

# Analysis of the vitamin D system in basal cell carcinomas (BCCs)

Tanja Mitschele<sup>1</sup>, Britta Diesel<sup>2</sup>, Michael Friedrich<sup>3</sup>, Viktor Meineke<sup>4</sup>, Ruth M Maas<sup>2</sup>, Barbara C Gärtner<sup>5</sup>, Jörn Kamradt<sup>1</sup>, Eckart Meese<sup>2</sup>, Wolfgang Tilgen<sup>1</sup> and Jörg Reichrath<sup>1</sup>

<sup>1</sup>Universitäts-Hautklinik; <sup>2</sup>Institut für Humangenetik; <sup>3</sup>Universitäts-Frauenklinik, Universitätskliniken des Saarlandes, Homburg, Germany; <sup>4</sup>Institut für Radiobiologie, Sanitätsakademie der Bundeswehr, München, Germany and <sup>5</sup>Institut für Medizinische Virologie, Universitätskliniken des Saarlandes, Homburg, Germany

**Using real-time PCR (LightCycler) and immunohistochemistry, we have analyzed expression of key components of the vitamin D system in basal cell carcinomas (BCCs) and normal human skin (NS). Increased VDR-immunoreactivity was demonstrated in BCCs using a streptavidin-peroxidase technique. RNA expression of vitamin D receptor (VDR) and of main enzymes involved in synthesis and metabolism of calcitriol (vitamin D-25-hydroxylase [25-OHase], 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase [1 $\alpha$ -OHase], 1,25-dihydroxyvitamin D-24-hydroxylase [24-OHase]) was detected in BCCs and NS. Expression levels were determined as ratios between target genes (VDR, 1 $\alpha$ -OHase, 25-OHase, 24-OHase) and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal control. Median of mRNA ratios for VDR/GAPDH (BCCs: 16.54; NS: 0.00021), 1 $\alpha$ -OHase/GAPDH (BCCs: 0.739; NS 0.000803) and 24-OHase/GAPDH (BCCs: 0.00585; NS 0.00000366) was significantly (Wilcoxon–Mann–Whitney *U*-test) elevated in BCCs. In contrast, median of mRNA ratio for 25-OHase/GAPDH (BCCs: 0.17; NS: 0.016) was not significantly altered in BCCs as compared to NS. Additionally, we report for the first time expression of 1 $\alpha$ -OHase splice variants in BCCs and NS, that were detected using conventional RT-PCR. In conclusion, our findings provide supportive evidence for the concept that endogenous synthesis and metabolism of vitamin D metabolites as well as VDR expression may regulate growth characteristics of BCCs. New vitamin D analogs that exert little calcemic side effects, their precursors, or inhibitors of 24-OHase may offer a new approach for the prevention or therapy of BCCs. The function of alternative transcripts of 1 $\alpha$ -OHase that we describe here for the first time in BCCs and NS and their effect on activity level has to be investigated in future experiments.**

*Laboratory Investigation* (2004) 84, 693–702, advance online publication, 12 April 2004; doi:10.1038/labinvest.3700096

**Keywords:** vitamin D; vitamin D receptor; basal cell carcinoma; skin; vitamin D-25-hydroxylase; 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase; 1,25-dihydroxyvitamin D-24-hydroxylase

1,25-Dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub> or calcitriol), the biologically active metabolite of vitamin D, has been shown to regulate the growth of various cell types, including human keratinocytes.<sup>1–3</sup> This potent seco-steroid hormone acts via binding to a corresponding intranuclear receptor (VDR), present in target tissues.<sup>4,5</sup> VDR belongs to the superfamily of trans-acting transcriptional regulatory factors, which includes the steroid and thyroid hormone receptors as well as the retinoid-X receptors and retinoic acid receptors.<sup>6–8</sup> Keratinocytes express VDR,<sup>1,9</sup> whose natural ligand, calcitriol, inhibits proliferation and induces differentiation of cultured

human keratinocytes *in vitro*.<sup>1,3</sup> Transcriptional regulation of cell cycle regulatory proteins including WAF-1, and other proteins involved in keratinocyte growth and differentiation, including  $\beta_3$ -integrin and fibronectin, by calcitriol has been shown.<sup>10–12</sup> Clinically, the antiproliferative and prodifferentiating effects of vitamin D analogs are used successfully in the treatment of the hyperproliferative skin disease psoriasis.<sup>13–15</sup> Combination of 1,25-dihydroxyvitamin D<sub>3</sub> and isotretinoin was reported to be effective in the chemotherapy of precancerous and cancerous skin lesions, including squamous and basal cell carcinomas (BCC).<sup>16</sup> Additionally, it has been shown that VDR ablation sensitizes skin to chemically induced carcinogenesis.<sup>17</sup> BCC is the most common malignant tumor of the skin.<sup>18,19</sup> Most theories propose that BCC arise from keratinocyte stem cells in the epidermal basal layer or from adnexal structures, but consensus concerning the

Correspondence: Priv.-Doz. Dr med. J Reichrath, Universitäts-Hautklinik und Poliklinik, Homburg/Saar, 66421, Germany.  
E-mail: hajrei@med-rz.uni-sb.de

Received 01 February 2002; revised 12 February 2004; accepted 13 February 2004; published online 12 April 2004

origin and differentiation of this tumor is still lacking. BCCs are semimalignant skin tumors characterized by a locally aggressive and invasive growth pattern, but, except for very rare cases, have nonmetastasizing behavior.<sup>18</sup> The hedgehog signaling pathway has been identified to be crucial to the development of basal cell carcinomas.<sup>20,21</sup> However, the biological mechanisms underlying the unique characteristics of BCC tumor growth are still completely unknown.<sup>22</sup>

There are two principal enzymes involved in the formation of circulating  $1\alpha,25(\text{OH})_2\text{D}_3$  from vitamin D, the hepatic microsomal or mitochondrial vitamin D-25-hydroxylase (25-OHase) and the renal mitochondrial enzyme  $1\alpha$ -hydroxylase ( $1\alpha$ -OHase) for vitamin D and  $25(\text{OH})\text{D}_3$ , respectively.<sup>23,24</sup> These hydroxylases belong to a class of proteins known as cytochrome P450 mixed function monooxidases. Extrarenal activity of  $25(\text{OH})\text{D}_3$ - $1\alpha$ -hydroxylase has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells.<sup>25–29</sup>

The aim of this study was to analyze the expression of the vitamin D system in BCCs. We addressed the following questions: (i) Do BCCs express the main enzymes involved in the formation or metabolism of calcitriol, indicating that endogenous synthesis or metabolism of vitamin D metabolites may be involved in growth regulation of BCCs? (ii) Are mRNA-levels for VDR,  $1\alpha$ -OHase, 24-OHase or 25-OHase in BCCs different as compared to normal human skin, indicating that vitamin D synthesis and metabolism are altered in BCCs? (iii) Are BCCs potential targets for prevention or therapy with new vitamin D analogs that exert little calcemic side effects or by pharmacological modulation of calcitriol synthesis/metabolism?

