Univerzita Karlova v Praze
3. lékařská fakulta

Dizertační práce

Ondřej Gojiš

Praha 2013

The role of selected molecules in biologic behaviour of human breast cancer

Dizertační práce

MUDr. Ondřej Gojiš

Ústav patologie 3. lékařské fakulty UK v Praze,
Department of Cancer Medicine, Imperial College London, UK

Školitel: Prof. MUDr. Václav Mandys, CSc.

Praha 2013

Dizertační práce byla vypracována v rámci postgraduálního studia Biomedicíny v Ústavu patologie 3. LF Univerzity Karlovy, a v Department of Cancer Medicine, Imperial College London, Cancer Research UK Laboratories, London, UK

Uchazeč: MUDr. Ondřej Gojiš

Adresa: Ústavu patologie 3. LF Univerzity Karlovy, Ruská 87, Praha 10, 100 00.

Telefon: +420 2 6716 2500

Email: gojismd@gmail.com

Oborová rada: Biologie a patologie buňky

Předseda oborové rady: Prof. MUDr. Karel Smetana, DrSc.

Školitel: Prof. MUDr. Václav Mandys, CSc.

Poděkování

Rád bych poděkoval svému školiteli prof. MUDr. Václavu Mandysovi, CSc., přednostovi Ústavu patologie 3. LF UK, za veškerou podporu a za odborné vedení po celou dobu postgraduálního studia.

Děkuji prof. Carlu Palmierimu za jeho pomoc při mé stáži na Imperial College London a velký dík mu také patří za jeho dohled při sepisování daného tématu.

Děkuji panu přednostovi Gynekologicko-porodnické kliniky 3. LF UK a FNKV doc. MUDr. Eduardu Kučerovi, CSc. a panu primáři MUDr. Janu Zmrhalovi, CSc. za jejich klinické konzultace a trpělivost při řešení technických problémů.

V neposlední řadě děkuji za pomoc řadě pracovníkům Ústavu patologie 3. LF UK bez jejichž odborných znalostí by tato práce nikdy nevznikla.

Contents:

Abbrevations:

ERα - estrogen receptor alpha
PgR - progesterone receptor
Pax2 - Pair box gene 2
CK5/6 - cytokeratin 5/6
Ki67 - protein encoded by the MKI67 gene
AML - acute myeloid leukemia
DCIS - ductal carcinoma in situ
SRC-1,2,3 - steroid receptor co-activator 1,2,3
AIB1 - Amplified In Breast cancer 1
CBP/p300 - CREB binding protein, p300 binding protein
P/CAF - p300/CBP associated protein
CARM1 - coactivator-associated arginine methyltransferase 1
PRMT1 - Protein arginine N-methyltransferase 1
NCoR - nuclear receptor co-repressor 1
SMRT - silencing mediator (co-repressor) for retinoid and thyroid-hormone receptors
HER-1,2,3 - Human Epidermal Growth Factor Receptor 1,2,3
EGFR - Epidermal growth factor receptor
CEBP - CCAAT/enhancer binding proteins
CACNA2D3 - the calcium channel regulatory subunit α2d-3

1. Introduction:

1.1 Background

Estrogens are implicated in the pathogenesis and progression of breast cancer. The cellular effects of estrogen are primarily mediated by estrogen receptor alpha (ERα). Given that ERα is expressed in 70% of breast cancers endocrine treatment is a key treatment modality in the management of the vast majority of breast cancer patients. However resistance to endocrine therapy (de novo resistance) or eventual unresponsiveness to endocrine therapy (acquired resistance) is a major clinical problem. Therefore, a greater understanding of the molecular mechanisms that regulate ERα activity in breast cancer cells, in particular with regard to endocrine resistance is vital if improvements are to be made in the management of endocrine-responsive and unresponsive breast cancer and if novel therapeutic strategies are to be developed.

1.2 Incidence.

Breast cancer is a leading cause of cancer-related morbidity in North America and Europe. It is the the most frequent malignancy in women in the UK and the Western hemisphere, and a leading cause of cancer related mortality (Ferlay et al.2002). Breast cancer is responsible for 26.5% of all new cancer cases among women in Europe and 17.5% of cancer deaths (Tyczynski et al., 2004), since the 1960s there has been a 2-3% increase per year in the incidence of breast cancer in Europe, with a decline in moratlity of 2% per year since 2000 (Hery et al., 2008). In the Czech Republic breast cancer is the most common cancer in females, there were 5533 new breast cancer

cases in 2005, an incidence of 105.5 per 100 000 (Institute of Health information and statistics of the Czech Republic), in 2000 there were 1,976 deaths from breast cancer, representing 15.9% of all deaths from malignant disease in females in the Czech Republic (Tyczynski et al., 2004).

1.3 Breast cancer belongs to the group of hormonal dependent cancers

Estrogens stimulate the growth, survival and differentiation of breast tissues, and are implicated in the pathogenesis and progression of malignancies arising from breast tissues (Russo & Russo.2006). The cellular effects of estrogen are primarily mediated by estrogen receptors (ERα and ERβ) which belong to the steroid/thyroid hormone superfamily of transcription factors. Upon hormone binding, activated estrogen receptors (ERs) regulate the expression of diverse target genes. Given that ERα is expressed in greater than 70% of breast cancers, it is an important therapeutic target, endocrine treatment therefore is a key modality in the management of ERα positive breast cancer (Goldhirsch et al. 2005).

1.4 The role of ER in breast cancer

Estrogen receptors act mainly by regulating the expression of target genes whose promoters contain specific sequences called estrogen-responsive element (ERE). After ERE-binding of ligand-bound ER dimers, modulation of transcription occurs via interaction with coactivators or corepressors. All together, these complexes play an important role in the recruitment of transcriptional machinery, the modulation of chromatine structure, and then in the regulation of ER target-gene expression. ER activity can also be modulated through indirect activation of the ER by growth factors or cytokines independently of the binding of natural or synthetic hormones. Positive

receptor status correlates with favorable prognostic features, including a lower rate of cell proliferation and histologic evidence of tumor differentiation. (Platet et al. 2005). Adjuvant hormonal therapy with tamoxifen improves survival by approximately 23%. (Early Breast Cancer Trialists' Collaborative Group,2000 and 2005). However resistance to endocrine therapy (*de novo* resistance) or eventual unresponsiveness to endocrine therapy (acquired resistance) is a major clinical problem (Ali and Coombes. 2002).

2. Co-regulators and Erα

ERα as a transcription factor acts by regulating gene expression either by direct binding to estrogen response elements or by recruitment to gene promoters through interaction with other transcription factors. Stimulation of gene expression by ERα requires the ordered recruitment of diverse protein complexes that facilitate chromatin remodelling and modification, leading to the recruitment of RNA polymerase II complexes, resulting in transcription initiation. Critical to ERα function are the p160 coactivators, SRC-1, SRC-2 and SRC-3, the latter also known as AIB1 (Amplified In Breast cancer 1). These coactivators facilitate the recruitment of CBP/p300 and P/CAF histone acetyl transferases, as well as the histone arginine methylases CARM1 and PRMT1, allowing modification of core histone tails in nucleosomes, events that are critical for transcription initiation. As such the p160 proteins are critical for the expression of estrogen responsive genes.The interaction between the LxxLL motif of SRC1 and the AF-2 domain of ERα occurs owing to a short amphipathic α helix that is recognized by a complementary groove formed by helices 3, 4, 5 and 12 on the surface of the ER. This conformational change to the ER protein allows for correct structural positioning for interactions with other co-factors and subsequent gene transcription (Green and Carroll 2007). In the absence of ligand, ERα is now known to be present at promoters of (at least some) estrogen responsive genes, together with transcriptional corepressors, most importantly NCoR and the highly related SMRT. (Horlein et al.1995) NCoR/SMRT in turn recruit histone deacetylases that deacetylate core histone tails and repress gene expression. Furthermore, binding of the antiestrogen tamoxifen to ERα also recruits NCoR/SMRT to inhibit the expression of estrogen-responsive genes.

A large proportion of patients who develop resistance to endocrine therapies respond to alternative endocrine agents, demonstrating that ERα continues to be critical in driving breast cancer cell proliferation in these breast tumours. A great deal of effort has been devoted to understanding how altered ERα activity could contribute to endocrine resistance and molecular events that alter ERα activity are likely to be important.

Steroid receptor coactivator-3 (**SRC-3**/**AIB1**/ACTR/pCIP/RAC3) is a member of the p160 coactivator family and plays an important role in cell growth, reproduction, metabolism, and cytokine signaling (Wang et al., 2000; Xu et al., 2000; Zhou et al., 2003). AIB1(amplified in breast cancer 1), was cloned during a search of chromosome 20q, an area known to be frequently amplified in breast cancer, it was subsequently shown to be a member of the SRC family and hence named SRC-3. (Anzick et al., 1997). There is growing evidence for the importance of AIB1/SRC-3 in cell growth and oncogenesis in breast cancer (Yan et al., 2006; Zhou et al., 2003). Transgenic mice that overexpress SRC-3 were found to have an extremely high breast tumor incidence (Torres- Arzayus et al., 2004), while in contrast, SRC-3 knockout mice have a significantly lower incidence of ras-induced mammary gland tumorigenesis (Kuang et al., 2004). Thus, it appears that the level of AIB1/SRC-3 protein is important in tumorigenesis and progression. In human breast cancer the SRC-3/AIB1 gene is amplified in up to 10% of breast cancers and high expression is found in 64% of cases (Anzick et al., 1997; List et al., 2001). AIB1 amplification is preferentially found in ERα and progesterone receptor-positive breast tumors (Bautista et al., 1998). SRC-3/AIB1 is highly expressed in cultured MCF-7 human breast cancer cells, and its activity is essential for the growth of these cells both *in vitro* and *in vivo* (List et al., 2001). AIB1, like the ER itself, is phosphorylated and

thereby functionally activated by Mitogen Activated Protein Kinase (MAPK); therefore, high levels of activated AIB1 could reduce the antagonist effects of tamoxifen, especially in tumors that also overexpress the HER-2 receptor, a member of the epidermal growth factor (EGFR) receptor family that activates MAPKs (Font de Mora et al., 2000). It has been subsequently shown that high levels of tumor AIB1 expression was associated with decreased risk of relapse in untreated patients. However, in tamoxifen-treated patients, AIB1 overexpression acts as a marker of disease relapse. Furthermore, when the expression of AIB1 and HER-2 were considered together, patients whose tumors expressed high levels of both AIB1 and HER-2 had worse outcomes with tamoxifen therapy than all other patients combined. Indicating that the antitumor activity of tamoxifen in breast cancer may be determined, in part, by tumor levels of AIB1 and HER-2 (Osbourne et al., 2003). Kirkegaard et al., (2006) subsequently found that high AIB1 expression in patients with human epidermal growth factor receptor (HER2) and HER3-overexpressing tumors or tumors expressing one or more of HER1, HER2, or HER3 (HER1-3 positive) was associated with an increased risk of relapse on tamoxifen. This data supports the notion that AIB1 can be modulated via growth receptor pathways, and be involved in endocrine resistance. Subsquenlty, a plausible mechanism in vitro for this observed in vivo resistance was shown in an experimental model system using ER-positive breast cancer cells that also overexpress both AIB1 and HER2. Utilising MCF-7/HER2-18, a cell line that is tamoxifen-resistant and engineered to overexpress HER2 as well as containing AIB1, it was found that tumours of this cell line had their growth inhibited by estrogen deprivation but were growth stimulated by tamoxifen. Molecular cross-talk between the ER and HER2 pathways was increased in the MCF-7/HER-2 cells compared with MCF-7 cells, with cross-phosphorylation and activation

of both the ER and the EGFR/HER2 receptors as well as phosphorylation of AIB1 itself with both estrogen and tamoxifen treatment. Tamoxifen recruited coactivator complexes (ER, AIB1, CBP, p300) to the ER-regulated pS2 gene promoter in MCF-7/HER2-18 cells and corepressor complexes (NCoR, histone deacetylase 3) in MCF-7 cells. Treatment with the tyrosine kinase inhibitor (TKI), Gefitinib blocked receptor cross-talk, reestablished corepressor complexes with tamoxifen-bound ER on target gene promoters, eliminated tamoxifen's agonist effects, and restored its antitumor activity both *in vitro* and *in vivo* in MCF-7/HER2-18 cells. This data therefore supports the notion that Tamoxifen behaves as an estrogen agonist in breast cancer cells that express high levels of AIB1 and HER2, resulting in *de novo* resistance, and that blockade with TKIs can eliminate this cross-talk and to restore tamoxifen's effects (Shou et al., 2004).

The CCAAT/enhancer binding proteins (CEBP) family is involved in a number of key cellular processes including differentiation, metabolism, inflammation, apoptosis and proliferation (Yamanaka et al., 1997; Wang et al., 1995; Zinszner et al., 1998; Robinson et al., 1998). CEBP are a highly conserved family of basic region leucine zipper (bZip) transcription factors, and comprises six family members (CEBPα to CEBP*δ*) (Ramji et al., 2002). CEBPδ has been proposed to have tumour suppressor function given its ability to decrease levels of cyclin D1 and cyclin E, while increasing p27 (Ikezoe et al., 2005; Gery et al., 2005; Parwar et al., 2010), as well as regulating pro-apoptotic gene expression during mammary gland involution (Tharangu et al., 2005; Stein et al.,2009). *In vivo* loss of CEBPδ results in increased mammary epithelial cell proliferation and ductal hyperplasia, supporting the importance of CEBPδ in regulating mammary epithelial growth *in vivo* (Gigliotti et al., 2003). This data is supported by the reduction observed in CEBPδ expression in

mammary tumor prone MMTV/c-neu transgenic mice and in carcinogen-induced rodent mammary tumors (Porter et al., 2001; Kuramoto et al., 2002). To date there have been no reports regarding the involvement of *CEBPδ* in metastasis in human cancer, nor of the utility of *CEBPδ* as a prognostic biomarker in breast cancer.

The calcium ion (Ca2+) is a key intracellular messenger and regulates a diverse range of cellular processes by activating or inhibiting cellular signalling pathways and Ca2+-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 Ca2+ channel expression, Ca2+ efflux pumps as well as the expression of proteins that control the Ca2+ content of the endoplasmic reticulum (Monteith et al, 2007) Given the evidence of the biological properties of the calcium channel regulatory subunit α2d-3 (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.

3. References:

Ferlay et al. Cancer Research UK 2003; Cancer Research UK 2004; 2002.

Tyczynski JE, Plesko I, Aareleid T, Primic-Zakelj M, et al. Breast cancer mortality patterns and time trends in 10 new EU member states: mortality declining in young women, but still increasing in the elderly. Int J Cancer. 2004;112:1056-64.

Héry C, Ferlay J, Boniol M, Autier P.Changes in breast cancer incidence and mortality in middle-aged and elderly women in 28 countries with Caucasian majority populations.Ann Oncol. 2008 May;19(5):1009-18

Institute of Health information and statistics of the Czech republic

Russo & Russo. The role of estrogen in the initiation of breast cancer. J Steroid Biochem Mol Biol. 2006. 102(1-5):89-96. Review.

Goldhirsch A, Gelber RD, Coates AS. What are the long-term effects of chemotherapy and hormonal therapy for early breast cancer? Nat Clin Pract Oncol. 2005; 2:440-1.

Platet N, Cathiard AM, Gleizes M, Garcia M. Estrogens and their receptors in breast cancer progression: a dual role in cancer proliferation and invasion. Crit Rev Oncol Hematol. 2004;51:55-67.

Early Breast Cancer Trialists' Collaborative Group,2000 and 2005

Ali, Coombes. Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer. 2002;2:101-12. Review.

Kelly A. Green, Jason S. Carroll :Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. Nat Rev Cancer. 2007;7:713-22

Hörlein AJ, Näär AM, Heinzel T, Torchia J et al. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor.Nature. 1995;377:397-404.

References

Wang Z, Rose DW, Hermanson O, Liu F et al. Regulation of somatic growth by the p160 coactivator p/CIP.Proc Natl Acad Sci U S A. 2000;97:13549-54.

Xu J, Liao L, Ning G, Yoshida-Komiya H The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. Proc Natl Acad Sci U S A. 2000;97:6379-84.

Zhou G, Hashimoto Y, Kwak I, Tsai SY Role of the steroid receptor coactivator SRC-3 in cell growth.Mol Cell Biol. 2003;23:7742-55.

Anzick SL, Kononen J, Walker RL, Azorsa DO et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer.Science. 1997; 277:965-8.

Yan J, Tsai SY, Tsai MJ SRC-3/AIB1: transcriptional coactivator in oncogenesis Acta Pharmacol Sin. 2006;27:387-94.

