Expression of Prostaglandin- and Vitamin D-metabolising Enzymes in Benign and Malignant Breast Cells

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Abstract. *Background: Cyclooxygenase-2 (COX-2) plays a crucial role in prognosis of malignancy and has been associated with carcinogenesis, particularly neoangiogenesis and tumor progression. 15-Hydroxyprostaglandin dehydrogenase (15-PGDH) is described as a tumour suppressor in cancer. The antiproliferative effects of calcitriol [1,25(OH)$_2$D$_3$] mediated via the vitamin D receptor (VDR) render vitamin D a promising target in breast cancer therapy. Materials and Methods: The expression of prostaglandin (PG)-metabolizing enzymes, vitamin D-metabolising enzymes and VDR were determined in benign and malignant breast cell lines using western blot analysis. Results: We detected an inverse correlation between the two types of metabolism, a reduced VDR expression in the malignant breast cell lines, and therefore an insufficient induction of 24-hydroxylase in the malignant cells. Conclusion: We suggest the possibility of dysregulation of vitamin D-metabolizing enzymes in malignant breast cell lines.*

There have been many advances in treatment options for breast cancer, such as targeted therapies with monoclonal antibodies, tyrosine kinase inhibitors, mammalian target of rapamycin (mTOR) antagonists and vaccines. Despite these advances, there are more targets that warrant further exploration. Two promising targets are cyclooxygenase-2 (COX-2), the key enzyme required to convert arachidonic acid to prostaglandins (PG), and calcitriol [1,25(OH)$_2$D$_3$] which is the biologically active form of vitamin D.

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COX-2 is highly expressed in a subset of breast carcinomas and is associated with increased proliferation, invasion, apoptotic resistance and angiogenesis (1, 2). Thus, it is also associated with a poor prognosis (3). Several epidemiologic, preclinical, and clinical studies support a protective role for non-steroidal anti-inflammatory drugs (NSAIDs) that target COX (4, 5). The key PG catabolic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH) is responsible for the degradation of PGs and is known to be a tumor suppressor for different malignancies (6), as well as in breast cancer (7). Multiple studies demonstrated an interaction between PGE_2/COX-2 and the oestrogen receptor (ER) signalling pathway. Salhab and co-workers found a correlation between COX-2 expression and the mRNA expression of the aromatase (8). Moreover, an association between 15-PGDH and the ER signalling pathway was found in breast cells and tissue (7).

It has been suggested that calcitriol could be a possible relevant factor in tumor prevention and therapy (9). Calcitriol is able to suppress tumour growth, inhibit metastasis and prolong survival *in vivo* in animal models (10). Numerous epidemiological studies have suggested that vitamin D has a protective effect against the formation and progression of breast cancer (11, 12). Concerning a correlation between the vitamin D intake and breast cancer risk, conflicting data exists (13-15), however, a recently published prospective randomized trial including 2569 breast cancer patients observed an inverse correlation (16).

Although vitamin D metabolism in normal extra renal cells has been thoroughly investigated (17), alterations of vitamin D metabolism in malignant cells remain to be further elucidated. The alteration of vitamin D metabolism in malignant breast cells in comparison to normal cells has been investigated *in vitro* in different breast cancer cell lines and benign breast cell lines and different types of breast cancer cell lines apparently exhibit different patterns of enzyme and metabolite expression (18).

The aim of the current study was to analyse the expression of PG- and vitamin D-metabolising enzymes, as well as the VDR, in breast cell lines according to their invasiveness. We also wanted to confirm the results from our earlier published data, where we found an expression of lowered VDR- and elevated PG-metabolising enzymes in tumourigenic breast cell lines, as well as in malignant breast tissue (19, 20).

