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Methylation of the calcium channel regulatory subunit α2δ-3 (CACNA2D3) predicts site-specific relapse in oestrogen receptor-positive primary breast carcinomas

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BACKGROUND: Calcium is an important intracellular messenger that mediates many biological processes that are relevant to the malignant process. Calcium ion channels are key in controlling the intracellular calcium, and little is known about their role in human cancer.

METHODS: We used qPCR and pyrosequencing to investigate expression and epigenetic regulation of the calcium channel regulatory subunit $\alpha_2\delta$-3 (CACNA2D3) in breast cancer cell lines, primary cancers and metastatic lesions.

RESULTS: Expression of CACNA2D3 mRNA is regulated in breast cancer cell lines by methylation in the CpG island located in the 5′ regulatory region of the gene. Expression is upregulated by azacytidine (AZA) in cells with CpG island methylation but unaffected in cells lacking methylation. In primary breast carcinomas, methylation is more common in cancers, which subsequently relapse with loco-regional and, particularly, visceral metastatic disease in both oestrogen receptor-α (ER)-positive and -negative cases. Furthermore, CACNA2D3 CpG island is frequently methylated in breast cancer that has metastasised to the central nervous system.

CONCLUSION: Methylation-dependent transcriptional silencing of CACNA2D3 may contribute to the metastatic phenotype of breast cancer. Analysis of methylation in the CACNA2D3 CpG island may have potential as a biomarker for risk of development of metastatic disease.

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The calcium ion (Ca^{2+}) is a key intracellular messenger and regulates a diverse range of cellular processes by activating or inhibiting cellular signalling pathways and Ca^{2+}-regulated proteins. These processes range from muscle contraction to apoptosis (Berridge *et al*, 2003; Monteith *et al*, 2007). Calcium ion has been implicated either directly or indirectly in many of the essential alterations key for malignant growth (Hanahan and Weinberg, 2000), having been shown to be involved in proliferation (Becchetti, 2011), cell motility, (Huang *et al*, 2004), angiogenesis (Patton *et al*, 2003), resistance to apoptosis (Rizzuto *et al*, 2003) and transcriptional regulation (Rizzuto and Pozzan, 2006). These effects could be modulated by changes in plasma membrane Ca^{2+} channel expression, Ca^{2+} efflux pumps as well as the expression of proteins that control the Ca^{2+} content of the endoplasmic reticulum (Monteith *et al*, 2007). Epigenetic mechanisms have been reported as one of the potential causes for changes in the expression of specific Ca^{2+} pumps and channels in human cancers (Toyota *et al*, 1999; Kim *et al*, 2003; Paz *et al*, 2003).

Voltage-gated (Ca_V) calcium channels of the Ca_V1 and Ca_V2 classes exist as heteromeric complexes, that is composed of a pore-forming α1 subunit and three regulatory subunits, α2δ, β and δ (Davies *et al*, 2007). There are four calcium channel voltage-dependent α2δ subunit genes, *CACNA2D1* to *CACNA2D4* (Qin *et al*, 2002). The importance of calcium ion channels in the malignant process has previously been shown by CACNA2D2 subunit, which is located in 3p21.3 region, a region deleted in breast and lung cancer (Wei *et al*, 1996). It has been shown to be expressed in normal lung tissue but lost in malignant tissue and its presence has been shown to mediate apoptosis (Carboni *et al*, 2003).

The CACNA2D3 subunit has been implicated to have a role in a number of cancers. An 80-fold decrease in expression of CACNA2D3 has been reported in the highly metastatic osteosarcoma cell line MG63-A1, when compared with the parental cell line (Xiong *et al*, 2009). CACNA2D3 is a putative tumour suppressor gene in both lung cancer, renal cell cancer neuroblastoma and squamous cell oesophageal cancer (Hanke *et al*, 2001;

