Marked Increase of *CYP24A1* mRNA Level in Hepatocellular Carcinoma Cell Lines Following Vitamin D Administration

EVELIN HORVATH[1], PÉTER LAKATOS[1], BERNADETT BALLA[1], JÁNOS PÁL KÓSA[1], BÁLINT TÓBIÁS[1], HASAN JOZILAN[1], KATALIN BORKA[2], HENRIK CSABA HORVÁTH[1], ILONA KOVALSZKY[3] and FERENC SZALAY[1]

[1]*First Department of Internal Medicine,* [2]*Second Department of Pathology,* [3]*First Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest, Hungary*

Abstract. *Aim: 1,25-Dihydroxyvitamin D_3 (1,25(OH)$_2$D$_3$) inhibits cell growth and induces apoptosis in numerous types of tumors. We aimed to examine the mRNA and protein expression of 1,25(OH)$_2$D$_3$-inactivating CYP24A1 and mRNA expression of the activating CYP27B1 enzymes, as well as that of vitamin D receptor (VDR), in hepatocellular carcinoma (HCC) cell cultures in response to 1,25(OH)$_2$D$_3$ administration. Materials and Methods: Increasing amounts of 1,25(OH)$_2$D$_3$ (0.256-10 nM) were added to cultures of HepG2, Huh-Neo, Hep3B, Huh5-15 human HCC cell lines and cells then incubated for various time periods (30 min–28 h). The mRNA expression was analyzed by real time reverse transcription-polymerase chain reaction (RT-PCR). CYP24A1 protein in HepG2 cells was detected by immuncytochemistry. Results: CYP24A1 mRNA expression significantly ($p<0.0001$) increased in response to 1,25(OH)$_2$D$_3$ administration in two cell lines: in HepG2 cells, the CYP24A1 mRNA level exhibited 5,300-fold elevation, reaching a maximum value at 8 h; in Huh-Neo cells, the increase was 152-fold that of the baseline value, with the maximum being reached at 14 h. There was no significant change in Hep3B and Huh5-15 cell lines, nor was there any change in CYP27B1 and VDR gene expression in any cell cultures. Immuncytochemistry in HepG2 cells proved that gene activation was followed by CYP24A1 protein synthesis. Conclusion: Our novel data indicate that administration of 1,25(OH)$_2$D$_3$ results in a marked increase of CYP24A1 mRNA expression in some, but not all, human HCC lines in vitro. These differences could be dependent upon the origin of the tumor cells.*

Correspondence to: Dr. Evelin Horvath, First Department of Internal Medicine, Semmelweis University, Korányi Sándor Street 2/A, H-1083 Budapest, Hungary. Tel: +36 14591500, Fax: +36 12101007, e-mail: horvath.evelyn@gmail.com

Key Words: Hepatocellular carcinoma, 1,25-dihydroxyvitamin D$_3$, *CYP24A1*, mRNA expression, HepG2.

Hepatocellular carcinoma (HCC) is one of the most common types of malignant tumors worldwide. Despite great progress in our understanding over the molecular mechanisms of hepatocarcinogenesis, many regulatory processes are still unknown. 1,25-Dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$), the active form of vitamin D, inhibits cell growth and induces apoptosis in numerous tumor types such as colon, breast and prostate cancer. An antitumor effect of 1,25(OH)$_2$D$_3$ on liver cancer cells has been detected *in vitro*. The clinical use of 1,25(OH)$_2$D$_3$ for HCC is limited since the dose of vitamin D exerting antitumor effects induces hypercalcemia *in vivo* (1). Several 1,25(OH)$_2$D$_3$ analogs without hypercalcemic effect were tested for the treatment of HCC in pre-clinical studies (2-4). The antitumor effect of vitamin D analogs was variable in patients with HCC but the cause of such individual differences in response remains unknown (5). The actual concentration of vitamin D available in tissues depends on the balance between activating and inactivating enzymes.