Torres-Arzayus MI, Font de Mora J, Yuan J, Vazquez F, Bronson R, Rue M, Sellers WR, Brown M. High tumor incidence and activation of the PI3K/AKT pathway in transgenic mice define AIB1 as an oncogene.Cancer Cell. 2004;6:263-74.

Kuang SQ, Liao L, Zhang H, Lee AV Cancer Res. AIB1/SRC-3 deficiency affects insulin-like growth factor I signaling pathway and suppresses v-Ha-ras-induced breast cancer initiation and progression in mice.2004;64:1875-85.

List HJ, Lauritsen KJ, Reiter R, Powers C, Wellstein A, Riegel AT. Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. J Biol Chem 2001;276:23763–8.

Bautista S, Valles H,Walker RL, et al. In Brest cancer, amplification of the steroid receptor coactivator gene AIB1is correlated with estrogen and progesterone receptor positivity. Clin Cancer Res 1998;4:

Font de Mora J, Brown M. AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. Mol Cell Biol 2000;20:5041–7.

References

Osborne CK, Bardou V, Hopp TA, Chamness GC et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer.J Natl Cancer Inst. 2003;95:353-61.

Tove Kirkegaard, Liane M. McGlynn, Fiona M. Campbell, Sven Miller.Amplified in breast cancer in human epidermal growth factor receptor positive tumors of tamoxifen-treated breast cancer patiens.Clin Cancer Res 2007;13.

Shou J, Massarweh S, Osborne CK, Wakeling AE et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst. 2004; 96:926-35.

Gomm JJ, Browne PJ, Coope RC, Liu QY et al.Isolation of pure populations of epithelial and myoepithelial cells from the normal human mammary gland using immunomagnetic separation with Dynabeads.Anal Biochem. 1995;226:91-9.

Singh A, Ali S, Kothari MS, De Bella MTet al. Reporter gene assay demonstrates functional differences in estrogen receptor activity in purified breast cancer cells: a pilot study.Int J Cancer. 2003;107:700-6.

Gomm JJ, Browne PJ, Coope RC, Bansal GS, Yiangou C, Johnston CL, Mason R, Coombes RC.A paracrine role for myoepithelial cell-derived FGF2 in the normal human breast.Exp Cell Res. 1997;234:165-73.

Kothari MS, Ali S, Buluwela L, Livni N et al. Purified malignant mammary epithelial cells maintain hormone responsiveness in culture. Br J Cancer. 2003;88:1071-6.

Affymetrix . Statistical Algorithms Reference Guide . Affymetrix Technical Note 2002 .

Li C, Wong WH . Model-based analysis of oligonucleotide arrays: expression index computation and outlier detection . Proc Natl Acad Sci U S A,2001;98:31–6.

Hosey AM,Gorski JJ, Murray MM, Quinn JE et al. Molecular basis for estrogen receptor alpha deficiency in BRCA1-linked breast cancer.Natl Cancer Inst. 2007;99:1683-94.

References

Yamanaka R, Kim GD, Radomska HS, Lekstrom-Himes J, Smith LT, Antonson P, Tenen DG and Xanthopoulos KG CCAAT/enhancer binding protein e is preferentially up-regulated during granulocyte differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. Proc. Natl. Acad. Sci. U.S.A. 1997: 94: 6462-6467

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR and Darlington GJ Impaired energy homeostasis in C/EBPa knockout mice. Science,1995: 269, 1108-1112

Zinszner H, Kuroda M, Wang X.-Z, Batchvarova N, Lightfoot R T, Remotti H, Stevens JL and Ron D CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev.1998:12:982-995

Robinson GW, Johnson PF, Hennighausen L and Sterneck E The C/EBPb transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes Dev.1998:12, 1907-1916

Ramji DP, Foka P CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J.2002: 365:561-575.

Ikezoe T, Gery S, Yin D, O'Kelly J, Binderup L, Lemp N, Taguchi H, Koeffler HP CCAAT/enhancer-binding protein delta: a molecular target of 1,25-dihydroxyvitaminD3 in androgen-responsive prostate cancer LNCaP cells. Cancer Res. 2005:65:4762–8.

References

Gery S, Tanosaki S, Hofmann WK, Koppel A, Koeffler HP C/EBPdelta expression in a BCR-ABL-positive cell line induces growth arrest and myeloid differentiation. Oncogene.2005: 24:1589–97.

Pawar SA, Roy Sarkar T, Balamurugan K, Sharan S, Wang J, Zhang Y, Dowdy SF, Huang AM, Sterneck E C/EBP{delta} targets cyclin D1 for proteasome-mediated degradation via induction of CDC27/APC3 expression. Proc Natl Acad Sci USA.2010:107: 9210–9215

Thangaraju M, Rudelius M, Bierie B, Raffeld M, Sharan S, Hennighausen L, Huang A-M and Sterneck E. C/EBPd is a crucial regulator of pro-apoptotic gene expression during mammary gland involution. Development. 2005:132: 4675-4685

Stein T, Salomonis N, Nuyten DS, van de Vijver MJ and Gusterson BA. A mouse mammary gland involution mRNA signature identifies biological pathways potentially associated with breast cancer metastasis. J Mammary Gland Biol Neoplasia.2009:14: 99-116

Gigliotti AP, Johnson PF, Sterneck E, DeWille JW (2003) Nulliparous CCAAT/ enhancer binding proteindelta (C/EBPdelta) knockout mice exhibit mammary gland ductal hyperlasia. Exp Biol Med 2003: 228:278-285.

Kuramoto T, Morimura K, Yamashita S, Okochi E, Watanabe N, Ohta T, Ohki M, Fukushima S, Sugimura T, Ushijima T. Etiology-specific gene expression profiles in rat mammary carcinomas. Cancer Res.2002: 62:3592-3597.

References

Porter DA, Krop IE, Nasser S, Sgroi D, Kaelin CM, Marks JR, Riggins G, Polyak K. A SAGE (serial analysis of gene expression) view of breast tumor progression. Cancer Res 2001:61:5697-5702.

Berridge MJ, Bootman MD, Roderick HL. Calcium signalling: dynamics, homeostasis and remodelling. Nature Rev Mol Cell Biol. 2003:4:517–529

Monteith GR, McAndrew D, Faddy HM, Roberts-Thomson SJ. Calcium and cancer: targeting Ca2ţ transport. Nat Rev Cancer. 2007:7:519–530

Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 100: 57–70 Hanke S, Bugert P, Chudek J, Kovacs G. Cloning a calcium channel alpha2delta-3 subunit gene from a putative tumor suppressor gene region at chromosome 3p21.1 in conventional renal cell carcinoma. Gene. 2000:264: 69–75

Becchetti A. Ion channels and transporters in cancer. 1. Ion channels and cell proliferation in cancer. Am J Physiol Cell Physiol. 2001:301: C255–C265

Huang JB, Kindzelskii AL, Clark AJ, Petty HR. Identification of channels promoting calcium spikes and waves in HT1080 tumor cells: their apparent roles in cell motility and invasion. Cancer Res.2004:64: 2482–2489

Patton AM, Kassis J, Doong H, Kohn EC. Calcium as a molecular target in angiogenesis. Curr Pharm Des. 2003:9: 543–551

Rizzuto R, Pinton P, Ferrari D, Chami M, Szabadkai G, Magalhaes PJ, Di Virgilio F, Pozzan T. Calcium and apoptosis: facts and hypotheses. Oncogene.2003:22: 8619–8627

References

Rizzuto R, Pozzan T. Microdomains of intracellular Ca2+: molecular determinants and functional consequences. Physiol Rev.2006:86: 369–408

4. Aims of the study

The p160 steroid receptor coactivator (SRC) family are critical to the transcriptional activation function of nuclear hormone receptors. A key member of this family is SRC-3 (AIB1). CCAAT/enhancer binding protein delta (CEBPδ) has properties consistent with a tumour suppressor, however other data suggests that CEBPδ may be involved in the metastatic process. Calcium is an important intracellular messenger that mediates many biological processes that are relevant to the malignant process. An understanding of the potential role of SRC-3, CEBPδ and Calcium in the pathogenesis and its possible prognostic role in breast cancer disease will improve our general understanding of carcinogenesis.

Aims:

- to assess immunohistochemical profiles in breast cancer brain metastases. (SRC3, Pax2, ER, PgR, Her2, EGFR, CK5/6, Ki67)
- to characterise association between immunohistochemical status in primary and secondary deposits, grade and histotype of the tumors
- to contribute to explanation of the role of *CEBPδ* in metastasis in human cancer
- to describe 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.

5. Results

5.1 Expression of selected proteins in breast cancer brain metastases – main study

Ondrej Gojiš, Martina Kubecová, Jozef Rosina, JanaVránová, Martin Čelko, Denisa Frajerová, Jan Zmrhal, Jozef Záhumenský, Tereza Báčová, Václav Mandys, Eduard Kučera**.**
Folia Histochem Cytobiol, 2013, accepted for publication
IF 1,1

Abstract

Objective: The aim of the study was to assess immunohistochemical profiles in breast cancer brain metastases. Immunohistochemical detection was performed for SRC3, Pax2, ER, PgR, Her2, EGFR, CK5/6, and Ki67. *Methods:* The study utilized tumor samples from 30 metastatic patients and correlations between all immunohistochemical variables were calculated. In fourteen cases, primary breast cancers paired with secondary deposits were analyzed. We evaluated the association between immunohistochemical status in primary and secondary deposits, grade and histotype of the tumors. *Results:* The examination of metastatic deposits in all 30 patients resulted in positive detection in the following cases: SRC3 in 20 cases (66.6%), Pax2 in 22 (73.3%), ER in 22 (73.3%), PgR in 25 (83.3%), Her2 in 10 (33.3%), EGFR in 12 (40%), CK5/6 in 7 (23.3%), and Ki67 in 23 (76.6%). Grade 2 was found in 13.3% and grade 3 in 86.7%. SRC3 and Pax2 were positive in both G2 and G3. Invasive lobular carcinoma was diagnosed in 23.3% of cases and invasive ductal carcinoma in 76.7%. There were no differences among the seven antigens in either grading or histotype of the tumors. In the immunohistochemical profiles which included SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67 and EGFR, we found no statistically significant differences between the primary cancer and brain metastases. *Conclusion:* In our study of metastatic breast carcinoma deposits, there was no correlation between SRC3, Pax2 status and histotype, and tumor grade. The immunohistochemical status of the paired primary and metastatic deposits did not differ in a statistically significant manner.

Key words*:* brain metastases, breast cancer, protein expression, SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67, EGFR.

Introduction

Breast cancer is the second most common cause of brain metastases. CNS metastatic deposit during the course of breast cancer is associated with indicators such as young age at diagnosis, disease-free interval less than one year, estrogen receptor negativity, Her2 receptor positivity and it has unfavourable prognosis. The higher incidence may be caused by clonal selection induced by new treatment like Herceptin**.** The development of breast cancer brain metastases depends on numerous factors, such as invasivity, migration, adhesion, extracellular matrix degradation, revascularization and proliferation. One of the most important characteristics of malignant breast cancer is penetration of the blood-brain barrier and the subsequent invasion of brain tissue.

Estrogen is a major factor in the pathogenesis, progression, growth, differentiation and survival of breast cancer. The cellular effects of estrogen are primarily mediated by estrogen receptor alpha (ERα). ERα is expressed in 70% of breast cancers and endocrine treatment is a key modality in treating of the vast majority of breast cancer patients.

ERα as a transcription factor acts by regulating gene expression, either through direct binding to estrogen response elements, or by recruitment of gene promoters through interactions with other transcription factors. The p160 co-activators are critical to ERα functioning: SRC-1, SRC-2 and SRC-3, the latter of which is also known as AIB1 (Amplified In Breast cancer 1). A large proportion of patients who develop resistance to endocrine therapies respond to alternative endocrine agents, demonstrating that ERα continues to play a critical role in breast cancer cell proliferation in breast tumors. Steroid Receptor Coactivator-3 (SRC-3/AIB1/ACTR/pCIP/RAC3) is a member of the p160 co-activator family and plays an important role in cell growth,

reproduction, metabolism, and cytokine signaling [1, 2, 3] There is growing evidence for the importance of SRC-3 in cell growth and oncogenesis in breast cancer [3, 4]. In human breast cancer, the SRC-3 gene is amplified in up to 10% of breast cancers, and high expression is found in 64% of cases [5, 6]. SRC-3 amplification is preferentially found in ERα and Progesterone Receptor (PgR) positive breast tumors [7]. It has been subsequently shown that high levels of SRC-3 expression in tumors were associated with a decreased risk of relapse in untreated patients. However, in tamoxifen-treated patients, SRC-3 overexpression acts as a marker of disease relapse. Furthermore, when the expression of SRC-3 and epidermal growth factor receptor 2 (Her2) were considered together, patients whose tumors expressed high levels of both SRC-3 and Her2 experienced worse outcomes with tamoxifen therapy than all other patients combined. This indicates that tamoxifen antitumor activity in breast cancer may be partly determined by SRC-3 and Her2 tumor levels [8]. The Pair box (Pax) gene family consists of nine genes which encode transcription factors [9]. Pax2 has been shown to compete with SRC-3 for the binding and regulation of Her2 transcription, which determines tamoxifen response in breast cancer cells [10]. Epidermal growth factor receptor (EGFR) activates the tumor cells through its specific surface receptor, and a higher expression of EGFR has been found in metastatic brain disease [11, 12]. Cytokeratin 5/6 (CK5/6) has been done to prove metastatic tumor cells and Ki67 was stained as nuclear antigen in cycling cells and as a cellular marker of proliferation.

Patients and methods

We studied 30 metastatic breast cancer deposits in cooperation with Imperial College London in the UK and the Charles University Teaching Hospital in Prague, Czech

Republic. The project was approved by the local ethics committees. All of the patients had been previously treated for brain metastases. 23 patients were treated for primary breast cancer tumors through the administration of standard anti-cancer drugs such as docetaxel, cisplatin and etoposide within the course of standard oncological regimes. None of these anti-cancer medications are able to penetrate the CNS blood-brain barrier. Histological data and the patient's age were used for statistical analysis. All histological slides were independently checked by two pathologists. Sections of paraffin-embedded tissues were studied by immunohistochemistry (IHC) using monoclonal antibodies to ER, PgR, Her2, SRC3, Pax2, CK5/6, Ki67 and EGFR. All primary and secondary breast cancer deposits underwent immunohistochemical processes at one time. Five µm sections of each paraffin-embedded specimen were stained with hematoxylin and eosin to verify adequate numbers of invasive tumor cells and tumor grade. Occasionally, some endothelial cells were stained, but no staining was observed in the control samples. Cases were considered positive for ER, PgR, Her2, SRC3, Pax2, CK5/6, Ki67, EGFR when at least 10% of tumor cells showed distinct positive staining. Immunohistochemistry for ER (Ventana, clone SP1), PgR (Ventana, clone IE2) and Her2 (Ventana, clone 4B5) was used to semi-quantitatively measure ER, PgR, Her2 protein expression (Ventana Benchmark XT, Ultraview detection kit)

Antigen retrieval was performed by heating the slides for 30 minutes in a CCI. Antibodies were applied at room temperature: ER for 28 minutes, PgR for 24 minutes and Her2 for 16 minutes. Immunohistochemistry for EGFR (Bondmax) was used to semi-quantitatively measure EGFR protein expression (Bondmax, Leica, and a Bond polymer detection kit DS9800). Antigen retrieval was performed by heating the slides

in Protease enzyme digestion (Leica) for 10 minutes. EGFR antibody was applied for 30 minutes in a 1:50 concentration. Immunohistochemistries for Ki67 (Bondmax) and CK5/6 were used to semi-quantitatively measure Ki67 and CK5/6 protein expression (Bondmax, Leica, and a Bond polymer detection kit DS9800). Antigen retrieval was performed by heating the slides for 30 minutes (Ki67) and 20 minutes (CK5/6) in ER1 (Leica). The antibodies were applied for 30 minutes in concentrations of 1:100 (Ki67) and 1:200 (CK5/6). [13]

Immunohistochemistry for AIB1 (BD transduction laboratories, San Diego) was used to semi-quantitatively measure AIB1 protein expression (Bondmax, Leica, and a Bond polymer detection kit DS9800). In summary, antigen retrieval was performed by heating the slides for 30 minutes in an ER2 (Leica). AIB1 antibody was applied for 30 minutes in a 1:500 concentration. [14] PAX2 immunohistochemistry was performed on an automated BondMax Immunostainer (Leica) with anti-PAX2 antibody (ab38738; Abcam) at a dilution of 1:100.