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De Preter *et al*, 2006; Tai *et al*, 2006; Qin *et al*, 2008). CACNA2D3 is highly expressed in neuroblasts and favourable prognosis neuroblastomas, with expression downregulated in unfavourable neuroblastomas (De Preter *et al*, 2006; Thorell *et al*, 2009). Comparison of 14 pairs of primary gastric carcinomas with matched normal gastric mucosa found that the *CACNA2D3* CpG island was unmethylated in all 14 normal tissues, whereas methylation was present in 36% of primary tumours. Assessment of a large group of unrelated gastric cancer and normal tissue found that the *CACNA2D3* CpG island was methylated in 30% of primary tumours, and 5.3% of normal tissue (Wanajo *et al*, 2008). A similar rate of methylation was reported in an independent cohort of gastric cancers (Yuasa *et al*, 2009). A significantly reduced survival time was found in tumours with methylation as compared with those with unmethylated *CACNA2D3*. *In-vitro* exogenous CACNA2D3 expression inhibited cell growth and adhesion and upregulated p21 and p27 expression in gastric cancer cell lines with inverse effects with CACNA2D3 small interfering RNA treatment (Wanajo *et al*, 2008).

Given the evidence of the biological properties of the CACNA2D3 subunit and its possible role in human malignant disease, we investigated the expression and epigenetic regulation of CACNA2D3 in human breast cancer cell lines as well as in clinical samples of primary and metastatic breast cancer.

MATERIALS AND METHODS

Breast cancer cell lines

Breast carcinoma cell lines (MDA-MB-231, MDA-MB-436, MDA-MB-453, MDA-MB-468, MCF-7, T47D, BT549, HCC1937, SKBR3, ZR75-1) were obtained from the Cancer Research UK Cell services (Claire Hall Laboratories, South Mimms, UK). All cells were maintained in Dulbecco's Modified Eagle Medium supplemented with 5 mM L-glutamine and 10% fetal bovine serum.

For pharmacological demethylation, cells were treated with 5 μM azacytidine (AZA; Sigma, Gillingham, UK) for 7 days. Cells were split every 2–3 days with the addition of fresh drug. After drug treatment, cells were harvested for qPCR.

Tumours

The study was performed with local research ethical approval. Primary breast carcinomas were from the pathology archives of S Croce General Hospital, Cuneo, Italy and all central nervous system (CNS) metastatic carcinomas were from the neuropathology archives of Imperial College Healthcare NHS Trust, London, UK. All cases were histologically confirmed as breast carcinoma, and analysed for expression of OEstrogen receptor-α (ER), progesterone receptor (PgR) and HER2 according to local protocols. As normal tissue controls, we used genomic DNA isolated from five pooled normal breasts obtained at reduction mammoplasty.

Nucleic acid isolation

Genomic DNA and RNA were isolated from cell lines using commercially available kits (Qiagen, Venlo, The Netherlands). Genomic DNA was isolated from archival cases in Cuneo by proteinase K digestion of 10 μm sticks cut from formalin-fixed paraffin-embedded tissue sections using standard xylene-phenol protocol. Total RNA was isolated from paraffin tissues using the RecoverAll Total Nucleic Acid Isolation kit (Ambion, Foster City, CA, USA).

Pyrosequencing analysis

Methylation in the CpG island of the *CACNA2D3* genes was analysed using Pyrosequencing to quantify the degree of methylation at each CpG site by measurement of the ratio between T and C. Primer sequences were as follows:

Forward primer 5′-GGTTAAGGATATTGGAGTTTT-3′
Reverse primer 5′-biot-CCTCTAACAACAACAACC-3′
Amplicon length 128 bp

PCR conditions were 95 °C for 10 min, 95 °C for 30 s/52 °C for 30 s/72 °C for 40 s for 40 cycles, 72 °C for 7 min. PCR products were then analysed by pyrosequencing using the Sample Prep kit (Diatech, Jesi, Italy) and the forward primer for sequencing. After pyrosequencing, analysis of percentage methylation at each CpG site was done using Pyromark QCpG Software (Qiagen). Placental DNA was used as negative control of methylation (0% average methylation) and a commercial methylated DNA (Millipore, Watford, UK) was used as positive control (98% average methylation).

Analysis of gene expression

For qPCR analysis, 25 μl PCR reactions were performed using 50 ng of cDNA obtained by reverse transcription of 1 μg of RNA. 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, Life Technologies Italia, Monza, Italy) and the pre-cast TaqMan Gene Expression Assays' (Applera; https://products.appliedbiosystems.com/) for CACNA2D3 (Hs00218157_m1). Quantification of target transcripts was performed in comparison to the reference transcript β2microglobulin (Hs99999907_m1), using the 'δ-δ Ct' method.

