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NT5E CpG island methylation is a favourable breast cancer biomarker

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BACKGROUND: Relapse risk assessment and individual treatment recommendations remain suboptimal for breast cancer patients. In the light of existing preclinical and clinical data, we studied *NT5E* (5′-nucleotidase, ecto) expression and *NT5E* CpG island methylation in breast cancer.

METHODS: We used RT–PCR, qPCR, methylation-specific PCR and pyrosequencing to analyse *NT5E* in breast carcinoma cell lines and primary and breast carcinomas.

RESULTS: *NT5E* CpG island methylation was inversely associated with *NT5E* expression in breast carcinoma cell lines. In clinical series, patients whose primary tumours had *NT5E* CpG island methylation were less likely to develop metastasis ($P = 0.003$, OR = 0.34, 95% CI: 0.17–0.69). In 3/4 paired samples, *NT5E* was methylated in primary tumours and demethylated in CNS metastases. Patients progressing to non-visceral as compared with visceral metastases were more likely to have *NT5E* CpG island methylation in primary tumours ($P = 0.01$, OR = 11.8). Patients with tumours lacking detectable methylation had shorter disease-free survival (DFS) ($P = 0.001$, HR = 2.7) and overall survival (OS) ($P = 0.001$, HR = 3). The favourable prognostic value of *NT5E* methylation was confirmed in oestrogen receptor negative ($P = 0.011$, HR = 3.27, 95% CI: 1.31–8.12) and in triple negative cases ($P = 0.004$; HR = 6.2, 95% CI: 1.9–20). Moreover, we observed a more favourable outcome to adjuvant chemotherapy in patients whose tumours were positive for *NT5E* CpG island methylation: DFS ($P = 0.0016$, HR = 5.1, 95% CI: 1.8–14.37) and OS ($P = 0.0005$, HR = 7.4, 95% CI: 2.416–23.08).

CONCLUSION: *NT5E* CpG island methylation is a promising breast cancer biomarker.

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Molecular Diagnostics

Prognostic and predictive factors used to inform optimal treatment approaches for individual breast cancer patients have gradually moved over the last three decades from clinico-pathological features defined by mathematical probabilistic approaches towards biological and eventually genetic biomarkers (Perou *et al*, 2000; Paik *et al*, 2004; Andreopoulou and Hortobagyi, 2008). Current state-of-the-art approaches that take into consideration oestrogen receptor (ER), progesterone receptor and HER-2 expression in primary tumours advanced, albeit modestly, personalised breast cancer therapy. However, numerous patients continue to derive little benefit from their treatment (Korde and Gralow, 2011; Perez, 2011). Recently, the introduction of microarray-based gene expression refreshed the prospects for more accurate personalised approaches (Sotiriou *et al*, 2003; Roukos *et al*, 2007). However, relapse risk profiling and thereof treatment recommendations still remain suboptimal. Existing microarray-based gene-expression profiling tests are only slow progressing in overtaking classical markers as more accurate prognostic systems and predictors of response to specific therapeutics and are not yet established in clinical practice (Sotiriou and Pusztai, 2009).

Translational cancer research has recently started focusing on epigenetics as a novel and rich source of potential biomarkers (Visvanathan *et al*, 2006). In this context, methylation markers appear particularly promising for being cost-effective, biologically plausible and technically straightforward, which conform to suggested principles for breast cancer biomarker evaluation (McGuire, 1991).

NT5E (5'-nucleotidase, ecto) is located at 6q14-q21 (NC_000006.11) and encodes CD73, a plasma membrane protein that

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catalyses the conversion of extracellular nucleotides to membrane-permeable nucleosides (Boyle *et al*, 1988). CD73 belongs to the 5′-nucleotidase family that have diverse physiological roles, especially in controlling nucleotide kinetics (Zimmermann, 1992). Evidence from a number of experimental systems suggests that expression of NT5E/CD73 may be important in increasing the invasive and metastatic properties of cancers and studies have shown that overexpression of NT5E may contribute to progression of breast cancer via generation of adenosine nucleoside (Zhou *et al*, 2007a; Zhou *et al*, 2007b). In breast cancer cell lines, NT5E overexpression results in an aggressive breast cancer phenotype and ectopic expression of NT5E in MCF-7 cells promotes cell viability, cell cycle progression and tumourigenesis (Wang *et al*, 2008). Conversely, depletion of NT5E mediated by inhibitory RNA causes reduction in cell growth rate, G0/G1 arrest and increased apoptosis in the triple-negative MDA MB 231 breast carcinoma cell line (Zhi *et al*, 2010). Moreover, animal models of cancer support a critical function for NT5E in mediating metastasis and hindering antitumour immunity (Stagg *et al*, 2011).

Given the proposed involvement of NT5E in malignant progression and apoptosis (Ujhazy *et al*, 1996; Mikhailov *et al*, 2008) there have been few translational studies of NT5E in breast cancer (Kruger *et al*, 1991; Spychala *et al*, 2004). In the latter study it was suggested that NT5E is suppressed by ERα, but little else is known about the mechanisms regulating expression of NT5E either in breast cancer or more generally in neoplasia.

Methylation-dependent transcriptional silencing is a key genome regulatory mechanism, crucial for organism development and cellular differentiation, by which gene promoters are repressed impeding the transcription of dependent genes and thereof the production of encoded proteins (Jaenisch and Bird, 2003). Characteristic gene promoter methylation/demethylation occurs in cancer, which can involve either cancer-promoting or tumour-suppressing genes, and may serve as novel cancer biomarkers (Lopez *et al*, 2009). Here, we assessed the DNA methylation status of the *NT5E* CpG island in breast cancer and investigated for clinical relevance.

MATERIALS AND METHODS

Cell lines

Fourteen breast carcinoma cell lines (MDA MB 231, MDA MB 361, MDA MB 436, MDA MB 453, MDA MB 468, MCF-7, GI101, T47D, MCF12A, ZR75.1, MB 157, NCI, BT20 and CAL51) and a human mammary epithelial cell line were grown as described previously (Shah *et al*, 2009). Genomic DNA was extracted from cell pellets using the DNeasy Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Total RNA was extracted using Trizol (Invitrogen, Paisley, UK). cDNA was synthesised from 1 μg total RNA using oligo (dT) primers and the ImProm-II reverse transcriptase kit (Promega, Southampton, UK). For demethylation, cells were treated with 5 μM 5′ azacytidine (5′ AZA; Sigma, St Louis, MO, USA) for 7 days. The cells were split every 2–3 days with the addition of fresh drug. After drug treatment, cells were harvested for qPCR.