Statistical analysis

To evaluate the association among all immunohistochemical variables, Spearman's and Kendall's correlation coefficients were calculated. To compare the immunohistochemical profiles of SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67 and EGFR among primary and brain metastases, both the Wilcoxon paired signed-rank test and McNemar's chi-square test were utilized.

Results:

Thirty patients with brain metastases were included in the present study. The median age at the time of primary tumor diagnosis was 54 years of age (range 42 – 83) and the average age was 57. Grade 2 was diagnosed in 4 out of 30 cases (13.3%) and grade 3 was diagnosed in 26 out of 30 cases (86.7%). Invasive lobular carcinoma was diagnosed in 7 out of 30 cases (23.3%) and invasive ductal carcinoma in 23 out of 30 cases (76.7%). One case of invasive ductal carcinoma was mixed with ductal carcinoma in situ. All patients had at least one positive lymphatic node for invasive cancer.

To assess the association between all immunohistochemically-observed variables, both Spearman's and (the more suitable) Kendall's correlation coefficients were calculated. Spearman's correlation coefficient only found one statistically significant correlation, and that was between EGFR and CK5/6 ($p < 0.004$); Kendall's correlation coefficient found statistically significant associations in the following cases: EGFR vs. CK5/6 ($p < 0.001$), ER vs. PR ($p < 0.01$), and AIB1 vs. CK5/6, PAX2 vs. Her2 and PAX2 vs. EGFR ($p < 0.04$ in all cases).

Of the metastatic deposits taken from all 30 patients, SRC3 was positive in 20 cases (66.6%), Pax2 in 22 (73.3%), ER in 22 (73.3%), PgR in 25 (83.3%), Her2 in 10 (33.3%), EGFR in 12 (40%), CK5/6 in 7 (23.3%), and Ki67 in 23 cases (76.6%). In fourteen cases, primary breast cancers paired with secondary deposits were analyzed. In primary tumors, SRC3 was positive in 8 out of 14 cases (57.1%), Pax2 in 11 out of 14 cases (78.5%), ER in 9 out of 14 cases (64.2%), PgR in 7 out of 14 cases (50%), Her2 in 4 out of 14 cases (28.6%), EGFR in 2 out of 14 cases (14.3%), CK5/6 in 6 out of 14 cases (42.9%), and Ki67 in 10 out of 14 cases (71.4%).

Results

In metastatic deposits, SRC3 were positive in 8 out of 14 cases (57.1%), Pax2 in 8 out of 14 cases (57.1%), ER in 3 out of 14 cases (21.4%), PgR in 2 out of 14 cases (14.3%), Her2 in 5 out of 14 cases (35.7%), EGFR in 8 out of 14 cases (57.1%), CK5/6 in 6 out of 14 cases (42.9%), and Ki67 in 13 out of 14 cases (92.9%) (Tab. 1). Both of the cohorts described above overlapped in 14 cases, allowing direct comparison of the primary tumor and corresponding metastases. Surprisingly, the majority of cases revealed unstable marker expression. Constancy in SRC3 status was observed in 10 cases out of 14 (71%), 2 cases converted to SRC3 negative (14.3%) and newly acquired SRC3 expression was detected in 2 cases (14.3%). Constancy of Pax2 was only observed in 7 cases out of 14 (50%). Five cases converted to Pax2 negative (35.7%) and 2 cases converted to Pax2 positive (14.3%). Constancy of ER was only observed in 4 cases (28.6%) and the switch was observed in 10 cases (71.4%). The switching of PgR, Her2, EGFR, CK5/6 and Ki67 was observed in 50%, 21.4%, 57.1%, 28.6% and 21.4% of cases, respectively. Interestingly, the highest constancy was observed in Her2 and Ki67 staining. For Her2, loss of expression was found in two cases (14.3%) and gain was encountered in one case (7.1%). For Ki67, gains were encountered in all three “switches” (21.4%) (Tab. 2). To prove whether the changes between primary tumors and secondary metastatic deposits were statistically significant, the Wilcoxon paired signed-rank test and McNemar’s chi-square test were used. There were no statistically significant differences among the observed variables at a p-value of 0.05. Differences at a significant level of $\alpha = 10\%$ (i.e. $p < 0.1$) were only found for EGFR ($p = 0.059$), PR ($p = 0.091$) and ER ($p = 0.093$) (Tab. 2).

Discussion and Conclusion

The incidence of brain metastases in breast cancer is increasing. The percentage of breast cancer involving the CNS is between 10 – 40%, depending on the study. Only 5% of patients with ER positive primary tumors developed CNS secondary deposits. 9% of ER negative breast cancer patients developed CNS metastases. The rate of discordance between primary tumor and secondary deposits were reported to range from 28% – 42% for ER and was 17% for PgR [15, 16]. Depending on the study and techniques utilized, discordance rates of Her2 ranged between 0% – 37% [17, 18, 19, 20, 21]. Hurtado et al. showed that PAX2 competes with SRC-3 for binding and regulation of HER-2 transcription. Human breast cancers that were PAX2 positive and SRC-3 negative had the lowest recurrence rate, and the relationship between PAX2 and SRC-3, with regard to levels that determine relapses, were found to be inversely dependent (P,0.03) [10]. This suggests a transcriptional link between the two subtypes of breast cancer, namely ER positive and HER2 positive tumors. This mechanism may also lead to the subsequent activation of SRC-3 via phosphorylation.

At the present time, there is no publication comparing SRC3 and Pax2 protein expression in primary and secondary deposits of breast cancer. Our study contained only a small number of patients, but it is nevertheless remarkable that the biological characteristics of the CNS deposits sometimes transformed from that of the primary tumor.

Immunohistochemical profiles were performed for 30 metastatic deposits using antigens for SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67 and EGFR. SRC was positive in 66.6% of cases and Pax2 in 73.3%. No correlation was observed between the patient’s age, cancer grade, histotype, lymphatic node status, or protein expression (Fig. 1-8).

Results

Using all seven antigens, the primary tumor's protein expression differed from that of the brain deposits in 13 cases out of 14 (93%). Constant expression of SRC3 and Pax2 was seen in 71% and 50%, respectively. The highest protein detection was observed with Ki67 in 10 cases out of 14 (71.4%); the biggest gain was observed with EGFR in 7 cases; and the biggest loss of protein was observed with ER in 8 cases.

According to the immunohistochemical results, our study suggests that not all distant metastases are biologically equal. It shows that re-assessment of protein expression may be useful to optimize oncological treatment.

Table 1: Summary of positive primary and metastatic breast cancer deposits

antigen	% of pos. primary deposits	% of pos. metastatic deposits
SRC3	57.10%	66.60%
Pax2	57.10%	73.30%
ER	21.40%	73.30%
PgR	14.30%	83.30%
Her2	35.70%	33.30%
EGFR	57.10%	40%
CK5/6	42.90%	23.30%
Ki 67	92.90%	76.60%

Table 2: Changes in protein expression in paired samples from primary breast cancer and secondary CNS deposits (n=14)

Primary/metastases	constant expression	pos/pos	neg/neg	gain	loss
SRC3	10(71%)	6	4	2	2
Pax2	7(50%)	6	1	2	5
ER	4(28.6%)	1	3	2	8
PgR	7(50%)	1	6	1	6
Her2	11(78.6%)	3	8	2	1
EGFR	6(42.3%)	1	5	7	1
CK5/6	10(71.4%)	4	6	2	2
Ki 67	11(78.6%)	10	1	3	0

Figures

Figure 1. Immunohistochemical staining sample - staining ER pos (x20)

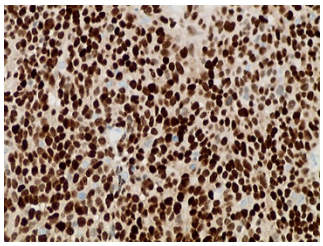


Figure 2. Immunohistochemical staining sample - staining PgR pos (x20)

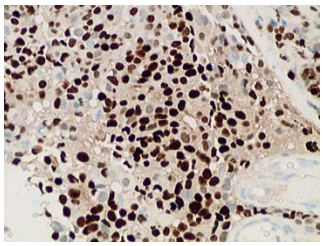


Figure 3. Immunohistochemical staining sample - staining Her2 pos (x20)

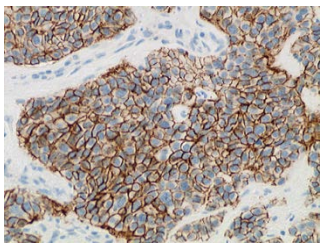


Figure 4. Immunohistochemical staining sample - staining Ki 67 pos**,** (x20)

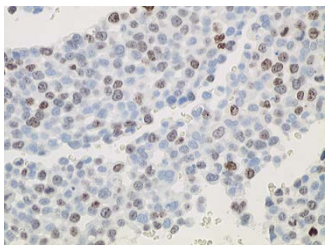


Figure 5. Immunohistochemical staining sample - staining EGFR pos (x20)

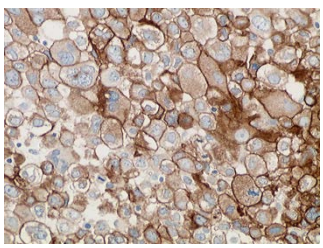


Figure 6. Immunohistochemical staining sample - staining CK 5.6 pos (x20)

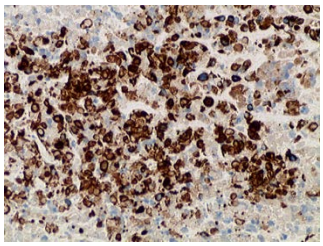


Figure 7. Immunohistochemical staining sample - staining AIB1 pos (x20)

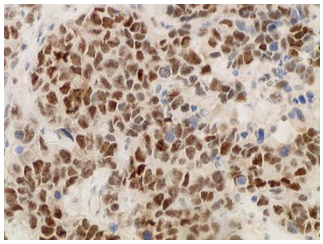
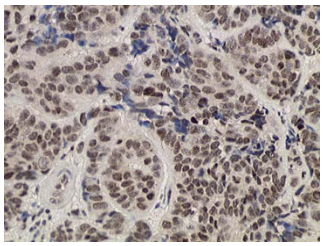


Figure 8. Immunohistochemical staining sample - staining Pax2 pos (x20)



Results

References:

1. Wang H, Zhang D, Wu W, et al. Overexpression and gender-specific differences of SRC-3 (SRC-3/AIB1) immunoreactivity in human non-small cell lung cancer: an in vivo study. *J Histochem Cytochem*. 2010;58(12):1121-7.

2. Xu J, Liao L, Ning G, Yoshida-Komiya H, Deng C, O'Malley BW. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc Natl Acad Sci USA*. 2000;97(12):6379-84.

3. Zhou G, Hashimoto Y, Kwak I, Tsai SY, Tsai MJ. Role of the steroid receptor coactivator SRC-3 in cell growth. *Mol Cell Biol*. 2003;23(21):7742-55.

4. Yan J, Yu CT, Ozen M, Ittmann M, Tsai SY, Tsai MJ. Steroid receptor coactivator-3 and activator protein-1 coordinately regulate the transcription of components of the insulin-like growth factor/AKT signaling pathway. *Cancer Res*. 2006;66(22):11039-46.

5. Anzick SL, Kononen J, Walker RL, et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*. 1997;277(5328):965-8.

6. List HJ, Reiter R, Singh B, Wellstein A, Riegel AT: Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. *Breast Cancer Res Treat*. 2001;68(1):21-8.

7. Bautista S, Valles H, Walker RL, et al. In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. *Clin Cancer Res*. 1998;4(12):2925-9.

8. Osborne CK, Bardou V, Hopp TA, et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst*. 2003;95(5):353-61.

9. Muratovska A, Zhou C, He S, Goodyer P, Eccles MR. Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. *Oncogene*. 2003;22(39):7989-97.

10. Hurtado A, Holmes KA, Geistlinger TR, et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. *Nature*. 2008;456(7222):663-6.

11. P OC, Rhys-Evans P, Modjtahedi H, Court W, Box G, Eccles S. Overexpression of epidermal growth factor receptor in human head and neck squamous carcinoma cell lines correlates with matrix metalloproteinase-9 expression and in vitro invasion. *Int J Cancer*. 2000;86(3):307-17.

12. Vihinen P, Nikkola J, Vlaykova T, et al. Prognostic value of beta1 integrin expression in metastatic melanoma. *Melanoma Res*. 2000;10(3):243-51.

13. Jiang J, Sarwar N, Peston D, et al.. Phosphorylation of estrogen receptor-alpha at Ser167 is indicative of longer disease-free and overall survival in breast cancer patients. *Clin Cancer Res*. 2007;13(19):5769-76.

14. List HJ, Reiter R, Singh B, Wellstein A, Riegel AT.
Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. *Breast Cancer Res Treat.* 2001;68(1):21-8.

15. Kamby C, Rasmussen BB, Kristensen B. Oestrogen receptor status of primary breast carcinomas and their metastases. Relation to pattern of spread and survival after recurrence. *Br J Cancer*. 1989;60(2):252-7.

16. Li BD, Byskosh A, Molteni A, Duda RB. Estrogen and progesterone receptor concordance between primary and recurrent breast cancer. *J Surg Oncol*. 1994;57(2):71-7.

17. Shimizu C, Fukutomi T, Tsuda H, et al. c-erbB-2 protein overexpression and p53 immunoreaction in primary and recurrent breast cancer tissues. *J Surg Oncol*. 2000;73(1):17-20.

18. Gong Y, Booser DJ, Sneige N. Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. *Cancer*. 2005;103(9):1763-9.

19. Gancberg D, Di Leo A, Cardoso F, et al. Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. *Ann Oncol*. 2002;13(7):1036-43.

20. Regitnig P, Schippinger W, Lindbauer M, Samonigg H, Lax SF. Change of HER-2/neu status in a subset of distant metastases from breast carcinomas. *J Pathol*. 2004;203(4):918-26.

21. Pectasides D, Gaglia A, Arapantoni-Dadioti P, et al. HER-2/neu status of primary breast cancer and corresponding metastatic sites in patients with advanced breast cancer treated with trastuzumab-based therapy. *Anticancer Res*. 2006;26(1B):647-53.

5.2 Site-specific CpG methylation in the CCAAT/enhancer binding protein delta (*CEBPδ*) CpG island in breast cancer is associated with metastatic relapse.

Carlo Palmieri, Martino Monteverde, Laura Lattanzio, **Ondrej Gojis**, Bharath Rudraraju, Mirella Fortunato, Nelofer Syed, Alastair Thompson, Ornella Garrone, Marco Merlano, Cristiana Lo Nigro and Tim Crook
Br J Cancer. 2012; 107 (4) :732-8
IF 4,3

Results

Abstract:

Background: CCAAT/enhancer binding protein delta (CEBPδ) is a member of a highly conserved family of basic region leucine zipper transcription factors. It has properties consistent with a tumour suppressor, however other data suggests that CEBPδ may be involved in the metastatic process.

Methods: We analysed the expression of CEBPδ and the methylation status of the CpG island in human breast cancer cell lines, in 107 archival cases of primary breast cancer and in two series of metastatic breast cancers using qPCR and pyrosequencing.

Results: Expression of CEBPδ is down-regulated in primary breast cancer by site-specific methylation in the CEBPδ CpG island. Expression is also down-regulated in 50% of cases during progression from primary carcinoma to metastatic lesions. The CEBPδ CpG island is methylated in 81% metastatic breast cancer lesions, while methylation in the CEBPδ CpG island in primary cancers is associated with increased risk of relapse and metastasis.

Conclusion: CEBPδ CpG island methylation is associated with metastasis in breast cancer. Detection of methylated CEBPδ genomic DNA may have utility as an epigenetic biomarker of primary breast carcinomas at increased risk of relapse and metastasis.

Introduction

The CCAAT/enhancer binding proteins (CEBP) are a highly conserved family of basic region leucine zipper (bZip) transcription factors, and comprises six family members (CEBPα to CEBP*δ*) (Ramji et al., 2002). CEBP proteins exhibit significant amino acid homology (>90%) in the bZip (C-terminal) domain, while the N-terminal regions are quite divergent exhibiting <20% sequence homology (Ramji et al., 2002). CEBP form homo- or heterodimers with each other as well as other bZip-containing proteins such as Jun and Fos (Vinson et al., 2002), with the dyad symmetrical repeat RTTGCGYAAY (where R is A or G, and Y is C or T) considered to be the optimal CEBP binding site (Osada et al., 1997). CEBPδ unlike other family members lacks an activation domain and, therefore, represses gene transcription by forming inactive heterodimers with other members (Cooper et al., 1995). The CEBP family is involved in a number of key cellular processes including differentiation, metabolism, inflammation, apoptosis and proliferation (Yamanaka et al., 1997; Wang et al., 1995; Zinszner et al., 1998; Robinson et al., 1998).

