

## 25-Hydroxyvitamin D<sub>3</sub> 1 $\alpha$ -Hydroxylase Splice Variants in Benign and Malignant Ovarian Cell Lines and Tissue

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**Abstract.** Calcitriol is judged to have a positive effect on control of the immune system, cell growth and differentiation and therefore, the prevention of cancer genesis. The aim of this study was to detect any possible differences in the 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1 $\alpha$ OHase)-expression between benign and malignant ovarian tissue and cell lines. The analysis was conducted quantitatively, by means of nested "touchdown" PCR and Western blot, and qualitatively, with the use of real-time PCR and Western blot. The gene structure was sequenced. Compared to the benign cell line, the malignant cell lines showed a significantly higher expression of 1 $\alpha$ OHase at the RNA level. A statistically lower expression of the 1 $\alpha$ OHase protein was found in the malignant tissue. In the malignant cell lines and tissues, divergent bands were detected, which led to various splice variants on sequencing. Their increased expression in malignancy is possibly bound to the reduction of enzyme activity, which may lead to the genesis of ovarian cancer. In the future, preventive and therapeutic activities may result from these findings.

Epithelial ovarian cancer is the most common cause of death from gynaecological malignancies for women in the U.S. and Europe. Many factors have been linked with various ovarian cancer risks, such as the intake of oral contraceptives, vitamin D receptor gene polymorphisms and dietary intake of calcium (1-3). Some studies have shown that the status of vitamin D<sub>3</sub> might be inversely associated with the risk of ovarian cancer in certain populations (4). The biologically

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active metabolite of vitamin D<sub>3</sub> is 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>). There are two principal enzymes involved in the formation of circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> from vitamin D<sub>3</sub>: the hepatic vitamin D-25-hydroxylase (25-OHase) and the renal 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1 $\alpha$ OHase) for vitamin D and 25-hydroxyvitamin D<sub>3</sub> (25(OH)D<sub>3</sub>), respectively (5). Both 25(OH)D<sub>3</sub> and 1,25(OH)<sub>2</sub>D<sub>3</sub> can be degraded through the catalysis of vitamin D 24-hydroxylase (24-OHase) (6).

Calcitriol causes an advancement of the innate as well as a dampening of the acquired immune reaction (7). The fact that 25(OH)D<sub>3</sub> can be metabolized to 1,25(OH)<sub>2</sub>D<sub>3</sub> by 1 $\alpha$ OHase in malignant tissue indicates the potential importance of 25(OH)D<sub>3</sub> in carcinogenesis (8) and details of the underlying mechanisms are partially known (9, 10).

It has been shown that various epithelial cells, such as those in the prostate, breast and colon, express 1 $\alpha$ OHase (11, 12), whereas circulating 1,25(OH)<sub>2</sub>D<sub>3</sub> produced by these extrarenal tissues could not be observed in anephric conditions (13). Vitamin D<sub>3</sub>-mediated growth control is assumed to be influenced in terms of autocrine and paracrine processes through the local production of 1,25(OH)<sub>2</sub>D<sub>3</sub>. According to the respective data, the extrarenal expression of 1 $\alpha$ OHase is encoded by the same gene (12q13.1-13.3) as in the proximal tubules. However, it is not assumed to be subject to the same exquisite autoregulation characteristics of the renal enzyme (14, 15). The tissue-specific expression of 1 $\alpha$ OHase may therefore act as the pivotal link between vitamin D status (25(OH)D<sub>3</sub> levels) and the anticancer effects of 1,25(OH)<sub>2</sub>D<sub>3</sub>.

Miettinen *et al.* proved a growth repressive effect of calcitriol at high concentrations (10 nM, 100 nM) in the ovarian cancer cell line OVCAR-3, whereas low concentrations induced growth (16).

The degree of 1 $\alpha$ OHase expression is linked to the immunological cells of an inflammatory infiltrate in connection with malignant tumours (17). In addition, splice variants may also play a role in carcinogenesis. They possess different biological functions and may cause tissue-specific

variations in healthy cells. A number of studies have shown that alternative splicing occurs frequently in cancer cells (e.g. breast, endometrial and ovarian cancer) (18-22). Sixteen splice variants (Hyd-V1 -Hyd-V16) of 1 $\alpha$ OHase mRNA have been described in glioblastoma and melanoma cell lines (23-25). Almost all these splice variants result in catalytically inactive enzymes (24, 26).

