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25-Hydroxyvitamin D_3 1α-Hydroxylase Splice Variants in Breast Cell Lines MCF-7 and MCF-10

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Abstract. *Background: It is known that 25(OH)D_3 can be metabolized to 1,25(OH)$_2D_3$ by 1α-OHase in breast tissue. This tissue-specific expression of 1α-OHase may act as the pivotal link between vitamin D status (25(OH)D_3 levels) and the anticancer effects of 1,25(OH)$_2D_3$. Alternative splicing frequently occurs in breast cancer cells; different splice variants of a given protein can display different biological functions and may cause tissue-specific variations. With this study it is the first time that expression and alternative splicing of 1α-OHase in the human breast cancer cell line MCF-7 and thebenign breast cell line MCF-10A are described. Materials and Methods: Expression of 1α-OHase RNA and protein was assessed using a real-time polymerase chain reaction (RT-PCR). The expression of 1α-OHase splice variants was detected by a highly specific PCR that combines nested and touchdown PCR. To determine which variants are translated in protein western blot analysis was carried out. Results: The expression of 1α-OHase was found to be 1.25-fold higher in MCF-7 compared to MCF-10A cells. In MCF-10A cells, at least 6 splice variants were detected whereas MCF-7 showed no or marginal expression levels of these variants. In MCF-7 cells the antibody detected a signal at 56 kDa corresponding to the size of normal 1α-OHase protein. In MCF-10A cells this signal was weaker. In western blot analysis at least two smaller variants at 45 kDa were found in MCF-7 cells. In MCF-10A cells at least 6 proteins between 37 and 56 kDa were detected with an only faint signal. Conclusion: We propose that alternative splicing of 1α-OHase can regulate the level of active enzyme. Splice variants may lead to a reduction of the protein. The significance of the smaller variants in MCF-7 cells has not been clarified either, but it is known that they are not able to use 25(OH)D_3 as a substrate to generate 1,25(OH)D_3. In MCF10A cells, more splice variants were identified, it may be that malignant cells contain inactive variants. How far they show a reduced activity remains unclear as no activity measurements were performed.*

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Breast cancer is the most common cancer and cause of death from cancer for women in the U.S. and Europe (1). Many factors have been related to various breast cancer risks including vitamin D_3 synthesis in the skin due to sunlight exposure or dietary intake. Several studies show that vitamin D_3 status might be inversely associated with breast cancer risk (2). The biologically active metabolite of vitamin D_3 is 1,25-dihydroxyvitamin D_3 (1,25(OH)$_2D_3$). There are two principal enzymes involved in the formation of circulating 1,25(OH)$_2D_3$ from vitamin D_3: the hepatic vitamin D-25-hydroxylase (25-OHase) and the renal 25-hydroxyvitamin D-1α-hydroxylase (1α-OHase) for vitamin D and 25-hydroxyvitamin D_3 (25(OH)D_3), respectively (3). Both 25(OH)D_3 and 1,25(OH)$_2D_3$ can be degraded through the catalysis of vitamin D 24-hydroxylase (24-OHase) (4).

In the kidney, 1α-OHase and 24-OHase form a classic feedback mechanism. Dietary intake and exposure to sunlight significantly influence the circulating concentration of 25(OH)D_3 which is considered to be a reliable indicator of the availability of vitamin D from diet and synthesis in the skin (5). In contrast, the circulating concentration of 1,25(OH)$_2D_3$ is tightly regulated by renal 1α-OHase and therefore kept within a relatively narrow range. Plasma levels of 25(OH)D_3 and risk of breast cancer were found to be inversely correlated in several studies (6-8). For women with plasma 25(OH)D_3 concentration <50 nmol/l the risk of breast cancer is more than 5-fold higher than for those with plasma concentration exceeding 150 nmol/l (7).