CEBPδ has been proposed to have tumour suppressor function given its ability to decrease levels of cyclin D1 and cyclin E, while increasing p27 (Ikezoe et al., 2005; Gery et al., 2005; Parwar et al., 2010), as well as regulating pro-apoptotic gene expression during mammary gland involution (Tharangu et al., 2005; Stein et al.,2009). Treatments *in vitro* which induce growth arrest such as serum and growth factor withdrawal increase *CEBPδ* expression and induce growth arrest in breast cancer cell lines as well as in human mammary epithelial cells.(O'Rourke et al.,1997;Sivko & DeWille, 2004). However, *in vivo* loss of CEBPδ results in increased mammary epithelial cell proliferation and ductal hyperplasia, supporting the importance of CEBPδ in regulating mammary epithelial growth *in vivo* (Gigliotti et al., 2003). This data is supported by the reduction observed in CEBPδ expression in mammary

tumor prone MMTV/c-neu transgenic mice and in carcinogen-induced rodent mammary tumors (Porter et al., 2001; Kuramoto et al., 2002). Further evidence that CEBPδ is a tumour suppressor comes from animal data utilising mice with a germ-line deletion of CEBPδ (on a MMTV-c-Neu background), with these animals developing significantly more breast tumours compared to controls (Balmarugan et al., 2010). Interestingly, in the context of this mouse knockout model absence of CEBPδ resulted in less efficient metastasis under hypoxia, implying that the protein may be required for metastasis at least under these conditions (Balmarugan et al., 2010).

CEBPδ is down regulated via methylation in cervical cancer, hepatocellular carcinoma and AML (Ko et al., 2008; Agarwal et al., 2007). CEBPδ protein expression also correlates with low-grade histology and disease-free survival in meningiomas (Barresi et al., 2009). CEBPδ has also been shown to be down-regulated in ductal carcinoma in-situ (DCIS) as compared to normal breast tissue (Porter et al., 2003). In a series of primary human breast cancers CEBPδ mRNA levels were very low in 32% (18/57) of cases, and in those cases with low mRNA levels and this was associated with CpG methylation in the CEBPδ gene promoter and 5' coding region (Tang et al., 2005). CEBPδ also formed part of 70-gene signature which predicted better survival of breast cancer patients (Naderi et al, 2007).

To date there have been no reports regarding the involvement of CEBPδ in metastasis in human cancer, nor of the utility of CEBPδ as a prognostic biomarker in breast cancer. Here, we have performed quantitative analysis of CEBPδ CpG island methylation to test these possibilities.

Methods

Breast cancer cell lines

Breast carcinoma cell lines SKBR3, MDA- MB231, MDA -MB 453, MDA -MB468, MDA-MB 435, MCF7, T47D, ZR75.1, HCC1937, HS578 were grown as described previously (Shah et al., 2009).

Two series of cases were analysed in the study. The first was 107 primary breast carcinomas. The characteristics of this patient population are shown in Table 1. These cases were randomly selected from the tissue archives of S. Croce General Hospital, Cuneo, Italy. For all 107 cases, genomic DNA was available and was analysed by pyrosequencing for CEBPδ CpG island methylation. For 26 of the 107 cases, mRNA was available and was used to analyse CEBPδ expression by qPCR. At the time of the study, metastatic relapse had occurred in 31 of the 107 patients. For 14 of 31 the relapsed cases, tissue from the metastasis was available and was analysed in parallel with matched tissue from the primary cancer for CEBPδ expression. The second series comprised 21 CNS metastatic lesions from Imperial Healthcare NHS Trust. These cases were identified from the neuropathology archives at Charing Cross Hospital, London. Tissue was originally obtained at neurosurgical resection of intracranial disease in patients with a pre-existing diagnosis of breast cancer and was confirmed by histopathology to be metastatic breast cancer. Genomic DNA from this series was analysed by pyrosequencing for CEBPδ CpG island methylation. The study received ethical committee approval in both centres. In all cases, the original diagnosis and adequate representation of neoplastic tissue was confirmed by histopathological review prior to inclusion in the study. Expression of the oestrogen receptor (ER), the progesterone receptor (PgR) and HER2 was determined according to local protocols.

Analysis of CEBPδ expression

Total RNA was extracted from formalin-fixed paraffin embedded (FFPE) tissue using Recover All kit (Ambion, Carlsbad, CA, USA). cDNA was synthesized from 1 µg total RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). For demethylation, cells were treated with 5 µM 5´azacytidine (5´AZA; Sigma) for 7 days. Cells were split every 2-3 days with the addition of fresh drug. After drug treatment, cells were harvested for qPCR. For qPCR analysis, 25l 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) and using the pre-cast "TaqMan® Gene Expression Assays" (Applera, https://products.appliedbiosystems.com/) for CEBPδ (Hs00270931_s1). Quantification of target transcripts was performed in comparison to the reference transcript $\beta_2$microglobulin (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, Forster City, CA, USA).

Pyrosequencing

Genomic DNA was extracted from cellular pellets and FFPE sections using the DNeasy Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions and from 10 micron sections of FFPE using phenol with the traditional protocol. Methylation in the CpG island of CEBPδ was analyzed by pyrosequencing technology, which allows the quantification of the degree of methylation at each CG site through the calculation of the ratio between T and C. PCR primers were as follows:

Results

Forward GGAGTGTTGGTAGAGGGAG – 5'biot

Reverse CCCTAAAAACCCCCAACCC

PCR conditions were as follows: 95ºC for 10 min, 95ºC for 30 s, 58°C for 30 s, 72ºC for 40 s for 40 cycles, 72ºC for 7 min. The PCR products were then analyzed by pyrosequencing using the Sample Prep kit (Diatech, Jesi, Italy).

After pyrosequencing, analysis of percentage methylation at each CG was determined using Pyromark Q CpG Software (Qiagen, Venlo, Netherland). DNA from 5 normal breast samples and placental DNA were used as a negative control for methylation (0% average methylation) and a commercial methylated DNA (Millipore, Billerica, MA, USA) was used as positive control (98% average methylation).

Statistical Analysis

CEBPδ CpG island methylation status and presence of metastatic profile were assessed for associations using the chi-square test, with Yates correction or Fisher exact test when appropriate. All the comparisons are two tailed.

Results

Site-specific CpG methylation correlates with silencing of CEBPδ in breast cancer cell lines

We analysed expression and epigenetic regulation of CEBPδ in a panel of breast carcinoma cell lines. Because a previous report has identified methylation-associated down-regulation of CEBPδ in the SUM-52PE breast carcinoma cell line (Tang et al., 2005), we were interested to further test and characterise the relationship between expression of CEBPδ mRNA and methylation in breast carcinoma cell lines. We wished to use a fully quantitative analytical technique rather than methylation-specific PCR (MSP) and we therefore used pyrosequencing to analyse a section of the CEBPδ CpG island (Figure 1). In breast cancer cell lines, methylation was predominantly but not exclusively seen at CG 5 in the fragment analysed by pyrosequencing (Figure 1). CEBPδ mRNA was detectable in many cell lines. Expression was highest in HCC1937 and ZR75.1 but was down regulated relative to normal breast cells in several cell lines (Figure 1). There was a good correlation between methylation at CG 5 of the analysed fragment and down-regulation of the mRNA expression (Figure 1).

CEBPδ is down-regulated in primary breast carcinomas compared to normal breast tissue

Next we sought to investigate whether there is down-regulation of CEBPδ mRNA in clinical cases of breast cancer, and we performed qPCR in 26 primary breast carcinomas. CEBPδ mRNA was reduced relative to normal breast tissue in many cases (Figure 2A). .Expression was reduced most strikingly in the series of primary cancers which later relapsed in comparison to cases which did not relapse: down-regulation by at least 50% compared to normal breast epithelium was observed in 1/7 non-relapsing cases and 11/19 relapsing cases (compare upper and lower panels in Figure 2A).

Down-regulation of CEBPδ in metastatic breast cancer lesions

We then analysed expression of CEBPδ in metastatic breast cancer lesions. We examined a series of 14 cases comprising the primary breast carcinoma together with the paired metastasis, which had been confirmed by histopathology. Clinico-pathological details and sites of metastasis for each pair are shown in Table 2. Using qPCR, we analysed expression of CEBPδ. In 7/14 (50%) cases, we observed a significant reduction in CEBPδ mRNA in the metastasis relative to the primary cancer, consistent with selective pressure for loss of CEBPδ expression with acquisition of a metastatic phenotype in breast cancer (Figure 2B).

CEBPδ CpG island methylation predicts breast cancer relapse

We next analysed a series of 107 cases of primary breast cancer from the same patient population to determine whether analysis of CpG island methylation in CEBPδ has utility as a biomarker predictive of clinical relapse. Clinico-pathological details of the study population are shown in Table 1. Representative analyses, showing the distribution of methylation in the amplified area of the CpG island are shown in Figure 3. Consistent with breast carcinoma cell lines, methylation at individual CG dinucleotides was variable. In the clinical cases, methylation was most dense in CG 4-7 in the analysed fragment (Figure 3A), CGs 2 and 3 being almost entirely unmethylated in all cases. Methylation correlated well with reduced expression of CEBPδ mRNA (Figure 3B). The distribution of CEBPδ CpG island methylation between cases relapsing with metastatic disease and non-relapsing cases is shown in Figure 4. At the time of censor, 29% (31/107) of cases had relapsed. Using a mean percentage CpG methylation cut off of 8% as determined by pyrosequencing, relapse was significantly more frequent in cases in which the CEBPδ CpG island was positive for methylation ($p=0.0006$ by Fisher's Exact test; $p=0.001$ with Yates correction), using a mean percentage CpG methylation cut off of 8% as determined by pyrosequencing.

CEBPδ CpG island methylation is associated with metastatic breast cancer

We had previously shown that expression of CEBPδ is down-regulated in metastatic breast cancer lesions (Figure 2B). We wished to test whether CEBPδ CpG island methylation is associated with increased risk of distant organ metastasis in breast cancer and we asked whether there was an association between CEBPδ CpG island methylation and metastasis at specific organ sites. Metastases in liver (p=0.01), lymph node (p=0.02) and skin (p=0.02) were more common in cases in which the primary cancer was positive for methylation (using a mean percentage CpG methylation cut off of 8% as determined by pyrosequencing). In contrast, metastases in bone and lung were not significantly affected by the methylation status of the CEBPδ CpG island (p=0.13 and p=0.24 respectively). The frequency of brain metastases was higher in cases in which the primary cancer was positive for CEBPδ CpG island methylation but this just failed to reach significance (p=0.06) due to the small number of cases with brain metastases. To extend these observations, we analysed CEBPδ CpG island methylation by pyrosequencing in a series of 21 CNS metastases confirmed by histopathology to be derived from primary breast carcinomas (Figure 5). As observed previously, methylation was most dense at CG 4 and CG 5 and was detected in 81% (17/21) of cases (Figure 5). For three of the cases the paired breast cancer primary was also available to us. In 1 of the 3 cases there was a change in methylation in the CNS metastasis in comparison to the primary, with acquisition of methylation at CGs 4, 5 and 6 in the metastasis (Figure 5).

Discussion

We have investigated the expression and regulation of CEBPδ in breast cancer. We show that the gene is a frequent target for down-regulation in primary breast carcinomas as a result of methylation in the CpG island. Furthermore, we demonstrate that methylation in CEBPδ is associated with metastasis and that methylation, when analysed with high resolution, quantitative methodology may have utility as a biomarker predictive of future metastatic relapse. We were initially interested to investigate this gene because there is experimental evidence that CEBPδ has tumour suppressor properties (Porter et al., 2001; Kuramoto et al., 2002) yet, at least in some animal models, may be involved in metastasis (Balmarugan et al., 2010). The data we present are consistent with and supportive of a tumour suppressor and metastasis suppressor function in human breast cancer.

We initially studied expression in a panel of established breast carcinoma cell lines and demonstrated that expression was reduced in several of the cell lines. Some studies of candidate epigenetically-regulated biomarker genes use non-quantitative, low resolution techniques such as methylation-dependent PCR (MSP) to analyse methylation. Here we have used pyrosequencing which allows high resolution quantification of percentage methylation at individual CG dinucleotides within a defined section of the CpG island. We observed using pyrosequenicng that methylation at CG5 in the analysed region of the CpG island showed a good correlation with transcriptional silencing in a panel of breast cancer cell lines. Our data showing correlation between methylation and down-regulation of expression in both breast cancer cell lines and primary carcinomas are supported by other studies in hepatocellular carcinoma (Ko et al., 2008).

We then extended these initial studies to investigate the possible role of down-regulation of CEBPδ in breast cancer metastasis. Several lines of evidence from our studies support loss of CEBPδ expression as a determinant of metastasis. Firstly, analysis of paired

primary:metastatic lesions showed clear down-regulation in the metastases in 50% of cases. Very few studies have specifically examined changes in expression of individual genes during metastasis in breast cancer. Our data are clearly consistent with selective pressure for loss of CEBPδ during acquisition of a metastatic phenotype. Secondly, we have shown that the presence of methylation in the CEBPδ CpG island in primary breast carcinomas is associated with an increased risk of relapse and of distant organ metastasis. Thirdly, we show that the CEBPδ CpG island is frequently methylated in CNS metastases shown to originate in primary breast carcinomas. Further we have also show herein, albeit in limited numbers of cases, that methylation in CEBPδ may be acquired during metastasis to the CNS, consistent with epigenetic evolution as cells acquire metastatic properties. Methylation of the CEBPδ CpG island as an important event in breast cancer metastasis is consistent with a previous report implicating down-regulation of CEBPδ (among other genes) in breast carcinoma cell lines with increased propensity for CNS metastasis (Bos et al., 2009).

Our current data in early breast cancer is consistent with CEBPδ being a tumour suppressor (Ikezoe et al., 2005; Gery et al., 2005; Parwar et al., 2010; Balmarugan et al., 2010). However, our data reporting fewer metastasis when CEBPδ is not methylated is at face value at odds with the in vivo data where loss of CEBPδ is associated with fewer metastasis (Balmarugan et al., 2010). Possible explanations for the diference include the fact that the study of Balmarugan et al., is from an animal experimental system (Balmarugan et al., 2010), while the present data is derived from human breast cancer samples. Furthermore, the model used was in a HER2 over-expressing background (Guy et al., 2010), whilst in the current series only 13% of cases were HER2 positive. In addition, only data relating to lung metastasis was reported, with no data with regard to the effect of CEBPδ on involvement of other common sites for metastasis or overall metastatic tumour burden. It is known within the context of human breast cancer that HER2 positive breast cancer not only has a predilection

to metastasize to the lung but also to the brain and liver (Kennecke et al., 2010). Therefore, the phenotype seen may be specific to the animal model in question. Furthermore, the underyling mechanism proposed for the effect observed in vivo was related to hypoxic HIF-1α accumulation and hypoxia adaptation. As such, these conditions may therefore be prerequiste for the effect observed and may in be part dependent on HER2 (Balmarugan et al., 2010).

It should be noted, of course, that CEBPδ is not the only gene contributing to a metastatic profile. The current data show that methylation of CEBPδ in the primary tumour (using a mean percentage CpG methylation cut off of 8% as determined by pyrosequencing) is associated with metastasis in the liver, lymph node and skin, with metastases in bone and lung not being significantly influenced by the methylation status of CEBPδ. Multiple additional genes must be at play and we have previously shown the importance of one such candidate CACNA2D3 in the metastatic process (Palmieri et al., 2012).

The patient population analysed in our study consisted predominantly of ER positive cases treated with adjuvant endocrine therapy. A key question in the management of early breast cancer continues to be risk stratification to identify patients likely to relapse despite being deemed to be at low risk by clinico-pathological parameters such as those in the St Gallen criteria. Such patients could then be offered appropriate systemic therapy such as adjuvant chemotherapy. While others could be spared such treatment if their risk could objectively be determined to be low. We have shown herein that methylation in the CEBPδ CpG island correlates with a significantly increased risk of metastatic relapse at distant organ sites including brain and liver. Our data imply that CEBPδ may have utility as a biomarker predictive of relapse and metastasis and thus in the identification of patients who may derive greater benefit from adjuvant treatment.

In conclusion, we show that transcriptional silencing of CEBPδ is associated with metastasis in breast cancer. Validation of these results in larger, independent cohorts of patients is required and if positive would encourage the inclusion of CEBPδ in molecular risk assessment algorithms to inform the use of adjuvant therapy in breast cancer.