Clinical material

The study was performed with local ethical committee approval. *NT5E* was analysed in three independent breast cancer clinical series.

- Eighty-three predominantly ER-positive primary breast carcinomas from northern Italy.
- Twenty-three surgically resected, histologically confirmed CNS metastases from patients with breast carcinomas. In four patients, paired primary cancers were available and also analysed.
- One hundred and fifty-seven primary breast carcinomas from Tayside, Scotland; of which, 119 were ER positive, 11 were HER-2 positive only and 26 were triple negative breast cancer (TNBC).

In series I and III, cancers were randomly selected from the tissue archives and only included in the study following confirmation by an expert, specialist breast pathologist of (i) original diagnosis (ii) sufficient neoplastic cell representation. Cases from series II (CNS metastases) were identified by searching the neuropathology archives for cases of resected space occupying lesions. Cases were confirmed by histopathology to be metastatic breast carcinomas and tumour cell representation was again verified by histopathology. In series I and series III, we investigated the effect of *NT5E* CpG island methylation on risk of future relapse with metastatic disease and (for series III) the effect on clinical outcome. In series II, we analysed the frequency of *NT5E* CpG island methylation in metastatic breast carcinomas and for a subset of the cases, we compared methylation in primary and metastatic lesions. In all cases, expression of ER, PgR and HER-2 was determined according to normal protocols of clinical care. Staging and clinical follow-up were done according to standard clinical guidelines in each institution, typically with 3 monthly follow-up post-surgery and imaging (mammography and CT scans) where indicated, according to local guidelines. In series I and III, patients were treated adjuvantly according to normal clinical protocols. ER-positive patients were treated adjuvantly with endocrine therapy according to clinical guidelines at the time of treatment. This was typically with tamoxifen for 5 years. Isolation of genomic DNA was using Proteinase K for the formalin-fixed, paraffin-embedded cases (series I and II) and as described previously for series III (Shah *et al*, 2009).

For statistical purposes, patients were grouped to those with non-visceral metastases, identified by bone-confined metastatic disease, lymph node only and bone + lymph node and those with visceral metastatic disease identified by brain, lung, liver and cutaneous metastases.

Methylation analysis

We identified a CpG island in the 5′ regulatory sequences of *NT5E* (http://www.genome.ucsc.edu/cgi-bin/hgGateway) and tested possible association between aberrant methylation in the CpG island and downregulation of mRNA expression using methylation-specific PCR (MSP) and pyrosequencing. DNA (0.5 μg) was modified by sodium bisulphite using the Zymo EZ DNA Methylation kit (Zymo Research, Irvine, CA, USA). This process converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged. Bisulphite-modified DNA was used as a template for methylation-specific PCR and pyrosequencing. CpGenome Universal Methylated DNA (Zymo Research) and normal human unmethylated DNA were used as positive and negative controls, respectively, in each experiment. Primers for methylation-specific PCR:

M forward primer: 5′-TATTTTATGAACGTTTTGCGTTACG-3′
M reverse primer: 5′-CTAAACTTACCACACTCTACCATCCG-3′
U forward primer: 5′-ATTTTATGAATGTTTTGTGTTATGA-3′
U reverse primer: 5′-AACTTACCACACTCTACCATCCACT-3′

Each PCR was performed at least three times and scored independently by two observers.

Primers for pyrosequencing amplified a 170-bp fragment from the *NT5E* CpG island.

Sequences were as follows:
PCR F 5′-GTATTAGGGTATTATTTGGTTTAT-3′
PCR R 5′- BIOT -CTTACCACACTCTACCATCC-3′

Polymerase Chain Reaction conditions were: 95 °C for 10 min, 95 °C for 30 s/54 °C for 30 s/72 °C for 40 s for 40 cycles, 72 °C for 7 min. PCR products were resolved on 2% agarose gels, visualised

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using a transilluminator, then analysed by pyrosequencing (Biotage Sample Prep kit, using forward primer). Analysis of % methylation at each CpG dinucleotide was performed using CpG Software (Qiagen). Placental DNA was used as negative control of methylation (0% average methylation) and a commercial methylated DNA (Millipore, Waltford, UK) was used as positive control (98% average methylation). The *RasL12* CpG island was analysed using bisulphite sequencing and methylation-specific PCR. Location of primers for methylation-specific PCR and bisulphite sequencing is shown in Figure 7.

NT5E expression analysis

For qPCR analysis of expression, total RNA was isolated using the RecoverAll Total Nucleic Acid Isolation (Ambion, Austin, TX, USA). Twenty-five microlitre PCR reactions were performed using 50 ng of cDNA obtained by reverse transcription. Amplification and analysis were done according to the manufacturer's protocol in 96-well plates in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the pre-cast 'TaqMan Gene Expression Assays' (Applera, https://products.appliedbiosystems.com/) for *NT5E* (Hs001573922_m1). Quantification of the target transcript was performed in comparison to the reference transcript β2microglobulin (Hs99999907_m1), using the 'delta-delta Ct method' for comparing relative expression results in real-time PCR as outlined by PE Applied Biosystems (Perkin Elmer, Foster City, CA, USA).

Statistics

CpG island methylation status and presence or metastatic profile were assessed for associations using Fisher's exact test. Kaplan–Meier curves were used to estimate the probabilities of overall survival (OS) and disease-free survival (DFS) and the log-rank test to assess the statistical significance of differences in event rates. All statistical analyses were performed using Prism 5 (GraphPad software, Inc., La Jolla, CA, USA). Overall survival was defined as time from treatment start to death from any cause, or last follow-up date. Disease-free survival was defined as time from treatment start to disease progression or death for any cause. Living patients without evidence of progression were censored at the last follow-up. Analysis of the risk of death for the 157 tumours from Tayside was conducted using Cox proportional-hazards regression modelling. We examined the relationship between OS and the methylation status of the *NT5E* CpG island and known clinico-pathological parameters, including tumour size, tumour grade, ER status, PgR status, nodal status and HER-2.