In order to study vitamin D metabolism in HCC, we examined the mRNA and protein expression of 1,25(OH)$_2$D$_3$-inactivating 24-hydroxylase (*CYP24A1*) and the mRNA expression of the activating 25-hydroxyvitamin D-1-alpha-hydroxylase (*CYP27B1*) enzymes, as well as that of vitamin D receptor (*VDR*) in four HCC cell lines in response to 1,25(OH)$_2$D$_3$ administration.

Materials and Methods

Ethics. This work has been carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. This study was approved ethically by the Regional Committee of Science and Research Ethics, Semmelweis University (ETT TUKEB 5637-0/2010-1018EKU – 402/PI/010).

Cell lines and culture conditions. Experiments were performed on four human hepatocellular carcinoma cell lines: HepG2, Huh-Neo, Huh5-15 and Hep3B. The Huh-Neo and Huh5-15 cell lines were received as gift from the Department of Molecular Virology,

University of Heidelberg, Germany. The Huh-Neo cell line contains the gene for neomycin phosphotransferase (NPT) and is resistant to neomycin (6). Huh5-15 cells, containing the subgenomic hepatitis C virus (HCV) replicon I389hyg-ubi/NS3-3' were provided by R. Bartenschlager at the University of Heidelberg (7, 8). HepG2 (ATCC® no. HB-8065 HepG2) was originally derived from the liver tissue of a 15-year-old Caucasian American male with a well-differentiated hepatocellular carcinoma (Sigma-Aldrich, St. Louis, MO, USA). Hep3B is a well-differentiated human hepatoma cell line from an 8-year-old black male, and contains an integrated HBV genome (Sigma-Aldrich) (9, 10).

Cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies – Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (Life Technologies – Invitrogen), 1 mM sodium pyruvate (Sigma-Aldrich), 100 IU penicillin, 100 g/ml streptomycin and 4 mM glutamine (all from Life Technologies – Invitrogen) at 37˚C, in a humidified atmosphere of 95% air and 5% CO_2. Media were changed every second day.

Subcultures were carried out as follows: After removal of medium, cells were rinsed with 2 ml of trypsin-EDTA solution, with sitting the flask at room temperature until the cells detached, followed by the addition of fresh culture medium, aspirating and dispensing cells into new culture flasks.

Incubation with 1,25(OH)$_2$D$_3$. 1,25-(OH)$_2$D$_3$ (Sigma-Aldrich) was dissolved in ethanol at 100 M and diluted in Opti-MEM (Life Technologies – Invitrogen) to give a final ethanol concentration of 0.1%. Control cultures were treated with Opti-MEM containing ethanol (0.1%) vehicle-only. Each HCC cell line was incubated with 1 nM and 10 nM vitamin D for 5 h in Opti-MEM at 37˚C in a humidified atmosphere of 95% air and 5% CO_2. Cells were harvested and *CYP24A1*, *CYP27B1* and *VDR* mRNA expressions were measured. In dose-response experiments, cells were incubated with different doses of 1,25-(OH)$_2$D$_3$ (0.256, 0.64, 1.6, 4.0, 10.0 nM) for 5 h in two parallel series. To measure the time course of mRNA responses, cells were incubated with 4 nmol of 1,25-(OH)$_2$D$_3$ for 30 min, and 1, 2, 5, 8, 10, 12, 14, 24, 26 and 28 h in two parallel series under normal growth conditions. Treatment solutions containing 1,25(OH)$_2$D$_3$ were prepared in serum-free Opti-MEM for all experiments. All experiments were repeated at least three times.