Statistical analysis

Methylation status of the CpG island of *CACNA2D3* and presence of or sites of metastasis were assessed for association using Fisher's exact test.

RESULTS

CACNA2D3 CpG island imethylation in breast cancer cell lines

A CpG island is located in the 5′ regulatory sequences of *CACNA2D3*. We used pyrosequencing to analyse methylation in this CpG island in the cell line panel. There was dense methylation in MDA-MB-231 and MDA-MB-453 and low-level methylation in T47D, but no evidence of methylation in normal breast epithelial cells or in the remaining cell lines in the panel (Figure 1A). Using qPCR, we studied expression of *CACNA2D3* mRNA in a panel of breast carcinoma cell lines. *CACNA2D3* mRNA was abundantly expressed in the majority of cell lines analysed but was downregulated in MDA-MB-231, MDA-MB-453 and T47D (Figure 1B) confirming a good correlation between methylation and downregulated expression.

To confirm the role of methylation in silencing expression of *CACNA2D3*, we grew MDA-MB-436 (unmethylated) and MDA-MB-453 (methylated) cells in the presence of the demethylating agent AZA and analysed CACNA2D3 mRNA using qPCR. In MDA-MB-453, exposure to AZA caused a strong upregulation of CACNA2D3 mRNA but AZA had no effect in MDA-MB-436 (Figure 2A). Consistent with upregulation, pyrosequencing revealed that AZA caused demethylation in the CpG island of MDA-MB-453 but the low level of methylation in MDA-MB-436 was unaffected (Figure 2B).

The *CACNA2D3* CpG island is methylated in metastatic breast cancer

Next, we wished to determine whether the *CACNA2D3* CpG island is methylated in clinical cases of breast cancer. In particular, we wished to determine whether methylation is present in metastatic

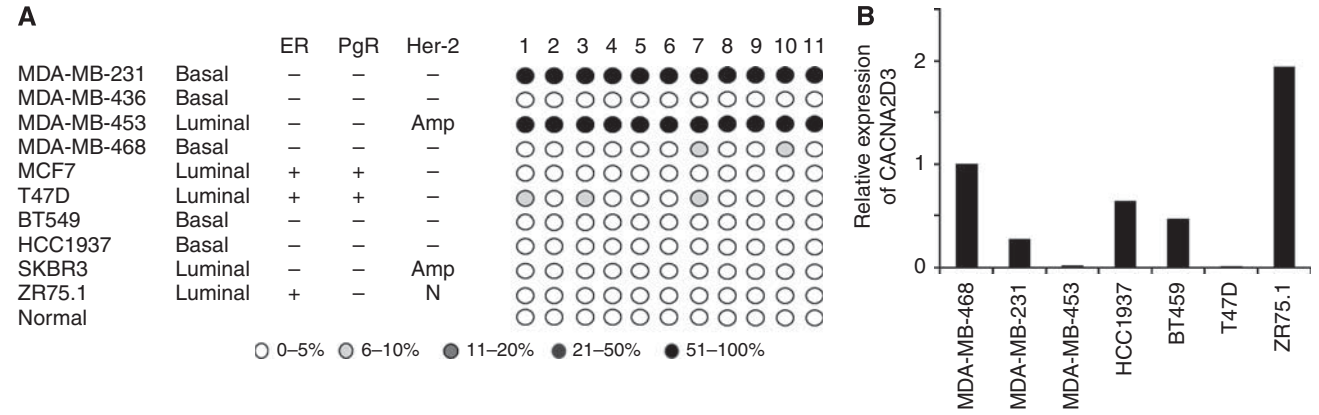

Figure 1 Methylation-dependent transcriptional silencing of *CACNA2D3* in breast cancer cell lines. (**A**) Quantitative pyrosequencing analysis of the *CACNA2D3* CpG island in breast carcinoma cell lines. Pyrosequencing was done as described in Materials and Methods. The level of methylation is represented 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 MSP analysis is also shown. Abbreviations: Amp = Amplified; ER = Oestrogen receptor; PgR = Progesterone receptor. (**B**) qPCR analysis of CACNA2D3 expression in breast cancer cell lines. qPCR was performed as described in Materials and Methods. Expression is shown relative to MDA-MB-468.