RESULTS

NT5E expression is regulated epigenetically in breast cancer

Methylation in the *NT5E* CpG island was detectable in MDA MB 361, MDA MB 436, MDA MB 453, MCF-7, GI101, T47D, MCF12A and ZR75.1 cell lines (Figure 1A). By methylation-specific PCR, methylation was complete (no detecteable amplification in the U-specific PCR) in MDA MB 453 and T47D and partial in the remaining cell lines (amplification of both U- and M-specific PCR). There was no detectable methylation in MDA MB157, MDA MB 231, MDA MB 468, NCI, BT20 and CAL51 (Figure 1A). To validate and extend these results, we performed pyrosequencing analysis of the *NT5E* CpG island in breast cancer cell lines. These included a subset of those analysed by methylation-specific PCR, together with additional lines not previously analysed (Figure 1B). Average methylation in the CpG island was 94% in MDA MB 453 and 88% in T47D, consistent with methylation-specific PCR (Figure 1B). There was partial methylation in MDA MB 436 (35%) and MCF-7 (26%) again consistent with methylation-specific PCR. MDA MB 231 and MDA MB 468 had average methylation levels of 2%, also in agreement with methylation-specific PCR (summarised in Table 1). Next, we assessed expression of *NT5E* mRNA using RT–PCR and correlated this with methylation status. These data (Figure 1, Table 1) imply a close correlation between methylation in the *NT5E* CpG island and absent/downregulated expression of *NT5E* mRNA. To further test this association, we used qPCR to analyse mRNA levels, initially in normal breast cells and cell lines with CpG island methylation. Expression was undetectable in MDA MB 453 and T47D (lines with almost complete methylation of the

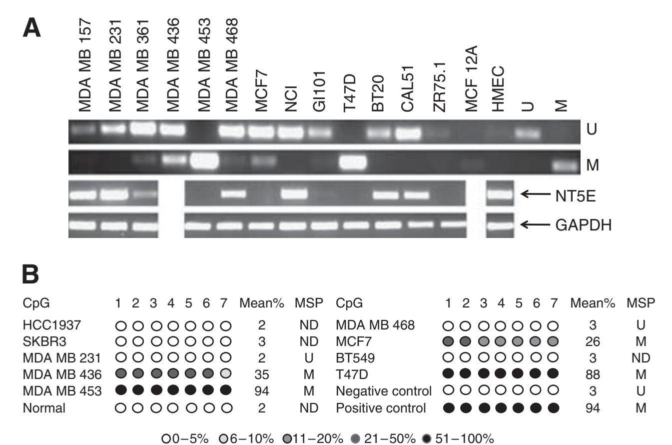

Figure 1 Methylation-dependent transcriptional silencing of NT5E in breast cancer cell lines. (**A**) Methylation in the NT5E CpG island correlates with downregulation of mRNA expression. The figure shows MSP analysis of the NT5E CpG island (upper panels) and semi-quantitative RT–PCR analysis of NT5E mRNA and the control gene *GAPDH* in breast carcinoma cell lines (bottom panels). MSP was performed as described in Materials and Methods. The figure shows unmethylated (U) and methylated (M) reactions for each cell line DNA. Also shown are control U and M DNA samples modified in parallel with the cell line DNA samples. (**B**) Pyrosequencing analysis of the *NT5E* CpG island in breast carcinoma cell lines. Pyrosequencing was done as described in Materials and Methods. The level of methylation is represented by the intensity of shading in the circles, each of which represents an individual CpG dinucleotide in the amplified fragment. The mean % CpG methylation in the amplified fragment, together with the methylation-specific PCR analysis is also shown. Abbreviation: ND = not determined.

Table 1 Methylation in the NT5E CpG island in breast carcinoma cell lines

Cell line	Phenotype	ER	PgR	HER-2	NT5E (MSP)	NT5E (pyro-sequencing %)
MDA MB 157	(Basal)	−	−	N	U	ND
MDA MB 231	(Basal)	−	−	N	U	2
MDA MB 361	(Luminal)	+	−	Amp	U/M	ND
MDA MB 436	(Basal)	−	−	N	U/M	35
MDA MB 453	(Luminal)	−	−	Amp	M	94
MDA MB 468	(Basal)	−	−	N	U	2
MCF-7	(Luminal)	+	+	N	U/M	26
MCF12A	(Basal)	−	−	N	U/M	ND
GI101	(Basal	+	−	N	U/M	ND
T47D	(Luminal)	+	+	N	M	88
BT20	(Basal)	−	−	N	U	ND
CAL51	(Basal)	−	−	N	U	ND
BT549	(Basal)	−	−	N	ND	3
ZR75.1	(Luminal)	+	−	N	U/M	ND
HCC1937	(Basal)	−	−	N	ND	2
SKBR3	(Luminal)	−	−	Amp	ND	3

Abbreviations: Amp = Amplified; ER = oestrogen receptor; M = methylated; MSP = methylation-specific PCR; ND = not determined; PgR = progesterone receptor; U = Unmethylated. Data on cell line phenotypes, ER, PgR and Her-2 are from Mackay *et al* (2009).

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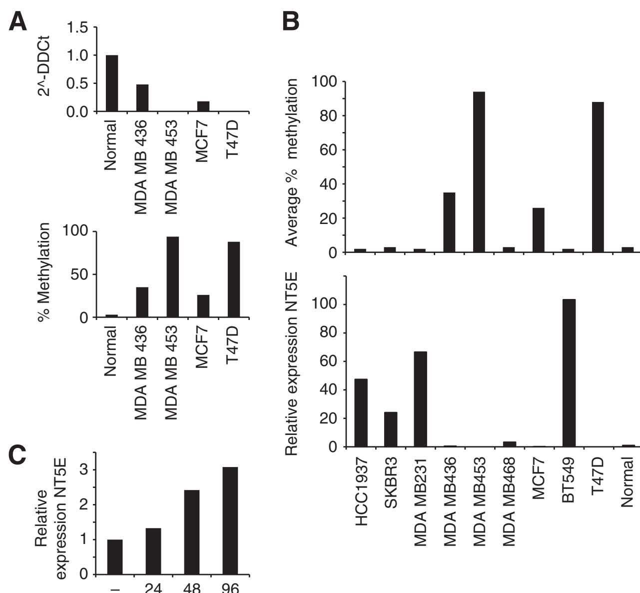