Table 1 Clinico-pathological features of primary breast cancers

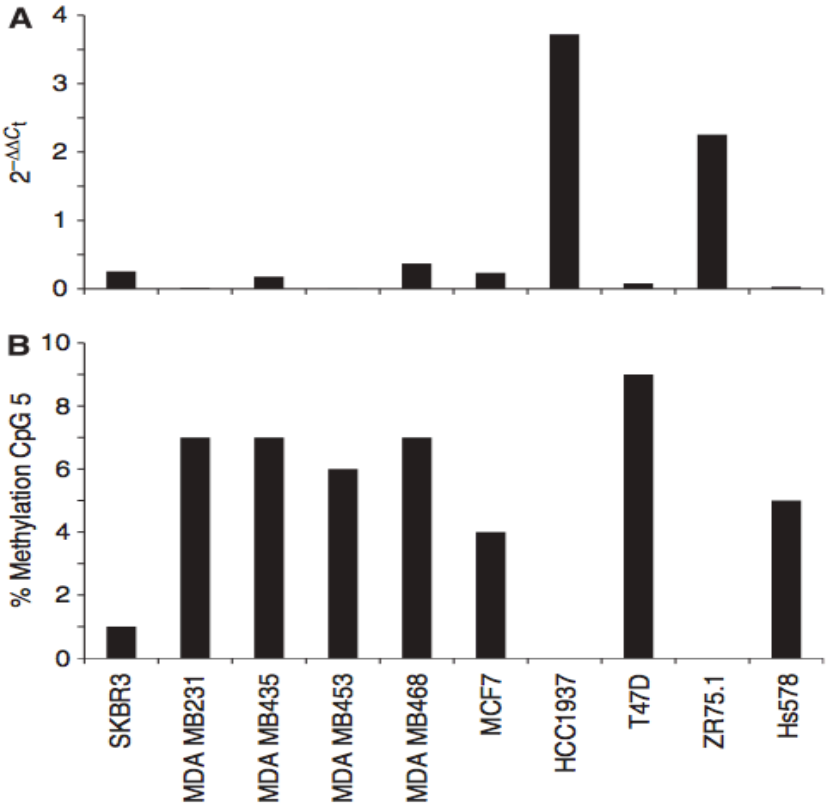
***N* = 107**	***N* (%)**
Age	
Median age (years)	63.0 (Range: 36–87)
Tumour size	
0–20 mm	42 (39)
>20 to 50 mm	40 (37)
>50 mm	3 (3)
Not known	22 (21)
Tumour grade	
Grade I	7 (7)
Grade II	82 (77)
Grade III	12 (11)
Not known	6 (6)
Nodal status	
Positive	41 (38)
Negative	51 (48)
Not known	15 (14)
Hormone receptor status	
ER +ve and PgR +ve unknown	70 (65)
ER +ve and PgR −ve	35 (33)
ER +ve and PgR unknown	2 (2)
HER2 status	
Positive (3+/2+ FISH positive)	14 (13)
Negative	86 (80)
Not known	7 (7)

Abbreviations: ER = oestrogen receptor; FISH = fluorescence *in situ* hybridisation; PgR = progesterone receptor.

Table 2 Receptor status of primary invasive breast cancer, site of initial relapse and site biopsied

Case	Primary tumour	Relapse sites	Relapse biopsied site
1	ER+ PgR+ HER2−	ST, Sk	Sk
2	ER+ PgR+ HER2−	LN, Sk	LN
3	ER+ PgR− HER2+	Br, Li, LN, Lu	LN
4	ER+ PgR+ HER2−	Bo, Lu	Lu
5	ER− PgR− HER2+	Li, LN, Sk	LN
6	ER− PgR− HER2+	LN	LN
7	ER−. PgR− HER2−	ST, Sk	Sk
8	ER+ PgR− HER2−	LN, ST	LN
9	ER+ PgR+ HER2−	Sk	Sk
10	ER+ PgR+ HER2−	Cw, Sk	Sk
11	ER+ PgR− HER2+	Cw, Sk	Sk
12	ER+ PgR+ HER2−	Bo, Li, Sk	Sk
13	ER+ PgR+ HER2−	Bo, Li, LN	LN
14	ER+ PgR+ HER2−	Bo	Bo

Abbreviations: Bo = bone; Cw = chest wall; ER = oestrogen receptor; Li = liver; LN = lymph node; Lu = lung; PgR = progesterone receptor; Sk = skin; ST = soft tissue.



C

Cell line	ER	HER2	1	2	3	4	5	6	7
SKBR3	–	Amp	4–8%	4–8%	0–3%	0–3%	0–3%	4–8%	0–3%
MDA MB231	–	–	4–8%	4–8%	0–3%	4–8%	4–8%	0–3%	0–3%
MDA MB435	+	–	0–3%	4–8%	0–3%	0–3%	4–8%	0–3%	0–3%
MDA MB453	–	Amp	0–3%	0–3%	0–3%	0–3%	4–8%	0–3%	0–3%
MDA MB468	–	–	0–3%	0–3%	0–3%	0–3%	4–8%	0–3%	0–3%
MCF7	+	–	0–3%	0–3%	0–3%	0–3%	4–8%	0–3%	0–3%
HCC1937	–	–	0–3%	0–3%	0–3%	0–3%	0–3%	0–3%	0–3%
T47D	+	–	4–8%	4–8%	0–3%	4–8%	9–12%	0–3%	0–3%
ZR75.1	+	–	4–8%	4–8%	0–3%	4–8%	0–3%	4–8%	0–3%
HS578	+	–	0–3%	4–8%	0–3%	4–8%	4–8%	0–3%	0–3%

0–3% 4–8% 9–12% 13–20% 20–50%

Figure 1 Downregulation of *CEBPδ* mRNA in breast carcinoma cell lines correlates with site-specific CpG methylation in the *CEBPδ* CpG island. (**A**) Expression of *CEBPδ* in breast carcinoma cell lines. qPCR was performed as described in Materials and methods. (**B**) Site-specific CpG island methylation in breast carcinoma cell lines. The percentage methylation at CG 5, as determined by pyrosequencing, is indicated. (**C**) Map of CpG methylation in the *CEBPδ* CpG island in breast cancer cell lines. Pyrosequencing was performed 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 CG dinucleotide in the amplified fragment.

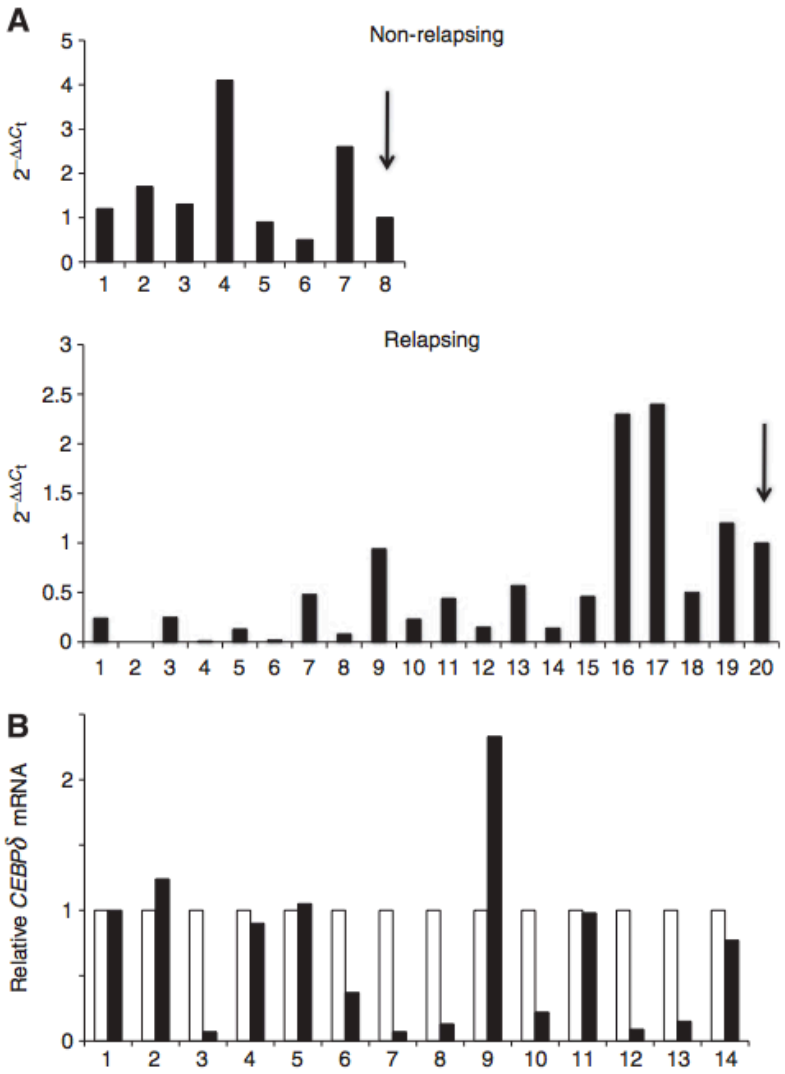


Figure 2 Expression of *CEBPδ* is downregulated in clinical cases of breast cancer. (**A**) Expression of *CEBPδ* in primary breast carcinomas. The figure shows mRNA levels determined by qPCR as described in Materials and Methods. $2^{-\Delta\Delta C_t}$ was calculated as described in Materials and Methods relative to *CEBPδ* expression in control normal breast tissue (arrowed). The upper panel shows expression in cases which had not relapsed at the time of censor, the lower panel shows cases which had relapsed at the time of censor. (**B**) Expression of *CEBPδ* is frequently downregulated in metastatic breast cancer. The figure shows 14 paired primary/metastasis cases. In each case, expression in the primary breast cancer (open box) is set at 1 and expression in the metastasis (black box) is relative to this. Expression is downregulated in cases 3, 6, 7, 8, 10, 12 and 13.

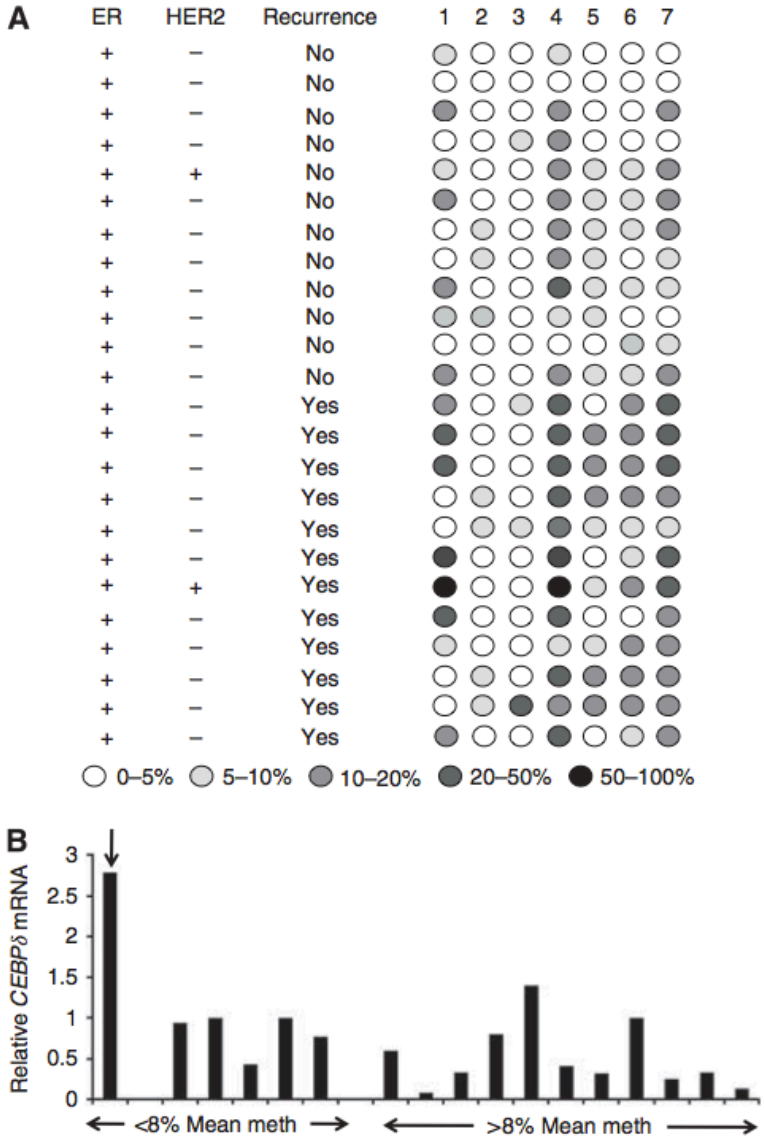


Figure 3 Methylation-associated transcriptional silencing of CEBPd in primary breast carcinomas. (A) Representative pyrosequencing analyses of the CEBPd CpG island in primary breast carcinomas. The upper 12 cases ere non-relapsing, the lower 12 cases later relapsed at distant metastatic sites. The level of methylation is represented by the intensity of shading in the circles, each of which represents an individual CG dinucleotide in the amplified fragment as indicated in the figure. (B) Association of CEBPd CpG island methylation with downregulation of CEBPd mRNA levels. Expression of CEBPd was determined by qPCR and CpG methylation by pyrosequencing as described in Materials and Methods. Cases are divided into those with mean % CG methylation below (o) or above (4) 8. Also shown is expression in normal breast epithelium (arrowed).

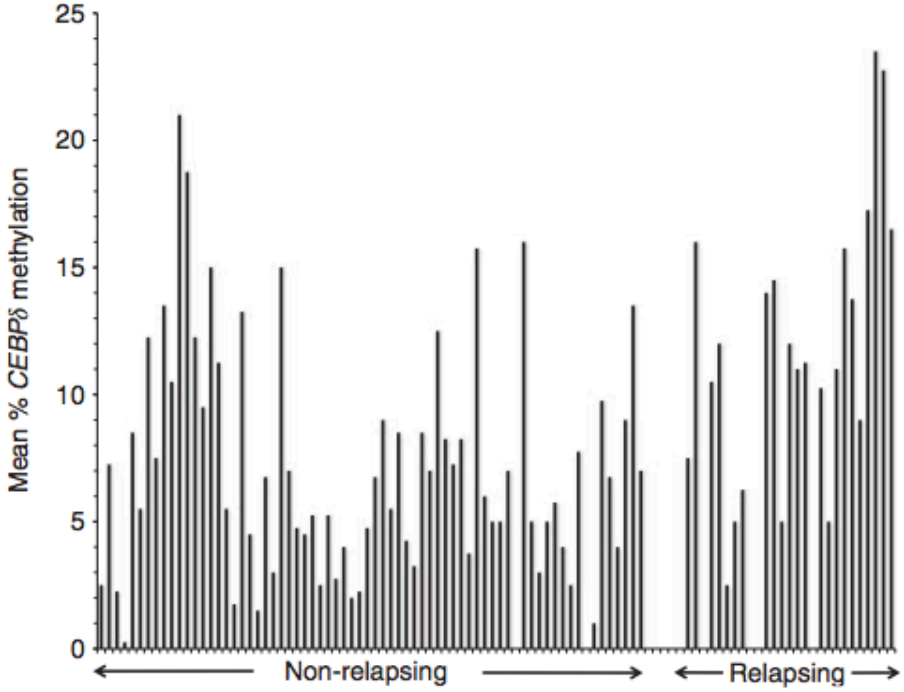


Figure 4 *CEBPδ* CpG island methylation is increased in primary breast carcinomas which subsequently relapse. The figure shows distribution of *CEBPδ* CpG island methylation in cases which at the time of censor had either relapsed or not relapsed. Mean percentage CpG methylation, determined by pyrosequencing, is shown in relapsing and non-relapsing cases.