## Materials and methods

### Skin Specimens

For immunohistochemistry and RNA analysis, freshly excised cutaneous BCC specimens ( $n=15$ ; male:  $n=9$ , female:  $n=6$ ; mean age: 60.9 years) and biopsies of normal skin ( $n=5$ ; male:  $n=3$ , female:  $n=2$ ; mean age 55.6 years; healthy volunteers, no history of skin disease) were immediately separated from surrounding connective and fat tissue, embedded in OCT Tissue-Tek II (Miles Laboratories, Naperville, IL, USA), snap-frozen in liquid nitrogen, and stored at  $-70^\circ\text{C}$ .

### Immunohistochemistry

#### Primary antibody directed against VDR

We used the rat monoclonal antibody 9A7 $\gamma$  (IgG<sub>2b</sub>; MU 193-UC, BioGenex, CA, USA) that is directed against partially purified vitamin D receptor from chicken intestine and cross reacts with human,

mouse, and rat VDRs, but does not bind to glucocorticoid or estrogen receptors.<sup>30</sup>

#### Preparation of sections and fixation

Serial sections ( $5\ \mu\text{m}$ ) were cut on a cryostat (Frigocut 2800, Reichert-Jung, Heidelberg, Germany) and mounted on pretreated glass slides. Pretreatment of slides with 2% aminopropylmethoxysilane (Sigma, München, Germany) in acetone for 5 min was performed to enhance sticking of sections during the staining procedure. Frozen sections to be stained for VDR were fixed in 3.7% paraformaldehyde (Merck 4005, Darmstadt, Germany) in phosphate-buffered saline (PBS) for 10 min at room temperature (RT), incubated in methanol (Merck 6009, 3 min,  $-20^\circ\text{C}$ ) and acetone (Merck 22, 60 s,  $-20^\circ\text{C}$ ), and transferred into PBS.

#### In situ detection of vitamin D receptor

Incubation steps were performed in a moist chamber at RT. The slides to be stained for VDR were incubated with the rat monoclonal antibody 9A7 $\gamma$  (16 h,  $4^\circ\text{C}$ ) at a dilution of 1:500. After intermediate washing steps (PBS/TBS,  $2 \times 5$  min), the sections were incubated with biotin-labeled rabbit anti-rat IgG (DAKO) at a dilution of 1:400 (30 min, RT), and then with streptavidin–peroxidase complexes (DAKO) at a dilution of 1:400 (30 min, RT), streptavidin-Cy3 (Dianova, Hamburg, Germany) or streptavidin-FITC (Dianova, Hamburg, Germany). After rinsing, streptavidin–peroxidase-labeled sections were incubated with 3-amino-9-ethylcarbazole (AEC, SIGMA, München, Germany) as a substrate for the peroxidase reaction, transferred into tap water, and mounted with AquaTex (Merck). Cy3- or FITC-labeled specimens were mounted with glycerin/PBS (1:10) after a final washing step. In control sections, primary antibody was replaced with polyclonal rat IgG or rabbit IgG (DAKO). No immunoreactivity was observed in these control sections. Specimens were analyzed under a Zeiss microscope (Zeiss, Oberkochen, Germany) or by confocal laser scanning microscopy (LSM2, Zeiss) equipped with a helium–neon-laser (543 nm) as described previously.<sup>31</sup> Photographs were taken on Kodak Ektachrome 64 or Agfa CTX 100 film.

### Conventional RT-PCR for the Analysis of 25-Hydroxyvitamin D- $1\alpha$ -Hydroxylase mRNA

The glioblastoma cell line TX3868 was established and cultured as described previously.<sup>32</sup> RNA isolation from the tumor cell line was performed with Trizol (GIBCO BRL, Rockville, MD USA), based on the same principle as TRI REAGENT.<sup>33</sup> The RNA was quantified spectrophotometrically, and its integrity was controlled by denaturing agarose gel electrophoresis.

Total RNA was DNaseI treated prior to RT-PCR. The absence of genomic DNA was confirmed by

Alu-PCR. First strand cDNA was synthesized with the Omniscript RT kit (QIAGEN, Hilden, Germany) using 2 µg of total RNA, oligo d(T)-Primer and Omniscript-Reverse Transcriptase in a volume of 20 µl. In all, 3 µl of the first-strand cDNA template were utilized in the following PCR. The mastermix for the reactions contained 0.5 µl Taq-DNA Polymerase (5 U/µl; GIBCO BRL, USA), 5 µl 10 × RT-buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.25 µl of 20 mM each dNTP, 1.5 µl MgCl<sub>2</sub> (50 mM), 0.25 µM of each primer (GAPDH and Gap; MWG Biotech, Germany) and 38.75 µl of RNase-free water.

RT-PCR standardization was performed with GAPDH specific primers, 34. The protocol (denaturation 94°C, 60 s; annealing 58°C, 45 s; elongation 72°C, 45 s) consisted of 24 cycles to ensure a linear range of the PCR-reaction. To analyze 1α-OHase splice variants, specific primers termed Gap were chosen from exon 3 and exon 8.<sup>34</sup> PCR conditions for this analysis was denaturation 94°C, 60 s; annealing 60°C, 60 s; elongation 72°C, 60 s for 34 cycles.

*Real-time RT-PCR for the analysis of VDR mRNA expression in NS and BCC*

*RNA isolation.* RNA from skin samples was isolated using the TRI REAGENT kit (2 ml reagent on 100–200 mg of tissue; SIGMA, Germany) as described in the manufacturer's protocol. The integrity of RNA was electrophoretically verified through agarose gels staining with ethidium bromide and the bands visualized under UV light.

*cDNA synthesis.* In all, 7 µl of eluted RNA from each sample were preincubated with 0.5 µg of

random hexamer primer (Promega, WI, USA) in a 8 µl solution for 5 min at 65°C. After chilling on ice, 5 µl of 5 × RT-buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl<sub>2</sub>, 50 mM DTT (1,4-Dithiothreitol)], 2.5 µl of 2.5 mM each dNTP, 8 µl of RNase-free water, 40 units of Rnasin ribonuclease inhibitor (Promega) and 0.5 µl of M-MLV-RT (Moloney Murine Leukemia Virus Reverse Transcriptase; 200 units/µl; Promega) were added to a final volume of 25 µl. The reaction mixture was then incubated for 60 min at 37°C and reverse transcriptase was finally inactivated at 4°C.

*Real-time RT-PCR (LightCycler).* Expression of target genes (VDR, 1α-OHase, 24-OHase and 25-OHase) was analyzed using a real-time quantification method (LightCycler) according to the manufacturer's recommendations (Roche Diagnostics, Mannheim, Germany). Hybridization probes, used as detection format, consist of two different oligonucleotides. One probe is labeled at the 5'-end with LC-Red 640, the second probe is labeled at the 3'-end with fluorescein. (TIB MOLBIOL, Berlin, Germany). To avoid amplification of contaminating genomic DNA, the two primers were placed in different exons. PCR sequence-specific primers and hybridization probes are shown in Table 1.