The fact that 25(OH)D_3 can be metabolized to 1,25(OH)$_2D_3$ by 1α-OHase in breast tissue also indicates the potential importance of 25(OH)D_3 in breast carcinogenesis (9). It has been shown that various epithelial cells, such as those in the prostate, breast and colon express 1α-OHase (10), whereas circulating 1,25(OH)$_2D_3$ produced by these extrarenal tissues were not be observed in anephric conditions (Welsh J *et al.,* 2003). Vitamin D_3-mediated growth control is

assumed to be influenced in terms of autocrine and paracrine processes by local production of $1,25(OH)_2D_3$. According to the respective data, extrarenal expression of 1α-OHase is caused by the same gene (CYP27B1) as that for the proximal tubules. However, it is not thought to be subject to same exquisite autoregulation characteristics of the renal enzyme (12, 13). Tissue-specific expression of 1α-OHase may therefore act as the pivotal link between vitamin D status ($25(OH)D_3$ levels) and the anticancer effects of $1,25(OH)_2D_3$.

Different splice variants of a given protein can display different biological functions and may cause tissue-specific variations in healthy cells. A number of studies show that alternative splicing occurs frequently in human cancer cells (*e.g.* breast and ovarian cancer) (14, 15). Recently, 16 splice variants (Hyd-V1 –Hyd-V16) of 1α-OHase mRNA were described in glioblastoma and melanoma cell lines (16-18).

In this study we describe for the first time expression and alternative splicing of 1α-OHase in the human breast cancer cell line MCF-7 and in the benign breast cell line MCF-10A.

Materials and Methods

Cell culture. The human benign breast cell line MCF-10F (ATCCC No. CRL-10318) and breast cancer cell line MCF-7 (ATCC No. HTB-22) were purchased from the European Collection of Cell Culture (Wiltshire, UK). MCF-7 cells were maintained in RPMI 1640 medium (GIBCO-BRL, Karlsruhe, Germany) supplemented with 25 mM HEPES, 1% L-glutamine and 10% foetal bovine serum (FBS); MCF-10F cells were kept in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium supplemented with 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone and 0.04 mM final calcium concentration, 5% horse serum (Invitrogen Karlsruhe, Germany).

RNA and poly (A)-RNA isolation. Total RNA from MCF-10F and MCF-7 cells were extracted from several passages of cell culture with TRIZOL (Invitrogen) according to the manufacturer's instruction. The total RNA amount was quantified spectrophotometrically and its integrity was ascertained using 1% agarose gel electrophoresis in MOPS buffer. A quantity of 150 μg of total RNA was used to isolate poly (A)-RNA according to the manufacturer's instructions (Oligotex mRNA, Qiagen, Hildesheim, Germany).

Reverse transcription. Prior to utilization, RNA was DNase I (Invitrogen, Karlsruhe, Germany) treated. First-strand cDNA was synthesized with Omniscript reverse transcriptase (Quiagen) and oligo-$d(T)_{15}$ primer (Invitrogen).

Nested "touchdown" PCR. The first PCR was performed using primers Sp1aFOR1 (5'-GGAGAAGCGCTTTCTTTCG-3') and Sp1aRev3 5'-TGGGGCAAACCCACTTAATA-3') with 10 cycles (2 min at 98˚C, 15 sec at 94˚C, 20 sec at 68˚C, 4 min at 68˚C, 15 min at 68˚C). The PCR product was purified (Nucleo Spin Extract II; Machery-Nagel, Düren, Germany) and 5 μl template were used for the second PCR using primers HE1 (5'-CAGACCCTCAAGTACGCC-3') and Sp1aRev2 (5'-AAACCAGGCTAGGGCAGATT-3'). This PCR consisted of 12 cycles (30 sec at 96˚C, 10 sec at 94˚C, 20 sec with a touchdown from 68˚C to 62˚C in 0.5˚C intervals, 4 min at 68˚C) followed by 18 cycles (10 sec at 64˚C, 20 sec at 62˚C, 4 min at 68˚C). The PCR reactions were performed using 2.5 Units RedACCu Taq™ LA DNA polymerase (Sigma, Munich, Germany). The obtained PCR products were separated on a 1% agarose gel.