Materials and Methods

Cell culture. The human non-tumorigenic breast cell line MCF-10F (ATCC No. CRL-10318) and the breast cancer cell lines MCF-7 (ATCC No. HTB-22) and MDA-MB-231 (ATCC No. HTB-26) were purchased from the LGC Standards GmbH (Wesel, Germany). MCF-7 and MDA-MB-231 cells were grown in RPMI-1640 medium (25 mM HEPES, 1% L-glutamine; Invitrogen, Karlsruhe, Germany) supplemented with 10% foetal bovine serum (FBS). MCF-10F cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, with 0.04 mM final calcium concentration supplemented with 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone and 5% horse serum (Invitrogen).

RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol. Subconfluently grown cells were trypsinated and pelleted before use. The isolated RNA was quantified spectrophotometrically and its quality was verified by agarose gel electrophoresis. Possible genomic DNA contamination was eliminated using the RNase-Free DNase Set followed by a QIAquick PCR Purification Kit (both by Qiagen)

Reverse transcription and real-time PCR. Total RNA (1 μg) was added to cDNA synthesis with the SuperScript™ II reverse transcriptase and random primers (Invitrogen). Two microlitres of a 1:10 dilution of the cDNA were used for quantitative real-time PCR with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). QuantiTect primer assays were purchased from Qiagen (QT01010170) for human VDR. The primers for the genes of the reference genes TATA box binding protein (*TBP*) and porphobilinogen deaminase (*PBDG*) had the following sequences: *TBP*-forward: 5'-CCA CTC ACA GAC TCT CAC AAC-3', -reverse: 5'-CTG CGG TAC AAT CCC AGA ACT-3'; *PBDG*-forward: 5'-AGC TAT GAA GGA TGG GCA AC-3', -reverse: 5'-TTG TAT GCT ATC TGA GCC GTC TA-3'. Amplification of a single product was verified in a melting curve analysis. A pool of MCF-7 cDNA was used for the experimental calculation of each primer pairs' efficiencies and as calibrator in different runs. The differences in gene expression were calculated according to Pfaffl (21).

Western blotting. Total protein samples were extracted after washing the detached cells with Laemmli-buffer [125 mM Tris, 8% SDS, 30% glycerine and complete mini protease inhibitor cocktail (Roche, Mannheim, Germany)). The lysate was heated for 5 min at 95˚C, cooled on ice, and centrifuged at 13000 rpm for 15 min at 4˚C. The supernatant contained the total protein extract. Protein concentrations were determined with the BCA Assay (Pierce, Bonn, Germany). Protein (40 μg) was applied to 10% SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher Schuell, Dassel, Germany). After blocking in 5% non-fat dry milk in PBST (phosphate-buffered saline, pH 7.2, 0.1% Tween-20) for 1 h at room temperature, the membranes were incubated in blocking reagent overnight at 4˚C with the primary antibodies for human COX-2 and 15-PGDH (IBL, Hamburg, Germany) both at a dilution of 1:1000; VDR antibody (clone 9A7, Dianova, Hamburg, Germany) at 1:10000, CYP-24 (24-OHase) antibody (S-20; SantaCruz, Heidelberg, Germany), the CYP27-B1 (1-α –OHase) antibody (Antibodies-online, Aachen, Germany) both at 1:2000; and a beta-actin antibody (SantaCruz, Heidelberg) was used for normalisation at a dilution of 1:100000. Three washing steps in PBST for 5 min each were followed by incubation with corresponding horseradish peroxidase conjugated antibodies (Amersham Biosciences, Freiburg, Germany) for 1 h (at a dilution of 1:8000, COX-2, 15-PGDH, CYP27-B1, CYP24; 1:20000, VDR; and 1:100000, beta-actin). The ECL detection system (Amersham Biosciences) was used to visualise the signals which were densitometrically analysed in relation to the beta-actin signal.

Measurement of prostaglandin-E_2 in culture media. A PGE_2 ELISA (IBL, Hamburg) was used to quantify the concentrations of PGE_2 in the media of cultivated cells.

Statistical analysis. The statistical analysis of the real-time PCR (q-PCR) and western blots results was performed using Student's *t*-test.