As the precise amount of total RNA added to each reaction and its quality are both difficult to determine, the target gene was normalized with an endogenous standard (GAPDH). To quantify the transcripts in this study, the housekeeping gene

**Table 1** Oligonucleotide primer and hybridization probe sequences for real-time PCR (LightCycler)

Gene	Oligonucleotides	Sequence	PCR product size (bp)
VDR <sup>1</sup>	Forward Primer	5'-CCAgTTCgTgTgAATgATgg-3'	383
	Reverse Primer	5'-gTCgTCCATggTgAaggA-3'	
	Flouescence- labeled probe	5'-CTCACCTCTgAggACCAgATCgTACT X-3'	
	LC-Red 640-labeled probe	5'-CTgAAgTCAAATgCCATTgAggTCA p-3'	
1α-OHase <sup>2</sup>	Forward Primer	5'-TgTTTgCATTTgCTCAgA-3'	226
	Reverse Primer	5'-CCgggAgAgCTCATAcAg-3'	
	Flouescence- labeled probe	5'-GCCCAgACTCCAggTCCTTCTC X-3'	
	LC Red 640-labeled probe	5'-CTgCCTCCgTTCCTCATggCTg p-3'	
24-OHase <sup>3</sup>	Forward Primer	5'-gCagCCTAgTgCAgATTT-3'	234
	Reverse Primer	5'-ATTCACCCAgAACTgTTg-3'	
	Flouescence- labeled probe	5'-TACgggATAAATgTAgAgAATCCACATT X-3'	
	LC Red 640-labeled probe	5'-ACTgTTTgCTgTCgTTTCCACCg p-3'	
25-OHase <sup>4</sup>	Forward Primer	5'-ggCAAATACCCAgTACgg-3'	291
	Reverse Primer	5'-AgCAAATAgCTTCCAAgg-3'	
	Flouescence- labeled probe	5'-CAGTggTgTCCTTCCgTggTgAAC X-3'	
	LC Red 640-labeled probe	5'-CCCATAggTCAggTgTgCTggTC p-3'	
GAPDH <sup>5</sup>	Forward Primer	5'-TTggTATCgTggAAggACTCA-3'	269
	Reverse Primer	5'-TgTCATCATATTTggCAggTTT-3'	
	Flouescence- labeled probe	5'-TgTCCCCACTgCCAACgTgTCag X-3'	
	LC-Red 640-labeled probe	5'-GgTggACCTgACCTgCCgTCTAgA p-3'	

<sup>1</sup>VDR: Vitamin D-receptor; <sup>2</sup>1α-OHase: 25-hydroxyvitamin D-1α-hydroxylase; <sup>3</sup>24-OHase: 1,25-dihydroxyvitamin D<sub>3</sub>-24-hydroxylase; <sup>4</sup>25-OHase: vitamin D-25-hydroxylase; <sup>5</sup>GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as internal RNA control. Target gene (VDR,  $1\alpha$ -OHase, 24-OHase and 25-OHase) expression of each sample was evaluated on the basis of its GAPDH content. Standard curves were used to determine the concentration of VDR,  $1\alpha$ -OHase, 24-OHase and 25-OHase and GAPDH gene products. Purified PCR products (High Pure PCR Product Purification Kit, Roche Diagnostics) for VDR,  $1\alpha$ -OHase, 24-OHase, 25-OHase and GAPDH (as shown below) were used in different concentrations to create external standard curves. Using this quantification method, 10-fold dilutions of the homologous standards and the target samples for the analysis of VDR,  $1\alpha$ -OHase, 24-OHase, 25-OHase and GAPDH gene expression were prepared in separate glass capillaries and amplified during the same run. DNA concentration of standards was quantified with a spectrophotometer at OD<sub>260</sub>. Each PCR run consisted of five external standards and a no-template control.

The 20  $\mu$ l reaction mixture consisted of a master-mix containing *Taq* DNA polymerase, dNTP mix and reaction buffer (LightCycler-Fast Start DNA Master Hybridization Kit, Roche). MgCl<sub>2</sub> was added in different concentrations: for  $1\alpha$ -OHase 2.5 mM MgCl<sub>2</sub>, for 25-OHase and GAPDH 3 mM MgCl<sub>2</sub>, for VDR and 24-OHase 4 mM MgCl<sub>2</sub>. An amount of 0.5  $\mu$ M of each primer, 0.25  $\mu$ M of each probe and 2  $\mu$ l of template cDNA was used in all assays except for the  $1\alpha$ -OHase where 0.15  $\mu$ M of each probe was applied. Amplification and detection were carried out as follows. After an initial preincubation and denaturation step of 10 min at 95°C, amplification was performed in a three-step cycle procedure (denaturation 95°C, 10 s, ramp rate 20°C/s; annealing 60°C, 10 s, ramp rate 20°C/s; and elongation 72°C, 5 s, ramp rate 20°C/s) for 50 cycles and the final cooling. With the exception of the  $1\alpha$ -OHase the amplification conditions were 95°C, 5 s, ramp rate 20°C/s for denaturation, 55°C, 8 s, ramp rate 20°C/s for annealing and 72°C, 12 s, ramp rate 20°C/s for elongation.

Quantification of target gene expression was obtained by direct comparison with external standards amplified in parallel reactions in the same run. After real-time data acquisition, the parameter  $C_t$  (threshold cycle) was calculated by determining the point at which the fluorescence exceeds an arbitrary threshold limit. The threshold limit was manually set to cross the fluorescent signal of all standards in the exponential phase. The target load in unknown samples was quantified by measuring  $C_t$  and by using a standard curve to determine the starting target message quantity.

For an accurate quantification of target DNA, the amplification efficiency of the target was the same as for the standard. The slope of the standard curve was converted to amplification efficiency  $E$  by the following algorithm:  $E = 10^{-1/\text{slope}}$ . The amplification efficiency of target (VDR,  $1\alpha$ -OHase, 24-OHase and

25-OHase) and housekeeping gene (GAPDH) did not differ more than  $\pm 0.05$ . Finally the target gene/GAPDH ratio was calculated in order to normalize the data.