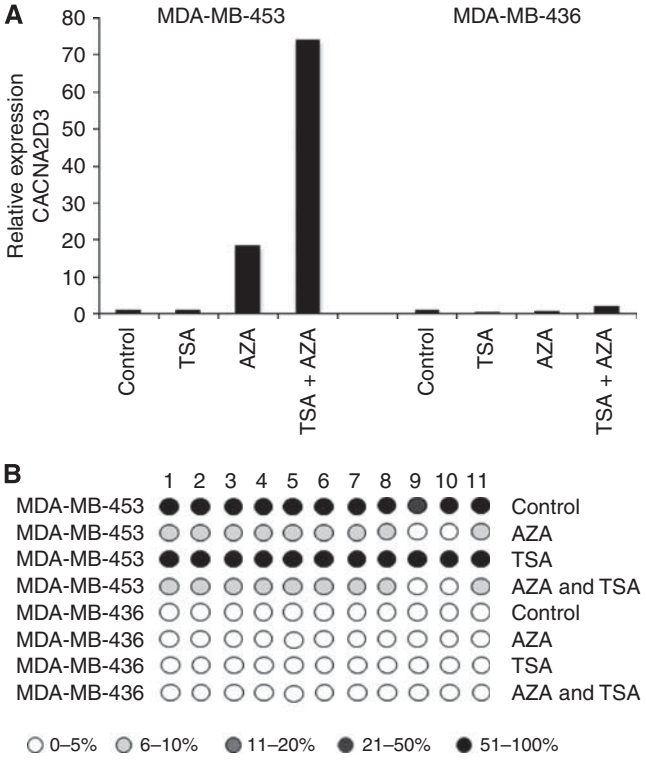

Figure 2 Demethylation reactivates expression of CACNA2D3. (**A**) MDA-MB-453 and MDA-MB-436 cells were grown in the presence of AZA, trichostatin A (TSA) or both. Expression of CACNA2D3 was determined by qPCR. (**B**) AZA-dependent demethylation of the CACNA2D3 CpG island correlates with re-expression of CACNA2D3. Cells were treated with AZA and TSA as above. CpG methylation was determined by pyrosequencing and 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.