RNA isolation, cDNA synthesis and quantitative reverse transcription-PCR (qRT-PCR). Total RNA was isolated from the cell cultures with Roche High Pure Total RNA Isolation Kit (Roche, Indianapolis, IN, USA). The procedure was performed following the manufacturer's instructions. Five-hundred nanograms of total RNA were reverse-transcribed to cDNA (Promega, Madison, WI, USA). Pre-designed and validated gene-specific TaqMan Gene Expression Assays from Applied Biosystems (Life Technologies – Invitrogen) were used in triplicate for quantitative real-time PCR according to the manufacturer's protocol. Every set contained gene-specific forward and reverse primers and fluorescence labeled probes. Probes span an exon junction and do not detect genomic DNA (Applied Biosystems TaqMan® Assays numbers were Hs00167999_m1 for *CYP24A1*, Hs99999905_m1 for glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), Hs00168017_m1 for *CYP27B1* and Hs00172113_m1 for *VDR*, respectively). PCR assays were used with the following protocol: 10 min denaturation at 95˚C, and 45 cycles of 15 s denaturation at 95˚C, followed by 1 min annealing and extension at 60˚C. The PCR reaction volume was 20 μl containing 2 μl cDNA, 10 μl TaqMan ×2 Universal PCR Master Mix NoAmpErase UNG (Applied Biosystems), 1 μl gene-specific TaqMan Gene Expression Assay Mix and 7 μl water. *GAPDH* was used as a housekeeping gene to normalize for RNA loading. Samples were analyzed using ABI Prism 7500 real-time PCR system (Applied Biosystems). Relative quantification (RQ) studies were carried out from collected data (threshold cycle numbers, referred to as Ct) with 7500 System SDS software 1.3 (Applied Biosystems).

Immunocytochemistry. Cells were cultured on special slides in the same way as described above (Cell lines and culture conditions). Lab-Tek® Chamber Slide™ and Lab-Tek® Chambered Coverglass (Thermo Fisher Scientific GmbH, Bremen, Germany) were used for these experiments.

Immunocytochemistry for *CYP24A1* was performed using the two-step indirect immunoperoxidase technique. WH0001591M7 (monoclonal anti-CYP24A1, clone 1F8 antibody produced in mouse; Sigma-Aldrich) antibody was used. Normal kidney sections were used as a positive control. Staining was carried out following the manufacturer's instructions. Expression of *CYP24A1* was evaluated semi-quantitatively. Ten randomly selected areas of each slide were manually analyzed using the high-power field objective (×40), with 500 cells counted per field. Immunoreactions were scored as positive where nuclear staining was seen.

Statistical analysis. Data were analyzed using the SPSS for Windows, release 18 (IBM, Armonk, NY, USA). Final data are presented as the means of two independent measurements. Results are expressed as mean±standard error of the mean (S.E.M.). Statistical analysis was performed using the unpaired Student's *t*-test; results with a *p*-value of 0.05 or less were considered statistically significant.

Results

CYP24A1, CYP27B1 and VDR mRNA changes following 1,25(OH)$_2$D$_3$ administration. Basal *CYP24A1* mRNA expression was very low in all of cell lines. Exposure to 1,25-(OH)$_2$D$_3$ both at doses of 1 nM and 10 nM for 5 h, resulted in a strongly significant ($p<0.0001$) expression of *CYP24A1* mRNA in HepG2 and Huh-Neo cell lines, but had no effect on Huh5-15 and Hep3B cells at the investigated time point (5 h). In HepG2 cells, the increase of *CYP24A1* mRNA expression was more pronounced, close to 1,000-fold (Figure 1A) compared to Huh-Neo cells, which exhibited a 100-fold increase after 5 h of incubation (Figure 1B). In HepG2 cells, the larger dose of vitamin D did not result in a further increase of mRNA expression compared to the effect of 1 nM. In Huh-Neo cells, the magnitude of the mRNA elevation was smaller ($p<0.001$), and in this set, 10 nM of vitamin D neither resulted in a further significant increase of mRNA expression.

There was no change in *CYP27B1* or *VDR* mRNA expressions in any of the examined cell lines after 5 h of 1,25-(OH)$_2$D$_3$ incubation neither at 1 nM nor at 10 nM.