Plasmid isolation and sequence analysis. Cloning of PCR products in vector pCR4-TOPO was performed using TOPO TA Cloning Kit for sequencing (Invitrogen) and carried out according to the manufacturer's instructions. A column purification was added for plasmids that were sequenced (Plasmid Mini Kit; Qiagen).

Sequencing was performed according to the manufacturer's instructions using the Big Dye Cycle Sequencing Kit (Applied Biosystems; Foster City, USA). Plasmid inserts were sequenced with an automated sequencer (ABI310). The obtained sequences were edited using Sequencher™ 3.0 program (Genecodes, Michigan, USA). Homology search was performed with the BLASTN algorithms.

Real time PCR. A volume of 2 μl of RT reaction mixture were used as the template for real-time PCR and 0.5 mmol/l primers (HPRT forward 5'-CCT GGC GTC GTG ATT AGT GAT-3', reverse 5'-CCA GCA GGT CAG CAA AGA ATT TA-3'; 1α-OHase forward: 5'-TGT TTG CAT TTG CTC AGA-3', reverse: 5'- CCG GGA GAG CTC ATA CAG-3'). After adding 25 μl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), the volume was adjusted to 50 μl with nuclease-free distilled water. The samples were amplified in the DNA Engine opticon 2™ (Biorad; Hercules, USA) System and PCR was performed by an initial denaturation step at 50˚C for 2 min and 95˚C for 2 min followed by 50 cycles with a denaturation step at 95˚C for 15 sec, primer annealing at 57.3˚C (1α-OHase) and 60˚C (HPRT) for 15 sec and an extension phase at 72˚C for 15 sec for 1α-OHase and HPRT.

A melting curve was generated after 50 cycles for the final PCR product of all genes investigated by decreasing the temperature to 65˚C for 15 sec followed by a slow increase in temperature to 95˚C. During the slow heating process the fluorescence was measured at 0.2˚C increments. To obtain relative gene expression data (fold change) between MCF10 and MCF7, the comparative $2^{-\Delta\Delta Ct}$ method (Livak KJ *et al.* 2001) was used. The fold change was determined with the formula: fold change = $2^{-\Delta(\Delta Ct)}$, where ΔCt = Ct,1α-OHase-Ct, HPRT; Δ(ΔCt) = ΔCt, MCF7-ΔCt, MCF10. The experiments were performed in triplicates for each gene.

Statistical significance. Statistical analysis of real-time PCR results was performed using Student's *t*-test and the normalized cycle threshold (delta/delta CT) values.

Western Blot. Cells were harvested, washed twice with PBS and lysed in sample buffer (125 mM Tris, 30% Glycerine, 8% SDS, PH 6.8). A total of 20 μg of protein was subjected to 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. After separation, proteins were transferred to a nitrocellulose membrane (Optitran BA-S 85; Schleicher Schuell, Dassel, Germany). The membranes were blocked with phosphate-buffered saline tween (PBST) containing 5% non-fat powdered milk for 1h at room temperature. Membranes were labelled with primary antibodies against human 1α-OHase (Biologo, Kiel, Germany) at a dilution of 1:2000 overnight at 4˚C. The secondary