Figure 2 (**A**) Downregulation of *NT5E* mRNA correlates with CpG methylation in ER-positive breast carcinoma cell lines. Expression of *NT5E* mRNA was determined by qPCR and % methylation by pyrosequencing. Downregulation correlates with methylation. (**B**) *NT5E* mRNA is overexpressed in breast carcinoma cell lines lacking CpG island methylation. Average % methylation in the *NT5E* CpG island was determined by pyrosequencing and expression of *NT5E* mRNA by qPCR (relative to normal breast epithelium). (**C**) Demethylation reactivates *NT5E* expression. MCF-7 cells were treated with AZA and harvested at the indicated times (in hours). Total RNA was isolated and levels of *NT5E* mRNA determined by qPCR as described in Materials and Methods.

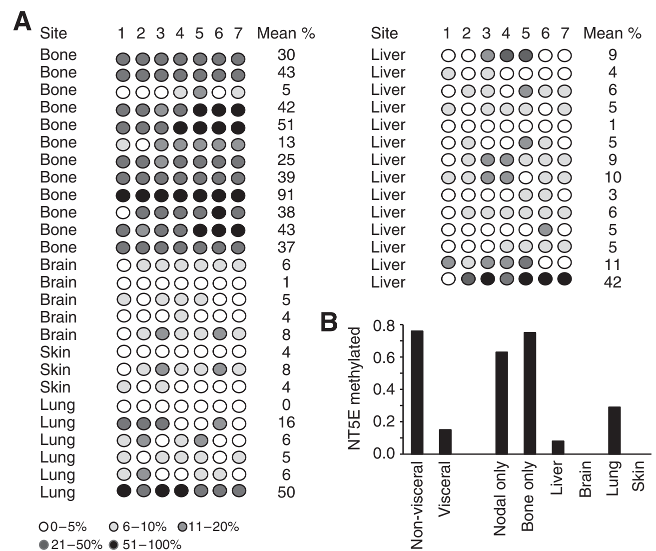

Figure 3 *NT5E* CpG island methylation is more common in cancers relapsing in non-visceral metastatic sites in ER-positive breast cancer. (**A**) Representative pyrosequencing analysis of the *NT5E* CpG island in primary breast carcinomas. The predominant site of metastatic relapse is indicated. Pyrosequencing analysis was performed as described in Materials and Methods. The % methylation at each analysed CpG dinucleotide is represented by the intensity of shading in the circles as shown in the figure, together with the mean % methylation for each case. (**B**) Graph showing proportion of methylation-positive cases in ER-positive primary breast cancers, treated with endocrine therapy, which subsequently relapsed at different metastatic sites. Primary carcinomas which relapsed with metastasis to brain, liver, lung and skin are designated 'visceral'. Bone and lymph node metastasis are designated as 'non-visceral'.

NT5E CpG island) and downregulated relative to normal breast epithelium in MDA MB 436 and MCF-7 (partially methylated *NT5E* CpG island) (Figure 2A). Having confirmed correlation between methylation and downregulation of mRNA, we next analysed a larger panel of cell lines again using qPCR. *NT5E* mRNA was expressed at high levels in HCC1937 (BRCA1-associated TNBC), MDA MB 231 (TNBC), BT549 (TNBC) and SKBR3 (ER −, PgR − and HER-2 +) (Figure 2B). All cell lines that overexpressed *NT5E* were unmethylated as determined by MSP and pyrosequencing. To further test the association of mRNA silencing with aberrant CpG methylation, we analysed the effect of the demethylating agent azacytidine (5′ AZA) in MCF-7 cells in which the CpG island was partially methylated and expression downregulated. Twenty-four hours after exposure to 5′ AZA there was an increase in NT5E mRNA levels and this increased further to a maximum three-fold upregulation after 96 h of 5′-AZA exposure (Figure 2C). Together, data from Figures 1 and 2 imply that the *NT5E* CpG island is unmethylated and the gene expressed at high levels predominantly but not exclusively in hormone receptor negative breast cancer cell lines, whereas, conversely, methylation-dependent silencing of *NT5E* occurs more commonly in hormone receptor-positive cell lines.

NT5E CpG island methylation associates with non-visceral predominant metastatic disease

The *in vitro* data prompted us to investigate methylation in the *NT5E* CpG island in clinical cases of breast cancer. We first analysed using pyrosequencing DNA extracted from 83 formalin-fixed, paraffin-embedded primary breast cancer cases from Northern Italy (Figure 3). These cases comprised 79 ER-positive cancers treated adjuvantly with endocrine therapy and 4 ER-negative cases. The *NT5E* CpG island was methylated in 40/79 (51%) ER-positive cases. We asked whether methylation of the *NT5E* CpG island in primary ER-positive cases was associated with loco-regional and/or distant metastatic relapse. Representative pyrosequencing is shown in Figure 3A. The *NT5E* CpG island was densely methylated in 16 of 21 (76%) patients who developed non-visceral metastatic disease compared with 6/28 cases with visceral metastases (21%) ($P = 0.0002$, OR = 11.73). Interestingly, the *NT5E* CpG island was unmethylated in all four ER-negative cases and visceral metastatic relapse occurred in 3/4 of these cases.

NT5E CpG island methylation in metastatic breast cancer

Data from primary cancers suggest that NT5E may influence the anatomical site of relapse. To further address this observation, we performed pyrosequencing analysis of 23 resected CNS metastases (series II) from patients with primary breast carcinomas. In each case, the diagnosis of metastatic breast cancer was confirmed by histopathological analysis and receptor expression studies (ER, PgR and HER-2). Of the 23 cases, 14 were ER negative and 8 of these 14 cases were TNBC. 10/14 ER-negative cases were negative for *NT5E* methylation and 6/8 TNBC were negative (Figure 4).