### Statistical Analysis

The nonparametric Wilcoxon–Mann–Whitney  $U$ -test was used to evaluate the target gene/GAPDH ratios in skin samples.<sup>35</sup>

## Results

### 1,25-Dihydroxyvitamin D<sub>3</sub> Receptor (VDR) mRNA and Protein Content is Elevated in BCC

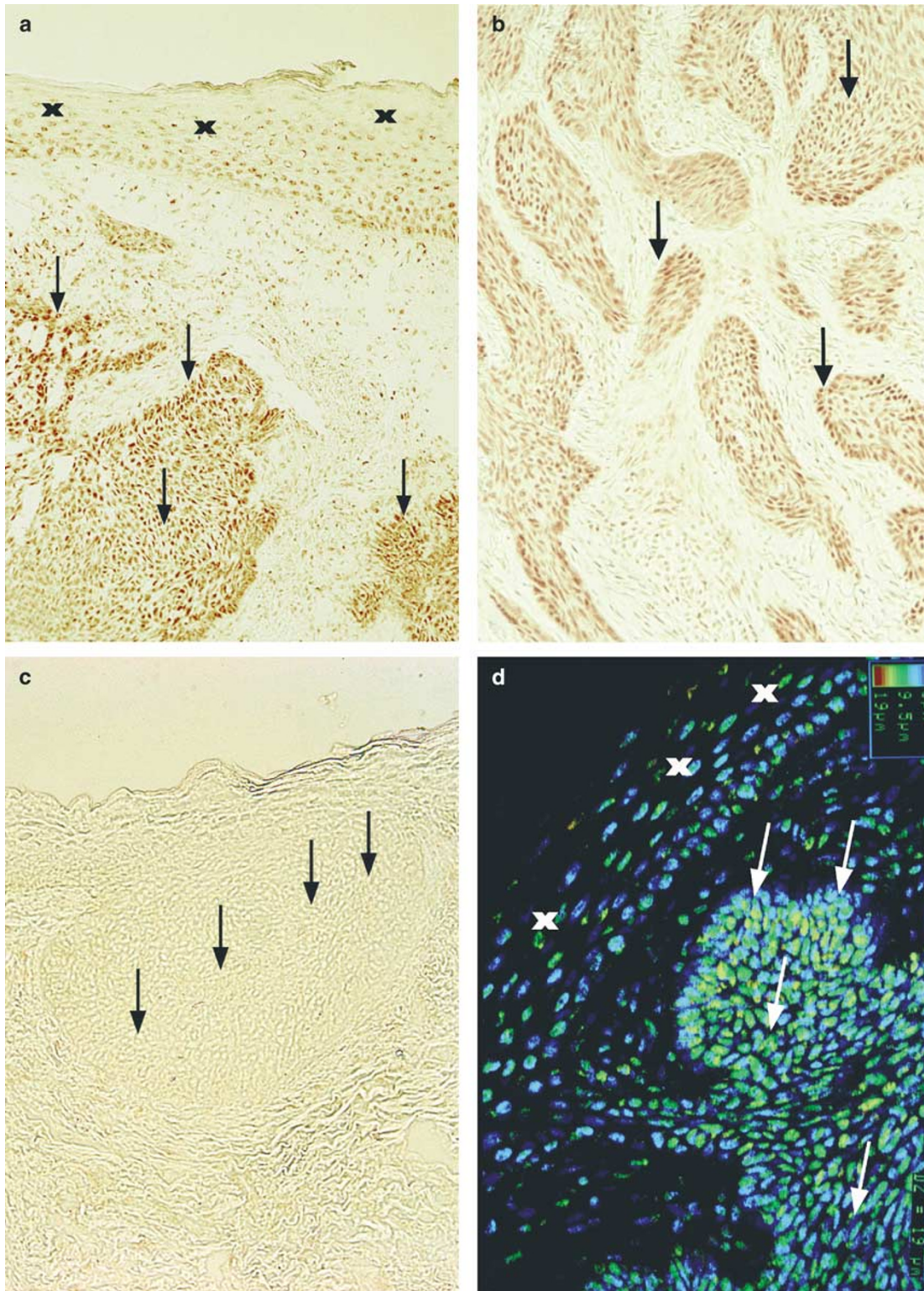
Strong VDR immunoreactivity was observed in all BCCs analyzed ( $n=15$ ) (Figure 1). Almost every tumor cell revealed nuclear immunoreactivity for VDR. There was no visual difference comparing VDR staining pattern in the different variants of BCCs (nodular type, superficial type, fibrosing type). In general, VDR immunoreactivity was pronounced in the palisaded array of peripheral tumor cells. This staining pattern was detected focally in seven specimens and continuously in eight biopsies. VDR staining intensity was markedly stronger in BCCs as compared to adjacent epidermis or to distant unaffected epidermis of the same section (Figure 1, Table 2). No differences in distribution or intensity of VDR staining were detected when comparing adjacent to distant unaffected epidermis. Keratinocytes of all viable epidermal cell layers revealed nuclear immunoreactivity for VDR, that was pronounced in some specimens in lower epidermal cell layers (Figure 1).

Real-time PCR analysis showed a markedly increased ratio of VDR/GAPDH gene expression in BCCs (median: 16.54; ranging from 0 to 114.0) as compared to normal human skin (median: 0.00021; ranging from 0 to 0.00008;  $P=0.0032$ ) (Figure 2a).

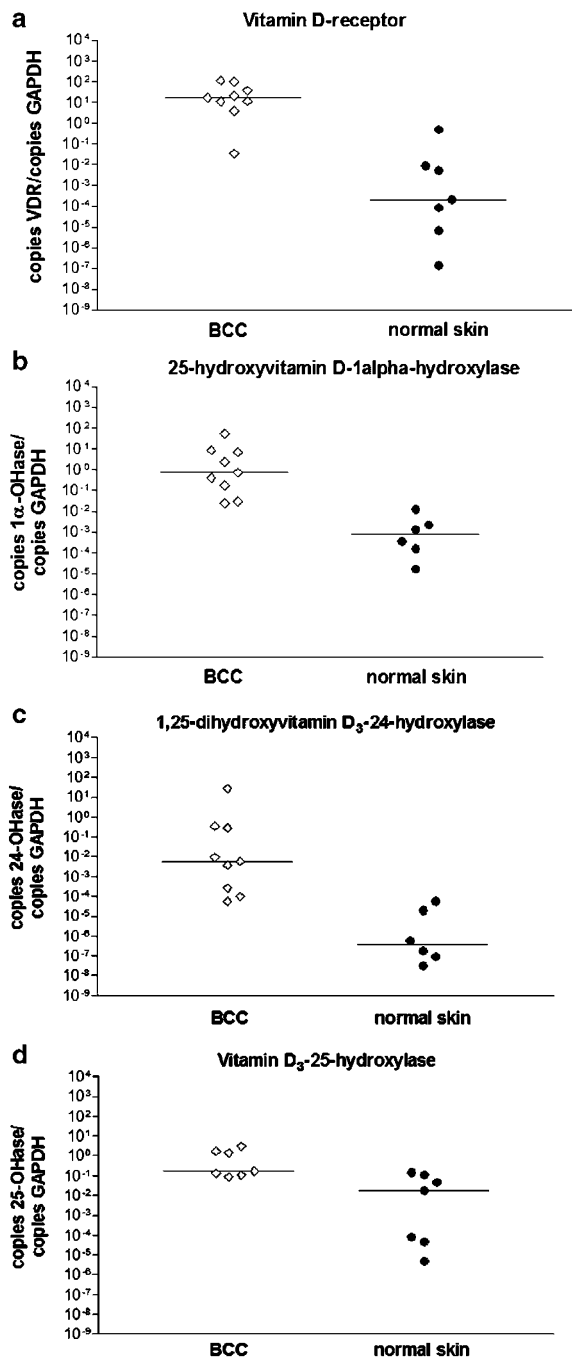
### RNA Levels of $1\alpha$ -OHase, 24-OHase and 25-OHase are Increased in BCC

