysis

Genes	Non-parametric paired test *p* value	Spearman correlation coefficient *p* value	
HOXD9	0.675	0.339	0.054
HOXA7	0.993	0.120	0.507
PENK	0.766	0.110	0.544
TMEFF2	0.280	0.399	0.021
HOXA1	0.614	0.347	0.048
MT1A	0.714	0.675	0.000
CRABP1	0.715	0.567	0.001
HOXD11	0.822	0.313	0.077
HOXD12	0.217	0.216	0.227
NEUROD1	0.814	0.361	0.039
GAD1	0.526	0.266	0.135
HOXA13	0.382	−0.035	0.847
PITX2 (*II*)	0.829	0.256	0.151

TUP Tumour taken from the periphery, *TUC* Tumour taken from the centre

p values are given from the 13 genes that were further confirmed in the validation set. Non-parametric paired test analysis comparing the rank order of values for TUC versus TUP to assess if there is a difference in their methylation levels and correlation coefficient analysis is also demonstrated

epithelium of the tumour. It also provides further evidence on our previous published data that DNA methylation of PCGT genes in cancer may result in a residual stem-cell memory rather than a selective pressure for silencing these particular genes during carcinogenesis [30].

Therefore, based on the following three observations: (1) there is no relationship between *NEUROD1* methylation and expression levels (2) lack of *NEUROD1* expression in the majority of the samples tested and (3) published findings that methylation of PCGT genes is a promising target for marker identification [27, 30–32], we wanted to further examine the predictive role of these genes in breast cancer and to examine whether they are affected by intra-tumour heterogeneity. MethyLight analysis of PCGT genes identified a constant panel of genes to be methylated in both central and peripheral tumour samples compared with controls, and non-parametric paired analysis indicated that there was no statistical significant difference between the methylation levels of the two zones within the cancer. This was true for both breast cancer specific genes and genes that were not specifically methylated in cancer. In order to investigate whether there is an association between the methylation changes observed in the two different tumour tissues, we performed correlation analysis showing 4 out of 13 genes to be positive associated.

NEUROD1 was one of the genes that did not show statistically significant differences in the methylation levels between TUC and TUP, suggesting it is homogeneously methylated within the tumour. This is an important finding as it further supports our previous report that *NEUROD1* methylation could be a good predictive marker in breast cancer as it is not affected by intra-tumour heterogeneity [27]. Moreover, this study provides further evidence for paired-like homeodomain transcription factor 2 (*PITX2*) which has been shown by Harbeck et al. [37] to be a good biomarker for breast cancer hormone therapy treatment and, having performed analysis of several different tissue sections, has also shown a low variability in methylation measurements.

To our knowledge there is no other study that has examined breast cancer intra-tumour heterogeneity and its effect on DNA methylation changes. In contrast, there are two studies analysing expression modifications in correlation to intra-tumour heterogeneity. Both were carried on micro-dissected tumour cells rather than core biopsies. The first study by Aubele et al. [14] confirmed heterogeneity by comparative genomic hybridization. The second study by Zhu et al. [15] described expression heterogeneity in sections that were obtained from morphologically dissimilar regions, one from the centre containing invasive breast tumourigenic cells, and the other from the periphery containing ductal carcinoma in situ (DCIS). The differences in the expression profile described in this study could be attributed to the different type of cells analysed i.e. comparing invasive with non-invasive cells. Our finding that methylated PCGT genes provide reliable data irrespective of sampling topography, suggests that methylation analysis of these genes could hold great potential for improving breast cancer management. In addition, we have also demonstrated that the technology for methylation analysis

can be easily applied in clinical routine as only a core biopsy would be required instead of purified cell population of cells.

Our results are in contrast to reports of DNA methylation changes of different candidate genes in other cancer types which have been shown to be affected by intra-tumour heterogeneity. In melanoma, when methylation changes and expression status of suppressor genes were analysed, tissue taken from the centre of the tumour found to represent the whole tumour more accurately than the tissue from the periphery [38, 39]. A more recent study of ovarian cancer suggested that both inter- and intra-tumour heterogeneity are allied with *NY-ESO-1* expression, which was correlated with promoter and global DNA-methylation alterations when micro-dissected cells were analysed [40]. Further studies are required before it can be determined whether these changes are cancer or gene specific.

In this study, we have identified and confirmed in the validation set, 13 PCGTs that can predict breast cancer. This includes the first report of hypermethylation of the transmembrane protein with EGF-like and two follistatin-like domains 2 (*TEMFF2*), the proenkephalin (*PENK*), glutamate decarboxylase-1 (*GAD1*) and cellular retinoic acid binding protein 1 (*GRABP1*) genes in breast cancer. Even though the role of *TMEFF2* gene methylation has been observed in other types of cancer such as colorectal, bladder [41, 42] and gastric adenocarcinomas [43], there are no reports for breast cancer. *PENK* gene expression has been shown to be down regulated in prostate and bladder cancer using, expression profiling [44]. Methylation of *GAD1* has not been previously shown to be associated with cancer and *CRABP1* methylation was only reported in association with colon [45] and ovarian [46] cancer. In contrast, there are reports of an association between methylation of *PITX2* and metallothionein 1A (*MT1A*) with breast carcinogenesis, further validating the data presented [37, 47].

Interestingly, 6 of the 13 methylated loci we identified are genes belonging to the homeobox (*HOX*) domain. These genes are known to control normal development and differentiation of many multi-cellular organisms [48]. For more than a decade the role of the *HOX* domain in carcinogenesis has also been highlighted. An example is *HOXB7* which has been implicated as an oncogene and is known to increase the expression of basic fibroblast growth factor (*bFGF*) in melanoma [49]. Indeed, previous data has confirmed that *HOX* gene cluster methylation is a common feature in cancer [50] including breast cancer [25, 51]. In the search for more specific and sensitive markers, a recent study by Fiegl et al. [52] has shown that *HOXA11* is methylated in ovarian cancer and a marker indicating poor prognosis.

It is worth mentioning despite the small sample size used in this study, our results are consistent and identified cancer specifically methylated genes characterized by high AUC values indicating a high sensitivity and specificity compared with studies that have used larger sample sizes [25].

For future work we plan to investigate methylation profiles of metastatic specimens compared with matched primary tissues in order to examine whether DNA methylation of PCGT genes is homogeneous in breast cancer metastases. A recent study by Wu et al. [53] demonstrated that samples taken from a patient's primary breast carcinoma and their metastatic breast cancer are characterized by extensive expression heterogeneity. The study confirmed that ER and/or PR status characterizing the primary cancer may be lost in the metastatic carcinoma. This observation is important as the metastasis will not be hormone sensitive as its primary carcinoma resulting in resistance to the therapy. Interestingly the methylation signature of the primary tumour tissue compared with the metastatic specimen was similar, with the latter only exhibiting a higher intensity of methylation.

This is a proof of principle study demonstrating for the first time that methylation of PCGT genes is unaffected by intratumour heterogeneity within a set of breast cancer samples. These findings suggest that methylation of specifically identified PCGT genes may present a more robust means with which to guide breast cancer management, particularly in instances when only small core biopsies are available for assessment. Further investigation of epigenetic intra-tumour heterogeneity within breast cancer, as well as other cancer types is necessary; nonetheless evidence is beginning to accumulate in recognition of the potential of DNA methylation markers in cancer assessment and treatment.

Acknowledgments The work described in this manuscript was supported by The Eve Appeal, the Jubilaeumsfonds der Oesterreichischen Nationalbank, project no. 11992 and a grant from the European Union (FP6-016467, Biognosis).

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