Methylation in the *NT5E* CpG island varies with acquisition of a metastatic phenotype

Our data imply selective pressure for overexpression of NT5E in at least a subset of cases of metastatic breast cancer, particularly in ER negative and TNBC cases. However, it is clear that some primary cancers, with high level of *NT5E* CpG island methylation, nonetheless ultimately relapsed in visceral sites. This prompted us to ask whether the methylation status of the *NT5E* CpG island changes in metastatic lesions relative to primary lesions. Using pyrosequencing, we analysed four cases in series II (all TNBC) for

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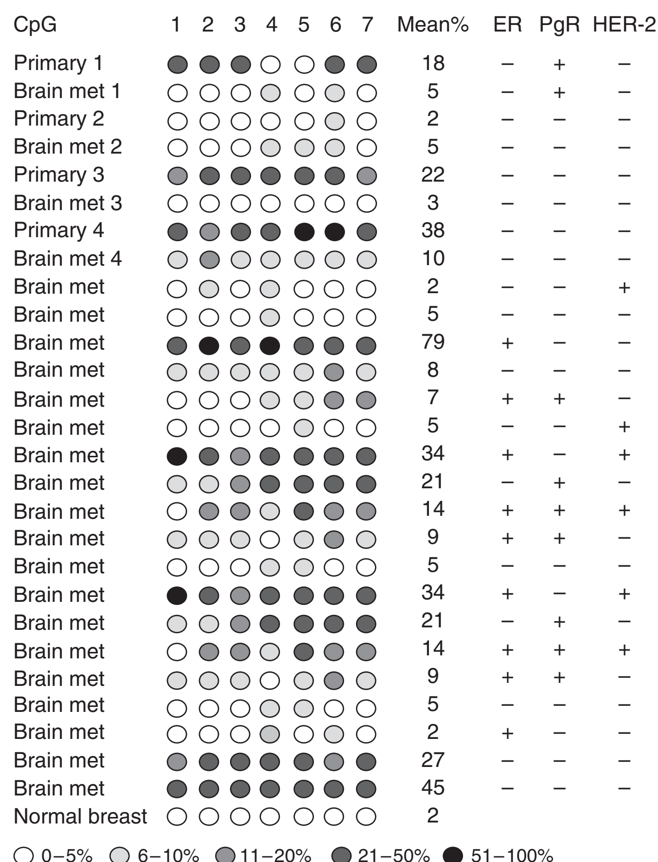

Figure 4 Pyrosequencing analysis of *NT5E* CpG island in CNS metastatic breast cancer lesions. Pyrosequencing was done as described in Materials and Methods. The level of methylation is represented by the intensity of shading in the circles, each of which represents an individual CpG dinucleotide in the amplified fragment. The mean % CpG methylation in the amplified fragment together with ER, PgR and Her-2 status of the lesion are indicated. Four cases of paired primary and CNS metastatic lesions are also shown to illustrate changes in methylation.

which paired primary and CNS metastatic tissue was available to us. There was no change in the status of ER, PgR or HER-2 between the primary and metastatic lesions in each case (Figure 4). Nevertheless in three cases of the CNS metastases there was a clear loss of methylation in the *NT5E* CpG island relative to the paired primary lesion, consistent with selection of cell clones lacking methylation (Figure 4). In the 4th case, methylation levels were low in the primary carcinoma and remained low in the CNS metastasis (Figure 4).

NT5E CpG island methylation is an independent breast cancer biomarker

The apparent association between the methylation status of the *NT5E* CpG island and the site specificity of metastasis prompted us to extend the investigation to an independent cohort of primary breast cancers, for which complete and long-term follow-up data were available. This series (series III) comprised 157 primary cancers cases (119 ER positive and 38 ER negative, of which 26 were TNBC; Table 2). Fifty-two patients had developed metastatic disease at the time of censoring. Methylation was detected in 96/157 (61%) cases (Figure 5A), which is comparable to the first series analysed (51%). Methylation was detected in both ER-positive (77/119; 0.65) and ER-negative (19/38; 0.5) cases. Patients whose primary tumours, were positive for *NT5E* CpG island methylation, were less likely to develop metastasis than those lacking

Table 2 (A) Clinico-pathological characteristics of patients ($n = 157$); (B) Multivariate analysis of prognostic factors

Characterstic	Number of cases (frequency)
(A)	
Grade 1	29 (0.19)
Grade 2	60 (0.39)
Grade 3	63 (0.41)
Missing	5
Stage 1	63 (0.41)
Stage 2	87 (0.56)
Stage 3	6 (0.03)
Missing	2
Node positive	69 (0.45)
Node negative	83 (0.55)
Missing	5
ER positive	119 (0.76)
ER negative	38 (0.24)
PgR positive	84 (0.54)
PgR negative	73 (0.46)
HER-2 positive	19 (0.12)
HER-2 negative	136 (0.88)
Missing	2
TP53 mutation	46 (0.29)
TP53 wild-type	111 (0.71)
NT5E methylated (ER positive)	77 (0.65)
NT5E methylated (ER negative)	19 (0.5)

(B) Variable	**Hazard ratio**	***P*-value**	**95% CI**
Tumour grade	1.548	0.002	1.168–2.052
Nodal status	1.099	0.000	1.043–1.160
ER positive	0.372	0.12	0.107–1.291
PgR positive	0.497	0.182	0.178–1.387
TP53 mutation	2.041	0.08	0.919–4.529
NT5E methylation	0.328	0.010	0.141–0.765

methylation ($P = 0.003$, OR = 0.34, 95% CI: 0.17–0.69). When analysing by histological subtype, we confirmed the association of CpG island methylation with lower probability of metastasis in ER negative ($P = 0.049$, OR = 0.21, 95% CI: 0.05–0.83) and in TNBC cases ($P = 0.017$, OR = 0.09, 95% CI: 0.01–0.55) (Figure 5B), but in the cohort of 119 ER-positive cases, this association just failed to reach statistical significance ($P = 0.08$, OR = 0.4271, 95% CI: 0.20–1.09) (Figure 5B). When all cases were analysed, *NT5E* CpG island methylation was more common in those with non-visceral compared with visceral metastatic disease ($P = 0.01$, OR = 11.8). When analysing by histological subtype, this association was significant in ER-negative cases ($P = 0.02$, OR = 22), but did not reach significance in ER-positive cases ($P = 0.33$). Consistent with this observation, in cases with visceral metastases, the frequency of unmethylated *NT5E* CpG island was lower in ER-positive cases than in ER-negative cases (12/49 *vs* 12/19; $P = 0.02$, OR = 4.28). We then investigated *NT5E* CpG island methylation as a determinant of disease outcome in the same series, using time-dependent endpoints. Patients with tumours lacking detectable *NT5E* CpG island methylation had increased probability of shorter DFS ($P = 0.001$, HR 2.7, Figure 5C) and OS ($P = 0.001$, HR = 3, Figure 5D). In subgroup analysis, unmethylated *NT5E* CpG island was also associated with increased risk for poorer survival in ER