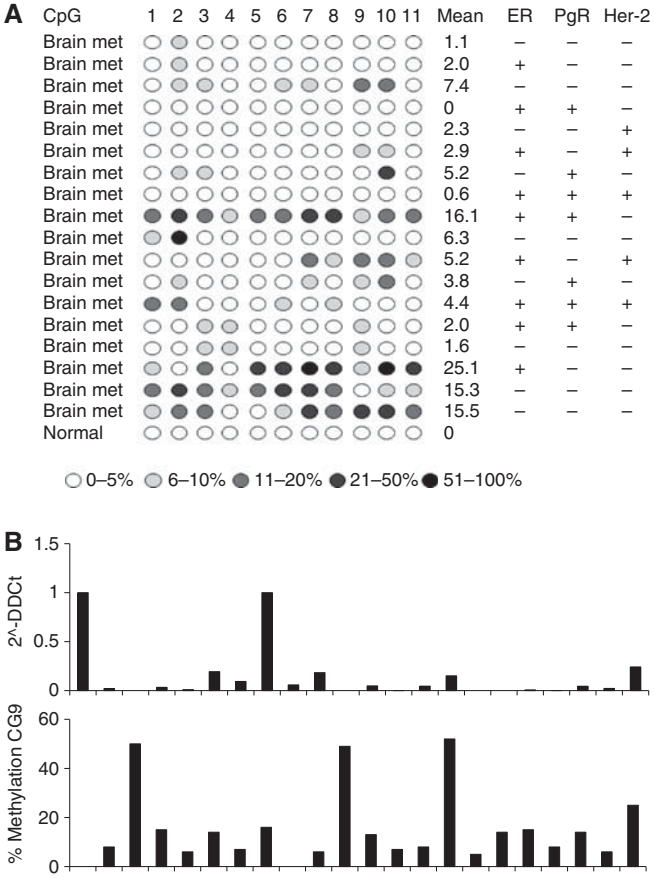

Figure 3 (**A**) The CACNA2D3 CpG island is methylated in metastatic breast cancer. The figure shows pyrosequencing analysis of 17 CNS metastases derived from CNS metastases derived from primary breast carcinomas. CpG methylation was determined by pyrosequencing and the level of methylation is represented by the intensity of shading in the circles, each of which represents an individual CpG dinucleotide in the amplified fragment. The ER, PgR and Her2 status is also shown for each case. (**B**) Methylation-associated downregulation of CACNA2D3 in breast cancers is associated with CpG island methylation. The upper panel shows qPCR analysis of CACNA2D3 in a series of primary breast carcinomas (each of which later relapsed with loco-regional or distant metastatic disease). The lower panel shows percentage methylation at CpG9 in the fragment of the CpG island analysed by pyrosequencing.

breast cancer. Accordingly, using pyrosequencing, we analysed in detail methylation in a series of 18 histological confirmed CNS breast cancer metastases (Figure 3). The fragment of the *CACNA2D3* CpG island analysed by pyrosequencing was entirely unmethylated in normal breast epithelium, with no methylation detected at any of the 11 analysed CpG dinucleotides (Figure 3). In the CNS metastases, the level of methylation at each CpG

Table 1 Clinico-pathological characteristics of all 142 patients

Age (at primary diagnosis) median: 64 (range: 36–87)	
Tumour size: n *(%)*	
<20 mm	21 (15)
20–49 mm	18 (13)
50 mm	0 (0)
NA	103 (72)
Tumour grade: n *(%)*	
Grade I	8 (6)
Grade II	102 (72)
Grade III	22 (15)
NA	10 (7)
Nodal status: n *(%)*	
Positive	53 (37)
Negative	68 (48)
NA	21 (15)
Hormone receptor status: n *(%)*	
ER +ve and PR +ve	85 (60)
ER +ve and PR –ve/unknown	41 (29)
ER –ve and PR –ve/unknown	13 (9)
ER unknown and PR unknown missing	3 (2)
HER2	
Positive	20 (0.14)
Negative	113 (0.80)
NA	9 (0.06)

Abbreviations: ER = oestrogen receptor; NA = not available; PR = progesterone receptor.

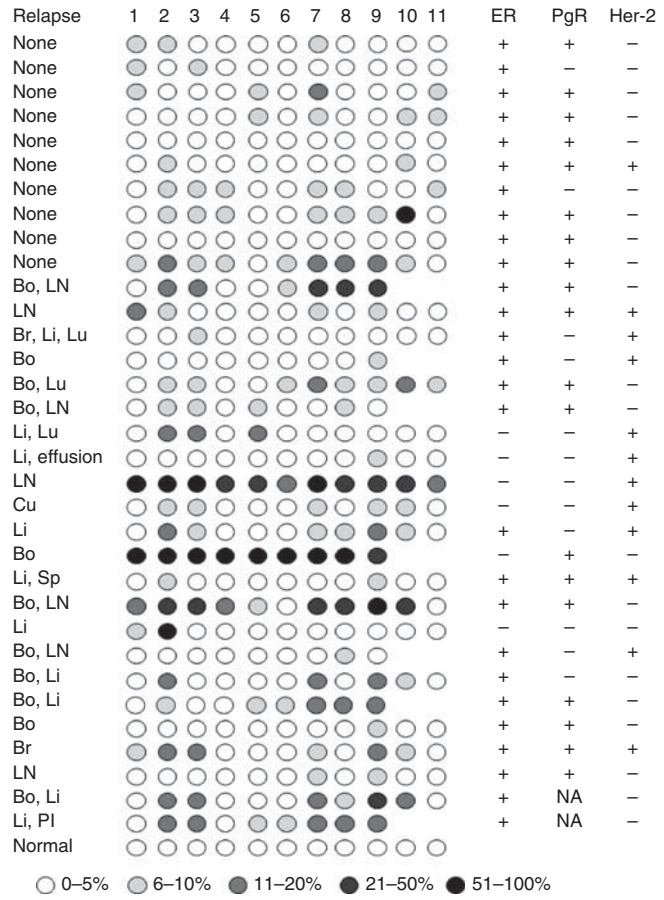

Figure 4 The CACNA2D3 CpG island is methylated in primary breast cancer. The figure shows representative pyrosequencing analysis of individual primary breast carcinomas which were either non-relapsing or relapsed at various distant organ sites. CpG methylation was determined by pyrosequencing and the 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 ER, PgR and Her2 status is also shown for each case. Abbreviations: Bo = bone; Br = brain; Cu = cutaneous; Li = liver; LN = lymph node; Lu = lung; Pl = pleura; Sp = spleen; NA = not available.