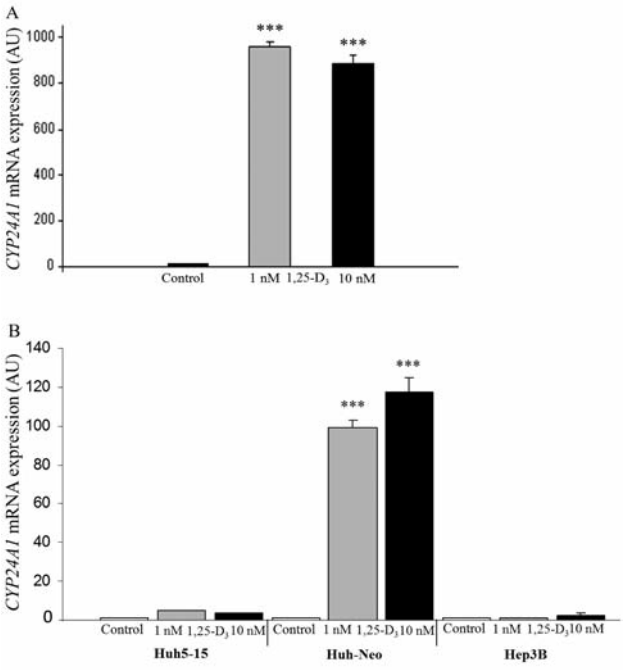

Figure 1. *24-hydroxylase (CYP24A1) mRNA expression changes in response to 1,25-dihydroxyvitamin [1,25(OH)$_2$D$_3$)]. HepG2 (A) and Huh-Neo (B) cell lines were incubated with 1 nmol/l and 10 nmol/l active vitamin D for a duration of 5 h. Cells without 1,25(OH)$_2$D$_3$ incubation were used as controls. ***p<0.0001. AU: Arbitrary units.*

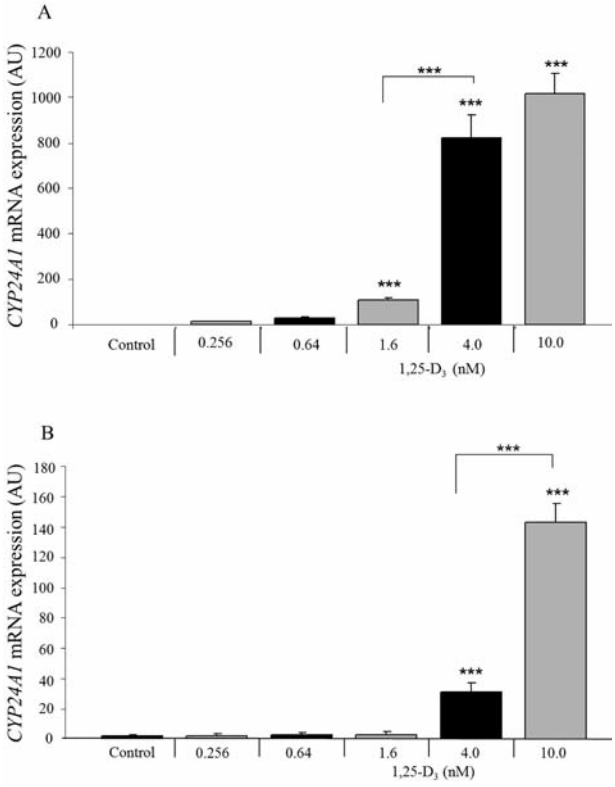

Figure 2. *24-hydroxylase (CYP24A1) mRNA dose-response curves of HepG2 (A) and Huh-Neo (B) cell lines in response to 1,25-dihydroxyvitamin [1,25(OH)$_2$D$_3$]. The cells were treated with increasing concentrations of 1,25(OH)$_2$D$_3$ from 0.256 nmol/l up to 10 nmol/l for 4 h. Cells without 1,25(OH)$_2$D$_3$ incubation were used as controls. ***p<0.0001. AU: Arbitrary units.*