Results

We present only the results of the protein expression (apart from the *VDR* mRNA data), as the RT-PCR data (not shown) are controversial and it seems likely that COX-2 undergoes complex post-transcriptional and post-translational modifications to become the active enzyme (22).

Expression of PG-metabolising enzymes in MCF-10F, MCF-7 and MDA-MB-231 cells. COX-2 protein expression in the MCF-7 cell line was 2.16-fold (±0.30; $p<0.01$) higher, while the COX-2 protein expression in MDA-MB-231 cells was not significantly increased (1.09±0.07) compared to the benign MCF-10F cells as detected in the Western blot analysis. The highest protein expression of 15-PGDH was detected in MCF-7 cells (1.29±0.08) but without statistical significance compared to MCF-10F (1.04±0.14); MDA-MB-231 cells expressed lower levels of 15-PGDH (0.74±0.05) compared to MCF-10F cells (Figure 1).

Quantitation of PGE_2 secretion. PGE_2 concentration in the culture media of the different breast cell lines was measured and found to be 2-fold higher in MCF-10F cells (30.86±3.34 pg/ml) compared to the media of the MDA-MB-231 cell line (72.75±1.90 pg/ml). No PGE_2 was detectable in the media of the MCF-7 cell line (Figure 2).

Expression of VDR and vitamin D-metabolising enzymes. In a quantitative RT-PCR analysis of the *VDR* the malignant MCF-7 cells, and MDA-MB-231 cells expressed 4-fold less (0.23±0.04) and 3-fold less *VDR* mRNA (0.30±0.04) compared to the non-tumorigenic cell line MCF-10F (1.00±0.11), respectively. In the western blot analysis of VDR protein

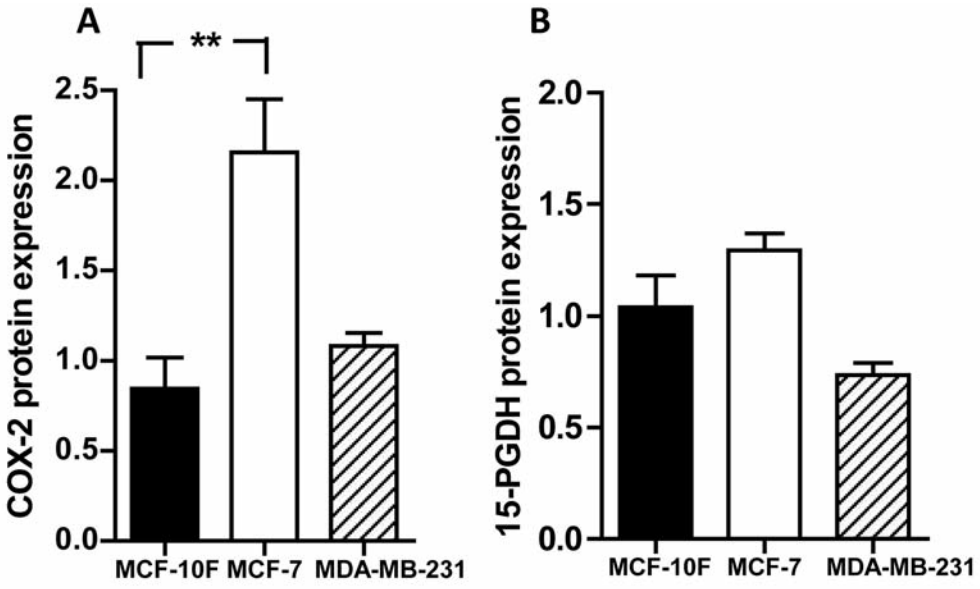

Figure 1. *Cyclooxygenase-2 (COX-2) (A) and 15 hydroxyprostaglandin dehydrogenase (15-PGDH) expression (B) in MCF-10F, MCF-7 and MDA-MB-231 breast cell lines. Protein expression was densitometrically analysed and normalised to β-actin as loading control (b). Differences are significant at **p<0.01.*