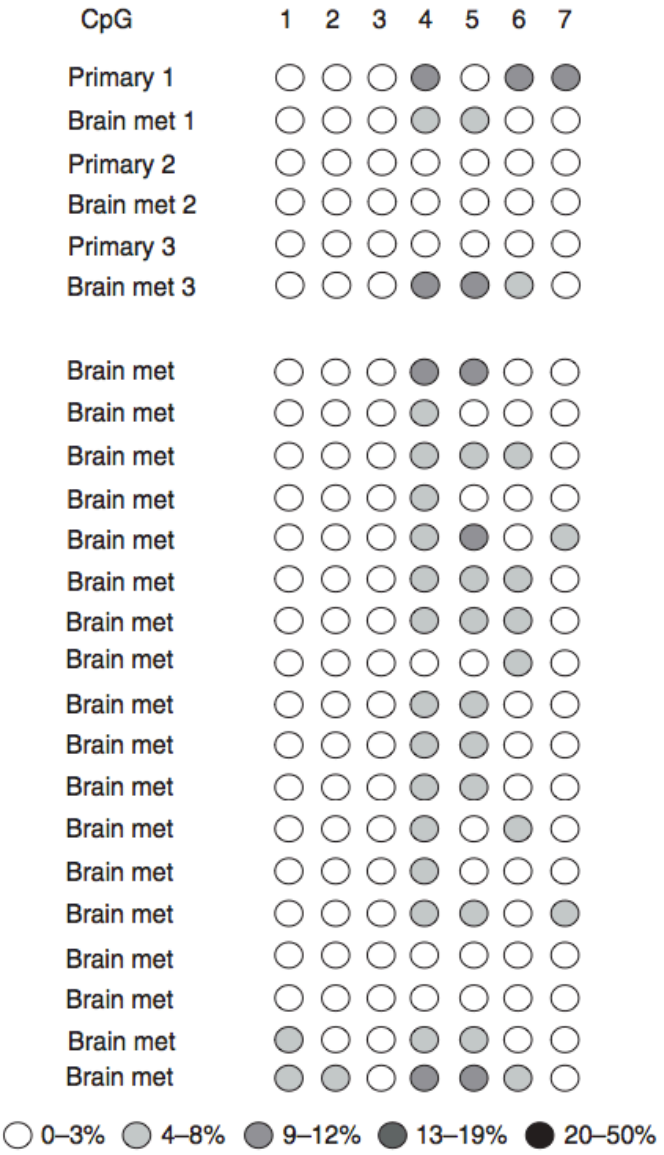


Figure 5 *CEBPδ* CpG island methylation in breast cancer central nervous system (CNS) metastases. The figure shows pyrosequencing analysis of the *CEBPδ* CpG island in CNS metastatic lesions, confirmed by histopathology to be metastatic breast cancer. Also shown (top) are three paired primary/CNS metastatic breast cancers. 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 CG dinucleotide in the amplified fragment.

Acknowledgements

Carlo Palmieri is the recipient of a Cancer Research UK Clinician Scientist award and also acknowledges support from Imperial College Healthcare Charity. Ondrej Gojis is in part funded by a grant from the Ministry of Education of the Czech Republic (Project “Oncology” MSM 0021620808) and is also a recipient of the Translational Research Fellowship from the European Society of Medical Oncology and a fellowship from the European Society for Surgical Oncology. The Division of Cancer at Imperial College London, Imperial College Healthcare NHS Trust is an Experimental Cancer Medicine Centre (ECMC) that is supported by funds from Cancer Research UK and the Department of Health (C37/A7283) and also forms part of the Imperial Cancer Research UK Centre (C42671/A12196). The authors would like to thank Prof Gerry Thomas, Sarah Chilcott-Burns and the Imperial College Healthcare NHS Trust, Human Biomaterials Resource Centre (Tissue Bank). Nelofer Syed acknowledges support from the Brain Tumour Research Campaign (BTRC). Tim Crook is a Scottish Senior Clinical Fellow in Medical Oncology. We thank De Federico Roncaroli for his contribution to the work.

References:

Agrawal S, Hofmann WK, Tidow N, Ehrich M, van den Boom D, Koschmieder S, Berdel WE, Serve H, Müller-Tidow C (2007) The C/EBPdelta tumor suppressor is silenced by hypermethylation in acute myeloid leukemia. Blood 2007 109: 3895–905.

Balamurugan K, Wang JM, Tsai HH, Sharan S, Anver M, Leighty R and Sterneck E (2010) The tumour suppressor C/EBPδ inhibits FBXW7 expression and promotes mammary tumour metastasis. EMBO J 29: 4106-4117.

Barresi V, Vitarelli E, Cerasoli S, Barresi G (2009) The cell growth inhibitory transcription factor C/EBPdelta is expressed in human meningiomas in association with low histological grade and proliferation index. J Neurooncol 97: 233–240

Bos PD, Zhang XH, Nadal C, Shu W, Gomis RR, Nguyen DX, Minn AJ, van der Vijver MJ, Gerald WL, Foekens JA and Massague J (2009). Genes that mediate breast cancer metastasis to the brain. Nature 459: 1005-1009.

Cooper C, Henderson A, Artandi S, Avitahl N and Calame K (1995) Ig/EBP(C/EBPδ) is a transdominant negative inhibitor of C/EBP family of transcriptionalactivators. Nucleic Acids Res. 23: 4371-4377.

Gery S, Tanosaki S, Hofmann WK, Koppel A, Koeffler HP (2005) C/EBPdelta expression in a BCR-ABL-positive cell line induces growth arrest and myeloid differentiation. Oncogene 24:1589–97.

Gigliotti AP, Johnson PF, Sterneck E, DeWille JW (2003) Nulliparous CCAAT/ enhancer binding proteindelta (C/EBPdelta) knockout mice exhibit mammary gland ductal hyperlasia. Exp Biol Med 2003, 228:278-285.

Guy CT,Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ (1992) Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. Proc Natl Acad Sci USA 89: 10578–10582

Ikezoe T, Gery S, Yin D, O'Kelly J, Binderup L, Lemp N, Taguchi H, Koeffler HP (2005) CCAAT/enhancer-binding protein delta: a molecular target of 1,25-dihydroxyvitaminD3 in androgen-responsive prostate cancer LNCaP cells. Cancer Res 2005 65:4762–8.

Kennecke H, Yerushalmi R, Woods R, Cheang MC, Voduc D, Speers CH, Nielsen TO, Gelmon K (2010). Metastatic behavior of breast cancer subtypes. J Clin Oncol. 2010 ;28:3271-7.

Ko C-Y, Hsu H-C, Shen M-R, Chang W-C and Wang J-M (2008) Epigenetic silencing of CCAAT Enhancer-binding Protein d activity by YY1/Polycomb Group/DNA methyltransferase complex. Journal Biol Chem 283: 30919- 30932.

Kuramoto T, Morimura K, Yamashita S, Okochi E, Watanabe N, Ohta T, Ohki M, Fukushima S, Sugimura T, Ushijima T (2002) Etiology-specific gene expression profiles in rat mammary carcinomas. Cancer Res 62:3592-3597.

Naderi A, Teschendorff AE, Barbosa-Morais NL, Pinder SE, Green AR, Powe DG, Robertson JF, Aparicio S, Ellis IO, Brenton JD, Caldas C (2007) A gene-expression signature to predict survival in breast cancer across independent data sets. Oncogene 26: 1507–1516.

O'Rourke J, Yuan R, DeWille J (1997) CCAAT/enhancer-binding protein delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. J Biol Chem 1997 272:6291–6.

Osada S, Yamamoto H, Nishihara T and Imagwa M (1997) DNA binding specifcity of the CCAAT/enhancer-binding protein transcription factor family. J. Biol. Chem. 271, 3891-3896.

Palmieri C, Rudraraju B, Monteverde M, Lattanzio L, Gojis O, Brizio R, Garrone O, Merlano M, Syed N, Lo Nigro C Crook T, Methylation of the calcium channel regulatory subunit α2δ-3 (CACNA2D3) predicts site-specific relapse in oestrogen receptor-positive primary breast carcinomas. Br J Cancer. 2012 May 29. doi: 10.1038/bjc.2012.231. [Epub ahead of print]

Pawar SA, Roy Sarkar T, Balamurugan K, Sharan S, Wang J, Zhang Y, Dowdy SF, Huang AM, Sterneck E (2010) C/EBP{delta} targets cyclin D1 for proteasome-mediated degradation via induction of CDC27/APC3 expression. Proc Natl Acad Sci USA 107: 9210–9215

Porter DA, Krop IE, Nasser S, Sgroi D, Kaelin CM, Marks JR, Riggins G, Polyak K (2001) A SAGE (serial analysis of gene expression) view of breast tumor progression. Cancer Res 2001, 61:5697-5702.

Porter D, Lahti-Domenici J, Keshaviah A, Bae YK, Argani P, Marks J, Richardson A, Cooper A, Strausberg R, Riggins GJ, Schnitt S, Gabrielson E, Gelman R, Polyak K (2003) Molecular markers in ductal carcinoma in situ of the breast. Mol Cancer Res 2003; 1:362–75.

Ramji DP, Foka P (2002) CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365:561-575.

Shah R, Smith P, Purdie C, Quinlan P, Baker L, Aman P, Thompson AM and Crook T (2009) The Prolyl 3-Hydroxylases P3H2 and P3H3 are novel targets for epigenetic silencing in breast cancer. Br J Cancer 100: 1687-1696.

Stein T, Salomonis N, Nuyten DS, van de Vijver MJ and Gusterson BA (2009) A mouse mammary gland involution mRNA signature identifies biological pathways potentially associated with breast cancer metastasis. J Mammary Gland Biol Neoplasia 14: 99-116

Sivko GS, DeWille JW. CCAAT/Enhancer binding protein delta (c/ EBPdelta) regulation and expression in human mammary epithelial cells (2004) I. "Loss of function" alterations in the c/EBPdelta growth inhibitory pathway in breast cancer cell lines. J Cell Biochem 93: 830–43

Tang D, Sivko GS, DeWille JW (2006) Promoter methylation reduces C/EBPdelta (CEBPD) gene expression in the SUM-52PE human breast cancer cell line and in primary breast tumors. Breast Cancer Res Treat 95:161–70

Thangaraju M, Rudelius M, Bierie B, Raffeld M, Sharan S, Hennighausen L, Huang A-M and Sterneck E (2005). C/EBPd is a crucial regulator of pro-apoptotic gene expression during mammary gland involution. Development 132: 4675-4685

Robinson GW, Johnson PF, Hennighausen L and Sterneck E (1998) The C/EBPb transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. Genes Dev. 12, 1907-1916

Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M (2002) Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol 22:6321-6335

Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR and Darlington GJ (1995) Impaired energy homeostasis in C/EBPa knockout mice. Science 269, 1108-1112

Yamanaka R, Kim GD, Radomska HS, Lekstrom-Himes J, Smith LT, Antonson P, Tenen DG and Xanthopoulos KG (1997) CCAAT/enhancer binding protein e is preferentially up-regulated during granulocyte differentiation and its functional versatility is determined by alternative use of promoters and differential splicing. Proc. Natl. Acad. Sci. U.S.A. 94: 6462-6467

Zinszner H, Kuroda M, Wang X.-Z, Batchvarova N, Lightfoot R T, Remotti H, Stevens JL and Ron D (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev. 12:982-995

5.3 Methylation of the calcium channel regulatory subunit α2d-3 (CACNA2D3) predicts site-specific relapse in oestrogen receptor-positive primary breast carcinomas

C Palmieri, B Rudraraju, M Monteverde, L Lattanzio, **O Gojis**, R Brizio, O Garrone, M Merlano,N Syed, C Lo Nigro and T Crook
Br J Cancer. 2012, 107 (2):375-81
IF 4,3

Results

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 α2d-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 50 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.

Keywords: breast cancer; metastasis; calcium channels; epigenetics

The calcium ion (Ca2+) is a key intracellular messenger and regulates a diverse range of cellular processes by activating or inhibiting cellular signalling pathways and Ca2+-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 Ca2+ channel expression, Ca2+ efflux pumps as well as the expression of proteins that control the Ca2+ 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 Ca2+ pumps and channels in human cancers (Toyota et al, 1999; Kim et al, 2003; Paz et al, 2003). Voltage-gated (CaV) calcium channels of the CaV1 and CaV2 classes exist as heteromeric complexes, that is composed of a poreforming a1 subunit and three regulatory subunits, a2d, b and d (Davies et al, 2007). There are four calcium channel voltagedependent a2d 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; 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 carcinoma cell lines (MDA-MB-231, MDA-MB-436, MDAMB- 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 5mM L-glutamine and 10% fetal bovine serum. For pharmacological demethylation, cells were treated with 5 mM 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 St. 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 estrogen 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 mm 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 50-GGTTAAGGATATTGGAGTTTT-30 Reverse primer 50-biot-CCTCTAACAACAACAACC-30 Amplicon length 128 bp PCR conditions were 95 1C for 10 min, 95 1C for 30 s/52 1C for 30s/ 72 1C for 40 s for 40 cycles, 72 1C for 7min. 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 ml PCR reactions were performed using 50 ng of cDNA obtained by reverse transcription of 1 mg 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 'd-d 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 50 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 A). 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 MDAMB-453 (methylated) cells in the presence of the demethylating agent AZA and analysed CACNA2D3 mRNA using qPCR. In MDAMB-453, exposure to AZA caused a strong pregulation 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 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 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 ethylation 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 downregulation 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, ERnegative 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 clinicopathological 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 clinico-pathological information, treatment and clinical outcome. These 100 cases comprised of ERpositive, tamoxifen-treated patients, clinico-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 nonrelapsing cases vs 30 out of 39 (77%) methylated in relapsing cases; Po0.0001. 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, PĽ0.012 and PĽ0.02, 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). We first demonstrated using quantitative analysis (pyrosequencing) that CACNA2D3 expression varies with the methylation status of the CpG island. There was dense methylation in MDAMB-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 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 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 α2d 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 pathomolecular 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.

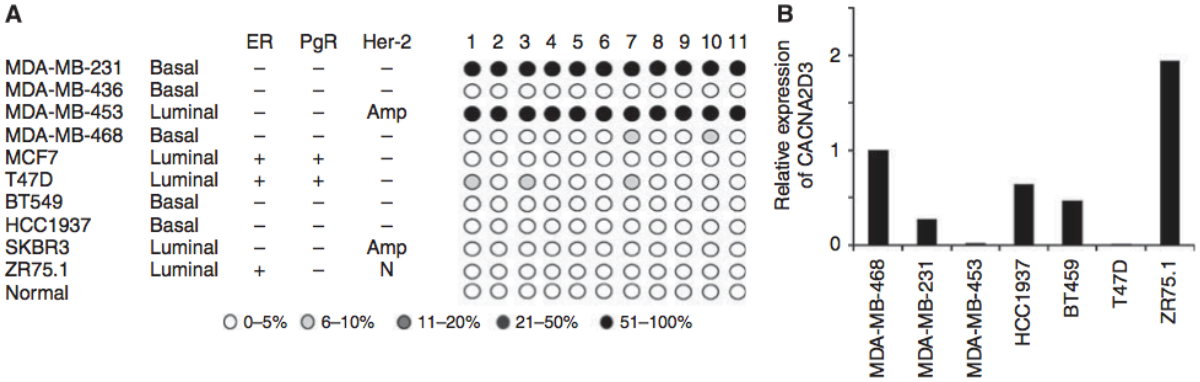


Figure 1 Methylation-dependent transcriptional silencing of *CACNA2D3* in breast cancer cell lines. (**A**) Quantitative pyrosequencing analysis of the *CACNA2D3* CpG island in breast carcinoma cell lines. Pyrosequencing was done as described in Materials and Methods. The level of methylation is represented 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.

