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No evidence for amplification of 25-hydroxyvitamin D-1α-OHase (1α-OHase) or 1,25-dihydroxyvitamin D-24-OHase (24-OHase) genes in malignant melanoma (MM)☆

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Abstract

Increasing evidence points at an important function of Vitamin D metabolites for growth regulation in various tissues, including MM. Using array CGH, amplification of 24-OHase was recently detected as a likely target oncogene of the amplification unit 20q13.2 in breast cancer cell lines and tumors. Additionally, amplification of 1α-OHase has been reported in human malignant glioma. Using immunohistochemistry, we have now detected nuclear Vitamin D receptor (VDR) immunoreactivity in primary cutaneous malignant melanoma (MM), indicating that Vitamin D metabolites may be of importance for the growth regulation in these tumors. Using Southern analysis, we have analyzed MM and metastases for evidence of amplification of 1α-OHase or 24-OHase genes. Our results do not support the hypothesis that amplification of 1α-OHase or 24-OHase genes may be of importance for pathogenesis or progression of MM.

Keywords: Vitamin D; Malignant melanoma; Gene amplification

1. Introduction

1,25-Dihydroxyvitamin D_3 (Calcitriol, $1,25(OH)_2D_3$) and analogs have been shown to inhibit proliferation and to induce differentiation in various cell types, including human melanocytes [1,2]. It is well known that $1,25(OH)_2D_3$ acts via binding to a corresponding intranuclear receptor (Vitamin D receptor, VDR), present in target tissues [3,4]. 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 [5,6]. There are two principal enzymes involved in the formation of circulating $1,25(OH)_2D_3$ from Vitamin D, the hepatic microsomal or mitochondrial Vitamin D 25-hydroxylase (25-OHase) and the renal mitochondrial enzyme 1α-hydroxylase (1α-OHase) for Vitamin D and $25(OH)D_3$, respectively [7,8]. $1,25(OH)_2D_3$ is metabolised in target cells at least in part by 1,25-dihydroxyvitamin D 24-hydroxylase (24-OHase), resulting in a specific C-24 oxidation pathway to yield the biliary excretory product calcitroic acid. These hydroxylases belong to a class of proteins known as cytochrome P450 mixed function monooxidases. Extrarenal activity of 1α-OHase has been reported in various cell types including macrophages, keratinocytes, prostate and colon cancer cells [9,10].

Using array CGH, amplification of 24-OHase was recently detected as a likely target oncogene of the amplification unit 20q13.2 in breast cancer cell lines and tumors [11]. It has been speculated that over-expression of 24-OHase due to gene amplification may abrogate Vitamin D_3-mediated growth control. Additionally, amplification of the 1α-OHase gene has been reported in human malignant glioma [12]. The significance of this finding remains to be investigated.

The aim of this study was to analyze immunohistochemically expression of VDR in primary cutaneous malignant melanoms and to analyze MM and metastases for evidence of amplification of 1α-OHase or 24-OHase genes using Southern analysis.

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2. Materials and methods

2.1. Skin specimens for immunohistochemistry

Biopsies of freshly excised primary cutaneous malignant melanomas (MM) ($n = 3$) and biopsies of normal skin ($n = 5$, healthy volunteers, no history of skin disease) were immediately embedded in OCT Tissue-Tek II (Miles Laboratories, Naperville, IL, USA) snap-frozen in liquid nitrogen, and stored at −80 °C. Diagnosis was confirmed histologically by a certified pathologist.

2.2. Immunohistochemical detection of VDR

Serial sections (5 μm) were cut on a cryostat (Frigocut 2800, Reichert-Jung, Heidelberg, Germany) and mounted on glass slides pretreated with 2% aminopropylmethoxysilane (Sigma, München, Germany). VDR was detected immunohistochemically using the rat monoclonal antibody 9A7γ [13] at a dilution of 1:500 (16 h, 4 °C) as desribed previously [14,18–20]. The rat monoclonal antibody 9A7γ (IgG_{2b}; MU 193-UC, BioGenex, CA, USA) 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 [13].