Dose-dependent increase of CYP24A1 expression in HepG2 and Huh-Neo cells. We found a dose-dependent increase in *CYP24A1* expression in HepG2 and Huh-Neo cell lines in response to 1,25(OH)$_2$D$_3$ administration for 4 h. In concordance with the first set of experiments, the response of HepG2 cells was larger than that of Huh-Neo cells at each dose (Figure 2). In Hep G2 cells, 1.6 nM 1,25(OH)$_2$D$_3$ resulted in significant elevation of mRNA (p<0.001), while in Huh-Neo cells, the elevation reached significance only at a dose of 4.0 nM. The difference between the two cell types in *CYP24A1* mRNA expression was one order of magnitude using 1.6, 4.0 and 10.0 nM 1,25(OH)$_2$D$_3$. In HepG2 cells, 180-, 820-, and 1,010-fold elevations were detected *versus* 2.0-, 38-, and 140-fold increases in Huh-Neo cells at 1.6, 4.0, and 10 nM, respectively.

Different time curves of CYP24A1 mRNA expression in HepG2 and Huh-Neo cells. Not only was the magnitude of *CYP24A1* mRNA expression found to be different in HepG2 and Huh-Neo cells but so was the kinetics of expression. In HepG2 cells, the *CYP24A1* mRNA expression exhibited a 5,300-fold elevation, reaching its maximum value at 8 h. In Huh-Neo cells, the increase was 152-fold that of the baseline and the maximum was reached at 14 h (Figure 3).

CYP24A1 protein expression by immunocytochemistry. Immunocytochemistry showed that gene activation with 1,25(OH)$_2$D$_3$ was followed by *CYP24A1* protein synthesis, indicating effective translation. Strong *CYP24A1* enzyme staining was shown in HepG2 cells after 32 h of incubation with 4 nM 1,25(OH)$_2$D$_3$, whereas no staining was observed in the untreated control cells (Figure 4). Expression of *CYP24A1* was confined to the cytoplasm; the punctuated pattern of the staining is consistent with mitochondrial localization of the enzyme.

Discussion

HCC is the fifth most common type of cancer in men and the seventh in women (11). Despite preventative and therapeutic efforts, the annual death rate is over seven million worldwide (12). The discovery of the antitumor effect of 1,25(OH)$_2$D$_3$ on various malignancies, such as breast and colon carcinoma, melanoma and also HCC opened up an opportunity for