In this study, the expression and alternative splicing of 1 $\alpha$ OHase in ovarian benign and malignant cell lines and analogous tissue is described.

## Materials and Methods

**Cell culture.** The human benign granulosa cells (GLZ) originated from women who had to be treated hormonally in the context of *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) at the Department of Obstetrics and Gynecology, University Hospital of Lübeck (Germany). Ovarian cancer cell lines HGL5 and COV434 were kindly provided by Mrs. Sonntag from the Department of Obstetrics and Gynecology, University Hospital of Münster (Germany).

The granulosa cells and HGL5 cells were maintained in RPMI (Gibco, Karlsruhe, Germany) supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin and 2% Ultrosor G (Pall Bio Septra, Cergy St Christophe, France) and the COV434 cells in DMEM medium (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin.

**Tissue samples.** Benign and malignant ovarian tissue was extracted from women whose ovaries had to be excised because of ovarian cancer or benign ovarian disease and were used with the consent of the women. The tissue samples were frozen in liquid nitrogen at  $-80^{\circ}\text{C}$ .

**RNA and poly (A)-RNA isolation.** The total RNA was extracted from three different passages of cell culture and of several benign and malignant tissue samples with Trizol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The total RNA was quantified spectrophotometrically and 100  $\mu\text{g}$  were used to isolate the poly (A)-RNA according to the manufacturer's instructions (Oligotex mRNA; Qiagen, Hildesheim, Germany).

**Reverse transcription.** First-strand cDNA was synthesized with Omniscript reverse transcriptase (Quiagen) and oligo-d(T)<sub>15</sub> primer (Invitrogen) according to manufacturer's instructions.

**Nested "touchdown" PCR.** The first PCR was performed using primers Sp1aFOR1 (5'-GGAGAAGCGCTTTCTTTTCG-3') and Sp1aRev3 (5'-TGGGGCAAACCCACTTAATA-3') with 10 cycles (2 min at  $98^{\circ}\text{C}$ , 15 s at  $94^{\circ}\text{C}$ , 20 s at  $68^{\circ}\text{C}$ , 4 min at  $68^{\circ}\text{C}$ , 15 min at  $68^{\circ}\text{C}$ ). The PCR product was then purified (Nucleo Spin Extract II, Machery-Nagel, Düren, Germany) and 5  $\mu\text{l}$  were used as the template for the second PCR using primers HE1 (5'-CAGACCCTCAA GTACGCC-3') and Sp1aRev2 (5'-AAACCA GGCTAGGGCA GATT-3'). This PCR consisted of 12 cycles (30 s at  $96^{\circ}\text{C}$ , 10 s at  $94^{\circ}\text{C}$ , 20 s with a touchdown from  $68^{\circ}\text{C}$  to  $62^{\circ}\text{C}$  in  $0.5^{\circ}\text{C}$  intervals, 4 min at  $68^{\circ}\text{C}$ ) followed by 18 cycles (10 s at  $64^{\circ}\text{C}$ , 20 s at  $62^{\circ}\text{C}$ , 4 min at  $68^{\circ}\text{C}$ ). 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.** The cloning of PCR products in vector pCR4-TOPO was performed using a TOPO TA Cloning Kit for sequencing (Invitrogen) and carried out according to the manufacturer's instructions. Column purification was added for the plasmids that were sequenced (Nucleo Spin Plasmid Kit; Macherey-Nagel, Bethlehem, USA). Sequencing was performed according to the manufacturer's instructions using a Big Dye Cycle Sequencing Kit (Applied Biosystems, Foster City, USA). The plasmid inserts were sequenced with an automated sequencer (ABI310). The obtained sequences were edited using the Sequencher™ 3.0 program (Genecodes, Ann Arbor, MI, USA). The homology search was conducted with the basic local alignment search tool (BLAST) algorithms, in this case with the nucleotide-nucleotide BLAST (BLASTN) that returns the most similar DNA sequences from the DNA database to a given DNA query.