2.3. Skin specimens for Southern analysis

Paraffin embedded samples of normal human tissues (colon and stomach, $n = 10$) and primary cutaneous MM (NMM, LMM, SSM; thickness of tumors between 0.4 and 5.00 mm; $n = 16$) were analyzed. In MM samples, DNA was extracted as described previously [12] after microscopic microdissection.

2.4. Southern analysis

Samples of 10 μg genomic DNA from melanoma tissues were restricted overnight with 80 U *Eco*RI and resolved on 0.8% agarose gels. Southern blots were prepared on nitrocellulose membranes and hybridized to the following probes: (A) 1α-OHase: a 226 bp PCR amplified cDNA fragment containing Exons 4–6. (B) 24-OHase: a 234 bp PCR amplified cDNA fragment containing Exons 7–8. Probes were labed using the PCR Dig-labeling kit (Roche, Mannheim) according to manufactors protocol. Prehybridization and hybridization reactions were performed in 0.5 M $Na_2HPO_4 \cdot 2H_2O$, pH 7.2, 5 g blocking reagent (Roche, Mannheim) 0.3 g SDS at 58 °C, and washed to a final stringency of 0.1× SSC, 0.1% (w/v) SDS, 1 mM EDTA at 65 °C. Hybridization was detected with anti-Dig-AP and CDP-Star (Roche, Mannheim) by chemiluminescence.

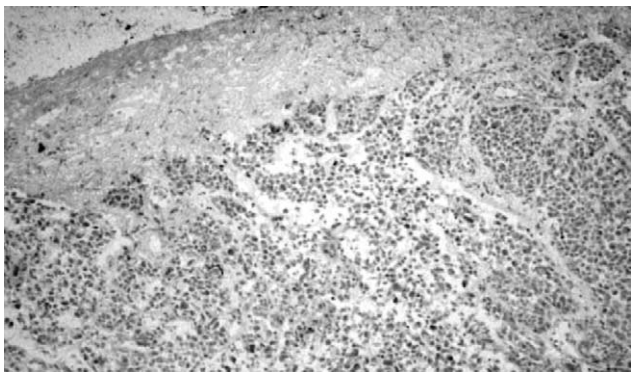

Fig. 1. Immunohistochemical detection of VDR in malignant melanoma. Note that most tumor cells reveal nuclear VDR-immunoreactivity.

3. Results

3.1. Expression of 1,25-dihydroxyvitamin D_3 receptors (VDR) in primary cutaneous malignant melanoma (MM) and normal human skin

Every specimen analyzed ($n = 5$) revealed nuclear VDR immunoreactivity consistently in all cell layers of the viable epidermis. In three of five biopsies analyzed, intensity of VDR immunoreactivity was stronger in keratinocytes of the basal layer as compared to upper layers. In the hair follicle VDR was most markedly expressed in keratinocytes of the outer root sheath, whereas staining of cells of the inner root sheath was heterogeneous (data not shown). Single scattered VDR-positive fibroblasts (identified by their shape) were found as well.

VDR immunoreactivity was observed in every MM analyzed ($n = 3$) (Fig. 1). Almost every tumour cell revealed nuclear immunoreactivity for VDR (Fig. 1). VDR staining intensity was stronger in MM as compared to adjacent epidermis or to distant unaffected epidermis of the same section. No differences in distribution or intensity of VDR staining were detected comparing adjacent to distant unaffected epidermis.