Real-Time PCR analysis showed ratios of  $1\alpha$ -OHase/GAPDH (Figure 2b; BCCs: median: 0.739; ranging from 0.025 to 53.1; NS: median: 0.000803; ranging from 0.000016 to 0.0114;  $P=0.0004$ ), and 24-OHase/GAPDH (Figure 2c; BCCs: median: 0.0058; ranging from 0.00005 to 27.2; NS: median: 0.000000366; ranging from 0.00000009 to 0.00005;  $P=0.0004$ ) gene expression in BCCs that was significantly increased as compared to NS (Figure 2b, 3.4). Ratio of 25-OHase/GAPDH (Figure 2d; BCCs: median: 0.17; ranging from 0 to 3; NS: median: 0.0166; ranging from 0 to 0.00008;  $P=0.62$ ) was not significantly altered in BCCs as compared to NS.

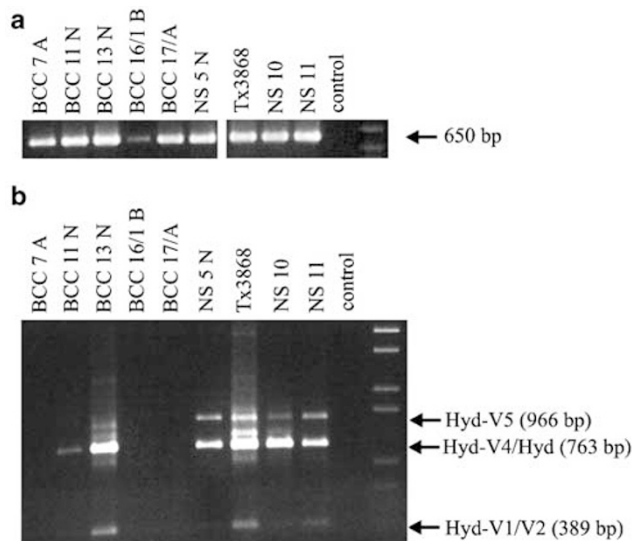




**Figure 1** Immunohistochemical detection (streptavidin–peroxidase technique) of VDR (brown, nuclear labeling, arrow) in nodular (a) and superficial (b) BCC. Note strong VDR immunoreactivity in tumor cells (arrow) as compared to adjacent unaffected epidermis (cross) that is absent. Negative control (c) BCC marked with arrows. (d) Immunohistochemical detection of VDR in a nodular BCC using confocal laser scanning microscopy (overlay technique: different colors (from red to blue) represent VDR immunoreactivity in different levels from 0 to 23  $\mu\text{m}$  of a relatively thick [30  $\mu\text{m}$ ] section). Note increased nuclear fluorescence for VDR in BCC (arrow) as compared to adjacent unaffected epidermis (cross). No counterstaining has been used to detect nuclei.



**Figure 2** Real-time PCR analysis (LightCycler) of VDR (a), 1 $\alpha$ -OHase (b), 24-OHase (c), and 25-OHase (d) genes in BCCs and NS using target gene-specific hybridization probes as described in Materials and methods. The median of *ratio target gene/GAPDH* indicates the relative expression of target gene-specific mRNA, normalized for RNA loading. Note that ratios of VDR/GAPDH (BCCs: median: 16.54; NS: median: 0.00021), 1 $\alpha$ -OHase/GAPDH (BCCs: median: 0.739; NS: median: 0.000803), and 24-OHase/GAPDH (BCCs: median: 0.585; NS: median: 0.00000365) gene expression were significantly increased in BCCs as compared to NS. In contrast, ratio of 25-OHase/GAPDH (BCCs: median: 0.167; NS: median: 0.0166) was not significantly altered in BCCs as compared to NS.



**Figure 3** Expression analysis of the 1 $\alpha$ -OHase gene in BCCs, NS and glioblastoma cell line Tx3868 using conventional PCR. (a): RT-PCR standardization of DNaseI-treated RNA with GAPDH-specific primers. (b) RT-PCR using primers from exon 3 and exon 8 of the 1 $\alpha$ -hydroxylase gene. Arrows, variants of the 1 $\alpha$ -hydroxylase cDNA.

### Detection of 25-Hydroxyvitamin D-1 $\alpha$ -Hydroxylase Splice Variants in NS and BCCs

We performed conventional RT-PCR with five samples from BCC and three samples of NS to examine the expression of the 1 $\alpha$ -OHase gene in BCC. The glioblastoma cell-line TX3868 showing overexpression of 1 $\alpha$ -OHase, due to amplification of the 1 $\alpha$ -OHase gene,<sup>34</sup> served as a control. RT-PCR standardization was with primers specific for the gene of GAPDH, that is known to be expressed equally (Figure 3a). 1 $\alpha$ -OHase specific RT-PCR was performed with primers that bind in exon 3 and 8, allowing in addition the identification of previously reported splice variants.<sup>34</sup> RT-PCR analysis showed three signals at 966, 763 and 389 bp (Figure 3b). The signals demonstrate for the first time in NS and BCC, splice variants of the 1 $\alpha$ -OHase gene named Hyd-V1–Hyd-V5 as we have reported before in glioblastomas and in a glioblastoma cell line.<sup>34</sup> The fragment of 389 bp can be produced by Hyd-V1 and Hyd-V2 lacking exons 4 and 5 whereas the product of 763 bp can be generated by Hyd-V4 or the normal cDNA of the 1 $\alpha$ -OHase gene. The 966 bp product can be assigned to the Hyd-V5 variant containing intron 5. Hyd-V3 could not be amplified with the primers used in this study due to the deletion of the binding site of the reverse primer in the beginning of exon 8. The glioblastoma cell line, TX3868 revealed the strongest expression of normal 1 $\alpha$ -OHase-RNA and all splicing variants, when compared to BCCs or NS. In three samples of normal skin, the expression profile of the 1 $\alpha$ -OHase and splicing variants was weaker in quantity, but comparable in quality to the

glioblastoma cell line. In contrast, no expression of  $1\alpha$ -OHase was detected in three out of five BCCs using conventional RT-PCR as described in Materials and methods. One BCC showed strong expression of the signal corresponding to the normal enzyme and weak signals of all splicing variants, while another BCC revealed the signals corresponding to the normal enzyme and Hyd-V4 by 763 bp. However, because of the weak signal of 763 bp, the overall intensity in this sample might be too weak to detect all splicing variants. Summarizing our results with conventional PCR, we report for the first time splicing variants of  $1\alpha$ -OHase in NS and BCCs. These splice variants were previously described in glioblastoma.