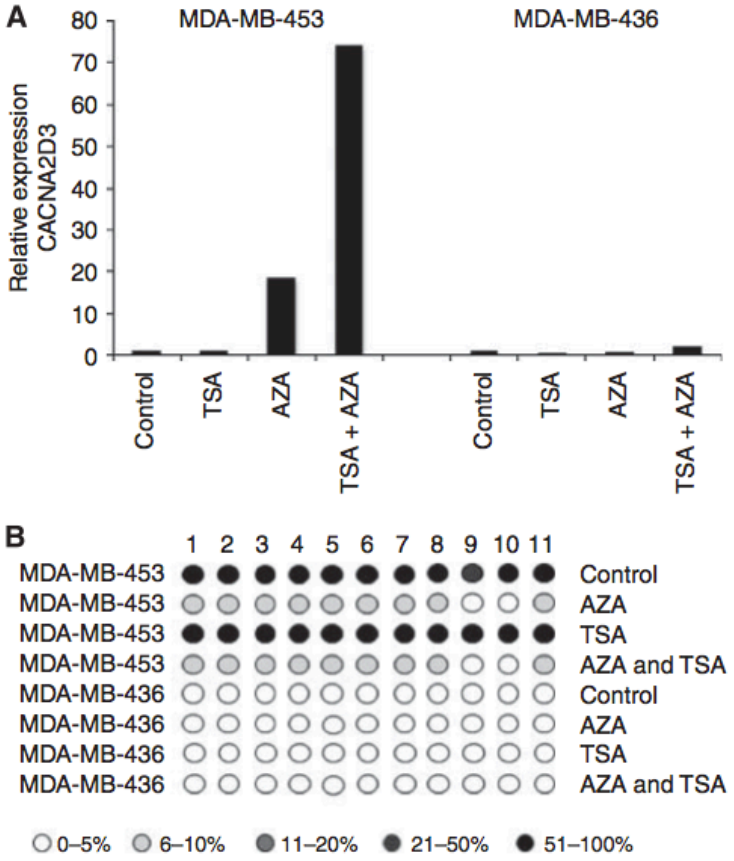


Figure 2 Demethylation reactivates expression of CACNA2D3. (**A**) MDA-MB-453 and MDA-MB-436 cells were grown in the presence of AZA, trichostatin A (TSA) or both. Expression of CACNA2D3 was determined by qPCR. (**B**) AZA-dependent demethylation of the CACNA2D3 CpG island correlates with re-expression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

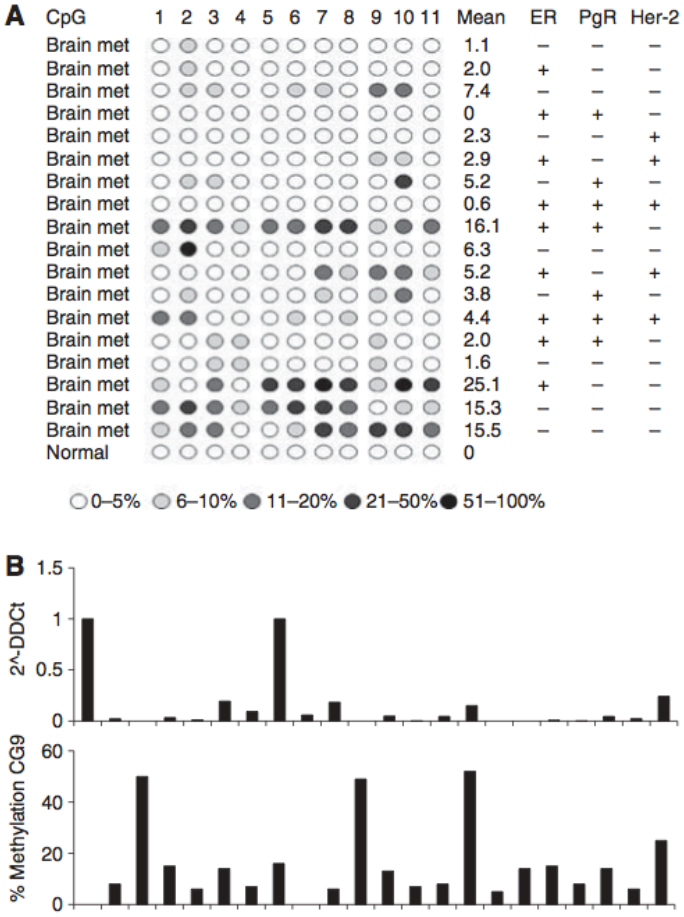


Figure 3 (**A**) The CACNA2D3 CpG island is methylated in metastatic breast cancer. The figure shows pyrosequencing analysis of 17 CNS metastases derived from CNS metastases derived from primary breast carcinomas. CpG methylation was determined by pyrosequencing and the level of methylation is represented 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.

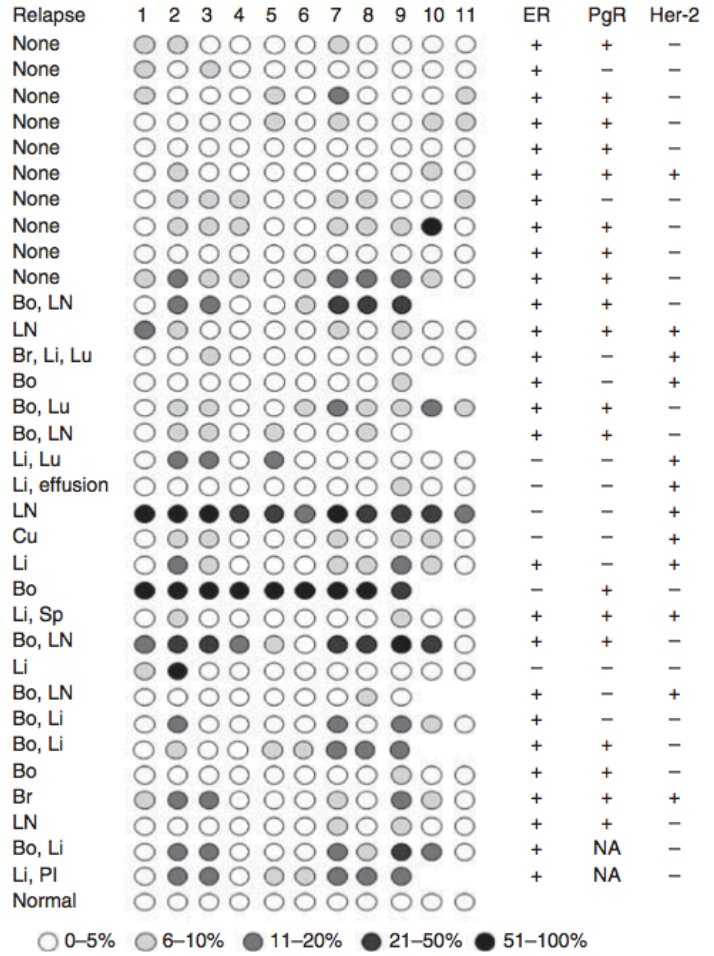


Figure 4 The CACNA2D3 CpG island is methylated in primary breast cancer. The figure shows representative pyrosequencing analysis of individual primary breast carcinomas which were either non-relapsing or relapsed at various distant organ sites. CpG methylation was determined by pyrosequencing and the 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; PI = pleura; Sp = spleen; NA = not available.

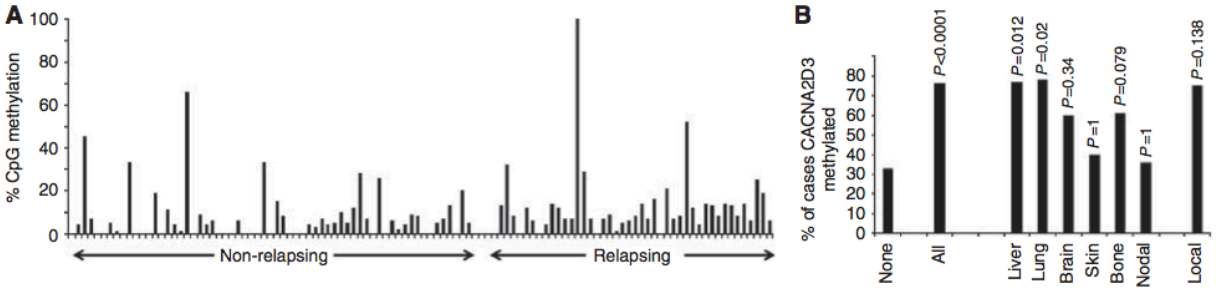


Figure 5 Methylation in the CACNA2D3 CpG island in primary breast cancers treated adjuvantly with endocrine therapy is associated with increased risk of visceral metastasis. (**A**) Percentage methylation at CpG9 in non-relapsing and relapsing primary breast carcinomas. The figure shows percentage methylation, 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.

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.

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.

References

Bai J, Uehara Y, Montell DJ (2000) Regulation of invasive cell behavior by taiman, a Drosophila protein related to AIB1, a steroid receptor coactivator amplified in breast cancer. Cell 103: 1047–1058

Becchetti A (2011) Ion channels and transporters in cancer. 1. Ion channels and cell proliferation in cancer. Am J Physiol Cell Physiol 301: C255–C265

Berridge MJ, Bootman MD, Roderick HL (2003) Calcium signalling: dynamics, homeostasis and remodelling. Nature Rev Mol Cell Biol 4:517–529

Buchhagen DL, Qiu L, Etkind P (1994) Homozygous deletion, rearrangement and hypermethylation implicate chromosome region 3p14.3-3p21.3 in sporadic breast-cancer development. Int J Cancer 15: 473–479

Carboni GL, Gao B, Nishizaki M, Xu K, Minna JD, Roth JA, Ji L (2003) CACNA2D2-mediated apoptosis in NSCLC cells is associated with alterations of the intracellular calcium signaling and disruption of mitochondria membrane integrity. Oncogene 22: 615–626

Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, Dolphin AC (2007) Functional biology of the alpha(2)delta subunits of voltage-gated calcium channels. Trends Pharmacol Sci 28: 220–228

De Preter K, Vandesompele J, Heimann P, Yigit N, Beckman S, Schramm A, Eggert A, Stallings RL, Benoit Y, Renard M, De Paepe A, Laureys G,Pahlman S, Speleman F (2006) Human fetal neuroblast and neuroblastoma
transcriptome analysisconfirms neuroblast origin and highlights neuroblastoma candidate genes. Genome Biol 7: R84. 1–17

Gojis O, Rudraraju B, Gudi M, Hogben K, Sousha S, RC Coombes, Cleator S, Palmieri C (2010) The role of SRC-3 in human breast cancer. Nat Rev Clin Oncol 7: 83–89

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100: 57–70 Hanke S, Bugert P, Chudek J, Kovacs G (2001) Cloning a calcium channel alpha2delta-3 subunit gene from a putative tumor suppressor gene
region at chromosome 3p21.1 in conventional renal cell carcinoma. Gene 264: 69–75

Huang JB, Kindzelskii AL, Clark AJ, Petty HR (2004) Identification of channels promoting calcium spikes and waves in HT1080 tumor cells: their apparent roles in cell motility and invasion. Cancer Res 64: 2482–2489

Kim SG, Chan AO, Wu TT, Issa JP, Hamilton SR, Rashid A (2003) Epigenetic and genetic alterations in duodenal carcinomas are distinct from biliary and ampullary carcinomas. Gastroenterology 124: 1300–1310

Labhart P, Karmakar S, Salicru EM, Egan BS, Alexiadis V, O'Malley BW, Smith CL (2005) Identification of target genes in breast cancer cells directly regulated by the SRC-3/AIB1 coactivator. Proc Nat Assoc Sci 102:
1339–1344

Li C, Liang YY, Feng XH, Tsai SY, Tsai MJ, O'Malley BW (2008a) Essential phosphatases and a phospho-degron are critical for regulation of SRC-3/AIB1 coactivator function and turnover. Mol Cell 31:835–849

Li LB, Louie MC, Chen HW, Zou JX (2008b) Proto-oncogene ACTR/AIB1 promotes cancer cell invasion by up-regulating specific matrix metalloproteinase expression. Cancer Lett 261: 64–73

Monteith GR, McAndrew D, Faddy HM, Roberts-Thomson SJ (2007) Calcium and cancer: targeting Ca2ţ transport. Nat Rev Cancer 7:519–530

Patton AM, Kassis J, Doong H, Kohn EC (2003) Calcium as a molecular target in angiogenesis. Curr Pharm Des 9: 543–551

Paz MF, Wei S, Cigudosa JC, Rodriguez-Perales S, Peinado MA, Huang TH, Esteller M (2003) Genetic unmasking of epigenetically silenced tumor suppressor genes in colon cancer cells deficient in DNA methyltransferases. Hum Mol Genet 12: 2209–2219

Qin N, Yagel S, Momplaisir ML, Codd EE, D'Andrea MR (2002) Molecular cloning and characterization of the human voltage-gated calcium channel alpha(2)delta-4 subunit. Mol Pharmacol 62: 485–496

Qin YR, Fu L, Sham PC, Kwong DL, Zhu CL, Chu KK, Li Y, Guan XY (2008) Single-nucleotide polymorphism-mass array reveals commonly deleted regions at 3p22 and 3p14.2 associate with poor clinical outcome in esophageal squamous cell carcinoma. Int J Cancer 123: 826–830

Rizzuto R, Pinton P, Ferrari D, Chami M, Szabadkai G, Magalhaes PJ, Di Virgilio F, Pozzan T (2003) Calcium and apoptosis: facts and hypotheses. Oncogene 22: 8619–8627

Rizzuto R, Pozzan T (2006) Microdomains of intracellular Ca2ţ: molecular determinants and functional consequences. Physiol Rev 86: 369–408

Tai AL, Mak W, Ng PK, Chua DT, Ng MY, Fu L, Chu KK, Fang Y, Qiang Song Y, Chen M, Zhang M, Sham PC, Guan XY (2006) High-throughput loss-of-heterozygosity study of chromosome 3p in lung cancer using single nucleotide polymorphism markers. Cancer Res 66: 4133–4138

Thorell K, Bergman A, Care´n H, Nilsson S, Kogner P, Martinsson T, Abel F (2009) Verification of genes differentially expressed in neuroblastoma tumours: a study of potential tumour suppressor genes. BMC Med

Genomics 53: 1–13

Toyota M, Ho C, Ohe-Toyota M, Baylin SB, Issa JP (1999) Inactivation of CACNA1G, a T-type calcium channel gene, by aberrant methylation of its 5' CpG island in human tumors. Cancer Res 59: 4535–4541

Wanajo A, Sasaki A, Nagasaki H, Shimada S, Otsubo T, Owaki S, Shimizu Y, Eishi Y, Kojima K, Nakajima Y, Kawano T, Yuasa Y, Akiyama Y (2008)

Methylation of the calcium channel-related gene, CACNA2D3, is frequent and a poor prognostic factor in gastric cancer. Gastroenterology 135:580–590

Wei MH, Latif F, Bader S, Kashuba V, Chen JY, Duh FM, Sekido Y, Lee CC, Geil L, Kuzmin I, Zabarovsky E, Klein G, Zbar B, Minna JD,Lerman MI (1996) Construction of a 600-kilobase cosmid clone contig and generation of a transcriptional map surrounding the lung cancer tumor suppressor gene (TSG) locus on human chromosome 3p21.3: progress toward the isolation of a lung cancer TSG. Cancer Res 56: 1487–1492

Whittaker CA, Hynes RO (2002) Distribution and evolution of von Willebrand/integrin A domains: widely dispersed domains with roles in cell adhesion and elsewhere. Mol Biol Cell 13: 3369–3387

Xiong W, Niu PY, Zhu WT, Chen J (2009) Tenascin-C as a prognostic biomarker in osteosarcoma? Chin Med J 122: 2737–2743

Yuasa Y, Nagasaki H, Akiyama Y, Hashimoto Y, Takizawa T, Kojima K, Kawano T, Sugihara K, Imai K, Nakachi K (2009) DNA methylation status is inversely correlated with green tea intake and physical activity in gastric cancer patients. Int J Cancer 124: 2677–2682

6 Summary

In human breast cancer the rate of immunohistochemical discordance between primary tumour and secondary deposits were reported to range from 28% – 42% for ER and was 17% for PgR. Depending on the study and techniques utilized, discordance rates of Her2 ranged between 0% – 37%. It has been showed that PAX2 competes with SRC-3 for binding and regulation of HER-2 transcription. Human breast cancers that were PAX2 positive and SRC-3 negative had the lowest recurrence rate, and the relationship between PAX2 and SRC-3, with regard to levels that determine relapses, were found to be inversely dependent. This suggests a transcriptional link between the two subtypes of breast cancer, namely ER positive and HER2 positive tumors. Immunohistochemical profiles were performed for 30 metastatic deposits using antigens for SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67 and EGFR. SRC-3 was positive in 66.6% of cases and Pax2 in 73.3%. No correlation was observed between the patient's age, cancer grade, histotype, lymphatic node status, and protein expression. Using all seven antigens, the primary tumor's protein expression differed from that of the brain deposits in 13 cases out of 14 (93%). Constant expression of SRC3 and Pax2 was seen in 71% and 50%, respectively. The biggest gain was observed with EGFR in 7 cases; and the biggest loss of protein was observed with ER in 8 cases.

CEBPδ study discovered that the gene is a frequent target for down-regulation in primary breast carcinomas as a result of methylation in the CpG island. The data we present are consistent with hypothesis of a tumour suppressor and metastasis suppressor function in human breast cancer. The data showed correlation between methylation and down-regulation of expression in both breast cancer cell lines. This

initial study was consequently extended to investigate the possible role of down-regulation of CEBPδ in breast cancer metastasis. Analysis of paired primary metastatic lesions showed clear down-regulation in the metastases in 50% of cases. The data are clearly consistent with selective pressure for loss of CEBPδ during acquisition of a metastatic phenotype. The presence of methylation in the CEBPδ CpG island in primary breast carcinomas is associated with an increased risk of relapse and of distant organ metastasis. CEBPδ CpG island is frequently methylated in CNS metastases originating from primary breast carcinomas. Methylation in CEBPδ may be acquired during the process of metastasis to the CNS. The current data in early breast cancer is consistent with CEBPδ being a tumour suppressor. Fewer metastasis when CEBPδ is not methylated. Methylation of CEBPδ in the primary tumour is associated with metastasis in the liver, lymph node and skin, while metastases in bone and lung are not significantly influenced by the methylation status of CEBPδ. These data give evidence that CEBPδ is not the only gene contributing to a metastatic profile, multiple additional genes must be at play. We have previously shown the importance of one such candidate CACNA2D3 in the metastatic process. It is also known HER2 positive breast cancer has a predilection to metastasize to the lung and also to the brain and liver. A key question in the management of early breast cancer continues to be risk stratification to identify patients likely to relapse despite being deemed to be at low risk by clinico-pathological parameters. Methylation in the CEBPδ CpG island correlates with a significantly increased risk of metastatic relapse at distant organ sites including brain and liver. In conclusion, the study demonstrated that transcriptional silencing of CEBPδ is associated with metastasis in breast cancer.