expression in the cancer cell lines MCF-7 and MDA-MB-231, expression of only 6% (±1%) and 5% (±1%) was detected in comparison to that of the MCF-10F cells (100%±0.14%). The expression of 1-α-hydroxylase in MCF-7 cells was significantly lower at 13% (±2%) compared to that in the MCF-10F cells. 1-α-Hydroxylase expression in MDA-MB-231 cells was 72% (±8%) that of MCF-10F cells but without statistical significance. The protein expression of 24-hydroxylase in the cancer cell lines MCF-7 and MDA-MB-231 was 22.5% (±3.8%) and 6.2% (±1%) that of the benign MCF-10F cell line, respectively (Figure 3).

Discussion

We observed sparse protein expression of the VDR and accordingly a lower expression of 24-hydroxylase in the malignant breast cell lines. This might imply a dysregulation of vitamin D metabolism and in turn might lead to a response failure to tumor-protective vitamin D in malignant breast cell lines.

We detected an elevated COX-2 expression in non-invasive MCF-7 cells and in the invasive MDA-MB-231 tumorigenic cell line compared to benign MCF-10F cells. Half and co-workers analysed MCF-7 and MDA-MB-231 cells and revealed no COX-2 expression in MCF-7 cells and only a very low expression in MDA-MB-231 cells (23). Similar results were presented by Denkert *et al*. (24). Two other studies by Basu *et al*. (25, 26) analysed malignant breast cancer cells using western blot analysis. In addition to MCF-7 and MDA-MB-231 cells, the authors examined moderate invasive and

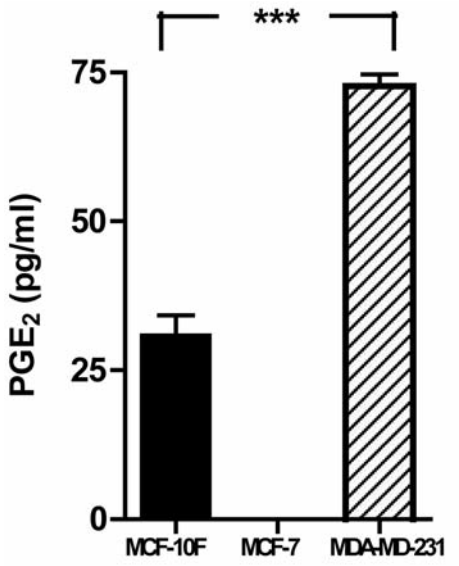

Figure 2. *Prostaglandin E_2 (PGE_2) secretion measured in the media of MCF-10F, MCF-7 and MDA-MB-231 breast cell lines. Differences are significant at p<0.001.*

ER-negative MDA-MB-435 cells and detected a correlation of the invasiveness with a decrease of COX-2 expression. MCF-7 cells expressed no COX-2 (25), moreover they analysed the moderately invasive ER-negative MDA-MB-468 and MDA-MB-231 cells, with the same result (26).

Regarding 15-PGDH expression, only marginal differences were shown between the three cell lines, however, we did observe a non-significant lower expression in MDA-MB-231