## Discussion

The monoclonal antibody 9A7 $\gamma$  has been used successfully in the immunohistochemical investigation of VDR in various tissues such as chicken intestine, rat brain, disaggregated rat bone cells, rat osteosarcoma 17/2.8 cells fibroblasts, normal and psoriatic human skin.<sup>9,31,36-40</sup> We have characterized the immunohistochemical staining pattern of MoAb 9A7 $\gamma$  in BCCs.<sup>41</sup> All BCC samples analyzed in this study revealed strong VDR-immunoreactivity that was comparable to results that we have published previously.<sup>41</sup> Additionally, VDR mRNA expression was analyzed using real-time PCR. VDR immunoreactivity and mRNA content was much stronger in tumor tissue as compared to NS, indicating increased levels of VDR protein and mRNA in these tumor cells. It appears that cellular VDR expression may be a function of the state of differentiation. In various cell cultures, including human keratinocytes, VDR displays highest maximal ligand binding in the early logarithmic phase of cell growth and diminishes as cells reach confluence.<sup>42</sup> Our observation of increased VDR expression in BCCs could reflect the altered differentiation pattern in these tumor cells. However, we did not find differences comparing VDR immunoreactivity or VDR mRNA content in different types of BCCs (nodular type, superficial type, fibrosing type). These results are in accordance with results that we have published previously.<sup>41</sup>

Regulation of proliferation and differentiation in various cell types, including keratinocytes, has been demonstrated by 1,25-dihydroxyvitamin D<sub>3</sub> and its corresponding receptor.<sup>3</sup> Vitamin D response elements are beginning to be identified in genes involved in cellular growth, differentiation, apoptosis, invasion and metastasis of tumor cells; that is, cell cycle regulators such as the human p21/WAF1, cyclin A and cyclin E genes, the human nm23.H1 gene, the human *c-fms*, *c-fos*, *c-jun*, and *c-myc* genes, the human retinoblastoma gene, the murine fibronectin gene, the human plasminogen activator inhibitor 2 gene, the human laminin and laminin

receptor ( $\alpha 6$ ) genes, and the chicken  $\beta 3$ -integrin gene.<sup>7,43,44</sup> Therefore, we conclude that modulation of VDR expression may be of importance for the unique growth characteristics of BCCs.

It has been demonstrated that VDR require heterodimerization with auxiliary proteins for effective DNA interaction.<sup>45,46</sup> These auxiliary proteins have been identified as the retinoid-X receptors (RXR)- $\alpha$ ,  $\beta$ , and  $\gamma$ .<sup>8,45,46</sup> Heterodimerization of VDR with auxiliary proteins has been shown to increase the transcriptional function and DNA binding to the respective response elements in target genes.<sup>8,45,46</sup> Different classes of vitamin D response elements have been identified that are activated either by VDR-homodimers or by heterodimers of VDR and RXRs or retinoic acid receptors (RAR).<sup>7</sup> It was demonstrated that ligands for RXRs and RARs can enhance the transcriptional activity of 1,25-D<sub>3</sub> mediated nuclear signaling pathways.<sup>7</sup> We have shown expression of RXR and RAR-isotypes in BCCs previously.<sup>47</sup> Therefore, we conclude that different vitamin D signaling pathways are present in BCCs that are determined by the RXR- or RAR-ligand and the nature of the response element.

Using real-time PCR analysis, we show significantly increased mRNA levels of the main enzymes involved in the synthesis and metabolism of calcitriol in these tumors. It can be speculated whether the increased content of VDR on the mRNA and protein levels that we have shown is due to an increased formation of calcitriol in BCCs. In contrast to over-expression of 24-OHase that abrogates calcitriol-mediated growth control, overexpression of functionally intact 25-OHase and  $1\alpha$ -OHase results in an increased synthesis of biologically active vitamin D (calcitriol) and should thereby exert antiproliferative and prodifferentiating effects. In various cell types, calcitriol has been shown to enhance VDR expression at the mRNA and protein levels *in vitro*.<sup>48,49</sup> Interestingly, this elevation of the VDR protein following calcitriol administration is due to an increased transcription of the VDR gene and/or an increased receptor protein lifetime.<sup>49</sup> It has been shown that human keratinocytes have the capacity to hydroxylate vitamin D at the C-1 and C-25 positions.<sup>50,51</sup> Therefore, keratinocytes are able not only to produce vitamin D by the action of UV light, but also to synthesize the biologically active vitamin D metabolite, 1,25(OH)<sub>2</sub>D<sub>3</sub>. After the time of the initial submission of this manuscript, we have reported additional findings documenting the critical importance of the vitamin D biosynthetic system in the pathogenesis of various cutaneous malignancies including squamous cell carcinomas and supporting the importance of the vitamin D system in BCCs.<sup>52</sup>

We and others have shown that cytokines and other factors that stimulate transmembrane-signaling pathways alter VDR levels in keratinocytes and other cell types *in vitro*.<sup>53,54</sup> Surrounding stroma of BCCs often contains an increased number of inflam-



matory cells. Excreted cytokines and inflammatory peptides of these cells may be a reason for increased VDR expression in adjacent tumor cells.

Investigations in numerous cancer cell lines have shown that high concentrations of calcitriol ( $10^{-9}$ – $10^{-7}$  M) inhibit the growth of tumor cells *in vitro*. Additionally, it has been demonstrated that calcitriol has beneficial effects in various *in vivo* models of different types of cancer (reviewed in Hansen *et al.*,<sup>43</sup> and Reichrath.<sup>44</sup> Therefore, if the overexpression of  $1\alpha$ -OHase results in an overproduction of biologically active calcitriol, the accumulation of this metabolite should inhibit growth and invasion of BCCs. It can be speculated whether overexpression of 25-OHase and  $1\alpha$ -OHase in BCCs represents a physiological feedback loop coupled to the increased proliferative activity in these tumors. However, one has to keep in mind that calcitriol may be rapidly metabolized via 24-OHase, that we show is overexpressed in BCCs as well. In conclusion, it is not known whether overexpression of 25-OHase,  $1\alpha$ -OHase and 24-OHase genes results in increased or reduced levels of calcitriol in BCCs. In this context, the function of alternative transcripts of  $1\alpha$ -OHase and their effect on activity level has to be investigated in future experiments. Additionally, the question of cellular or systemic consequences of the endogenous overexpression of  $1\alpha$ -OHase and 24-OHase in BCC remains to be clarified.

Using array CGH, amplification of 24-OHase was detected as a likely target oncogene of the amplification unit 20q13.2 in breast cancer cell lines and tumors.<sup>55</sup> It has been speculated that overexpression of 24-OHase due to gene amplification may abrogate vitamin D<sub>3</sub>-mediated growth control. We have detected overexpression of 24-OHase mRNA in cutaneous BCCs that may be of importance for the growth characteristics and metastatic behavior of these tumors. It is not known whether overexpression of the 24-OHase gene in BCCs is a result of gene amplification or different mechanisms such as transcriptional regulation. It can be speculated whether pharmacological inhibition of 24-OHase activity in BCCs may be effective in the treatment of these tumors, increasing their sensitivity to the antiproliferative effects of endogenous or therapeutically applied vitamin D analogs.

