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Ondřej Gojiš

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

MUDr. Ondřej Gojiš

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Uchazeč: MUDr. Ondřej Gojiš

Adresa: Ústav 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.

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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. 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 calcium channel regulatory subunit α2d-3 (CACNA2D3) in human breast cancer cell lines as well as in clinical samples of primary and metastatic breast cancer.

4. Materials and methods

Patients: In AIB1 and Pax2 study we analysed 30 metastatic breast cancer deposits. 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. In the CEBPδ study we analysed 107 primary breast carcinomas. 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.
Immunohistochemistry: 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. All histological slides were independently checked by two pathologists. 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). 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. 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.
Breast cancer cell lines: In the study were used: SKBR3, MDA-MB231, MDA -MB 453, MDA -MB468, MDA-MB 435, MCF7, T47D, ZR75.1, HCC1937, HS578. 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. Genomic DNA from the metastatic serie was analysed by pyrosequencing for CEBPδ CpG island methylation.
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" for CEBPδ.

Quantification of target transcripts was performed in comparison to the reference transcript $\beta_2$microglobulin 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: 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).

In CACNA2D3 study 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,Reverseprimer:50-biot TCTAACAACAACAACC-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.

5. Results

5.1 SRC-3, Pax2 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. 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 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%). 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%). 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%). 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 ($p = 0.093$).

5.2 CEBPδ study

In CCAAT/enhancer binding protein delta (CEBPδ) study we analysed expression and epigenetic regulation of CEBPδ in a panel of breast carcinoma cell lines. In breast cancer cell lines, methylation was predominantly but not exclusively seen at CG 5 in the fragment analysed by pyrosequencing. Expression was highest in HCC1937 and ZR75.1. Speaking of CEBPδ down-regulation in primary breast carcinomas compared to normal breast tissue, 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. Observing down-regulation of CEBPδ in metastatic breast cancer lesions, we 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. 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. 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. 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. 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. In contrast, metastases in bone and lung were not significantly affected by the methylation status of the CEBPδ CpG island. 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. Than 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. As observed previously, methylation was most dense at CG 4 and CG 5 and was detected in 81% (17/21) of cases.

5.3 CACNA2D3 study

In calcium channel regulatory subunit α2d-3 (CACNA2D3) study 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. CACNA2D3 mRNA was abundantly expressed in the majority of cell lines analysed but was downregulated in MDA-MB-231, MDA-MB-453 and T47D 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. 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. The CACNA2D3 CpG island is methylated in metastatic breast cancer. Using pyrosequencing, we analysed in detail methylation in a series of 18 histological confirmed CNS breast cancer metastases. 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. In the CNS metastases, the level of methylation at each CpG dinucleotide varied markedly within individual lesions. The most frequently and densely methylated CpGs in the amplified fragment were dinucleotides 9 and 10. 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. Using pyrosequencing we analysed CACNA2D3 CpG island methylation. There was increased methylation in almost all cases with downregulation of the mRNA. CACNA2D3 CpG island methylation predicts site-specific relapse in primary breast carcinomas treated with endocrine therapy. Again using pyrosequencing, we analysed ER-positive, ERnegative and triple negative cancers. 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 then performed further analysis in 100 of the cases for which we had complete clinico-pathological information, treatment and clinical outcome. 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. 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. Metastasis to liver and lung was significantly more common in primary carcinomas with methylated CACNA2D3 than in cases lacking methylation. 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. There was no evidence that metastasis to skin or lymph nodes was increased in cases with CACNA2D3 CpG island methylation.

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 charakterizován 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.

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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