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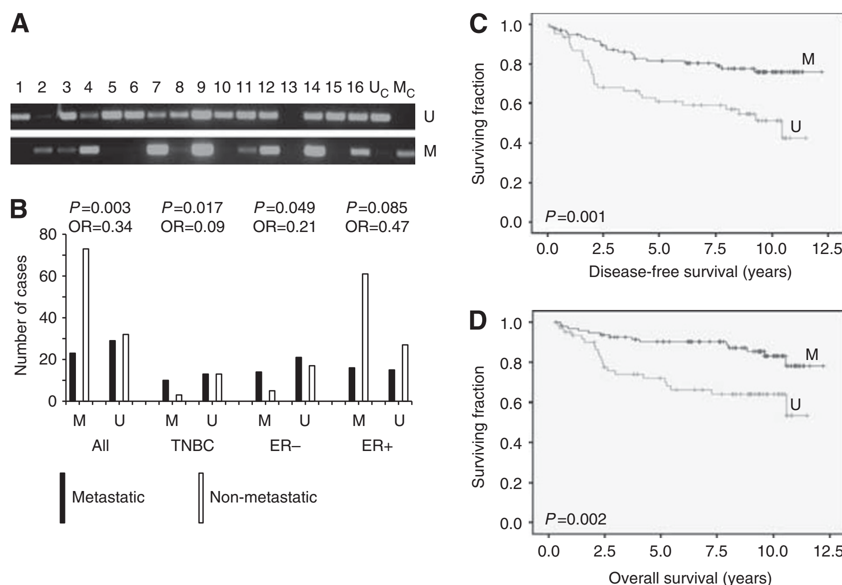

Figure 5 Methylation of the *NT5E* CpG island in primary breast carcinomas influences clinical outcome. (**A**) Representative methylation-specific PCR analysis of the *NT5E* CpG island in series III breast carcinomas. The figure shows unmethylated (U) and methylated (M) reactions for each individual case DNA (numbered 1–16). Also shown are control U and M DNA samples modified in parallel with the cell line DNA samples. (**B**) Metastatic relapse is more frequent in patients whose primary breast carcinoma lacks methylation in the *NT5E* CpG island. The figure shows number of patients with metastatic relapse as a function of the methylation status of the *NT5E* CpG island (M, U). All: $N=157$; TNBC: $N=26$; ER $-$: $N=38$; ER $+$: $N=119$. (**C**) Kaplan–Meir analysis of disease-free survival (DFS) in primary breast carcinomas according to methylation status of *NT5E* CpG island. Methylation in the *NT5E* CpG island was analysed by MSP and statistical analysis done as described in Materials and Methods. The figure shows DFS in primary cancers either M or U. (**D**) Kaplan–Meir analysis of OS in primary breast carcinomas according to methylation status of *NT5E* CpG island. Methylation in the *NT5E* CpG island was analysed by methylation-specific PCR and statistical analysis done as described in Materials and Methods. The figure shows OS in primary cancers either M or U. Abbreviations: ALL = entire patient population; ER $-$ = oestrogen receptor negative; ER $+$ = oestrogen receptor positive; MSP = methylation-specific PCR; TNBC = triple-negative breast cancer.

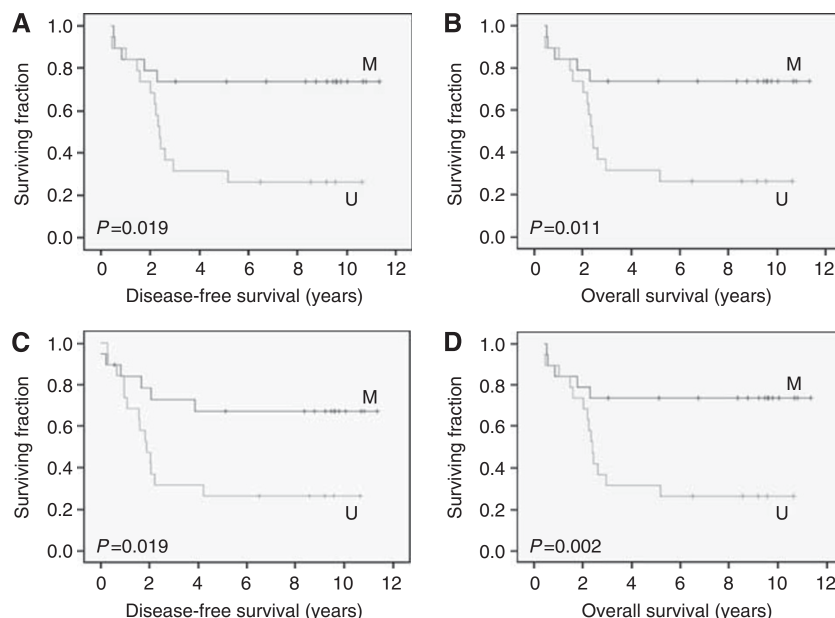

Figure 6 Kaplan–Meir analysis of outcome in ER negative and triple-negative primary breast carcinomas according to methylation status of *NT5E* CpG island. Methylation in the *NT5E* CpG island was analysed by methylation-specific PCR and statistical analysis done as described in Materials and Methods. The top two panels show DFS (**A**) and OS (**B**) in ER-negative primary cancers either methylated (M) or unmethylated (U) in the *NT5E* CpG island. The lower two panels show DFS (**C**) and OS (**D**) in triple-negative breast carcinomas either M or U in the *NT5E* CpG island.