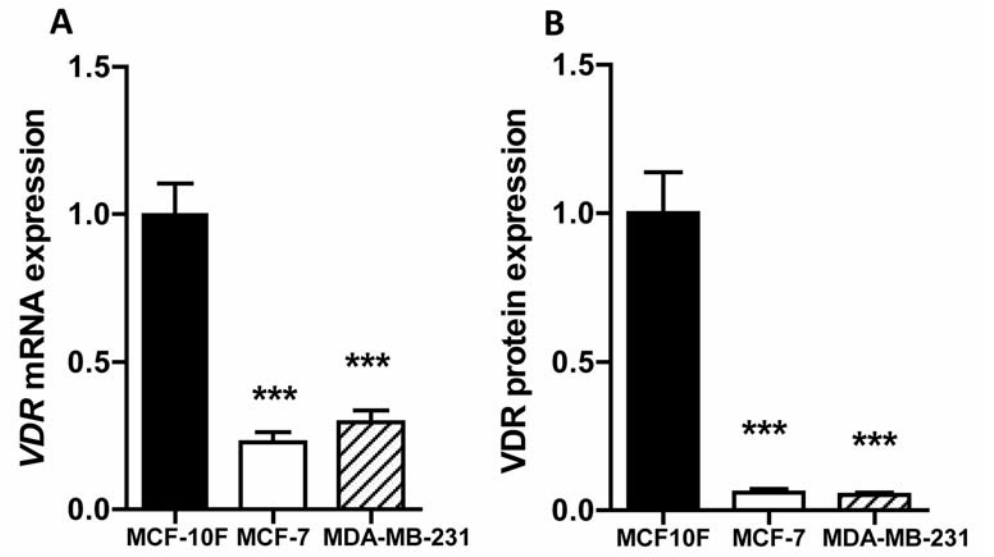

Figure 3. *Vitamin D receptor (VDR) expression in MCF-10F, MCF-7 and MDA-MB-231 breast cell lines. VDR mRNA level was quantified using real-time PCR (A) and protein level (B) was densitometrically analysed and normalised to β-actin as loading control. Differences are significant at ***p<0.001.*

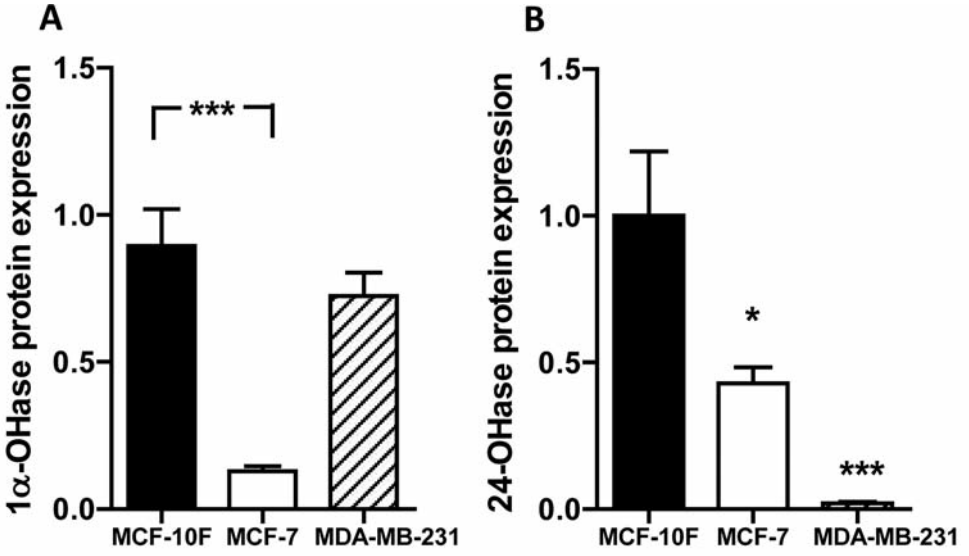

Figure 4. *1-α-Hydroxylase (A) and 24-hydroxylase expression (B) in MCF-10F, MCF-7 and MDA-MB-231 breast cell lines. Protein expression was densitometrically analysed and normalised to β-actin as loading control. Differences are significant at ***p<0.001.*

cells compared to the other two cell lines, but the highest expression was measured in MCF-7 cells. In contrast to these results, Wolf and co-workers found a significantly lower expression in all but MCF-7 cells (7) and Celis *et al*. were unable to detect 15-PGDH expression in MDA-MB-231 and MCF-7 cells by 2D PAGE analysis (27).