Overexpression of  $1\alpha$ -OHase and 25-OHase genes in BCCs suggests that precursors of biologically active calcitriol may be used for the treatment of BCCs. A great deal of work has been done evaluating the chemotherapeutic and/or chemopreventive effects of retinoids on BCCs. The steroid hormone responsiveness is directly proportional to the number of corresponding receptors.<sup>48</sup> As VDR mediates the biological effects of calcitriol and analogs on proliferation and differentiation in target cells, increased VDR content in cutaneous BCCs may indicate a high sensitivity to endogenous or therapeutically applied calcitriol. Combination

of 1,25-dihydroxyvitamin D<sub>3</sub> and retinoids was reported to be effective in the treatment of precancerous and cancerous skin lesions, including actinic keratoses, squamous cell carcinomas, cutaneous T-cell lymphomas, and BCCs.<sup>16</sup> The results reported herein are encouraging; however, much more work is required to establish a role for endogenously produced or therapeutically applied vitamin D analogs in the chemoprevention of these tumors.

## References

- 1 Smith EL, Walworth NC, Holick MF. Effect of  $1\alpha,25$ -dihydroxyvitamin D<sub>3</sub> on the morphologic and biochemical differentiation of cultured human epidermal cells. *J Invest Dermatol* 1986;86:709–714.
- 2 Bikle DD, Pillai S. Vitamin D, calcium and epidermal differentiation. *Endocr Rev* 1993;14:3–19.
- 3 Gniadecki R. Stimulation versus inhibition of keratinocyte growth by 1,25-dihydroxyvitamin D<sub>3</sub>: dependence on cell culture conditions. *J Invest Dermatol* 1996;294:510–516.
- 4 Stumpf WE, Sar M, Reid FA, *et al.* Target cells for 1,25-dihydroxyvitamin D<sub>3</sub> in intestinal tract, stomach, kidney, skin, pituitary and parathyroid. *Science* 1979;209:1189–1190.
- 5 Baker AR, McDonnell DP, Hughes M. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc Natl Acad Sci USA* 1988;85:3294–3298.
- 6 Evans RM. The steroid and thyroid hormone receptor superfamily. *Science* 1988;240:889–895.
- 7 Haussler MR, Mangelsdorf DJ, Komm BS, *et al.* Molecular biology of vitamin D hormone. *Recent Prog Horm Res* 1988;44:263–305.
- 8 Kliewer SA, Umesono K, Mangelsdorf DJ, *et al.* Retinoic X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D<sub>3</sub> signalling. *Nature* 1992;355:446–449.
- 9 Milde P, Hauser U, Simon T, *et al.* Expression of 1,25-dihydroxyvitamin D<sub>3</sub> receptors in normal and psoriatic skin. *J Invest Dermatol* 1991;97:230–239.
- 10 Cao X, Ross FP, Zhang L, *et al.* Cloning of the promoter for the avian integrin  $\beta_3$  subunit gene and its regulation by 1,25-dihydroxyvitamin D<sub>3</sub>. *J Biol Chem* 1993;268:27371–27380.
- 11 Liu M, Lee MH, Cohen M, *et al.* Transcriptional activation of the Cdk inhibitor p21 by vitamin D<sub>3</sub> leads to induced differentiation of the myelomonocytic cell line U937. *Genes Dev* 1996;10:142–153.
- 12 Polly P, Carlberg C, Eisman JA, *et al.* Identification of a vitamin D<sub>3</sub> response element in the fibronectin gene that is bound by vitamin D<sub>3</sub> receptor homodimers. *J Cell Biochem* 1996;60:322–333.
- 13 Kragballe K. Vitamin D analogues in the treatment of psoriasis. *J Cell Biochem* 1992;49:46–52.
- 14 Perez A, Chen TC, Turner A, *et al.* Efficacy and safety of topical calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>) for the treatment of psoriasis. *Br J Dermatol* 1996;134:238–246.
- 15 Perez A, Raab R, Chen TC, *et al.* Safety and efficacy of oral calcitriol (1,25-dihydroxyvitamin D<sub>3</sub>) for the treatment of psoriasis. *Br J Dermatol* 1996;134:1070–1078.