negative ($P=0.011$, HR $=3.27$, 95% CI: 1.31–8.12, Figure 6) and in TNBC ($P=0.004$, HR $=6.2$, 95% CI: 1.9–20 Figure 6), but not in patients with ER-positive tumours ($P=0.35$, HR $=1.64$, 95% CI: 0.57–4.68). To further confirm that the effects of *NT5E* CpG island methylation are not the result of a non-specific 'methylator' phenotype, we tested the effects on clinical outcome of

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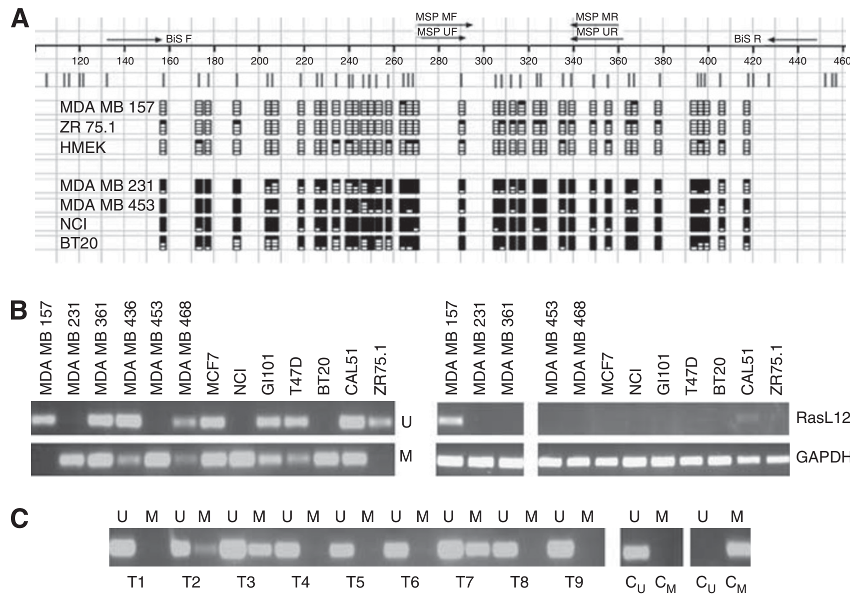

Figure 7 Methylation-dependent transcriptional silencing of *RasL12* in breast cancer. (**A**) Bisulphite sequencing analysis of *RasL12* CpG island. The figure shows a map of the *RasL12* CpG island with the position of primers indicated for bisulphite sequencing (Bis F and Bis R) and methylation-specific PCR (MSP MF, MSP UF, MSP MR and MSP UR). Vertical lines below the scale represent individual CpG dinucleotides within the CpG island. The density of methylation for each cell line is represented by a quartile of blocks corresponding to each CpG. Black shading represents up to 25% methylation. Open blocks indicate no methylation. (**B**) Methylation-specific PCR analysis of *RasL12* CpG island methylation (left panel) and RT–PCR analysis of expression (right panel) in breast cancer cell lines. The figure shows U- and M-specific PCR reactions for each cell line and RT–PCR. (**C**) MSP analysis of *RasL12* CpG island methylation in primary breast carcinomas from series III. Representative analysis of nine cases (T1–T9) is shown, together with control unmethylated (C_U) and control methylated (C_M) DNA modified and analysed in parallel with the test samples.

a second novel epigenetically regulated gene, *RasL12*, in the same patient population. We initially showed using bisulphite sequencing and methylation-specific PCR that *RasL12* is subject to CpG island methylation in many breast carcinoma cell lines, with a perfect correlation between bisulphite sequencing and methylation-specific PCR and downregulated expression of *RasL12* (Figure 7A and Figure 7B). We then analysed the same patient population using methylation-specific PCR (Figure 7C). There was methylation in the *RasL12* CpG island in 48/157 (31%) cases. Methylation in the *RasL12* CpG island did not significantly affect median DFS or median OS. For DFS: $P = 0.5339$, HR = 0.7939, 95% CI: 0.3836–1.643; for OS: $P = 0.3854$, HR = 0.7608, 95% CI: 0.4104–1.410.

NT5E CpG island methylation status and outcome after adjuvant chemotherapy

We then asked whether the methylation status of *NT5E* affects clinical outcome in patients who received adjuvant chemotherapy. Disease-free survival in patients who received chemotherapy was significantly shorter in patients with unmethylated *NT5E* CpG island ($P = 0.0016$, HR = 5.1, 95% CI: 1.8–14.37). Similarly, OS was shorter in cases with unmethylated *NT5E* CpG island ($P = 0.0005$, HR = 7.4, 95% CI: 2.416–23.08).

NT5E CpG island methylation is inversely correlated with *TP53* mutation

Finally, we performed multivariate analysis to assess the prognostic significance of *NT5E* CpG island methylation in series III (Table 2). This analysis revealed that *NT5E* methylation retained significance after multivariate analysis of known prognostic variables (HR = 0.328, 95% CI: 0.141–0.765; $P = 0.010$). Further, we found that *NT5E* CpG island methylation was inversely associated with *TP53* mutation ($P < 0.005$).

DISCUSSION

Here, we report that *NT5E* (CD73) expression is regulated in breast cancer by the methylation status of the CpG island and that methylation is a prognostic indicator of favourable clinical outcome. Previous work has identified involvement of NT5E expression in cancer behaviour (Zhi *et al*, 2010), but this is to the best of our knowledge the first demonstration that *NT5E* is an independent prognostic marker in human cancer.

We have shown using two techniques, methylation-specific PCR and (quantitative) pyrosequencing, that downregulation of *NT5E* mRNA correlates well with methylation in the CpG island in breast cancer. In addition, we found that the *NT5E* CpG island is unmethylated and mRNA expressed in normal breast epithelial cells, while the hypomethylating compound 5′ AZA restores expression in breast cancer cell lines. Together, these observations imply that aberrant CpG methylation is an important mechanism of *NT5E* silencing in breast cancer and that silencing is specific for neoplasia. The expression analysis we report in breast cancer cell lines is consistent with previous work (Spychala *et al*, 2004; Mackay *et al*, 2009). We confirm and extend these expression analyses by showing that CpG island methylation is a key regulator of *NT5E* expression and the predominant mechanism of downregulation in breast cancer cell lines. Our results also further extend previous data by showing that expression in ER-positive (predominantly luminal) breast carcinoma cell lines is downregulated by methylation-dependent transcriptional silencing and that in the absence of methylation (most commonly, but not

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exclusively, seen in ER negative and triple-negative cell lines), *NT5E* is often greatly overexpressed.