PGE_2 is synthesised by COX-2 and therefore, a higher PGE_2 serum level should be expected when overexpression of COX-2 occurs (20, 28). Accordingly, PGE_2 secretion was doubled in MDA-MB-231 tumor cells compared to MCF-10F cells. Interestingly, no secretion of PGE_2 was detected in MCF-7 cells. These results confirm the findings of Schrey and Patel who detected PGE_2 secretion solely in the MDA-MB-231 cells when analysing seven different breast cell lines (29).

In the present study the VDR expression was significantly lower in the malignant cell lines compared to that in the MCF-10F cell line, and both were analysed by western blot and RT-PCR. These data are confirmed by the results of Kemmis *et al*., who analysed different cell lines (MCF-7, HKC-8, human mammary epithelial cell HMEC) and found the highest VDR expression in the benign HMEC (30). Furthermore, Buras *et al*. suggested that VDR expression is lower in oestrogen-

independent, less differentiated lines compared to oestrogen-dependent, well-differentiated lines, such as MCF-7 (31). Therefore, this is another confirmation of our findings. Based on the current data, we suggest that increasing invasiveness of breast cell lines leads to a significant reduction or loss of VDR.

The VDR plays a role both in the activation of *CYP24* and in the deactivation of *CYP27B1*. On one hand, high serum levels of calcitriol block its own synthesis, and on the other hand, high levels induce its own inactivation in terms of a classic feedback mechanism. Calcitriol binds to the VDR, induces 24-hydroxylase, which leads to an inactivation of calcitriol. Regarding 1-α-hydroxylase protein expression, the highest expression was detected in the MCF-10F cells and the lowest in the MCF-7 cell line. The difference between both the malignant cell lines was significant because the MDA-MB-231 cell line had a higher expression than did the MCF-7 cells. These different findings might be explained by the existence of splicing variants of 1-α-hydroxylase (32, 33), 24-hydroxylase, as well as of VDR (34, 35), as an alternative splicing of 1-α-hydroxylase may regulate the level of active enzymes and splice variants could lead to a reduction of the protein (32).

Compared to the MCF-10F cell line a significantly lower 24-hydroxylase expression was measured in MCF-7 cells; in MDA-MB-231 cells, the expression was only sporadically detectable. These findings correlate especially with the VDR protein expression, as the VDR had the lowest expression in the MDA-MB-231 cell line. Regarding the positive feedback between VDR and 24-hydroxylase, we did not expect any 24-hydroxylase expression because of the low VDR expression. These findings are corroborated by Cordes *et al*., as they described a significantly reduced 24-hydroxylase expression in breast cancer tissue (36).

COX inhibitors have been shown to suppress breast cancer cell growth both *in vivo* and *in vitro* (37). The hypothesis of breast cancer prevention after constant intake of NSAIDs is supported by a recently published meta-analysis of 38 epidemiological studies that showed a decrease in breast cancer risk on their use (38). Additionally, calcitriol or vitamin D analogs have an antiproliferative and proapoptotic effect on cancer cells and tissues (39). Moreover, our own data suggest a possible link between the PG and vitamin D metabolism in breast cancer (19, 20). Therefore, the possibility of a synergistic action using a combination of calcitriol and NSAIDs for treating cancer cells should be considered. There is data regarding prostate cancer proposing that a combination of calcitriol and COX inhibitors might be a useful therapeutic strategy (40).

In the light of our findings, we suggest a possible dyregulation of vitamin D metabolism in breast cancer cell lines. Regarding this suggestion, the therapeutic value of calcitriol or vitamin D analogs might be less than expected and the idea of a chemopreventive approach is more appropriate. Data on the clinical use of calcitriol are sparse and the results are disappointing (41). Thus, further work is required to examine the effect of calcitriol, COX inhibitors and the combination of both on breast cell lines and tissue, and moreover to establish how NSAIDs, calcitriol and vitamin D analogs can best be applied for therapeutic or even better for a chemopreventive benefit.

Conflict of Interest Statement

The Authors declare no conflict of interest relevant to this article.

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