**Real-time PCR.** Two  $\mu\text{l}$  of reverse transcription (RT) reaction mixture were used as the template for real-time PCR and 0.5 mmol/l primers were added, using hypoxanthine-guanine phosphoribosyltransferase (*HPRT*) as a housekeeping gene: *HPRT* forward (5'-CCT GGC GTC GTG ATT AGT GAT-3'), *HPRT* reverse (5'-CCA GCA GGT CAG CAA AGA ATT TA-3'), *1 $\alpha$ OHase* forward (5'-TGT TTG CAT TTG CTC AGA -3'), *1 $\alpha$ OHase* reverse (5'-CCG GGA GAG CTC ATA CAG -3'). After adding 25  $\mu\text{l}$  of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), the volume was adjusted to 50  $\mu\text{l}$  with nuclease-free distilled water. The samples were amplified in a DNA Engine Opticon 2™ System (Biorad, Munich, Germany) and the PCR was performed with an initial denaturing step at  $50^{\circ}\text{C}$  for 2 min and  $95^{\circ}\text{C}$  for 2 min followed by 50 cycles with a denaturing step at  $95^{\circ}\text{C}$  for 15 s, primer annealing at  $57.3^{\circ}\text{C}$  (*1 $\alpha$ OHase*) and  $60^{\circ}\text{C}$  (*HPRT*) for 15 s and an extension phase at  $72^{\circ}\text{C}$  for 15 s for *1 $\alpha$ OHase* and *HPRT*. A melting curve was generated after 50 cycles for the final PCR product of all the examined genes by decreasing the temperature to  $65^{\circ}\text{C}$  for 15 sec followed by a gradual increase in temperature to  $95^{\circ}\text{C}$ . During the gradual heating process, the fluorescence was measured at increments of  $0.2^{\circ}\text{C}$ . To obtain the relative gene expression data (fold change) between the benign and malignant cell lines and tissue, the comparative  $2^{-\Delta\Delta\text{Ct}}$  method (27) was used. The fold change was determined by the formula: fold change =  $2^{-\Delta(\Delta\text{Ct})}$ , where  $\Delta\text{Ct} = \text{Ct}_{1\alpha\text{OHase}} - \text{Ct}_{HPRT}$ ; in the case of the benign GLZ cell line. The experiments were performed in triplicates for each gene.

**Statistical significance.** The statistical analysis of the real-time PCR results was performed using Student's *t*-test and the normalized cycle threshold ( $\Delta\Delta\text{Ct}$ ) values.

**Western blot.** The cells were harvested, washed twice with PBS and lysed in sample buffer (125 mM Tris, 30% glycerine, 8% SDS, pH 6.8), the tissue samples were homogenized in sample buffer and the protein was extracted. Twenty  $\mu\text{g}$  of proteins were subjected to 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions. Upon separation, the proteins were transferred to a nitrocellulose membrane (Optitran BA-S 85; Schleicher Schuell, Dassel, Germany). The membranes were blocked with PBST containing 5% non-fat powdered milk at room temperature for one hour. The membranes were labelled with primary antibodies against human *1 $\alpha$ OHase* (Biologo, Kiel, Germany) at a dilution of 1:2,000 overnight at  $4^{\circ}\text{C}$ . The secondary antibodies conjugated to horseradish peroxidase (anti-mouse IgG, Amersham Biosciences, Freiburg, Germany) were added at a