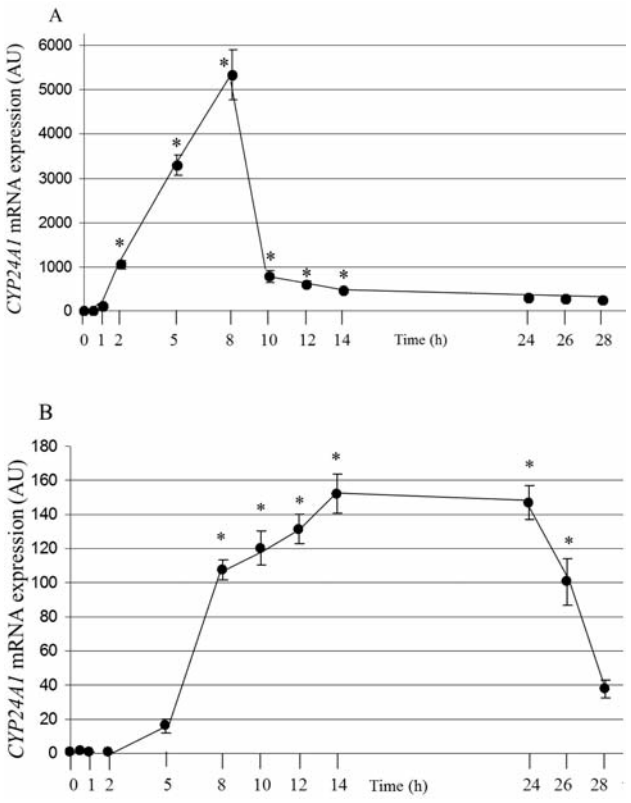

Figure 3. *24-hydroxylase (CYP24A1) mRNA time course curves of HepG2 (A) and Huh-Neo (B) cell lines in response to 1,25-dihydroxyvitamin [1,25(OH)$_2$D$_3$]. *p<0.0001. AU: Arbitrary units.*

treatment (13-16). However, the clinical use of 1,25(OH)$_2$D$_3$ in HCC is limited by its hypercalcemic effect due to the relatively large doses required in order for it to exert its antitumor effect (1). The use of vitamin D analogs without hypercalcemic effect is the object of pre-clinical and clinical studies (17, 18).

Increased activity of *CYP24A1*, the key neutralizing enzyme of 1,25(OH)$_2$D$_3$ was found in colon, lung, prostate, thyroid, breast and ovarian cancer cells (19-22). To our knowledge, this is the first study on *CYP24A1* mRNA expression in HCC lines *in vitro*. Our novel data on the marked increase of *CYP24A1* mRNA and protein expression in HepG2 and Huh-Neo cell lines in response to 1,25(OH)$_2$D$_3$ indicate that these HCC cells are capable of metabolizing 1,25(OH)$_2$D$_3$ autonomously. The differences in *CYP24A1* mRNA response to 1,25(OH)$_2$D$_3$ among HCC cell lines of different origin might explain individual differences in responses of patients to vitamin D treatment.

Similarly, differences were reported among cervical and ovarian cancer cells (23). In our study, only two out of four HCC cell lines exhibited an increase in *CYP24A* mRNA following incubation with 1,25(OH)$_2$D$_3$. We found a one-magnitude higher increase of *CYP24A* mRNA in HepG2 cells than that which others observed in ovarian cancer cells (OVCAR-3), despite the fact that a much higher concentration of 1,25(OH)$_2$D$_3$ was used in those experiments (100 nM as compared to 4 nM in our study). These data suggest that these HCC cell lines are much more sensitive to 1,25(OH)$_2$D$_3$ in responding with *CYP24A1* expression. The cause of differences in magnitude and kinetics of *CYP24A1* mRNA expression between HepG2 and Huh-Neo cells needs further study.

The magnitude of the increase of *CYP24A1* mRNA expression in HepG2 cells (5,300-fold) following 1,25(OH)$_2$D$_3$ administration is in concordance with the expert of response of DU-145 prostate cancer cells and some colon cancer cell lines (Caco-2 and COGA-1) which also exhibit high expression of this metabolizing enzyme (19, 24).

The antiproliferative effect of 1,25(OH)$_2$D$_3$ on HepG2 cells has been published and 100 μM was found to be the lowest effective inhibiting dose (3). We have shown here, that a significantly smaller dose of 1,25(OH)$_2$D$_3$ induces activation of *CYP24A* mRNA, which could lead to an accelerated metabolism and a reduced antitumor effect of 1,25(OH)$_2$D$_3$.

Based on our results, the antitumor effect of 1,25(OH)$_2$D$_3$ or vitamin D analogs could be increased in HCC by combination with *CYP24A1* enzyme inhibitors. This has been reported in human prostate cancer cells *in vitro* and in an animal experiment (24). As *CYP24A1* was suggested as a candidate oncogene in colon and breast cancer, the same possibility could arise in some HCC cell lines (25, 26).