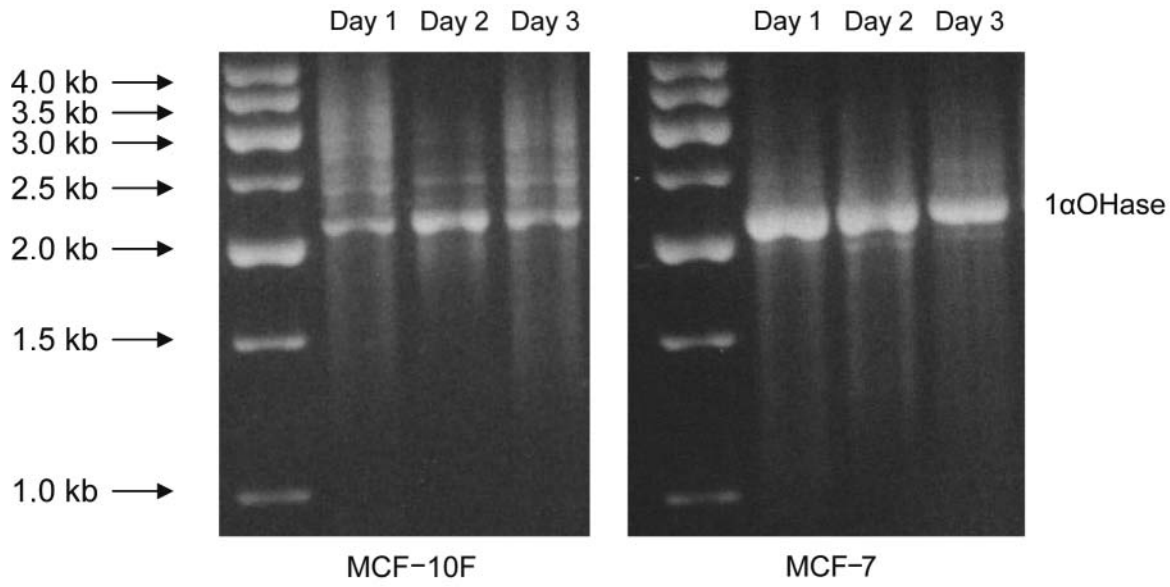

Figure 1. *Nested touch-down PCR of MCF-10 and MCF-7 cell PCR-products at 2.2 kb correspond to the normal 1α-OHase transcript. In MCF-10 cells there are many PCR products between 2.2 and 4.0 kb representing variants with insertion of different introns. MCF-7 cells lack these PCR products but there is a smaller variant at 1.9 kb which represents the new variant Hyd-V17. RNA was isolated at different time-points to analyze whether there was any change of splice pattern at different stages of confluence in the cell culture.*

antibodies conjugated to horseradish peroxidase (anti-mouse IgG; Amersham Biosciences, Freiburg, Germany) were added at a dilution of 1:6000. After several washing steps, bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, Freiburg, Germany). The obtained signals were compared to β-actin as internal standard.

Results

Quantitative RT-PCR was used to analyze the expression of 1α-OHase in MCF-7 and MCF-10A cell lines. RNA was isolated at 5 different passages of both cell lines and measured using real-time PCR in triplicates. The relative amount of transcript in MCF-7 cell was 0.6-fold (0.35-1.00) less than in MCF-10 cells but with low statistical significance ($p=0.057$).

To investigate the expression of 1α-OHase splice variants in MCF-7 and MCF-10A cells, we used a highly specific PCR that combines nested and touchdown PCR (16). Various splice variants were found in both cell lines but the overall pattern was different (Figure 1). MCF-7 cells showed a higher level of normal transcript (2.15 kb). Only faint bands of smaller variants with deletions in different exons were seen in both cell lines. The greatest difference in the splice pattern could be detected between 2.5 and 4 kb. This could represent splice variants HydV8 to HydV16, with insertions of one or several entire introns. In MCF-10A cells, at least 6 splice variants were detected, whereas MCF-7 showed no or marginal expression levels of these same variants. To analyze if the PCR-products were real transcripts of 1α-OHase we isolated PCR products and sequenced them. We identified variants with Intron 1 and a new one (Hyd-V17) with deletion of Exon 3 to 5 (17.6 kDa) that has not yet been described.

To determine which variants are translated in protein we carried out Western blot analysis using a polyclonal antibody generated by utilizing a peptide located in exon 5 of murine 1α-OHase (Figure 2). In MCF-7 cells the antibody provides a signal at 56 kDa that corresponds to the size of normal 1α-OHase protein. In MCF-10A cells, this signal is weaker indicating a lower level of active enzyme in these cells. Just like the nested PCR, the overall pattern of Western blot analysis was different. In MCF-7 cells there were at least two smaller variants, one at 45 kDa, possibly representing variants HydV3 and/or HydV5, and another at 17 kDa possibly representing variants HydV17. In MCF-10A cells, at least 6 proteins between 37 and 56 kDa were be detected but the signals were only faint.

Discussion

The aim of this study was to investigate whether there is a difference in the splice pattern in cell culture of malignant and normal breast cell lines.

We found MCF-7 cells to show a different splice pattern from MCF-10 cells. At the RNA level as well as at the protein level, both cell lines differed from each other. It is known that MCF-7 cells express 1α-OHase and that they are also able to convert $25(OH)D_3$ or analoga (EB1285) into $1,25(OH)_2D_3$ or EB1089, respectively (19, 20).

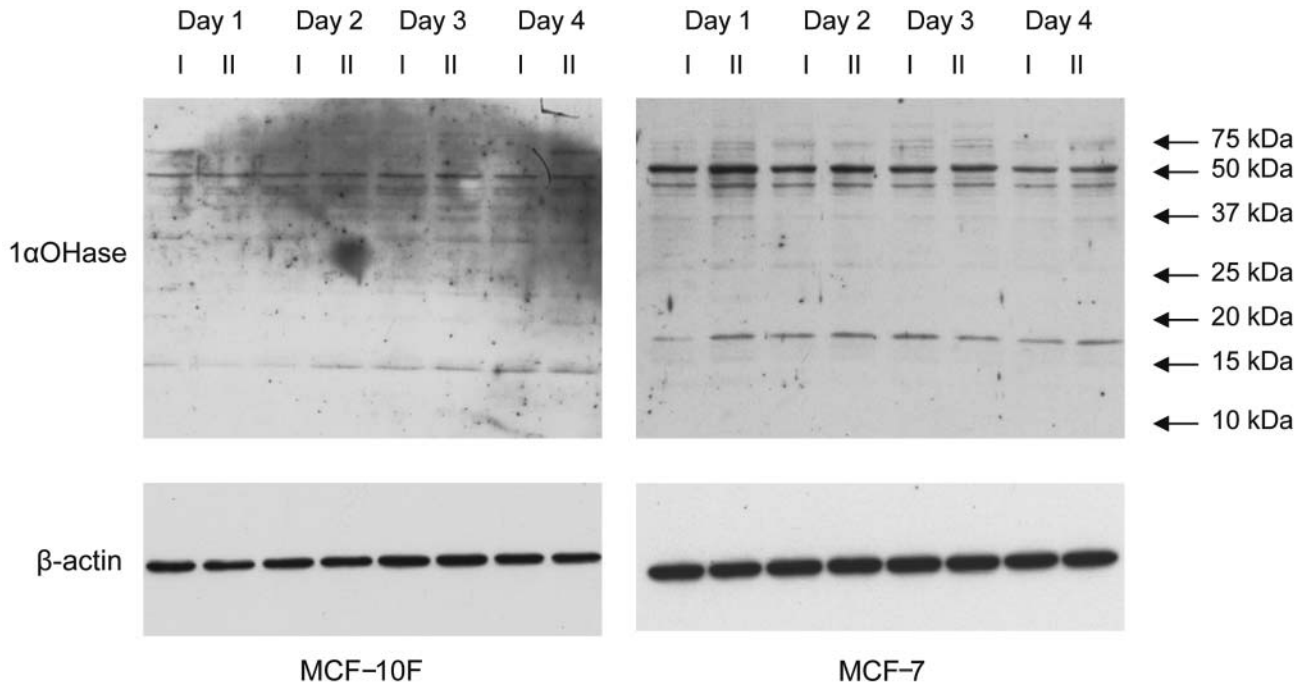

Figure 2. *Western blot of MCF-10F and MCF-7 cells. The signal at 56 kDa corresponds to the size of normal 1α-OHase protein. As shown for the nested touch-down PCR, there is a clear difference in the splice pattern of MCF-10 and MCF-7 cells. In MCF-7 cells, the full length active enzyme is obvious and a smaller protein at 17 kDa is apparent. Cells were cultivated for four days. No differences were seen in the splice pattern.*

We suggest that alternative splicing of 1α-OHase can regulate the level of active enzyme either through generating inactive shorter protein variants or generating variants with intron sequences and an early stop codon. In accordance with earlier studies, we did not detect a short 9 kDa peptide in MCF-7 or in MCF-10A even though we see PCR products representing variants with intron 1. However, this approach did not allow detailed quantitative measurements due to the high product variation.

The significance of variants with Intron 1 remains unclear. Intron retention is common in plants (21) but rare in higher eukaryotes (22, 23). Often retention of internal introns makes them the targets for degradation by the cellular nonsense-mediated decay machinery if they contain premature stop codons. However, an alternative spliced noncoding transcript of the steroid receptor RNA activator (SRA), containing the first intron, has been identified and the relative proportion of these RNAs varied within breast cancer cell lines (24). Therefore, a post transcriptional regulation or a so-called fine tuning of the transcript amount can be assumed. This finally regulates the amount of active enzyme. Splice variants, which appear as mRNA in the cell, compete for the ribosome's binding site. This way they may lead to a reduction of the protein. This theory is supported by a weaker band at 56 kDa in MCF-10 cells in the analysis *via* Western blot; these represent the active enzyme. From these data we can conclude a lower amount of protein. In contrast, the relative amount of transcript measured with real time PCR in MCF-7 cell is 0.6-fold less than in MCF-10 cells. The existence of many splice variants may be a reason for the different results, so that the real-time PCR will not detect all transcripts and will not reflect the total amount of mRNAs.

The significance of the smaller variants (45 and 17 kDa) in MCF-7 cells has not been clarified either. As all variants lack the active centre, they are not able to use $25(OH)D_3$ as an substrate to generate $1,25(OH)D_3$. It is unknown to what extent these proteins influence proliferation, differentiation or calcium metabolism. Examinations regarding the activity of splice variants only showed that HydV2 (without Exon 4 and 5) as well as HydV4 with an inframe insertion of Intron 2 are not able to convert $25(OH)D_3$ (16).

It is known that $1,25(OH)_2D_3$, depending on its concentration, either stimulates proliferation (low concentration, 10^{-12} molar) or represses it (10^{-6} to 10^{-8} molar) (25). Changes in splice patterns and the generation of inactive variants therefore could lead to different effects of $1,25(OH)_2D_3$, without regulating the expression *via* the promotor activity.

In an earlier study, we investigated the expression of 1α-OHase in the MCF-7 cell line and breast cancer tissue (20). Consistent with other studies, mRNA levels of 1α-OHase were significantly increased in breast cancer compared to normal breast tissue and incubation of MCF-7 cells with [3H]-$25(OH)D_3$ resulted in its conversion to [3H]-$1,25(OH)_2D_3$

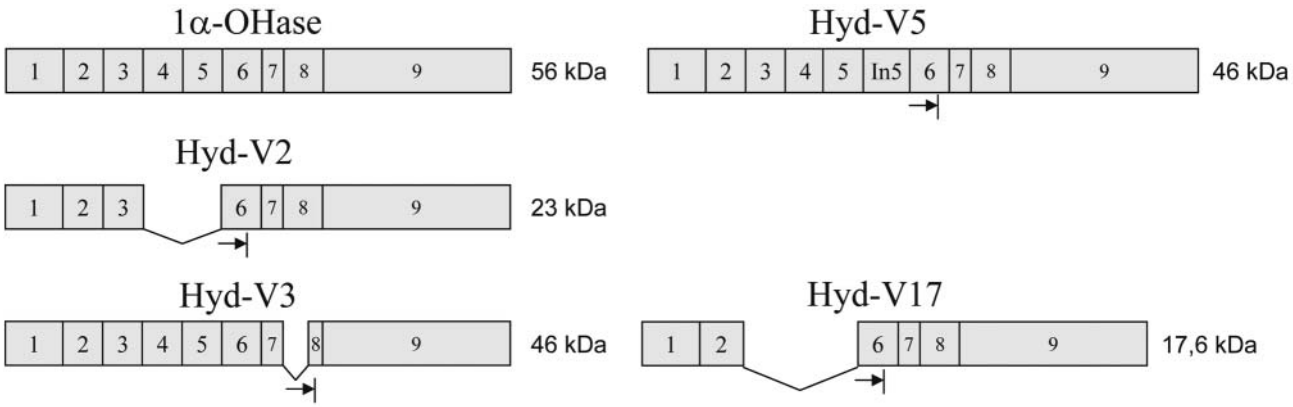

Figure 3. *Exon organisation of different splice variants including the new splice variant Hyd-V17. Arrows are premature stop codons and sizes of potential resulting proteins are indicated in kDa.*

(20, 26, 27). Enzyme activity of 1α-OHase was also increased in tumors compared with non-neoplastic tissue as measured with the same assay (28). In addition, it was shown that MCF-7 cells which express the 1α-OHase enzyme are able to convert the non 1α-hydroxylated prodrug of EB1089 (EB1285) and activate transcription *via* vitamin D_3 response elements in a reporter construct (19). Finding the activation of transcription being ketoconazole-sensitive, it was highly expected that the observed effects were due to 1α-hydroxylation. However, the transcription activation potential of EB1285 is significantly lower than that of EB1089 in MCF-7 cells. This may be caused by a low 1α-OHase enzyme activity in the cells, or a possibly inefficient hydroxylation due to steric hindrance between the substrate and the enzyme. It could also be provoked because of an alternative splicing (19). However, Segersten *et al.* demonstrated no increase of 1α-OHase expression in breast cancer compared to normal tissues (19).

Hewison *et al.* found that 1α-OHase in malignancies show more activity than in benign tissue (27). At the same time, a higher expression of enzymes was verified than in healthy tissue. However, Segersten (19) did not find an increase compared to the normal tissue, admittedly without performing any activity measurements.

A reason for the different results could be that Hewison (27) did not use benign breast tissue as reference tissue. In cancer research, a major issue is the lack of standardization in sample preparation and analysis, which affects the comparability of data. This would change the results of expression analyses. 1α-OHase expression has been shown using immunohistochemistry and at the same time the higher activity was proved in case of a higher expression. The significance of immunohistochemistry on its own would be limited, being a qualitative not quantitative procedure.

Our examination showed only a slight increase in expression in MCF-7 cells in comparison to MCF-10A cells. How far the MCF-10A cells show a lower activity because of a higher share of inactive splice variants compared to MCF-7 cells is questionable as we did not perform any activity measurements. The discrepancy between the respective examinations could also be explained by the existence of the different splice variant pattern: In MCF-7 cell lines only one splice variant was identified *via* cloning and sequencing. Therefore it may be that malignant cells contain inactive variants (data not published).

Conclusion

The examination regarding autocrine or paracrine effects of $1,25(OH)_2D_3$ respectively turns out to be more and more interesting in connection with protective and growth reducing attributes of the hormone. A reduced absorption or synthesis of vitamin D_3, characterized by a low $25(OH)D_3$ concentration in the blood is considered to be related to the development of breast cancer. It is unknown if breast tissue in general has a reduced 1α-OHase activity in comparison to other native cells, which would explain the higher potential for malignant degeneration. Low concentrations of $25(OH)D_3$ in connection with a reduced activity of 1α-OHase could significantly increase the risk of breast cancer. In women exposed to higher UV-radiation, the lower activity of 1α-OHase was partially neutralized by a higher supply of vitamin D_3 (29). As 1α-OHase in normal breast cells firstly expresses less and secondly is spliced differently, it shows a lower activity there. Especially with regard to the high number of splice variants, each therapeutic concept for the use of vitamin D analogs in breast cancer treatment should take into consideration the complexity of vitamin D metabolism.

Acknowledgements

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