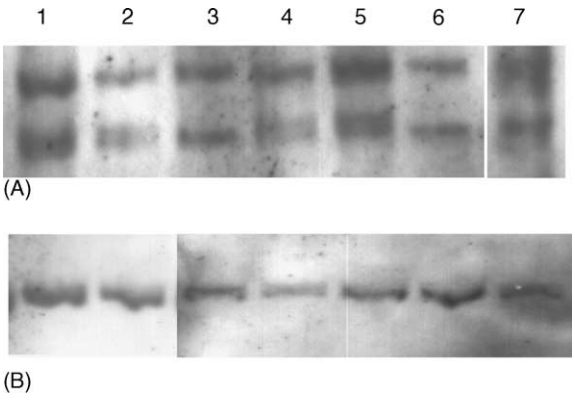

Fig. 2. Southern blot analysis of seven representative samples of normal (1) and MM (2–7) tissue DNA samples. Data show no signs for amplification of 24-OHase (A) or 1α-OHase (B) genes.

3.2. Southern analysis

Southern analysis revealed no evidence for amplification of 1α-OHase or 24-OHase genes in primary cutaneous MM (Fig. 2).

4. Discussion

Regulation of proliferation and differentiation by $1,25(OH)_2D_3$ and its corresponding receptor has been demonstrated in various cell types, including melanoma cells [1]. To our knowledge, this is the first report demonstrating VDR-immunoreactivity in cutaneous MM. The monoclonal antibody 9A7γ 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, and basal cell carcinomas [14–20]. Our evaluation of 9A7γ immunoreactivity in normal human skin is in agreement with previous observations [14,15,18–20]. Vitamin D response elements have been identified in genes involved in cellular growth, differentiation, apoptosis, invasion and metastasis of tumor cells; i.e. 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 retinoblastom gene, the murine fibronectin gene, the human plasminogen activator inhibitor 2 gene, the human laminin and laminin receptor (α6) genes, and the chicken β3-integrin gene (review in [21,22]). Therefore, it can be assumed that VDR-expression may be of importance for growth regulation in MM.

Evidence for a protective influence of Vitamin D has emerged in a number of systemic cancers, particularly colon, prostate and breast (review in [21,22]). This is mainly based on epidemiological studies related to diet, skin type and geographical factors, serum 25-OH-D levels and, recently, functionally significant polymorphisms of the Vitamin D nuclear receptor (VDR) gene (review in [21,22]). There is now accumulating evidence for a similar role of the Vitamin D system in MM. Low serum levels of $1,25(OH)_2D_3$ have been reported in MM patients, but this did not reach statistical significance [23]. It has been shown that polymorphism at the *Fok*I restriction site of the VDR is associated with increased susceptibility to MM but a stronger association was found with MM outcome, as predicted by Breslow thickness [24]. The risk reduction for MM attributable to the *FF* genotype was estimated at 33.6%, and the combined *ttff* genotype, and to a lesser degree heterozygote genotypes, were associated with thicker tumours, particularly those >3.5 mm thick. It has been speculated that the effect on tumour thickness could be the result of an effect on cell proliferation or alternatively on tumour cell invasion [25]. These findings for MM are closely similar to those reported for the occurrence of carcinoma of the breast, and outcome, but not occurrence, of cancer of the prostate (review in [25]). Thus, there are close parallels in the evidence of the involvement of Vitamin D_3 in MM and certain systemic cancers, in terms of VDR expression, growth and death responses of malignant cells in culture, malignant cell migration and metastasis, association with low circulating levels of Vitamin D_3 and the effect of VDR polymorphisms (review in [25]).

Using array CGH, amplification of 24-OHase was recently detected as a likely target oncogene of the amplification unit 20q13.2 in breast cancer cell lines and tumors [11]. It has been speculated that over-expression of 24-OHase due to gene amplification may abrogate Vitamin D_3-mediated growth control. Additionally, amplification of 1α-OHase has been reported in human malignant glioma [12]. In this study, we did not find evidence that amplification of 1α-OHase or 24-OHase genes may be involved in pathogenesis or progression of MM. In conclusion, there is increasing evidence for a contribution of the Vitamin D system to the pathogenesis and progression of MM. However, there is no evidence for amplification of 1α-OHase or 24-OHase genes in MM or metastases.

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