dinucleotide varied markedly within individual lesions (Figure 3). The most frequently and densely methylated CpGs in the amplified fragment were dinucleotides 9 and 10 (Figure 3). With a cutoff of 7% methylation, 8 out of 18 (44%) CNS metastases were positive for methylation at CpG9 and 8 out of 18 (44%) positive for methylation at CpG10. These results show that the *CACNA2D3* CpG island is methylated in metastatic breast cancer lesions. To confirm that expression of CACNA2D3 was affected by CpG island methylation, we used qPCR to measure steady-state mRNA levels in 21 primary breast carcinomas that later relapsed with either loco-regional or distant metastatic disease. CACNA2D3 mRNA was downregulated in the majority of these cases (Figure 3B). Using pyrosequencing we analysed CACNA2D3 CpG island methylation. There was increased methylation in almost all cases with down-regulation of the mRNA (Figure 3B).

CACNA2D3 CpG island methylation predicts site-specific relapse in primary breast carcinomas treated with endocrine therapy

The presence of CACNA2D3 CpG island methylation in CNS metastatic breast carcinomas prompted us to determine whether methylation in primary carcinomas is predictive of future recurrence and/or metastasis. The clinico-pathological parameters of the study population are shown in Table 1. Again using pyrosequencing, we analysed an archival series of 142 cases from our clinical practice, which contained both ER-positive, ER-negative and triple negative cancers. Patients were treated adjuvantly according to normal protocols of clinical care. As seen with the CNS lesions, the level of methylation was variable between specific CpG dinucleotides in individual patients (see Figure 4 for representative pyrosequencing data). Using a cutoff of mean 7% methylation for the amplified fragment analysed by pyrosequencing, 38 out of 142 (27%) cases were positive for CACNA2D3 methylation. We wished to explore in more detail, whether the presence of methylation in the CACNA2D3 CpG island in primary cancers was associated with an increased probability of future metastasis. We therefore excluded those cases in which full clinico-pathological parameters were not available and those patients who had been lost to follow-up and determined the frequency of metastatic relapse as a function of CACNA2D3 CpG island methylation. We then performed further analysis in 100 of the cases for which we had complete clinic-pathological information, treatment and clinical outcome. These 100 cases comprised of ER-positive, tamoxifen-treated patients, clinic-pathological details of this group are presented in Table 2. Detailed analysis of each individual CpG dinucleotide within the amplified fragment of the CACNA2D3 CpG island revealed that methylation at CpG9 was a sensitive predictive biomarker of future metastatic relapse and a specific discriminator between cases which did and did not relapse (Figures 4 and 5A). At the time of analysis, 51 patients had relapsed with either loco-regional or distant metastatic disease. Applying a methylation cutoff of 7% at CpG9, the frequency of methylation was significantly higher in cases that relapsed than in cases with no relapse: 21 out of 61 (34%) methylated in non-relapsing cases *vs* 30 out of 39 (77%) methylated in relapsing cases; $P<0.0001$. Next, we asked in the same patient population whether methylation in the CACNA2D3 CpG island in primary breast carcinomas affected the risk of later metastasis in specific

anatomical sites. Metastasis to liver and lung was significantly more common in primary carcinomas with methylated CACNA2D3 than in cases lacking methylation, $P = 0.012$ and $P = 0.02$, respectively (Figure 5B). Metastasis to bone and brain was also more common in primary cancers, with methylation in CACNA2D3 but because of the small number of cases these did not reach statistical significance (Figure 5B). There was no evidence that metastasis to skin or lymph nodes was increased in cases with CACNA2D3 CpG island methylation (Figure 5B).