To assess the clinical significance of our cell line observations, we extended the study to primary breast carcinomas. Given the potential importance of NT5E as a mediator of malignant progression and metastasis, studies of NT5E expression in clinical cases of breast cancer have been remarkably limited. One such study, performed 20 years ago, employed immunohistochemical analysis of NT5E in an unselected series of breast carcinomas and showed that expression was detected in ~10% (Kruger *et al*, 1991). More recently, it was shown in a small series of clinical cases that NT5E was underexpressed predominantly in ER-positive breast carcinomas and more commonly overexpressed in TNBC (Spychala *et al*, 2004). Our data, in three independent series of breast carcinomas, are clearly consistent with both previous studies. Furthermore, we show in a series of ER-positive cases treated with adjuvant endocrine therapy that bone predominant disease, a clinical entity with a typically indolent clinical course (Andrade *et al*, 2011), is more common in cases with dense *NT5E* CpG island methylation, whereas visceral and CNS metastases predominate in cases lacking dense *NT5E* methylation. We confirmed these results in a second-independent clinical series containing ER positive, ER negative and TNBC. Aggressive disease, with visceral and/or brain metastases, is more likely in cases that lack methylation in the *NT5E* CpG island, than in those with methylation. Our data are consistent with the observation that overexpression of NT5E in an experimental model of breast cancer promotes metastasis to the lungs via activation of A2B adenosine receptors (Stagg *et al*, 2011).

Despite this strong propensity for cases lacking methylation to metastasise to viscera and brain, it is clear that (in a minority of cases) clinically aggressive disease develops from primary breast cancers in which the *NT5E* promoter CpG island is methylated. We propose two potential explanations for this. First, knock-down of NT5E causes increased cell migration in some cell types (Andrade *et al*, 2011). It is therefore possible that, perhaps in early disease, methylation-dependent silencing of *NT5E* might promote enhanced migration in a subset of breast carcinomas through as yet undefined mechanisms. Second, we have shown in the present study that the methylation status of the *NT5E* CpG island often changes from methylated to unmethylated in cases which have metastasised to brain, when compared with the primary breast carcinoma from which they derived, implying a strong selective pressure favouring (re-) expression of NT5E in aggressive breast cancers. The numbers of such paired cases available to us was limited. However, to the best of our knowledge, this is the first demonstration of dynamic epigenetic change in a specific gene from primary carcinoma to brain metastasis in breast cancer. Changes in expression of steroid hormone receptors and HER-2 between primary and recurrent disease are now well described (Aitken *et al*, 2010; Thompson *et al*, 2010) and changes in the epigenetic status of *NT5E* provide further evidence of a dynamic transcriptional process underlying metastasis.

To validate methylation in *NT5E* as an independent prognostic marker, we tested a second novel epigenetically regulated gene, *RasL12*. We showed using bisulphite sequencing and methylation-specific PCR that expression of *RasL12* is silenced by methylation in breast carcinoma cell lines and in primary breast carcinomas. Understanding of the precise role of *RasL12* in breast cancer awaits additional studies. However, the absence of any predictive effect of *RasL12* on clinical outcomes in our series of cases strongly endorses the specific biomarker candidacy of *NT5E* methylation.

In addition to influencing metastatic potential in breast cancer, we demonstrate that the methylation status of *NT5E* also affects outcome in cases treated with (predominantly anthracycline) chemotherapy. These results are supported by previous work in cell lines resistant to anthracyclines (Ujhazy *et al*, 1994; Mikhailov *et al*, 2008). Depletion of NT5E mediated by inhibitory RNA causes increased apoptosis and reduction in cell growth rate in MDA MB 231 cells consistent with the clinical importance of *NT5E* CpG island methylation in TNBC and particularly with its strong predictability of favourable outcome to adjuvant chemotherapy, potentially by intervening in accumulation of intracellular nucleotides that have been shown to act as critical pro-survival factors (Chandra *et al*, 2006). A further potential mechanism linking absence of methylation with less favourable outcome after chemotherapy is the highly significant association between absence of *NT5E* CpG island methylation and mutation in *TP53*.

The recognised role of NT5E in promoting invasion and metastasis (Wang *et al*, 2008) taken together with the frequent absence of methylation in *NT5E* in ER negative and TNBC, which we demonstrate in the present work, implies that inhibition of NT5E may be an attractive therapeutic strategy in these subtypes of breast cancer. Strategies to inhibit or block NT5E clearly merit consideration in breast cancer particularly in the difficult to treat TNBC and ER-negative cases. Conversely, our results may have implications in the potential use of demethylating agents in breast (and other) solid tumours. This approach has been considered because the majority of genes affected by methylation-dependent transcriptional silencing in cancer are tumour suppressor genes or genes silenced by methylation in cancers with acquired chemotherapy resistance. Re-expression of such genes would have likely therapeutic value. Our data imply that reactivation of *NT5E* would have deleterious effects by promoting a more aggressive phenotype and potentially resistance to chemotherapy. Identification of patients likely to benefit from use of demethylating agents might be informed, at least in part, by profiling of the methylation status of key genes, such as *NT5E*.

In conclusion, we show for the first time that *NT5E* is regulated epigenetically in breast cancer, the epigenetic status of this gene influencing metastasis and clinical outcome. We suggest that *NT5E* promoter CpG island methylation may serve as a promising favourable breast cancer epigenetic biomarker. NT5E is expressed in normal breast tissue (http://www.proteinatlas.org/ENSG00000135318). As such, the sensitivity of methylation analysis and its specificity for neoplasia together suggest that *NT5E* methylation analysis may be more informative and clinically useful than immunohistochemistry or qPCR as a prognostic biomarker. Our results warrant independent confirmation by other groups before considering its investigation as a metastasis predictor in clinical trials of adjuvant breast cancer therapies.

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