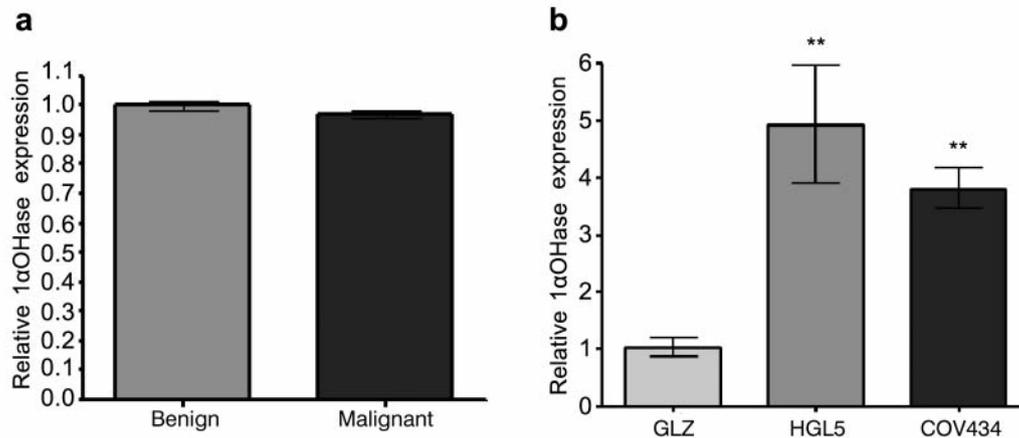


Figure 1. Quantitative analysis of 1 $\alpha$ OHase mRNA expression in relation to HPRT by real-time PCR in ovarian tissue (a) and cell lines (b). No statistically significant difference between benign and malignant tissue ( $p=0.1822$ ); statistically significantly higher expression in HGL5 and COV434 cells ( $p<0.001$ ) in relation to GLZ cells.

dilution of 1:6,000 for the benign and malignant tissue, and 1:8,000 for COV434, and 1:12,000 for GLZ and HGL5 cells. After several washing steps, the bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The obtained signals were compared to  $\beta$ -actin as the internal standard.

**Immunohistochemistry.** Samples of normal and malignant ovarian tissue were obtained from two patients and immediately fixed with 4% formaldehyde. Paraffin-embedded tissue sections were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub>/PBS and the tissue sections were incubated with rabbit serum (1:80) for 20 min. Thereafter, primary polyclonal 1 $\alpha$ OHase antibody (Biologo) was applied at a dilution of 1:1,000. Primary antibody binding was detected using a two-step biotin/streptavidin-based antibody detection system employing peroxidase-mediated DAB staining (DAKO, Hamburg, Germany). Finally, the tissue sections were counterstained with haematoxylin and mounted in permanent mounting medium. The evaluation of immunohistochemical staining was performed semi-quantitatively comparing the visual differences of staining intensities in normal and cancer tissues compared to negative controls stained with the secondary antibody.

## Results

By quantitative real-time RT-PCR, no difference was found in the mRNA expression of 1 $\alpha$ OHase between the benign and malignant tissues ( $p=0.1822$ ) (Figure 1a). However, at the protein level a significantly lower expression of the enzyme was detected in the cancer tissue (Figure 2a).

Statistically significantly higher mRNA expression in the HGL5 ( $p<0.01$ ) and COV434 ( $p<0.01$ ) cells was shown in relation to the benign GLZ cell line (Figure 1b), but no significant differences were found at the protein level (Figure 2b).

To investigate the expression of 1 $\alpha$ OHase splice variants in the cells, a highly specific PCR that combines nested and touchdown PCR was used. Various splice variants were found except in the GLZ cell line (data not shown). The overall patterns differed, the malignant tissue showed more bands, especially of the larger variants (Figure 3). The malignant HGL5 and COV434 cells had a higher level of abnormal transcripts; most of them were larger as well. Only faint bands of smaller variants were seen in the malignant tissue.

The greatest difference in the splice pattern was detected between 2.5 and 4 kb. This could represent splice variants HydV8 to HydV16, with insertions of one or several entire introns. To analyze whether the PCR products were real transcripts of 1 $\alpha$ OHase, the PCR products were isolated and sequenced. Variants with intron 1, a variant with deletion of exon 4 and 5 and a variant with intron 1 as well as a deletion of exon 4 and 5 were identified.

To determine which variants were translated into protein, Western blot analysis using a polyclonal antibody generated by utilizing a peptide located in exon 5 of murine 1 $\alpha$ OHase was carried out. In the malignant and benign tissue, the antibody detected a signal at 56 kDa that corresponded to the size of normal 1 $\alpha$ OHase protein. There was a noticeably strong signal at 42 kDa in the malignant tissue sample. Together with a slightly weaker signal at 23 kDa in the malignant and at 15 kDa in the benign samples, this band showed the most evident difference. Quantitative analysis showed statistically significantly reduced 1 $\alpha$ OHase expression in the malignant tissue in relation to the benign ( $p<0.05$ ).

Overall, the cell line signals were consistent, however, the signal at 13 kDa in the benign GLZ cell line was notable. The 22 kDa band was strongly distinctive in GLZ and HGL5 cells only. The 48 kDa band appeared especially strong in

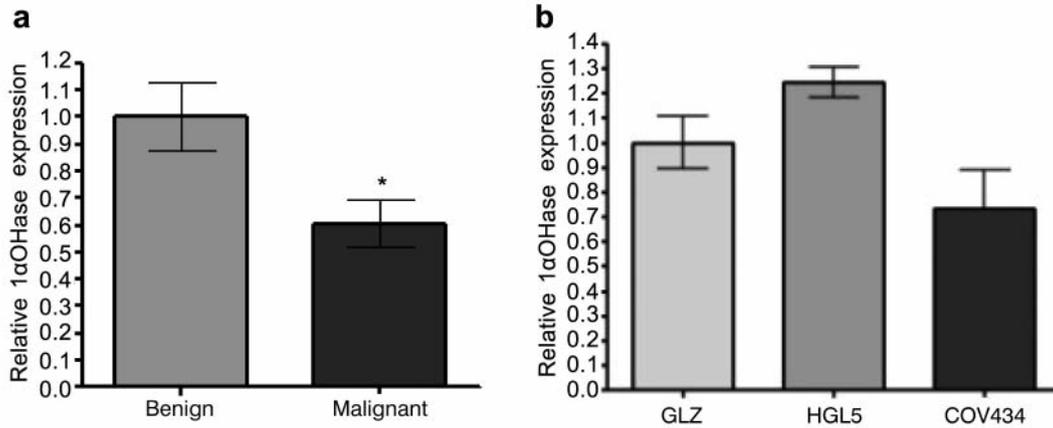


Figure 2. Quantitative analysis of protein expression in relation to  $\beta$ -actin in ovarian tissue (a) and cell lines (b). Statistically significantly lower expression in malignant tissue ( $p < 0.005$ ), no statistically significant difference between HGL5 ( $p = 0.0629$ ) or COV434 ( $p = 0.1795$ ) and GLZ cells.

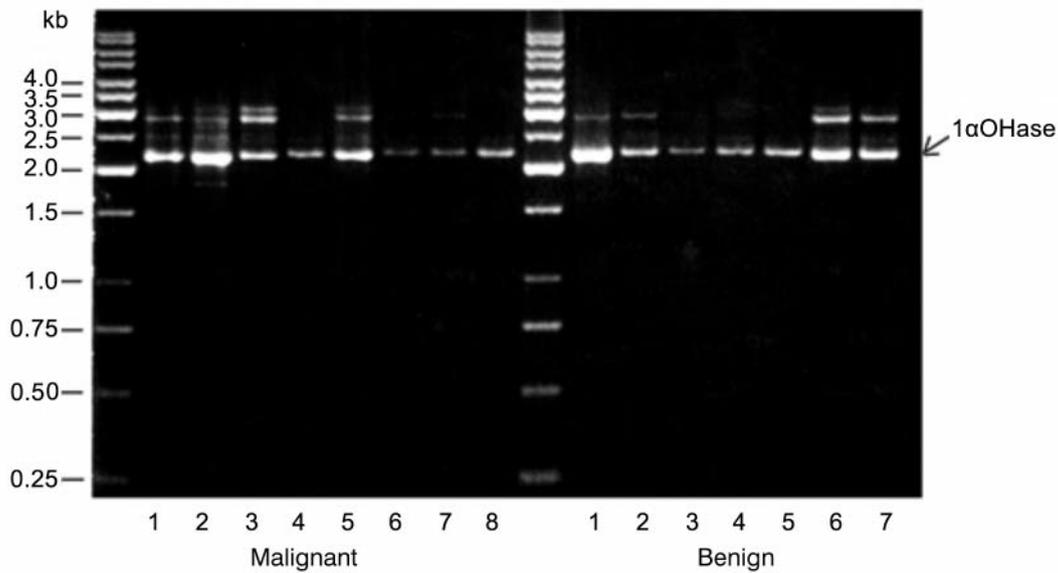


Figure 3. *1αOHase* splice variants in benign and malignant ovarian tissue. PCR products at 2.2 kb correspond to the normal *1α-OHase* transcript.

the HGL5 cells; in the COV434 cells, the signals above the 15 kDa band were characteristic. The quantitative analysis of the *1αOHase* expression showed no statistically significant differences in relation to GLZ.

The cloning and sequencing demonstrated three different variants with the insertion of intron 1 in the COV434 cells as well as in the benign and malignant tissues, a deletion of exon 4 and 5 in the COV434 cells and in the malignant tissue, and a combination of both alterations in the HGL5 cells (Figure 4).

Immunohistochemically intensive staining with an accordingly high expression of the *1αOHase* protein was

seen in the epithelial layer surrounding a benign follicle cyst as well as in the adenocarcinoma cells of the malignant tissue sample.

**Discussion**

An increased expression of *1αOHase* at the RNA level was demonstrated in the malignant cell lines. An elevated *1αOHase* expression was previously described, for example in prostate carcinoma (28) and breast cancer (18). However, studies by Hsu *et al.* also showed a clearly reduced activity of *1αOHase* in malignant prostate cells compared to healthy

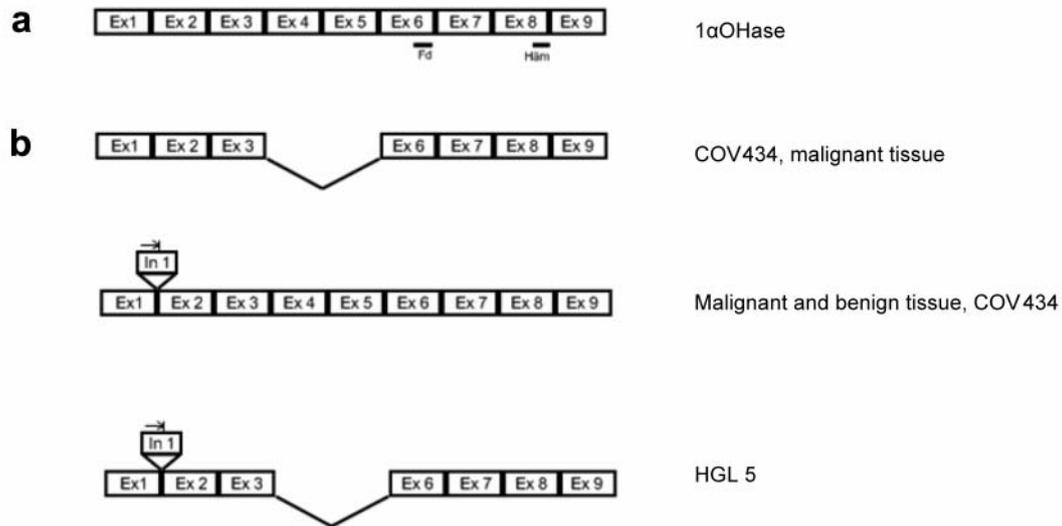


Figure 4. *1 $\alpha$ OHase* sequence in normal (a) and splice variants (b). Insertion of intron 1 occurred in COV434 cells as well as in benign and malignant tissue, a deletion of exon 4 and 5 in COV434 cells and in malignant tissue, and a combination of both alterations in HGL5 cells.

cells (28). The increased RNA expression of *1 $\alpha$ OHase* in the HGL5 and COV434 cells would not necessarily lead to increased protein synthesis in these cells. Enzymatically inactive *1 $\alpha$ OHase* splice variants at the RNA level could presumably not be translated into detectable protein. Various splice variants were found in the malignant cell lines, whereas no splice variants were detected in the GLZ cells. Abnormal transcripts were detected earlier by Diesel *et al.* in glioblastoma and melanoma samples and partially resulted from the insertion of intron 1 in an 8 kDa large enzyme that presented neither a haem- nor a ferredoxin-binding site and was therefore enzymatically inactive (24). Deletion of exon 4 and 5, or a combination of the deletion and intron insertion would result in frameshifts and premature termination signals.

An elevated expression of *1 $\alpha$ OHase* RNA in malignant cells combined with a similar expression at the protein level might also be explained by reduced protein stability and therefore a faster degradation of the protein in the malignant cell line (29).

The *1 $\alpha$ OHase* expression in the malignant cell lines at the RNA level might also be increased in reaction to a reduced response of the cells to vitamin D metabolites. In studies with prostate tissue, benign cells reacted to stimulation with calcitriol as well as with 25(OH)D<sub>3</sub> with reduced growth, whereas malignant cells showed sensitivity to calcitriol only (28). Among other things, the sensitivity of the cells to calcitriol depended on the expression level of the vitamin D receptor (VDR). Studies by Ahonen *et al.* on the ovary showed a reduced VDR level in the epithelium and stroma compared to the granulosa and theca cells of the ovarian follicle (30). Enzyme inactive splice variants of *1 $\alpha$ OHase*

and an additionally reduced VDR expression could therefore explain decreased calcitriol effects and a compensating increased *1 $\alpha$ OHase* expression.

In the present study in the tissue samples no differences in the mRNA level were found, but a significantly reduced *1 $\alpha$ OHase* expression of the protein in the malignant tissue compared to benign tissue was demonstrated. According to previous studies, a higher expression of *1 $\alpha$ OHase* in the malignant tissue would be expected (8). The reduced *1 $\alpha$ OHase* protein expression without a reduction in the mRNA level in the malignant tissue that was analyzed in this study could be explained as follows.

Particularly with regard to the high diversity of cell types in ovarian tissue it can be assumed that apart from the carcinoma cells, other cells in relevant quantities were included in the analysis, which led to a lower expression of *1 $\alpha$ OHase* at the protein level. However, an important reason for the reduced expression of *1 $\alpha$ OHase* might be the low differentiation of the analyzed carcinomas, 81% of the samples were of low differentiation and the remaining 19% were moderately differentiated. In malignant colon, breast and prostate cells Lechner *et al.* showed a dependency between the vitamin D metabolism and the grading of the tumour cells (31). Highly differentiated cells of colon carcinoma responded in a dose-dependent manner to calcitriol by up-regulation of the *1 $\alpha$ OHase* expression, whereas in less differentiated colon cell lines, negative regulation was observed (32). Therefore the down-regulation of *1 $\alpha$ OHase* in the analysed tissues might be explained by the low differentiation of the carcinomas. In contrast, the immunohistochemistry showed an apparently high protein expression in the malignant tissue, possibly

because of relatively higher differentiation in the sample. It has to be considered that the immunohistochemical results were based on one malignant and one benign sample only and that no quantitative analysis was conducted in the context of this study.

Almost identical mRNA levels in benign and malignant tissue were detected by Ogunkolade *et al.* in the colon (33).

The higher incidence of splice variants in the malignant tissue could potentially have influenced the result. It is assumed that the variants have a modified function and are not translated into the corresponding protein. This would explain a relative decrease of expression at the protein level.

The reduced evidence of  $1\alpha$ OHase protein in the malignant tissue could also have been caused by lower protein stability. All the detected splice variants were the result of nonsense mutations because of a premature termination codon (PTC), which in eukaryotic creatures leads to a general genetic regulation through the dissolution of the respective mRNA (29). This mechanism was also described for a cytochrome p450-hydroxylase (34). Using real-time PCR, mRNA could have been detected in the tissue samples which was not translated into  $1\alpha$ OHase protein afterwards.

The VDR expression in the tissue is relevant; studies by Anderson *et al.* showed a very low VDR expression in benign ovarian tissue (35). In relation to other organs such as the colon and lungs, only 2% of the VDR were expressed in the ovary, however, a significantly increased expression was found in ovarian cancer ( $p < 0.0001$ ), which pointed to a strongly increased sensitivity to calcitriol. Presumably, a low level of calcitriol suffices to inhibit growth and to cause differentiation. Therefore, the reduced  $1\alpha$ OHase expression could also have been a reaction to an assumed high VDR level. On the other hand, the functionally inactive  $1\alpha$ OHase splice variants detected in the malignant ovary might have caused the hydroxylation of  $25(\text{OH})\text{D}_3$  not to be catalyzed into calcitriol thus causing it to have no effect in spite of the VDR expression.

Based on the present data, a reduced availability of calcitriol in ovarian cancer can be assumed. This is supported by the detected splice variants of  $1\alpha$ OHase. For verification of the results, a larger sample population will have to be examined. In addition, an activity test after stimulation with  $25(\text{OH})\text{D}_3$  as well as calcitriol will need to be performed.

Should the protective effect of vitamin D for the ovary be confirmed, vitamin D analogs may gain increased significance in carcinoma prevention and therapy. For example, deltanoid derivative EB1089 showed a significantly strong growth inhibition effect in OVCAR-3 cells (36). However, studies in this area have been negative thus far (37).

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