DISCUSSION

Calcium ion channels mediate many biological processes potentially relevant to the malignant process including metastasis (Patton *et al*, 2003; Huang *et al*, 2004). CACNA2D3 in particular has a number of properties consistent with a tumour and/or metastasis suppressor function as demonstrated by its ectopic expression inhibiting cell growth and adhesion in gastric cancer cell lines, whereas knockdown with inhibitory RNA resulted in increased proliferation (Wanajo *et al*, 2008). Consistent with these *in-vitro* findings, methylation was more common in gastric cancer tissue as compared with normal gastric tissue and in those cancers where methylation was present it was associated with a shorter overall survival (Wanajo *et al*, 2008). Further evidence supporting a role of CACNA2D3 lung cancer, renal cell cancer, neuroblastoma and osteosarcoma has been previously reported (Hanke *et al*, 2001; De Preter *et al*, 2006; Tai *et al*, 2006; Thorell *et al*, 2009; Xiong *et al*, 2009). With regard to breast cancer, CACNA2D3 lies in 3p21 a region implicated in sporadic breast cancer development (Buchhagen *et al*, 1994), but no study has addressed these issues in breast cancer. We show in the present work that CACNA2D3 is subject to epigenetic regulation in breast cancer cell lines and primary and metastatic lesions via aberrant methylation in the CpG island located in the regulatory elements of the gene, consistent with such a tumour suppressor function in breast cancer and consistent with the previous data in other tumour types (Hanke *et al*, 2001; De Preter *et al*, 2006; Tai *et al*, 2006).

Table 2 Clinico-pathological characteristics of 100 ER-positive tamoxifen-treated patients

Age (at primary diagnosis) median: 64.5 (range: 36–87)	
Tumour size: n *(%)*	
<20 mm	16 (16)
20–49 mm	17 (17)
>50 mm	0 (0)
NA	67 (67)
Tumour grade: n *(%)*	
Grade I	6 (6)
Grade II	75 (75)
Grade III	13 (13)
NA	6 (4)
Nodal status: n *(%)*	
Positive	38 (38)
Negative	46 (46)
NA	16 (16)
Hormone receptor status: n *(%)*	
ER +ve and PR +ve	68 (68)
ER +ve and PR −ve	32 (32)
HER2	
Positive	11 (11)
Negative	84 (84)
NA	5 (5)

Abbreviations: ER = oestrogen receptor; NA = not available; PR = progesterone receptor.

We first demonstrated using quantitative analysis (pyrosequencing) that CACNA2D3 expression varies with the methylation status of the CpG island. There was dense methylation in MDA-MB-231 and MDA-MB-453 cell lines with concomitant downregulated expression. In contrast, the CpG island was unmethylated in normal breast epithelium and the remaining cell lines examined. Interestingly, both cell lines with methylation-dependent silencing of CACNA2D3 were ER-negative, although several other ER-negative cell lines did not show methylation and expressed CACNA2D3. In the clinical series analysed, the CACNA2D3 CpG island was methylated in both ER-positive and ER-negative cases. Expression of CACNA2D3 mRNA was efficiently restored by demethylation with AZA. Together, these observations are consistent with methylation-dependent transcriptional silencing being the mechanistic basis of CACNA2D3 downregulation in breast cancer.

We then analysed CACNA2D3 methylation in three well-characterised clinical series of breast cancers. The first comprised a panel of CNS metastases derived from patients with breast cancer and all confirmed by histopathology to be metastatic deposits of breast cancer. Detailed, quantitative pyrosequencing analysis of genomic DNA from this series revealed that methylation in the CpG island of CACNA2D3 was heterogeneous within individual cancers, as evidenced by variable levels of methylation at specific CpG dinucleotides within the analysed fragment. However, methylation at CpG9 within the amplified fragment was most

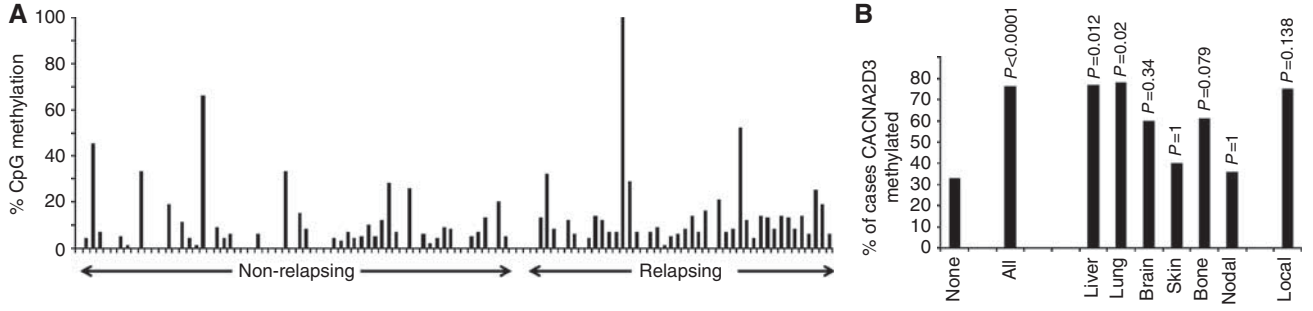

Figure 5 Methylation in the CACNA2D3 CpG island in primary breast cancers treated adjuvantly with endocrine therapy is associated with increased risk of visceral metastasis. (**A**) Percentage methylation at CpG9 in non-relapsing and relapsing primary breast carcinomas. The figure shows percentage methylation, determined by pyrosequencing, in individual cases which remained disease free or which later relapsed at various distant organ sites. (**B**) Methylation in CACNA2D3 predicts visceral relapse in tamoxifen-treated primary breast carcinomas. The figure shows the percentage of cases with methylation in primary cancers that did not relapse ('none'), in all relapsing cases ('all') and in the indicated metastatic sites. The *P*-value, calculated as described in Materials and Methods is shown above each column.

strongly associated with brain metastatic lesions. The presence of a relatively high frequency of methylation in CACNA2D3 in CNS metastases prompted us to determine whether methylation in primary breast carcinomas is associated with increased risk of recurrence and/or metastasis. In a series of 100 predominantly ER-positive primary breast carcinomas treated adjuvantly with tamoxifen, we demonstrated that in primary cancers with *CACNA2D3* CpG methylation, there is a significantly increased risk of recurrence, particularly at visceral sites of liver and lung, whereas there was no increased risk of nodal metastases. Our results are consistent with studies in gastric cancer, which have suggested that downregulation of *CACNA2D3* is associated with clinically more aggressive disease.

Previous *in-vitro* data in non-small cell lung cancer has shown that the A2D2 subunit can induce apoptosis by disrupting mitochondrial membrane integrity via its effect on intracellular calcium (Carboni *et al*, 2003). However, the loss of the A2D2 subunit *in vitro* is associated with abnormal growth, abnormalities in cell adhesion and downregulation of key cell-cycle regulators (Wanajo *et al*, 2008). The abnormalities in cell adhesion may be linked to the fact that the A2D3 subunit contains a von Willebrand A domain (Whittaker and Hynes, 2002), which is known to contain metal-ion–dependent adhesion sites responsible for binding to extracellular matrix protein. Therefore, it appears that the two $\alpha_2\delta$ subunits implicated in malignant disease have their own unique function and are important for normal functioning of calcium channels. Further work is required to understand the normal physiological role of CACNA2D3 in a non-excitable cells such as breast epithelium, as well as an understanding of the patho-molecular effects of perturbation or loss of these calcium channel subunits.

Interestingly, chromatin immunoprecipitation-based assay in MCF-7 cells has shown that, following treatment with estradiol, CACNA2D3 is negatively regulated by the co-activator steroid receptor co-activator-3 via ER (Labhart *et al*, 2005). Steroid receptor co-activator-3 is known to be an oncogene, which is involved in mammary tumourigenesis, endocrine resistance and is associated with a poorer outcome in breast cancer (Gojis *et al*, 2010). Furthermore, it can modulate cell motility and invasion in breast cancer cell lines (Bai *et al*, 2000; Li *et al*, 2008a, b). This phenotype could be via its ability to downregulate the expression of CACNA2D3.

In summary, the association of methylation in the CACNA2D3 CpG island with breast cancer metastasis and in particular visceral disease implies that analysis of this gene may be utilised as a biomarker for metastasis and warrants evaluation in larger independent clinical series. Further work is required to understand the normal physiological role of CACNA2D3 in a non-exciable cells such as breast epithelium, and the molecular effects and mechanisms underlying CACNA2D2 role in malignant disease mediated regulation of cell proliferation and cell death in the pathogenesis of lung cancers and other human cancers.

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Conflict of interest

The authors declare no conflict of interest.

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