Major risk factors for hepatocellular carcinoma include infection with HBV or HCV. Recent evidence shows that the serum vitamin D level could have a possible role in predicting the outcome of antiviral therapy in patients with chronic HCV infection (27). Thus, the higher risk for HCC in antiviral treatment-resistant patients might also be related to the low vitamin D levels. However, we did not detect *CYP24A1* mRNA response to 1,25(OH)$_2$D$_3$ in Huh5-15 HCC cells, which contain integrated HCV genome.

Despite the increase in *CYP24A1* mRNA expression, we did not detect significant changes in *VDR* mRNA expression in our experimental design. This is consistent with a previous report that in human breast and colon cancer cells, 1,25(OH)$_2$D$_3$ profoundly stimulated *CYP24A1* mRNA but did not affect *VDR* mRNA expression (28). However, based on observations on melanoma and breast cancer cell lines, the regulation of expression of 1,25(OH)$_2$D$_3$ target genes is very complex and not only depends on the type of vitamin D response element but also on a multitude of co-activators and co-repressors, and on other unknown mechanisms (29, 30).

In concordance with findings on other carcinoma cell lines, we also did not find changes in *CYP27B1* mRNA expression after 1,25(OH)$_2$D$_3$ administration (19).

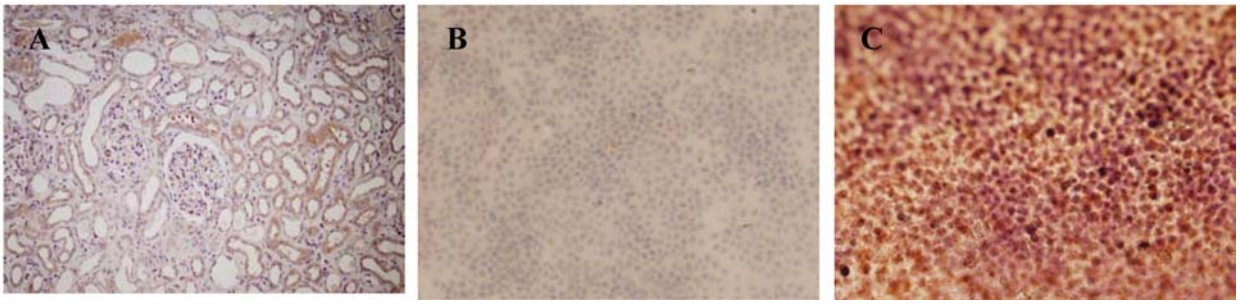

Figure 4. *Immunochemical detection of 24-hydroxylase (CYP24A1) enzyme expression. A: Normal kidney sections served as positive controls. A brown color indicates a positive reaction. Nuclei are counterstained with hematoxylin (blue) (×200 magnification). B: Negative control: HepG2 cells without 1,25-dihydroxyvitamin [1,25(OH)$_2$D$_3$] administration (×20 magnification). C: Detection of CYP24A1 enzyme protein in HepG2 cell cultures by immunochemical staining at 32 hours after administration of 4 nM 1,25(OH)$_2$D$_3$ (×20 magnification).*

In conclusion, our novel data indicate that administration of 1,25(OH)$_2$D$_3$ results in a marked increase of *CYP24A1* mRNA expression in some, but not all HCC cell lines *in vitro*. The differences in response to 1,25(OH)$_2$D$_3$ might be dependent upon of the origin of the tumor cells. It can be speculated that increased expression of *CYP24A1* protects cancer cells from the anticancer effects of 1,25(OH)$_2$D$_3$. Combining 1,25(OH)$_2$D$_3$ with *CYP24A1* enzyme inhibitors could be an approach to new antitumor drug development.

Conflicts of Interest

The Authors declare no financial or commercial conflict of interest.

Acknowledgements

The study was approved by the Regional Committee of Science and Research Ethics, Semmelweis University (ETT TUKEB 5637-0/2010-1018EKU – 402/PI/010.) and supported by a Scientific Project Grant, Semmelweis University.

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Received July 13, 2012
Revised September 16, 2012
Accepted September 18, 2012