- 16 Majewski S, Skopinska M, Bollag W, *et al*. Combination of isotretinoin and calcitriol for precancerous and cancerous skin lesions. *Lancet* 1994;344:1510–1511.
- 17 Zinser GM, Sundberg JP, Welsh J. Vitamin D<sub>3</sub> receptor ablation sensitizes skin to chemically induced tumorigenesis. *Carcinogenesis* 2002;23:2103–2109.
- 18 Miller SJ. Biology of basal cell carcinoma (Part I). *J Am Acad Dermatol* 1991;24:1–13.
- 19 Miller SJ. Biology of basal cell carcinoma (Part II). *J Am Acad Dermatol* 1991;24:161–175.
- 20 Bale AE, Yu KP. The hedgehog pathway and basal cell carcinomas. *Hum Mol Genet* 2001;10:757–762.
- 21 Lacour JP. Carcinogenesis of basal cell carcinomas: genetics and molecular mechanisms. *Br J Dermatol* 2002;146:17–19.
- 22 Ramachandran S, Fryer AA, Smith AG, *et al*. Basal cell carcinomas: association of allelic variants with a high-risk subgroup of patients with the multiple presentation phenotype. *Pharmacogenetics* 2001;11:247–254.
- 23 Nebert DW, Gonzalez FJ. P450 genes: structure, evolution, and regulation. *Ann Rev Biochem* 1987;56:945–993.
- 24 Henry HL. Vitamin D hydroxylase. *J Cell Biochem* 1992;49:4–9.
- 25 Bikle DD, Nemanic MF, Gee E, *et al*. 1,25-Dihydroxyvitamin D<sub>3</sub> production by human keratinocytes. Kinetics and regulation. *J Clin Invest* 1986;78:557–566.
- 26 Mawer EB, Hayes ME, Heys SE, *et al*. Constitutive synthesis of 1,25-dihydroxyvitamin D<sub>3</sub> by a human small cell lung cancer cell line. *J Clin Endocrinol Metab* 1994;79:554–560.
- 27 Cross HS, Peterlik M, Reddy S, *et al*. Vitamin D metabolism in human colon adenocarcinoma-derived Caco-2 cells: expression of 25-hydroxyvitamin D<sub>3</sub>-1 $\alpha$ -hydroxylase activity and regulation of side chain metabolism. *J Steroid Biochem Mol Biol* 1997;62:21–28.
- 28 Jones G, Ramshaw H, Zhang A, *et al*. Expression and activity of vitamin D-metabolizing cytochrome P450s (CYP1 $\alpha$  and CYP24) in human non-small cell lung carcinomas. *Endocrinology* 1999;140:3303–3310.
- 29 Tangpricha V, Flanagan JN, Whitlatch LW, *et al*. 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase in normal and malignant colon tissue. *Lancet* 2001;26:357:1673–1674.
- 30 Pike JW, Donaldson CA, Marion SL, *et al*. Development of hybridomas secreting monoclonal antibodies to the chicken intestinal 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> receptor. *Proc Natl Acad Sci USA* 1982;79:7719–7723.
- 31 Reichrath J, Classen UG, Meineke V, *et al*. Immunoreactivity of six monoclonal antibodies directed against 1,25-dihydroxyvitamin D<sub>3</sub> receptors in human skin. *Histochem J* 2000;32:625–629.
- 32 Fischer U, Gracia U, Elkahlon A, *et al*. Isolation of genes amplified in human cancers by microdissection mediated cDNA capture. *Hum Mol Genet* 1996;5:595–600.
- 33 Chomczynski P. A reagent for the single-step simultaneous isolation of RNA, DNA and protein from cell and tissue samples. *BioTechniques* 1993;15:532–534.
- 34 Maas RM, Reus K, Diesel B, *et al*. Amplification and expression of splice variants of the gene encoding the P450 cytochrome 25-hydroxyvitamin D<sub>3</sub>-1,  $\alpha$ -hydroxylase (CYP 27B1) in human malignant glioma. *Clin Cancer Res* 2001;7:868–875.
- 35 Sachs L. Der Vergleich zweier unabhängiger Stichproben: *U-Test* nach Wilcoxon, Mann und Whitney. In: Sachs L (ed.) *Angewandte Statistik*, Springer: Berlin, Heidelberg, New York, Tokyo, 1997, pp 230–237.
- 36 Berger U, Wilson P, McClelland RA. Immunohistochemical detection of 1,25-dihydroxyvitamin D<sub>3</sub> receptors in normal human tissues. *J Clin Endocrinol Metab* 1988;67:607–613.
- 37 Clemens TL, Garrett KP, Zhou XY, *et al*. Immunocytochemical localization of the 1,25-dihydroxyvitamin D<sub>3</sub> receptor in target cells. *Endocrinology* 1988;122:1224–1230.
- 38 Barsony J, Pike JW, DeLuca HF, *et al*. Immunocytology with microwave-fixed fibroblasts shows 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>-dependent rapid and estrogen-dependent slow reorganization of vitamin D receptors. *J Cell Biol* 1990;111:2385–2395.
- 39 Reichrath J, Collins ED, Epple S, *et al*. Immunohistochemical detection of 1,25-dihydroxyvitamin D<sub>3</sub> receptors (VDR) in human skin: a comparison of five antibodies. *Pathol Res Pract* 1996;192:281–289.
- 40 Reichrath J, Müller SM, Kerber A, *et al*. Biologic effects of topical calcipotriol (MC903) treatment in psoriatic skin. *J Am Acad Dermatol* 1997;36:19–28.
- 41 Reichrath J, Kamradt J, Zhu XH, *et al*. Analysis of 1,25-dihydroxyvitamin D<sub>3</sub> receptors (VDR) in basal cell carcinomas. *Am J Pathol* 1999;155:583–589.
- 42 Mangelsdorf DJ, Pike J, Haussler MR. Avian and mammalian receptors for 1,25-dihydroxyvitamin D<sub>3</sub>: *in vitro* translation to characterize size and hormone-dependent regulation. *Proc Natl Acad Sci USA* 1987;84:354–358.
- 43 Hansen CM, Binderup L, Hamberg KJ, *et al*. Vitamin D and Cancer: effects of 1,25(OH)<sub>2</sub>D<sub>3</sub> and its analogs on growth control and tumorigenesis. *Front Biosci* 2001;6:820–848.
- 44 Reichrath J. Will analogs of 1,25-dihydroxyvitamin D<sub>3</sub> (calcitriol) open a new era in cancer therapy? *Onkologie* 2001;24:128–133.
- 45 Yu VC, Deisert C, Andersen B. RXR $\delta$ : A coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 1991;67:1251–1266.
- 46 Leid M, Kastner P, Lyons R. Purification, cloning and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* 1992;68:377–395.
- 47 Dill-Müller D, Kamradt J, Reichrath J. Expression of retinoic acid receptors (RAR- $\alpha$ ,  $\beta$ ,  $\gamma$ ) and retinoid-X receptor- $\alpha$  (RXR- $\alpha$ ) in basal cell carcinomas. In: Norman AW, Bouillon R, Thomasset M (eds.). *Vitamin D Chemistry, Biology and Clinical Applications of the Steroid Hormone*. University of California Printing and Reographics: Riverside, CA, 1997, pp 493–494.
- 48 Costa EM, Feldman D. Measurement of 1,25-dihydroxyvitamin D<sub>3</sub> receptor turnover by dense amino acid labeling: changes during receptor up-regulation by vitamin D metabolites. *Endocrinology* 1987;120:1173–1178.
- 49 Wiese RJ, Uhland-Smith A, Ross TK, *et al*. Up-regulation of vitamin D receptor in response to 1,25-dihydroxyvitamin D<sub>3</sub> results from ligand-induced stabilization. *J Biol Chem* 1992;267:20082–20086.
- 50 Lehmann B. HaCaT cell line as a model system for vitamin D<sub>3</sub> metabolism in human skin. *J Invest Dermatol* 1997;108:78–82.

- 51 Rudolph T, Lehmann B, Pietsch J, *et al*. Normal human keratinocytes in organotypic culture metabolize vitamin D<sub>3</sub> to 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub>. In: Norman AW, Bouillon R, Thomasset M (eds.) Vitamin D Chemistry, Biology and Clinical Applications of the Steroid Hormone. University of California Printing and Reprographics, Riverside, CA, 1997, pp 581–582.
- 52 Kamradt J, Rafi L, Mitschele T, *et al*. Analysis of the vitamin D system in cutaneous malignancies. *Recent Results Cancer Res* 2003;164:259–269.
- 53 Reichrath J, Hügel U, Klaus G, *et al*. Modulation of 1,25-dihydroxyvitamin D<sub>3</sub> receptor (VDR) expression in HaCaT keratinocytes. In: Norman AW Bouillon R Thomasset M (eds.). Vitamin D: Gene Regulation, Structure-Function Analysis and Clinical Application. Walter de Gruyter: Berlin, 1991, pp 445–446.
- 54 Krishnan AV, Cramer SD, Bringhurst FR, *et al*. Regulation of 1,25-dihydroxyvitamin D<sub>3</sub> receptors by parathyroid hormone in osteoblastic cells: role of second messenger pathways. *Endocrinology* 1995;136:705–712.
- 55 Albertson DG, Ylstra B, Segraves R, *et al*. Quantitative mapping of amplicon structure by array CGH identifies CYP24 as a candidate oncogene. *Nat Genet* 2000;25: 144–146.