As mentioned before, calcium ion channels mediate many biological processes relevant to the malignant potential including metastasis. 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. With regard to breast cancer, CACNA2D3 lies in 3p21 a region implicated in sporadic breast cancer development. The study showed that CACNA2D3 is a 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. This suggests a tumour suppressor function in the breast cancer and it is consistent with the previous data in other tumour types such as gastric and lung cancer. In the clinical series analysed, the CACNA2D3 CpG island was methylated in both ER-positive and ER-negative breast cancers. The results of the study showed methylation-dependent transcriptional silencing being the mechanistic basis of CACNA2D3 downregulation in breast cancer. CACNA2D3 methylation in three well-characterised clinical series of breast cancers was analysed. 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. The methylation at CpG9 within the amplified fragment was most strongly associated with brain metastatic lesions. The presence of a relatively high frequency of methylation in CACNA2D3 in CNS metastases prompted determination 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 was demonstrated that in primary cancers with CACNA2D3 CpG methylation, there was a significantly increased risk of recurrence, particularly at visceral sites of liver and lung, whereas there was no increased risk of nodal metastases. This finding suggests downregulation of CACNA2D3 is associated with clinically more aggressive disease. Further work is required to understand more

closely physiological role of CACNA2D3 in a non-excitable cells such as breast epithelium, and improve understanding of the 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(SRC-3) via ER. 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.

Conclusions:

According to the immunohistochemical results, the studies suggest that not all distant metastases are biologically equal. It gives evidence that re-assessment of protein expression may be useful to optimize oncological treatment. Such patients could then be offered appropriate systemic therapy such as adjuvant chemotherapy.

The CEBPδ may have utility as a biomarker predictive of relapse and metastasis and thus in the identification of patients who may derive greater benefit from adjuvant treatment. Validation of these results in larger, independent cohorts of patients is required.

Further work is also required to understand the physiological role of CACNA2D3 in a non-excitable cells such as breast epithelium, and the molecular effects and mechanisms underlying CACNA2D2 role in malignant breast disease.

7 Souhrn

V různých imunohistochemických studiích kracinomu prsu se neshoda mezi primárním a sekundárním ložiskem v detekci estrogenového receptoru pohybuje od 28 do 42%, u progesteronového receptoru kolem 17% a u Her2 receptoru se neshoda exprese pohybuje mezi 0-37%. Předcházející práce prokázaly, že Pax2 soutěží s SRC-3 o regulaci Her2 v transkripčním procesu. Karcinomy prsu, které vykazovaly Pax2 pozitivitu a SRC-3 negativitu, měly nízké riziko relapsu onemocnění. Tento poznatek naznačuje možnost dvou "subtypů" karcinomu prsu: pozitivní pro ER a pozitivní pro Her2. V první studii byl chrakterizován imunohistochemický profil SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67, EGFR u 30 metastatických cerebrálních ložisek karcinomu prsu. SRC-3 byl pozitivní v 66,6% případů a Pax2 v 73,3%. Vztah mezi imunohistochemickým nálezem, věkem pacientky, gradem nádoru, histologickým typem a metastatickým postižením lymfatických uzlin byl statisticky nevýznamný. Při hodnocení přítomnosti sledovaných znaků v primárním tumoru a sekundárním metastatickém ložisku se proteinová exprese lišila v 93%. U SRC-3 byl stejný imunohistochemický nález v 71% a u Pax2 v 50% vzorků. Při porovnávání primárního nádoru a metastatického ložiska byl největší nárůst pozorován u EGFR (50%) a největší ztráta proteinové exprese u ER, v 57%.

Ve studii CEBPδ byla potvrzena role tohoto proteinu jako tumorsupresoru v metastazování karcinomu prsu. Ve zvýšeném riziku tvorby metastáz se uplatňuje metylace CpG. Naše analýza ukazuje "down-regulaci" tohoto genu v 50% případů metastatických ložisek karcinomu prsu. Bylo prokázáno, že při metylaci CpG CEBPδ v primárním ložisku nádoru je vyšší riziko relapsu onemocnění či metastatického rozsevu nádoru. Velké procento metylace bylo prokázáno v sekundárních ložiscích v CNS, nicméně v některých metastatických ložiscích v CNS metylace CpG prokázána

nebyla. Možným vysvětlením tohoto faktu, je skutečnost, že 13% metastatických ložisek CNS nádorů bylo HER2 pozitivních a z předchozích studií je známo, že HER2 pozitivní nádory prsu často metastazují do jater a do CNS. Metylace CEBPδ je dále spojena s metastatickým procesem do lymfatických uzlin, jater a kůže. V CEBPδ studii, kde byly zahrnuty pacientky s ER+ nádorem, kterým byla aplikována adjuvantní hormonální terapie, se ukázalo, že CEBPδ metylace koreluje se zvýšeným rizikem relapsu onemocnění a metatatickým procesem.

Poslední studie, zabývající se metastatickým rozsevem karcinomu prsu, byla zaměřena na vapníkové kanály, respektive na podjenotku vápníkového kanálu α2d-3 (CACNA2D3). Tato podjednotka má rovněž tumorsupresorovou funkci, spočívající zejména v inhibici buněčného růstu a snížení buněčné adheze. V této studii bylo prokázáno, že metylace CACNA2D3 je v karcinomu prsu spojena s vyšším rizikem metastatického procesu, stejně tak jako u zhoubného onemocnění žaludku či plic. CACNA2D3 metylace byla zjištěna u ER pozitivních i ER negativních nádorů prsu. První skupinu vzorků představovala metastatická ložiska v CNS, kde byla prokázána metylace CpG9. Druhá část vzorků zahrnovala 100 ER pozitivních karcinomů prsu u žen, které byly léčeny adjuvantně tamoxifenem. U této skupiny byla metylace CACNA2D3 spojena s vyšším rizikem metastatického procesu do plic a jater, ale s nižším rizikem metastazování do spádových lymfatických uzlin. Tento fakt vede k domněnce, že právě metylace CACNA2D3 CpG je spojena s vyšší agresivitou nádorového procesu. Dalším pozoruhodným nálezem je, že nádorová linie MCF-7 substituovaná estradiolem vykazovala “utlumení” CACNA2D3 cestou estrogenového receptoru pomocí AIB1 (SRC-3).

Závěr:

Z uvedených tří studií zaměřených na regulační mechanismy metastatického procesu u karcinomu prsu vyplývá, že opakovaná detekce nádorových proteinů má velký význam pro klinickou praxi i pro optimalizaci léčby nemocných s tímto onemocněním.

CEBPδ se jeví jako perspektivní onkologický znak, který může přispět k predikci rizika relapsu onemocnění či metastatického procesu. Z výsledků jeho hodnocení by mohly profitovat především pacientky indikované k adjuvantní chemoterapii. Zhodnocení klinického impaktu ve využití tohoho znaku bude zřejmě ještě vyžadovat provedení větší studie.

Alterace podjenotky vápníkového kanálu α2d-3 (CACNA2D3) je zřejmě spojena s vyšší agresivitou nádorového procesu. Další upřesňující poznatky bude potřeba získat zejména o jeho biologickém významu v nenádorové tkáni mléčné žlázy, v benigních nádorech prsu a o jeho uplatnění v regulačních mechanismech buněčné proliferace či buněčné smrti.

8 List of author´s publications:

Papers related to theses:

1. **Gojis O**, Kubecova M, Rosina J, Mandys V et al.:
   Expression of selected protein expression in breast cancer brain metastases
   Folia Histochem Cytobiol, 2013 (accepted for publication)
   IF 1,1

2. Palmieri C, Monteverde M, Lattanzio L, **Gojis O** et al. :
   Site-specific CpG methylation in the CCAAT/enhancer binding protein delta (*CEBPδ*) CpG island in breast cancer is associated with metastatic relapse.
   Br J Cancer, 2012, 7:132-8
   IF 4,3

3. Palmieri C, Rudraraju B, Monteverde M, Lattanzio L, **Gojis O**, Brizio R, Garrone O, Merlano M, Syed N, Lo Nigro C, Crook T. Methylation of the calcium channel regulatory subunit α2δ-3 (CACNA2D3) predicts site-specific relapse in oestrogen receptor-positive primary breast carcinomas.
   Br J Cancer. 2012,107: 375-381
   IF 4,3

Other publications related to theses:

1. **Gojis O**, Rudraraju B, Gudi M, Hogben K et al.: The role of SRC-3 in human breast cancer.
   Nature Reviews Clinical Oncology. 2010, 7:83-9.
   IF 9,11

2. Palmieri C, **Gojis O**, Rudraraju B, Stamp-Vincent C et al.: Expression of steroid receptor coactivator 3 in ovarian epithelial cancer is a poor prognostic factor and a marker for platinum resistance.
   Br J Cancer. 2013 (publication accepted)
   IF 4,3

3. Palmieri C, **Gojis O**, Rudraraju B, Cleator S. Does ER-{beta}cx Really Have No Clinical Importance in Tamoxifen-Treated Breast Cancer Patients? J Clin Oncol. 2008, 26:5824.
   IF 15.48

4. Shousha S, **Gojis O,** Peston D, Palmieri C: An Unusual Triple Negative Breast Carcinoma, Histopathology, 2009, 29:1235.
   IF 3.79

5. Francis RE, Myatt SS, Krol J, Wang J, Guest SK, Filipovic A, **Gojis O**, Palmieri C, et al.: FoxM1 is a downstream target and marker of HER2 overexpression in breast cancer. Int J Oncol. 2009,35:57-68.
   IF 2,3

6. Alifrangis C, Harcourt JP, **Gojis O**, Palmieri C: Poor reception.
   The Lancet. 2009,374:948
   IF 28,6

7. Phan S, Shousha S, **Gojis O**: CD10 An important marker in the profiling of triple negative breast cancer.
Virchows Archiv. 2009, 455:95-96.
IF 2.0

8. Palmieri C, Shah D, Krell J, **Gojis O**, et al.: The management and outcome of HER2-positive early breast cancer treated with or without trastuzumab in the adjuvant trastuzumab era. Clinical Breast Cancer. 2011, 11:93-102
IF 2,5

9. Krell J, James C, Shah D, **Gojis O**, et al.: Human Epidermal Growth Factor Receptor 2 Positive Breast Cancer Relapsing Post-Adjuvant Trastuzumab: Pattern of recurrence, Treatment and Outcome.
Clinical Breast Cancer, 2011, 11:153-60
IF 2,5

10. C Lo Nigro, D Vivenza, M Monteverde, L Lattanzio, **O Gojis**, et al.: High frequency of complex TP53 mutations in CNS metastases from breast cancer. British Journal of Cancer, 2011, 106:397-404
IF 4,3

11. Ross-Innes CS, Stark R, Teschendorff EA, **Gojis O**, et al.: Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. Nature,2011, 481: 389-93
IF 36,1

12. Lo Nigro C, Monteverde M, Lee S, Lattanzio L, **Gojis O** et al.: NT5E CpG island methylation is a favourable breast cancer biomarker.
Br J Cancer. 2012,107:75-83
IF 4,3

13. **Gojis O**, Rudraraju A, Alifrangis C, Krell J, Libalova P, Palmieri C: The role of steroid receptor coactivator -3 (SRC-3) in human malignant disease. European Journal of Surgical Oncology. 2009, 36:224-9.
    IF 2,5

Other papers:

1. Duskova M, Leamerova E, Sosna B, **Gojis O**. Guided tissue regeneration, barrier membranes and reconstruction of the cleft maxillary alveolus. J CraniofacSurg.2006,17:1153-60.
   IF 0.739

2. Duskova M, Kotova M, Urban F, Sosna B, Jirkalova R, Strnadel T, Kristen M, Leamerova E, **Gojis O**. Reconstructions of maxilla alveolus for application of dental implant in patients with cleft defect.
   Acta Chir Plast. 2004, 46:115-21.

3. Záhumensky J, Feldmar P, Kucera E, Zmrhal J, **Gojis O**, Kosova T, Bendova M, Stejskal D. Reproductive functions in women after cancer therapy. Klin Onkol. 2012,25:173-7

4. Zahumensky J, Gojis O, Kiss I, Kucera E. Velamentous insertion of the umbilical cord of twin B as a cause of vasa previa in monochorionic diamniotic twins. Int J Gynaecol Obstet. 2013 [Epub ahead of print, publication accepted]
   IF 2,8

List of author´s publications

Published abstracts:

A. Purohit[1], H. J. Tutill[1], J. M. Day[1], **O. Gojis[2]**, C. Palmieri[2] and M. J. Reed[1]: The Role of Steroid Sulfatase in Regulating Stromal-Epithelial Interactions in the Breast and Prostate. [1]Endocrinology and Metabolic Medicine and Sterix Ltd, Imperial College London, St Mary's Hospital, London W2 1NY, UK; [2]Cancer Research UK Laboratories, Imperial College London, Hammersmith Hospital, London W12 0NN, UK. The endocrine society annual meeting, 2009, USA.

Posters:

Phan, Shousha, **Gojis**: CD10- an important marker in the profiling of triple negative breast cancer. 22nd European congress of Pathology – European Society of Pathology,2009, Florence, Italy.

Shah, **Gojis**, Krell, Ahmad, Riddle, Coombes, Leonard, Fox, Cleator, Palmieri: Management and outcome of HER2-positive breast cancer treated with and without adjuvant trastuzumab: The Imperial College London experience. J Clin Oncol 28:7s, 2010 (suppl; abstr 650). ASCO 2010

Marla, Cardale, Dodwell, Skene, **Gojis**, Palmieri, Abram, Cleator, Bowman, Doughty: HER2-positive early breast cancers: What proportion are receiving adjuvant trastuzumab therapy? A multicenter audit. J Clin Oncol 28:7s, 2010 (suppl; abstr 668) ASCO 2010

Alifrangis, Shipway, **Gojis**, Emson, Mackie, Coombes, Palmieri: A phase II cross-over study of docetaxel versus vinorelbine in anthracycline-resistant, advanced breast cancer. J Clin Oncol 28:7s, 2010 (suppl; abstr 1097). ASCO 2010

List of author´s publications

Congress oral presentation:

2010 European Society for Surgical Oncology (ESSO) - Bordeaux 2010: **O.Gojis**: The role of AIB1 in human breast cancer.

2010 International Postgraduate Conference Hradec Králové 2010: **O.Gojis**: The role of AIB1 in human breast cancer.

2008 University of Cambridge: **Gojis O**: Breast Cancer management – Imperial College London (Master class of oncology – Breast cancer management)

2011 Conference of Czech gynecological and obstetrical society: **Gojiš O,** Zahumenský J**.** HPV infection in pregnancy

Grants and fellowships:

2008: European Society for Surgical Oncology (**ESSO**) **Major Award – research fellowship**.

2008: European Society for Medical Oncology (**ESMO**) - **Fellowship Award – Training fellowship in clinical oncology**.

2011: Hradec Králové – 3rd place Award for oral presentation. **ORPHEUS** - Organisation for PhD Education in Biomedicine and Health Sciences in the European System.

2011: Ministry of education of Czech Republic and Charles University award.

Trainings and courses:

2008: Pokroky v biologii buňky
2008: Základy vědecké práce

> Od: "Professor Zbigniew Kmieć" <zkmiec@gumed.edu.pl>
> Komu: "Dr. Ondrej Gojis" <anatom1@centrum.cz>
> Datum: 29.07.2013 14:33
> Předmět: [FHC] Folia Histochemica et Cytobiologica Editorial Office - Editor Decision
>
Dear Dr. Ondrej Gojis:

Ref.: Ms. entitled:
"Expression of selected proteins in breast cancer brain metastases".
FOLIA HISTOCHEMICA ET CYTOBIOLOGICA

I am pleased to tell you that your work has now been accepted for publication in FOLIA HISTOCHEMICA ET CYTOBIOLOGICA.

Comments from the Editor and Reviewers can be found below.

Thank you for submitting your work to this journal.

With kind regards

Professor Zbigniew Kmieć
zkmiec@gumed.edu.pl
FOLIA HISTOCHEMICA ET CYTOBIOLOGICA

Comments from the Editors and Reviewers: