# Expression of 25-Hydroxyvitamin D$_3$-24-Hydroxylase in Benign and Malignant Ovarian Cell Lines and Tissue

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**Abstract.** *Calcitriol (1,25-dihydroxyvitamin D$_3$, 1,25(OH)$_2$D$_3$) plays a pivotal role in maintaining calcium and phosphate homeostasis. Aside from that, it supports the native and attenuates the acquired immune system and has positive effects on cell growth, differentiation and the prevention of carcinogenesis. The goal of this study was to detect possible differences in the expression of the calcitriol-degrading enzyme 24-hydroxylase (24-OHase) between malignant and benign ovarian cell lines and tissue. The analyses were based on real-time PCR, nested touchdown PCR and Western blot. When compared to benign granulose GLZ cells, the malignant HGL5 cells showed a significantly higher 24-OHase expression at the protein level (p<0.01). In the malignant ovarian tissue, the expression was significantly higher in RNA (p<0.001), but lower at the protein level (p<0.01). An increased 24-OHase expression could indicate a decrease in available calcitriol.*

The etiology of epithelial ovarian cancer has not been clarified thus far. It is the most frequent cause of death among patients with gynaecological carcinomas, with a significantly higher incidence rate in the Western world compared to that in Asia and Africa. Frequent ovulations are a proven risk factor for the illness and the intake of oral contraceptives as well as numerous pregnancies have been shown to reduce the risk of ovarian cancer (1). In addition, it is believed that the risk of ovarian cancer varies with vitamin D receptor (*VDR*) gene polymorphisms and dietary intake of calcium (2-4). Some studies have demonstrated an inverse correlation between the vitamin D status and the risk of ovarian cancer (5, 6) and evidence has indicated antitumor effects of vitamin D analogs, especially when they are combined with other agents (7).

The most active form of vitamin D is generated by two enzymes: hepatic vitamin D 25-hydroxylase (25-OHase) catalyzes the hydroxylation of vitamin D$_3$ to 25-hydroxyvitamin D$_3$ (25(OH)D$_3$, calcidiol) and renal vitamin D 1α-hydroxylase (1α-OHase) catalyzes the hydroxylation of 25(OH)D$_3$ to 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$, calcitriol), the latter being regarded as the key reaction of vitamin D metabolism. Both calcidiol and calcitriol can be degraded through the catalysis of vitamin D 24-hydroxylase (24-OHase) (8).

Accordingly, the calcitriol level depends on the expression and activity of the key enzyme 1α-OHase as well as the degrading enzyme 24-OHase. To maintain the calcium and phosphate homeostasis, the enzymes are regulated by a negative feedback mechanism (9).

The majority of the effects of calcitriol are mediated through the intracellular VDR. Besides the primary vitamin D metabolism in the kidneys, an extra-renal expression of 1α-OHase has been described in the epithelial cells of various tissues, such as the prostate, breast and colon (10). Calcitriol synthesized in the extra-renal tissues is believed to act in an autocrine and paracrine fashion, and it is not subjected to the same exquisite autoregulation characteristics of the renal enzyme (11, 12) so that a high level of calcitriol does not increase the activity of 24-OHase (13) its expression is actually increased (14). Alternative splicing of 24-OHase has been proposed as a regulative mechanism for its extra-renal activity (15). An increased expression of 24-OHase enzyme has been reported in several malignant tumors, *e.g.* breast and colon, lung and ovarian carcinoma compared to normal tissue (16, 17). An increased amplification of the *24-OHase* gene (20q13.2) has been described in breast and ovarian cancer (18, 19). Inhibition of 24-OHase in the ovarian cancer cell line OVCAR-3 intensifies cell differentiation and growth inhibition mediated by calcitriol (20). Accordingly, *24-OHase* is regarded as a potential oncogene.

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In this study, the *24-OHase* expression in malignant and benign ovarian cells and tissues was compared at both the genomic and protein level.

## Materials and Methods

*Cell culture*. The human benign granulosa cells (GLZ) were derived from women who had been 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). The human malignant granulosa cell lines HGL5 and COV434 cells were kindly provided by Mrs Sonntag of the Department of Obstetrics and Gynecology, University Hospital of Münster (Germany).

The granulosa and HGL5 cells were maintained in RPMI medium (Gibco, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 2% Ultroser G (Pall Bio Sepra, 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 tissues were taken from women whose ovaries had to be excised because of ovarian carcinoma or benign ovarian disease and were used with the consent of the women. The tissue samples were frozen in liquid nitrogen at –80˚C.

*RNA and poly (A)-RNA isolation*. The total RNA was extracted from five different passages of the COV434 and HGL5 cells, six different passages of the GLZ cells and eight different samples of the benign and malignant tissues with Trizol (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. The total RNA was quantified spectrophotometrically and 100 μg were used to isolate poly (A)-RNA according to the manufacturer's instructions (Oligotex mRNA, Qiagen, Hildesheim, Germany).

*Reverse transcription*. First-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen) and oligo-d(T) primer (Invitrogen) according to the manufacturer's instructions.

*Real-time PCR*. Two μl of reverse transcription reaction mixture were taken as the template for real-time PCR and 0.5 mmol/l primers were added, using hypoxanthine-guanine phosphoribo-syltransferase (*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'; *24-OHase* forward: 5'-ATT CAC CCA GAA CTG TTG -3'; *24-OHase* reverse: 5'-GCA GCC TAG TGC AGA TTT -3'. After the addition of 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 by the DNA Engine Opticon 2™ System (Biorad, Munich, Germany) and PCR was performed by an initial denaturizing step at 50˚C for a period of 2 min and at 95˚C for another 2 min followed by 50 cycles of a denaturizing step at 95˚C for 15 s, primer annealing at 57.3˚C (*24-OHase*) and 60˚C (*HPRT*) for 15 s, and an extension phase at 72˚C for 15 s for *24-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˚C for 15 s followed by a gradual increase of temperature to 95˚C. During the gradual heating process, the fluorescence was measured in 0.2˚C increments. To obtain the relative gene expression data (fold change) between the benign and malignant cells and tissues, the comparative $2^{-\Delta(\Delta Ct)}$ method (21) was used. The fold change was determined by the formula: fold change=$2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct$, 24-*OHase*–Ct, *HPRT*; $\Delta(\Delta Ct) = \Delta Ct$, *HGL–5*-$\Delta Ct$, *GLZ* in the case of the benign GLZ and malignant HGL-5 cell lines. The experiments were performed in triplicate for each gene.

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

*Western Blot*. The cells were harvested, washed twice with PBS and lysed in a sample buffer (125 mM Tris, 30% glycerine, 8% SDS, pH 6.8) and the tissue samples were homogenized in the sample buffer and the proteins were extracted. Twenty μg of proteins were subjected to 12.5% SDS–polyacrylamide gel electrophoresis (PAGE) under reduced conditions. After the separate proteins were transferred to a nitrocellulose membrane (Optitran BA-S 85; Schleicher Schuell, Dassel, Germany), the membranes were blocked with PBS with tween 20 (PBST) containing 5% non-fat powdered milk for one hour at room temperature. The membranes were labeled with primary antibodies against human 24-OHase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:2,000 for the malignant and benign tissues and for the GLZ and COV434 cells, and at a dilution of 1:4,000 for the HGL5 cells overnight at 4˚C. The secondary antibody, a polyclonal antibody generated *via* the use of a peptide located in exon 2 of murine *24-OHase*, conjugated to horseradish peroxidase (anti-mouse IgG; Amersham Biosciences, Freiburg, Germany) was added at a dilution of 1:12,000 for the benign and malignant tissues, as well as for all the cell lines. After several washing steps, the bands were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). The obtained signals were compared to *β-actin* as an internal standard.

*Statistical analysis*. Regarding the tissue, the statistical analysis of the Western Blot results was performed using Student's *t*-test and the normalized cycle threshold ($\Delta/\Delta$ Ct) values. The results of the cell lines were analyzed using the one way ANOVA-test which allows to compare more than two sample groups. Dunnett's test was chosen for the post test. The malignant cell lines were compared to the benign granulosa cells which represented the standardised control group.

## Results

Statistically, the malignant tissue (Figure 1) showed a significantly higher expression of *24-OHase* mRNA compared to the benign tissue ($p < 0.001$).

With regards to the cell lines (Figure 2) and the relative amounts of transcript, there were no significant differences between the COV434 ($p=0.5596$) or HGL5 ($p=0.0505$) cells and the GLZ cells.

To determine whether the *24-OHase* mRNA transcripts were translated into protein, Western blot analysis was carried out. In the benign tissue, the antibody detected a signal at 55 kDa corresponding to the size of the normal 24-OHase
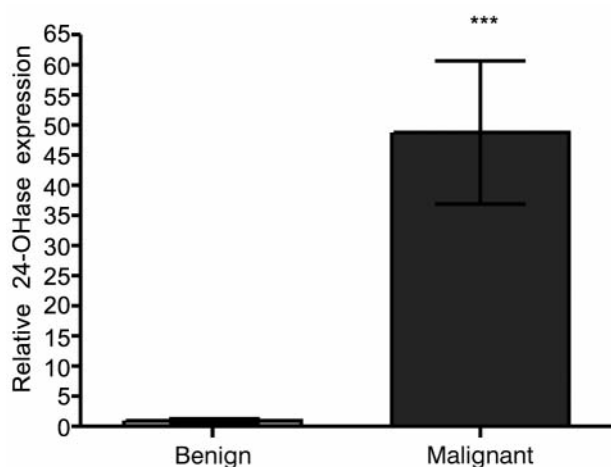
Figure 1. *Quantitative analysis of 24-OHase mRNA expression in relation to HPRT in malignant and benign ovarian tissue (each n=8).*



Figure 2. *Quantitative analysis of 24-OHase mRNA expression in relation to HPRT in HGL5 and COV434 cells (each n=6) compared to benign GLZ cells (n=5).*

protein, whereas no signal appeared in most of the malignant tissue samples at 55 kDa (Figure 3). The malignant tissues showed consistent signals at 48 kDa and at 46 kDa and occasionally, a signal at 27 kDa was observed. These potential *24-OHase* splice variants were only sporadically detected in the benign tissue. A quantitative analysis showed a statistically significantly lower 24-OHase expression in the malignant tissue (Figure 4) in relation to that of the benign ($p<0.01$).

Regarding the cell lines, a signal of the wild-type 24-OHase protein at 55 kDa as well as a band at 48 kDa was detected in all of them. Furthermore, a signal at 35 kDa appeared in the GLZ and HGL5 cells, and a signal at 30 kDa in the GLZ and COV434 cells (data not shown). Statistically, the quantitative analysis resulted in a significantly higher expression of 24-OHase protein in the HGL5 cells ($p<0.01$) and a significantly lower expression in the COV434 cells ($p<0.05$) in relation to the GLZ cells (Figure 5).

## Discussion

The high level of *24-OHase* mRNA in the malignant tissue indicates an increased degradation and, therefore, a decreased availability of calcitriol. Potentially limited effects of calcitriol on cell growth and differentiation could result in a malignant progression. Several studies have revealed an association of vitamin D with the prevention of ovarian carcinogenesis. Polymorphisms in the *VDR* gene influence ovarian cancer susceptibility, but the relevance of several of the polymorphisms differ between ethnic groups (3). Furthermore, an inverse association between solar UVB irradiation and thus vitamin D level and the incidence of ovarian carcinoma was detected in a study including 175 countries with different degrees of solar irradiation (6).
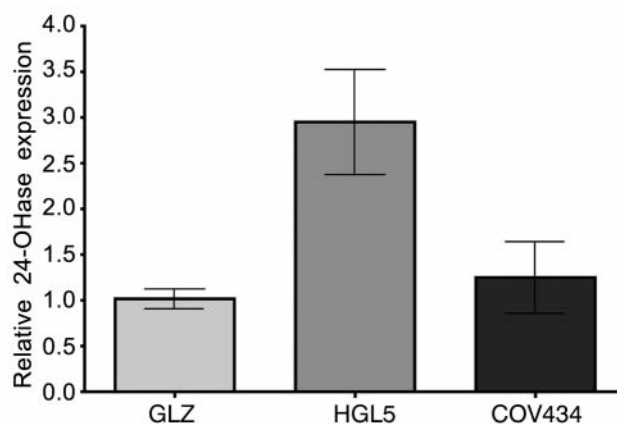
In the present study, the ovarian origin of the cell lines should be considered. The benign GLZ as well as the malignant granulosa HGL5 and COV434 cells surround the ovule of the ovarian follicle and comparison of these cell lines with the malignant and benign ovarian tissue must be regarded critically, as the cell lines were dissimilar from the epithelial origin of ovarian cancer.

The increased expression of *24-OHase* mRNA in the malignant ovarian tissue conformed to the results of another investigation (17), supporting the suggestion of *24-OHase* as a potential oncogene.

In the present study, the malignant tissue showed statistically significantly lower protein expression in relation to the benign tissue. This could very well have been due to decreased expression of the 1α-OHase enzyme in malignant ovarian tissue (24) followed by a reduced level of calcitriol available for degradation. Additionally, possible splice variants of the 24-OHase enzyme could explain the decreased 24-OHase protein expression as well. A *24-OHase* variant without exon 1 and 2, but containing intron 2 has been described for macrophages (15) and resulted in a catalytically inactive enzyme. As described in another study, non-coding RNA (ncRNA) resulting from alternative splicing potentially regulates the level of wild-type protein expression (13). Malignant ovarian tissue contains inflammatory infiltrate. The expression of regulatory active *24-OHase* splice variants in macrophages could explain the decreased level of wild-type 24-OHase protein in the malignant tissue. Additionally, a splice variant without exon 2 would have lead to a low 24-OHase protein expression compared with the level of mRNA, as the antibody used binds to exon 2.

The 24-OHase expression in the cell lines and ovarian tissue did not match in detail. In the HGL5 cells, the 24-OHase expression was increased both at mRNA and protein
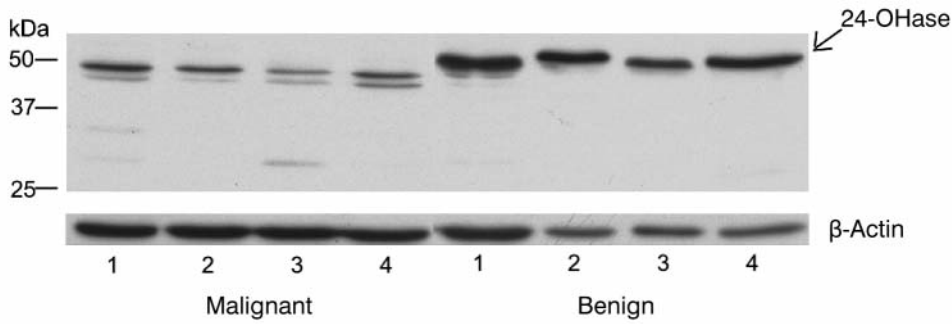
Figure 3. *Expression of 24-OHase protein and possible splice variants in benign and malignant ovarian tissue, exemplified by four malignant and benign tissue samples, respectively.*
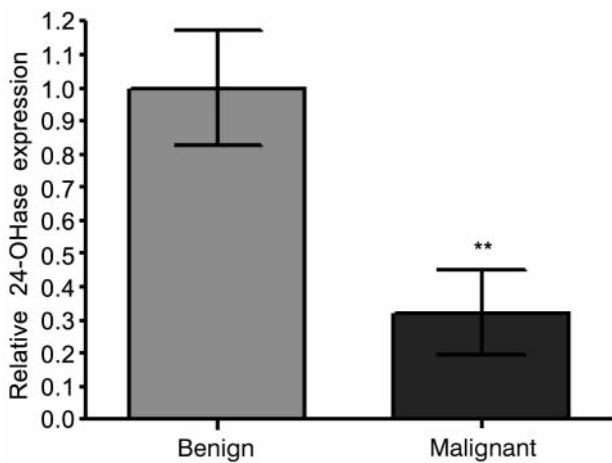


Figure 4. *Quantitative analysis of 24-OHase protein expression in relation to β-actin in malignant (n=17) and benign (n=16) ovarian tissue.*



Figure 5. *Quantitative analysis of 24-OHase protein expression in relation to β-actin in HGL5 and COV434 cells compared to benign GLZ cells (each n=8).*

levels (mRNA level statistically not significant). The relatively high protein level compared to the malignant tissue could be explained by the absence of an inflammatory infiltrate with macrophages containing regulatory active *24-OHase* splice variants. Differing results were seen in the COV434 cells. These cells showed similarities to the benign granulosa cells and, unlike the HGL5 cells, the COV434 cells were not exposed to luteinization, which could possibly have resulted in a lower grade of malignity, as a high release of luteinizing hormone causes pathological changes in the ovary (22, 23). Furthermore, the external influences on cell cultivation have to be considered. The adherent cell lines GLZ and HGL5 were cultivated with FBS as well as Ultroser G, a synthetic efficient alternative to FBS, whereas COV434, as a non-adherent cell line, was cultivated without Ultroser G.

In summary, both quantitative and qualitative analyses indicate decreased availability of calcitriol in malignant ovarian tissue and further qualitative investigations including the sequencing of the 24-OHase enzyme are needed to
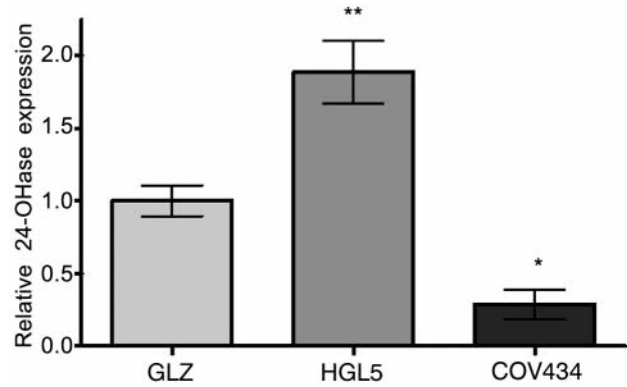
clarify the underlying mechanisms. The results with the ovarian cell lines were not clear and the inclusion of benign and malignant epithelial ovarian cell lines could possibly resolve the open questions.

In the future, calcitriol and its analogues, as well as a selective influence on the enzymes involved in the metabolism of calcitriol, could potentially offer new options for the treatment and prevention of ovarian carcinoma.

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