Published in final edited form as:

Hum Pathol. 2013 March ; 44(3): 374–387. doi:10.1016/j.humpath.2012.03.031.

Expression of Vitamin D-Activating Enzyme 1 α -Hydroxylase (CYP27B1) Decreases during Melanoma Progression**

Anna A. Brożyna^a, Wojciech Jóźwicki^a, Zorica Janjetovic^b, and Andrzej T. Slominski^{b,c,*}

^aDepartment of Tumor Pathology and Pathomorphology, Oncology Centre, Prof. Franciszek Łukaszczyk Memorial Hospital, The Ludwik Rydygier Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland ^bDepartment of Pathology and Laboratory Medicine, University of Tennessee Health Science Center, Memphis, TN, USA ^cDivision of Dermatology, Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, USA

Summary

1 α -Hydroxylase (CYP27B1), the enzyme responsible for the synthesis of the biologically active form of vitamin D (1,25(OH)₂D₃), is expressed in the skin. To assess the correlation between progression of melanocytic tumors and CYP27B1, we analyzed its expression in 29 benign nevi, 75 primary cutaneous melanomas, 40 metastases, and 4 re-excision and 6 normal skin biopsies. Immunoreactivity for CYP27B1 was significantly lower in the vertical growth phase (VGP) and metastatic melanomas (0.6 and 0.5 arbitrary units [AU], respectively) in comparison with nevi and radial growth phase (RGP) tumors (1.2 and 1.1 AU, respectively); and expression was reduced in more advanced lesions (Clark levels III–V, Breslow thickness $\geq$ 2.1 mm; 0.8 and 0.7 AU, respectively). There was an inverse correlation between CYP27B1 and Ki-67 expression. Furthermore, CYP27B1 expression was reduced in primary melanomas that created metastases in comparison with non-metastasizing melanomas. Reduced CYP27B1 expression in RGP was related to shorter overall survival (810 vs 982 vs 1151 days in melanomas with absent, low, and high CYP27B1 immunoreactivity), and low CYP27B1 expression in RGP and VGP was related to shorter disease-free survival (114 vs 339 vs 737 days and 129 vs 307 vs 737 days, respectively, in melanomas with absent, low, and high CYP27B1). Also, CYP27B1 expression was inversely related to melanin in melanoma cells in vivo and melanoma cells cultured in vitro. Thus, reduction of CYP27B1 correlates with melanoma phenotype and behavior, and its lack affects the survival of melanoma patients, indicating a role in the pathogenesis and progression of this cancer.

Introduction

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃; calcitriol) is an active form of vitamin D that exerts its phenotypic effects in target tissues through activation of vitamin D receptor (VDR) with following upregulation of gene expression after binding to the vitamin D response elements (VDREs) in their promoter regions [1,2]. The classical action of vitamin D₃ was

**This work was supported by NIH [Grant R01AR052190] to AS, and by the internal funds of the Ludwik Rydygier Collegium Medicum, Nicolaus Copernicus University [grant no 64/2009].

© 2012 Elsevier Inc. All rights reserved.

*Corresponding author and address for reprints: Andrzej Slominski, MD, Department of Pathology, 930 Madison Avenue, Memphis, TN 38163, Tel: 901-4483741; Fax: 901-4486979, aslominski@uthsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

said to be the regulation of calcium and phosphate homeostasis [3]. However, its activity actually is more complex, and studies initiated in the 1980s showed that vitamin D₃ regulates the proliferation and differentiation of normal and cancer cells and has endocrine and immunoregulatory functions [2,4,5].

The active form of vitamin D₃, 1,25(OH)₂D₃, is generated by two-step hydroxylation of cholecalciferol, first in the liver at position C25 to produce 25(OH)D₃ and second at position 1α by CYP27B1 in the distal convoluted tubule, the cortical and medullary part of the collecting ducts, and the papillary epithelia of the kidneys [6]. In the skin, vitamin D also is activated through sequential hydroxylation at positions C25 and C1α, making this organ independent of systemic activation [7]. Furthermore, CYP27B1 is expressed widely in the body, being found in normal and cancer tissues, of which examples are the lungs, liver, placenta, thymus, stomach, brain, lung cancer [8], breast [9], and skin [7, 10].

Under normal conditions, the expression of CYP27B1 is regulated by 1,25(OH)₂D₃ in a negative feedback loop, which, in addition to its inactivation by CYP24, prevents uncontrolled increases in the tissue calcitriol concentration [11]. This mechanism can be deregulated or fail under pathological conditions. For example, in tumors, both the metabolism of vitamin D₃ and vitamin D-regulated molecular signaling can be altered. This process appears to be complex; eg, in basal cell carcinomas, VDR and CYP27B1 expression is elevated [12], whereas in other tumors such colon cancers, VDR expression is decreased in parallel with decreases in tumor differentiation [13].

Vitamin D can regulate the activity of pigment cells (reviewed by Pinczewski and Slominski [14] and Slominski et al [15]). Also, melanoma cells respond to the anti-proliferative actions of vitamin D₃ derivatives; however, this response is heterogeneous. For example, only melanoma lines expressing VDR abundantly are sensitive to inhibition of proliferation [4]. In addition, the responsiveness of melanoma cells to novel vitamin D analogs is dependent on the expression of VDR [16,17].

There are clinical and epidemiologic data indicating that defects in VDR or reduced concentrations of 25(OH)D₃ are involved in melanoma formation [14,18]. Similarly, with the help of in situ analyses, we have shown that expression of VDR decreases during progression of skin melanocytic lesions and that primary advanced melanomas and melanoma metastases express the lowest amount of VDR [18]. We also have found that significant reduction or loss of VDR expression is associated in shorter overall survival of patients with melanoma. This finding indicates that attenuation of VDR signaling can promote melanoma progression. This hypothesis is consistent with epidemiologic data showing that a higher serum concentration of 25(OH)D₃ is positively associated with longer survival of melanoma patients and less advanced stages of the cancer [19] and that genetic defects in VDR increase the probability of melanoma development (reviewed by Pinczewski and Slominski [14]). Finally, experiments performed with transgenic mice in which retinoid X receptor (RXR), partner of VDR, or VDR itself was deleted showed a higher incidence of melanoma [20].

Therefore, VDR signaling appears to be an important factor in the progression of melanocytic lesions and in defining melanoma behavior. Because CYP27B1 plays a crucial role in the activation of vitamin D (a necessary step for VDR signaling), we investigated changes in its local expression. The expression was tested in melanocytic nevi and melanomas at different degrees of progression, and correlation was made with overall survival time (OS) and disease-free survival time (DFS). Because previous studies showed that VDR expression was attenuated by melanogenesis [18,21], we also looked for a correlation between melanin content and CYP27B1. To our knowledge, this is the first

comprehensive study linking CYP27B1 expression with progression of melanocytic tumors and correlating it with melanoma behavior.

Materials and methods

Patients and tissue samples

This study was approved by the Committee of Ethics of Scientific Research of Collegium Medicum of Nicolaus Copernicus University, Poland. We analyzed 154 samples obtained from 92 patients who were treated in the Oncology Centre-Prof. Franciszek Łukaszczyk Memorial Hospital, Bydgoszcz, Poland, from 2003 to 2009. The nevi and melanoma specimens were chosen randomly from the archive of the Department of Tumor Pathology and Pathomorphology of the Oncology Centre. Normal skin was obtained from patients who underwent other operations. The characteristics of the patients and pigmented lesions are presented in Table 1. Tissue samples were evaluated independently by two Board-certified pathologists. Melanoma class and stage according to the American Joint Committee on Cancer (AJCC) [22] are summarized in Table 2.

All local recurrences and 38 of 40 metastases were matched with their primary lesions.

Immunohistochemistry technique

Expression of CYP27B1 was evaluated in formalin-fixed sections by immunocytochemistry staining with the help of rabbit anti-CYP27B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:75. Detailed protocols are described in the supplemental material.

Melanin content assessment and Ki-67 and Bcl2 immunostaining was done as previously described [18, 21].

Assessment of immunostaining

Staining intensity was assessed semiquantitatively using a scale from 0 to 3 arbitrary units (AUs). Detailed protocols for CYP27B1, Ki-67, and Bcl2 immunohistochemical evaluation are described in the supplemental material. The staining intensity of VDR was scored from 0 to 3 AU as previously described [18].

Cell culture

Human SKMEL-188 melanoma cells (a kind gift from Dr Ashok Chokrabarty, Yale University), established from a human metastatic melanoma, were maintained as described [23]. Other metastatic melanoma cells—YUWERA, YUTICA, YUKSI, YULAC, YUSIV, YUAME, YUROB, YUCOT, YUKIM, YUMUT, and YUKOLI—were obtained from the cell culture facility of the Yale Skin Disease Research Core Center (YSDRCC) and were gifts of Dr. Ruth Halaban. Normal human epidermal melanocytes and keratinocytes were isolated, respectively, from adult skin or neonatal foreskin of African American donors [21]. Detailed protocols are given in the supplemental material.

Preparation of cell extracts

Nuclear and cytoplasm extracts were prepared according to the manufacturer's protocol (Active Motif, Carlsbad, CA), which is described in the supplemental material.

Western blotting analysis

Both CYP27B1 and β -actin were assessed by Western blotting. A detailed protocol is provided in the supplemental material.

Real-Time RT PCR

The detailed protocol is available in the supplemental material.

Statistical Analysis

Statistical analyses were performed using Prism 5.0 (GraphPad Software, San Diego, CA). For additional details, see the supplemental material.

Results

Clinicopathological characteristic

Overall (OS) and disease-free (DFS) survival times were studied in relation to standard prognostic factors such as lesion ulceration, mitotic index, percentage of Ki-67-positive cells, tumor-infiltrating lymphocytes (TILs), and AJCC pTNM stage [22, 24, 25]. Both OS and DFS were significantly longer in melanomas without ulceration or with a low mitotic index (< 1 mitoses/mm²) than in melanomas with ulceration or a high mitotic index (> 1 mitoses/mm²). We did not find differences in OS or DFS between female and male groups or between younger (< 50 years) and older (> 50 years) patients (Supplementary Table S1). In general, the location of melanoma did not affect the OS or DFS, and we found better survival only for melanomas localized on the extremities vs. acral melanomas ($\chi^2 = 7.826$; $P = .0051$; hazard ratio 0.1479; median survival 1068 and 582 days, respectively). In our cohort, TILs also affected OS; eg, in melanomas with brisk TILs, we observed significantly better survival than in patients with inactive or absent TILs (Supplementary Table S1). Furthermore, better OS was observed in less advanced melanomas (pTis, pT1, and pT2 or pN0) than in more advanced lesions (pT3 and pT4 or pN1, pN2, and pN3), and overall stage was related to both OS and DFS, with significant differences found between stages 0, I, and II vs. III and IV (Supplementary Table S1).

CYP27B1 immunolocalization in skin pigmented lesions

Immunostaining of CYP27B1 was found in all samples of normal skin, in 26 of 29 melanocytic nevi (89.7%), 61 of 75 primary melanomas (81.3%), and 19 of 44 metastases or recurrent lesions (43.2%) (Fig. 1 A–G). Cytoplasmic CYP27B1 was slightly but significantly elevated by *t* test ($P < .05$) but not ANOVA ($P > .05$) in melanocytic nevi compared with normal skin (Fig. 2). There was no difference in CYP27B1 expression between melanocytic nevi and melanomas at RGP. However, expression was significantly lower in VGP and metastatic melanomas than in nevi and RGP melanomas (Fig. 2). Furthermore, it was significantly lower in metastatic than in VGP melanoma. When melanomas were analyzed according to the histologic type, CYP27B1 was lower in superficial spreading (SSM) and nodular (NMM) melanomas than in melanocytic nevi by *t* test ($P < .05$), and it was lower in metastases than in SSM and VGP as analyzed by ANOVA (Fig. 2B). Paired analysis of primary melanomas and metastases in 28 of 38 cases showed higher CYP27B1 expression, whereas in 6 cases, expression was lower, and in 4 cases, the staining intensity was similar. The ANOVA test in this group showed that for RGP, CYP27B1 expression was higher than in metastases ($P < .001$), whereas VGP and metastases displayed similar staining intensity.

The detailed analysis of CYP27B1 immunoreactivity in melanomas stratified according Clark and Breslow levels showed similar enzyme expression in melanocytic nevi and melanomas in the less advanced stages (Clark levels I and II and Breslow thickness ≤ 2 mm) (Fig. 3 A, B). However, expression decreased significantly during transition to more advanced stages (Clark levels III–V and Breslow thickness ≥ 2.1 mm) (Fig. 3A, B), being the lowest at Clark level V (Fig. 3A).

To better define the association between CYP27B1 immunostaining and melanoma progression, the data were analyzed using Pearson's correlation. When melanoma progression was assessed using Clark level, a statistically significant negative correlation was found for CYP27B1 expression in both the RGP ($r = -0.248$; $P = .0117$) and VGP ($r = -0.3574$; $P = .0013$) melanomas. Similar negative correlation between CYP27B1 expression and melanoma progression was found using Breslow thickness ($r = -0.2212$; $P = .00236$ and $r = -0.3265$; $P = .003$ for RGP and VGP, respectively).

We found a similar relation when melanomas were classified according to AJCC pTNM stage (Supplemental Fig. 1A). Less advanced melanomas (pTis, pT1, and pT2) showed significantly higher ($P < .05$) CYP27B1 expression than more advanced melanomas (pT3 and pT4).

We also analyzed relative expression in relation to the ability to develop metastases and found reduced CYP27B1 expression in primary melanomas associated with metastases (MM) in comparison with NMM (Fig. 4). When melanomas were classified according to pN features (according to AJCC), significant differences ($P < .05$) were observed between pN0 and other stages (pN1, pN2, and pN3) of melanomas (Supplemental Fig. 1B). In addition, CYP27B1 was lower in VGP vs RGP of SSM and in deeper levels of invasion of NMM; eg, papillary dermis vs reticular dermis (Fig. 5A–D).

We found also that regional and distant metastatic melanomas (stage III and IV) had lower CYP27B1 immunostaining than localized melanomas (stage 0, I, and II). For RGP, significant differences were seen between stage III and I and III and II, whereas for VGP, these differences were observed for stages I and II vs stages III and IV (Supplemental Fig. 1C, D).

Lastly, we analyzed the cytoplasmic CYP27B1 immunoreactivity in the skin surrounding pigmented lesions and found that CYP27B1 expression was significantly lower in this skin than in normal skin and skin surrounding melanocytic nevi (Fig. 6).

There were no statistically significant relations of CYP27B1 with ulceration, presence of TILs, or age (data not shown). However, we observed a trend to more frequent ulceration in melanomas with no or low CYP27B1 expression. Similarly, a trend was noted for a higher rate of TIL invasion in melanomas with higher CYP27B1 concentrations ($P > .05$).

Correlation between CYP27B1 and proliferative potential

We also analyzed the correlation between CYP27B1, proliferation (as assessed by Ki-67 immunocytochemistry), and apoptosis (as assessed by Bcl-2 immunocytochemistry). In primary melanomas, the percentage of Ki-67-positive tumor cells correlated inversely with CYP27B1 expression, and in melanomas with the highest CYP27B1 immunolabeling, proliferative activity was reduced (Fig. 7). For melanomas at RGP, the percentage of Ki-67-positive cells in cases having low or no CYP27B1 expression was significantly higher than in melanomas with a large amount of CYP27B1 (Fig. 7A). For melanomas at VGP, the trend was similar, although the differences were not statistically significant (Fig. 7B). The latter was most likely attributable to the presence of one case of melanoma showing extensive CYP27B1 immunostaining.

Expression of Bcl-2 in nevi, melanomas, and metastases did not correlate with CYP27B1 immunostaining.

CYP27B1 expression affects OS and DFS

Finally, we analyzed whether there is a correlation between the immunostaining intensity of CYP27B1 in primary melanomas and OS. Reduced CYP27B1 expression in RGP was related to shorter overall survival (log-rank [Mantel-Cox] test: $\chi^2 = 9.102$; $P = .0106$; log-rank test for trend: $\chi^2 = 7.929$; $P = .0049$) (Fig. 8A; Table 3). For CYP27B1 expression in melanoma cells at VGP, we found only a significant trend ($\chi^2 = 5.714$; $P = .0168$) for shorter OS. We found no correlation between CYP27B1 immunostaining in metastases and OS.

Low CYP27B1 also was related to shorter DFS, and statistically significant correlations were found for both CYP27B1 immunostaining in RGP (log-rank [Mantel-Cox] test: $\chi^2 = 10.440$; $P = .0054$; log-rank test for trend: $\chi^2 = 5.201$; $P = .0221$) (Fig. 8B; Table 3) and VGP (log-rank [Mantel-Cox] test: $\chi^2 = 6.483$; $P = .0391$; log-rank test for trend: $\chi^2 = 6.087$; $P = .0138$) (Fig. 8C; Table 3) melanomas. The amount of CYP27B1 in melanoma metastases and recurrent lesions had no impact on DFS. The median OS and DFS are summarized in Table 3. The *t* test analysis confirmed the Mantel-Cox test results for both OS and DFS.

CYP27B1 gene expression in cultured normal keratinocytes and melanocytes and melanoma cell lines

Next, we evaluated whether a similar pattern of CYP27B1 gene expression is conserved in cultured epidermal keratinocytes and melanocytes in comparison with melanoma lines using real-time PCR (Fig. 9). In 8 of 11 melanoma cell lines (72.7%), we observed significant reduction of CYP27B1 mRNA compared with normal keratinocytes (Fig. 9A) and melanocytes (Fig. 9B), whereas in 3 (YUTICA, YUMUT, and YUKOLI), expression was increased.

Melanin synthesis affects CYP27B1 synthesis

We also tested the correlation between melanin pigmentation and CYP27B1 (Fig. 10) and found that its expression correlated inversely with melanin; eg, in highly pigmented melanomas, the expression of CYP27B1 was significantly reduced in comparison with amelanotic and moderately pigmented melanomas (Fig. 10A, D, E). To confirm the negative correlation between high melanin content and CYP27B1 expression, we performed molecular analyses on SK-Mel188 melanoma cells in which melanogenesis was stimulated by medium containing high concentrations of melanin precursors. Western blot analysis showed that melanotic melanoma cells (P) had significantly reduced CYP27B1 protein compared with amelanotic cells (Fig. 10B, C).

Discussion

Because CYP27B1 is required to activate 25(OH)D₃ to generate biologically relevant 1,25(OH)₂D₃ [2, 11], we analyzed its expression in 154 samples of melanocytic tumors and found that CYP27B1 decreased during progression of melanoma, being lower in more advanced lesions (Clark levels III–V and Breslow thickness ≥ 2.1 mm), as well as in VGP and metastatic melanomas, in comparison with nevi and RGP melanomas. This decline was accompanied by an inverse correlation between CYP27B1 and Ki-67 expression and ability to form metastases. Furthermore, shorter OS and DFS were found in melanomas lacking CYP27B1. The above data together indicate a role for downregulation of CYP27B1 in the pathogenesis and progression of melanomas.

The necessary step for anti-tumor and anti-proliferative activity of vitamin D hydroxyl derivatives is activation of 25(OH)D₃ by CYP27B1 to produce 1,25(OH)₂D₃, as the latter is

responsible for the activation of VDR with ensuing tumorostatic effects [5]. Vitamin D₃ and local regulation of its biologically active form can affect tumor biology, which is dependent on tumor type or the histologic nature of the tissue from which the tumor originated [5, 26]. The local synthesis of CYP27B1 in pathologic conditions can be disturbed, and its local expression can be heterogeneous, depending on the tumor type (9, 13, 26, 27). For example, Bises and colleagues found higher CYP27B1 expression in well-differentiated colon cancers compared with normal mucosa; however, in tumor areas showing considerable poor differentiation, this expression was lost [26]. Immuno-fluorescence studies showed also the lack of CYP27B1 expression in 6 of 8 high-grade (G3) undifferentiated tumors. Similarly, Matusiak et al. [13] observed lowering of CYP27B1 expression in poorly differentiated colon carcinomas, with almost complete loss of its synthesis in lymph node metastases. The CYP27B1 expression pattern in colon cancers and their lymph node metastases is in agreement with our results of decreased CYP27B1 expression during progression of melanocytic tumors.

Similar to the lowering of CYP27B1 in melanomas, Lopes and associates [9] saw a reduction of CYP27B1 immunostaining in tandem with the progression from in situ to invasive breast cancer when the percentage of CYP27B1-positive cases were evaluated (66.4 % vs. 44.6%). For normal breast (63.6%), benign (55.8%), and in situ tumors, no difference was found. However, Townsend et al. [28] saw elevated CYP27B1 mRNA level in breast cancers in comparison with normal tissue. Conversely, using cell lines, they found that CYP27B1 expression was lower in more aggressive breast cancer lines than in a normal cell line. Interestingly, Friedrich and coworkers [29] found elevated expression of CYP27B1 mRNA in breast cancer cell lines in comparison with normal breast tissue. In our studies, 3 of 11 melanoma cell lines showed significant upregulation of CYP27B1 mRNA in comparison with normal keratinocytes and melanocytes. However, the majority of cell lines showed a significant reduction in the expression of this enzyme. Similarly, Hsu et al. [30] using normal, benign, and cancerous primary-cell cultures, and Chen and colleagues [31], using primary and established prostate cancer cells lines, observed a significant reduction of CYP27B1 in malignant cells.

There is a shortage of information concerning CYP27B1 expression in normal melanocytes and melanoma cells. The first study indicating the expression of active 1 α -hydroxylase was published by Frankel et al. [32] in human primary melanin-producing melanoma cell lines. Seifert and coworkers [33], using Western blot analysis, found expression of CYP27B1 in normal melanocytes and established melanoma cell lines. However, in normal melanocytes, the expression pattern of the full-length active form and inactive variants of CYP27B1 was different than in melanoma cell lines: expression of the active form was more intense in melanocytes. Moreover, Radermacher et al. [8] observed that CYP27B1 splice variants in normal melanocytes and melanoma cells differed from those in HaCaT keratinocytes and other human tissue samples and cell lines. In addition, Reichrath and coworkers [34] discovered that CYP27B1 expression depended on the cell type, with a virtual lack of expression in two lines. However, these authors did not analyze normal skin cells. Similarly, our in vitro studies, which showed that 3 melanoma lines have increased and 8 decreased CYP27B1 in comparison with normal melanocytes, are not conclusive. However, they demonstrated a trend to decreased expression in melanomas. We did not perform in-depth molecular studies to clarify the mechanism of this heterogeneity, topics outside the scope of this manuscript. Nevertheless, the finding that 11 melanoma lines express CYP27B1 is important in the context of testing the anti-melanoma activity of novel non-calcemic vitamin D analogs, as those compounds might be activated by CYP27B1 in melanoma cells [16, 17, 21].

To our knowledge, our studies are the first to describe changes in the in situ expression of CYP27B1 in a representative cohort of melanoma patients in relation to tumor behavior and OS and DFS. However, the first report describing CYP27B1 in melanoma samples was published by Reichrath et al. [35], although those authors analyzed only gene amplification using Southern blotting in 16 melanomas, and they did not find amplification of CYP27B1. In contrast, our studies, performed on a large panel of melanomas, showed a clear negative correlation between CYP27B1 protein expression and progression of cancer to more advanced stages. Furthermore, absence of CYP27B1 was associated with shorter OS and DFS. This result indicates that immunocytochemical analysis of CYP27B1 expression in melanomas would be an excellent diagnostic and prognostic tool.

Our present results also are in accordance with our previous data concerning VDR expression in pigmented lesions of human skin [18]. Thus, decreases in both CYP27B1 and VDR expression in VGP melanomas could reflect a defect in vitamin D signaling secondary to low production of its active form and a decrease or lack of its receptor that could facilitate melanoma progression and invasion. This conclusion appears to be substantiated by the shorter overall survival of patients with low CYP27B1 and VDR expression. Moreover, lack of or reduced CYP27B1 promoted the development of metastases and correlated with shorter diseases-free survival.

For tumor development and evasion of immunosurveillance, interactions between cancerous cells and the microenvironment as well as appropriate microenvironment status are essential. Melanoma cells modulate the vitamin D endocrine system in surrounding tissues, and within skin surrounding melanocytic lesion, both VDR and CYP27B1 expression was significantly reduced. The low level of these proteins probably promotes melanoma development and invasion resulting from lack of anti-proliferative action and native immune activity of vitamin D.

In tumors, regulation of proliferation and apoptosis and the balance between these opposite processes usually are disrupted. As reviewed by others [36], the highest Bcl-2 expression was observed in nevi and was decreased in melanomas and metastases (data not shown). However, we did not find any correlation between CYP27B1 and Bcl-2 expression. The expression of pro- and anti-apoptotic proteins can be regulated by vitamin D₃, which can promote apoptosis through the mitochondrial pathway. The lack of any correlation between CYP27B1 and Bcl-2 suggests either more complex nonlinear regulation of apoptosis in melanoma cells by vitamin D₃ or pro-apoptotic responses of melanoma cells regulated predominantly by a vitamin D-independent pathway.

Interestingly, induction of melanogenesis in cultured melanoma cells, as well as high melanin production in situ in melanoma biopsy specimens, was inversely associated with CYP27B1 protein expression, indicating lower production of 1,25(OH)₂D₃ in melanotic melanomas. This observation is consistent with an increased resistance of melanotic melanomas to radiotherapy or chemotherapy and the immunoevasive properties of melanogenesis [21, 23, 36–38]. In agreement with above findings, we have found that induction of melanin pigmentation is associated with decreased VDR expression and decreased responsiveness to active vitamin D compounds [18, 21]. Therefore, we propose that melanogenesis [36] or synthesis of the pigment intermediates [23] affects VDR signaling in melanomas with a final attenuation of their responsiveness to vitamin D.

In summary, we demonstrated a decrease in CYP27B1 expression during melanoma progression to more advanced stages and showed that low or absent expression is associated with shorter overall survival and diseases free-survival time. Our data emphasize the beneficial role of active forms of vitamin D₃ in melanoma biology. Specifically, decreased

local expression of CYP27B1 may play a role in the pathogenesis and progression of melanomas by decreasing the supply of calcitriol (1,25(OH)₂D₃).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH [Grant R01AR052190] to AS and by the internal funds of the Ludwik Rydygier Collegium Medicum, Nicolaus Copernicus University [grant no 64/2009].

References

1. Wang TT, Tavera-Mendoza LE, Laperriere D, Libby E, MacLeod NB, Nagai Y, et al. Large-scale in silico and microarray-based identification of direct 1,25-dihydroxyvitamin D₃ target genes. *Mol Endocrinol*. 2005; 19:2685–2695. [PubMed: 16002434]
2. Bikle DD. The vitamin D receptor: a tumor suppressor in skin. *Discov Med*. 2011; 11:7–17. [PubMed: 21276406]
3. Chen H, Hu B, Allegretto EA, Adams JS. The vitamin D response element-binding protein: a novel dominant-negative regulator of vitamin D-directed transactivation. *J Biol Chem*. 2000; 275:35557–35564. [PubMed: 10948206]
4. Colston K, Colston MJ, Feldman D. 1,25-Dihydroxyvitamin D₃ and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. *Endocrinology*. 1981; 108:1083–1086. [PubMed: 6257495]
5. Fleet JC. Molecular actions of vitamin D contributing to cancer prevention. *Mol Aspects Med*. 2008; 29:388–396. [PubMed: 18755215]
6. Zehnder D, Bland R, Walker EA, Bradwell AR, Howie AJ, Hewison M, et al. Expression of 25-hydroxyvitamin D₃-1 α -hydroxylase in the human kidney. *J Am Soc Nephrol*. 1999; 10:2465–2473. [PubMed: 10589683]
7. Bikle DD, Nemanic MK, Gee E, Elias P. 1,25-Dihydroxyvitamin D₃ production by human keratinocytes: kinetics and regulation. *J Clin Invest*. 1986; 78:557–566. [PubMed: 2426308]
8. Radermacher J, Diesel B, Seifert M, Tilgen W, Reichrath J, Fischer U, et al. Expression analysis of CYP27B1 in tumor biopsies and cell cultures. *Anticancer Res*. 2006; 26:2683–2686. [PubMed: 16886678]
9. Lopes N, Sousa B, Martins D, Gomes M, Vieira D, Veronese LA, et al. Alterations in vitamin D signalling and metabolic pathways in breast cancer progression: a study of VDR, CYP27B1 and CYP24A1 expression in benign and malignant breast lesions. *BMC Cancer*. 2010; 10:483. [PubMed: 20831823]
10. Flanagan JN, Whitlatch LW, Chen TC, Zhu XH, Holick MT, Kong XF, et al. Enhancing 1 α -hydroxylase activity with the 25-hydroxyvitamin D-1 α -hydroxylase gene in cultured human keratinocytes and mouse skin. *J Invest Dermatol*. 2001; 116:910–914. [PubMed: 11407980]
11. Holick MF. Vitamin D: A millenium perspective. *J Cell Biochem*. 2003; 88:296–307. [PubMed: 12520530]
12. Reichrath J, Rafi L, Rech M, Mitschele T, Meineke V, Gartner BC, et al. Analysis of the vitamin D system in cutaneous squamous cell carcinomas. *J Cutan Pathol*. 2004; 31:224–231. [PubMed: 14984574]
13. Matusiak D, Murillo G, Carroll RE, Mehta RG, Benya RV. Expression of vitamin D receptor and 25-hydroxyvitamin D₃-1-hydroxylase in normal and malignant human colon. *Cancer Epidemiol Biomarkers Prev*. 2005; 14:2370–2376. [PubMed: 16214919]
14. Pinczewski J, Slominski A. The potential role of vitamin D in the progression of benign and malignant melanocytic neoplasms. *Exp Dermatol*. 2010; 19:860–864. [PubMed: 20872994]
15. Slominski A, Tobin DJ, Shibahara S, Wortsman J. Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev*. 2004; 84:1155–1228. [PubMed: 15383650]

16. Slominski AT, Janjetovic Z, Fuller BE, Zmijewski MA, Tuckey RC, Nguyen MN, et al. Products of vitamin D3 or 7-dehydrocholesterol metabolism by cytochrome P450scc show anti-leukemia effects, having low or absent calcemic activity. *PloS One*. 2010; 5:e9907. [PubMed: 20360850]
17. Slominski AT, Kim TK, Janjetovic Z, Tuckey RC, Bieniek R, Yue J, et al. 20-Hydroxyvitamin D₂ is a noncalcemic analog of vitamin D with potent antiproliferative and prodifferentiation activities in normal and malignant cells. *Am J Physiol Cell Physiol*. 2011; 300:C526–C541. [PubMed: 21160030]
18. Brozyna AA, Jozwicki W, Janjetovic Z, Slominski AT. Expression of vitamin D receptor decreases during progression of pigmented skin lesions. *Hum Pathol*. 2011; 42:618–631. [PubMed: 21292298]
19. Newton-Bishop JA, Beswick S, Randerson-Moor J, Chang YM, Affleck P, Elliott F, et al. Serum 25-hydroxyvitamin D₃ levels are associated with Breslow thickness at presentation and survival from melanoma. *J Clin Oncol*. 2009; 27:5439–5444. [PubMed: 19770375]
20. Hyter S, Bajaj G, Liang X, Barbacid M, Ganguli-Indra G, Indra AK. Loss of nuclear receptor RXR α in epidermal keratinocytes promotes the formation of Cdk4-activated invasive melanomas. *Pigment Cell Melanoma Res*. 2010; 23:635–648. [PubMed: 20629968]
21. Janjetovic Z, Brozyna AA, Tuckey RC, Kim TK, Nguyen MN, Jozwicki W, et al. High basal NF- κ B activity in nonpigmented melanoma cells is associated with an enhanced sensitivity to vitamin D₃ derivatives. *Br J Cancer*. 2011; 105:1874–1884. [PubMed: 22095230]
22. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR, et al. Final version of 2009 AJCC melanoma staging and classification. *J Clin Oncol*. 2009; 27:6199–6206. [PubMed: 19917835]
23. Slominski A, Zbytek B, Slominski R. Inhibitors of melanogenesis increase toxicity of cyclophosphamide and lymphocytes against melanoma cells. *Int J Cancer*. 2009; 124:1470–1477. [PubMed: 19085934]
24. Clark WH Jr, Elder DE, Guerry DT, Epstein MN, Greene MH, Van Horn M. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. *Hum Pathol*. 1984; 15:1147–1165. [PubMed: 6500548]
25. Clark WH Jr, Elder DE, Guerry DT, Braitman LE, Trock BJ, Schultz D, et al. Model predicting survival in stage I melanoma based on tumor progression. *J Natl Cancer Inst*. 1989; 81:1893–1904. [PubMed: 2593166]
26. Bises G, Kallay E, Weiland T, Wrba F, Wenzl E, Bonner E, et al. 25-hydroxyvitamin D₃-1 α -hydroxylase expression in normal and malignant human colon. *J Histochem Cytochem*. 2004; 52:985–989. [PubMed: 15208365]
27. Chen TC, Holick MF. Vitamin D and prostate cancer prevention and treatment. *Trends Endocrinol Metab*. 2003; 14:423–430. [PubMed: 14580762]
28. Townsend K, Banwell CM, Guy M, Colston KW, Mansi JL, Stewart PM, et al. Autocrine metabolism of vitamin D in normal and malignant breast tissue. *Clin Cancer Res*. 2005; 11:3579–3586. [PubMed: 15867263]
29. Friedrich M, Diesing D, Cordes T, Fischer D, Becker S, Chen TC, et al. Analysis of 25-hydroxyvitamin D₃-1 α -hydroxylase in normal and malignant breast tissue. *Anticancer Res*. 2006; 26:2615–2620. [PubMed: 16886671]
30. Hsu JY, Feldman D, McNeal JE, Peehl DM. Reduced 1 α -hydroxylase activity in human prostate cancer cells correlates with decreased susceptibility to 25-hydroxyvitamin D₃-induced growth inhibition. *Cancer Res*. 2001; 61:2852–2856. [PubMed: 11306457]
31. Chen TC, Wang L, Whitlatch LW, Flanagan JN, Holick MF. Prostatic 25-hydroxyvitamin D-1 α -hydroxylase and its implication in prostate cancer. *J Cell Biochem*. 2003; 88:315–322. [PubMed: 12520532]
32. Frankel TL, Mason RS, Hersey P, Murray E, Posen S. The synthesis of vitamin D metabolites by human melanoma cells. *J Clin Endocrinol Metab*. 1983; 57:627–631. [PubMed: 6603466]
33. Seifert M, Tilgen W, Reichrath J. Expression of 25-hydroxyvitamin D-1 α -hydroxylase (1 α -OHase, CYP27B1) splice variants in HaCaT keratinocytes and other skin cells: modulation by culture conditions and UV-B treatment in vitro. *Anticancer Res*. 2009; 29:3659–3667. [PubMed: 19667162]

34. Reichrath J, Rech M, Moeini M, Meese E, Tilgen W, Seifert M. In vitro comparison of the vitamin D endocrine system in 1,25(OH)₂D₃-responsive and -resistant melanoma cells. *Cancer Biol Ther.* 2007; 6:48–55. [PubMed: 17172823]
35. Reichrath J, Rafi L, Rech M, Meineke V, Tilgen W, Seifert M. No evidence for amplification of 25-hydroxyvitamin D-1 α -OHase (1 α -OHase) or 1,25-dihydroxyvitamin D-24-OHase (24-OHase) genes in malignant melanoma (MM). *J Steroid Biochem Mol Biol.* 2004; 89–90:163–166.
36. Carlson JA, Slominski A, Linette GP, Mysliborski J, Hill J, Mihm MC Jr, et al. Malignant melanoma 2003: predisposition, diagnosis, prognosis, and staging. *Am J Clin Pathol.* 2003; 120(Suppl):S101–S127. [PubMed: 15298147]
37. Slominski A, Paus R, Mihm MC. Inhibition of melanogenesis as an adjuvant strategy in the treatment of melanotic melanomas: selective review and hypothesis. *Anticancer Res.* 1998; 18:3709–3715. [PubMed: 9854482]
38. Brozyna AA, VanMiddlesworth L, Slominski AT. Inhibition of melanogenesis as a radiation sensitizer for melanoma therapy. *Int J Cancer.* 2008; 123:1448–1456. [PubMed: 18567001]

Figure 1. Representative CYP27B1 immunostaining in benign junctional (A) and dermal (B) melanocytic nevi, melanoma in situ (C), invasive superficial spreading (SSM) (D) and nodular (NMM) (E) melanomas, lymph node metastases (F), and recurrent melanomas (G). Nevus sections immunoprocessed without primary antibodies were the negative control (H). Arrows indicate CYP27B1 immunostaining; arrowheads mark lymphocytes, asterisks show melanin. Scale bar: 50 μ m.

Figure 2.

Changes in expression of CYP27B1 in normal skin, melanocytic nevi, and metastases in comparison with melanomas classified according growth phase (A) or histologic type (B). Radial growth phase (RGP), vertical growth phase (VGP), superficial spreading (SSM), and nodular (NMM) melanomas. Statistically significant differences are denoted with P values after ANOVA and with asterisks as $*P < .05$ or $**P < .01$ with t test.

Figure 3.
CYP27B1 expression in cytoplasm of nevi and melanomas stratified according to Clark level (A) and Breslow thickness (B). Statistically significant differences are denoted with P values after ANOVA and with asterisks as $*P < .05$, $**P < .01$, and $***P < .001$ with t test.

Figure 4. CYP27B1 immunostaining in metastasizing (M) and non-metastasizing (NM) melanomas. Statistically significant differences are denoted with *P* values after ANOVA.

Figure 5.

Expression of CYP27B1 decreases during melanoma progression. Comparison of CYP27B1 immunostaining in RGP and VGP of SSM and in papillary (PD) and reticular (RD) dermis compartments of NMM (A). Representative CYP27B1 immunostaining of RGP (B) and VGP (C) of SSM and in PD (D) and RD (E) compartments of NMM. Statistically significant differences are denoted with *P* values after ANOVA. Arrows indicate CYP27B1 expression. Scale bar: 50 μ m.

Figure 6.

Comparison of CYP27B1 immunostaining in normal skin and skin surrounding nevi and melanomas. Quantification of relative expression is in panel A, whereas representative immunostains for CYP27B1 in normal skin and skin surrounding melanomas are in panels B and C, respectively. D–E, High magnification views of areas indicated in squares in normal skin (D) and skin surrounding melanoma (E). Statistically significant differences are denoted with *P* values after ANOVA and with asterisks as $*P < .05$ with *t* test. Arrows indicate CYP27B1 staining. Scale bar: 50 μ m.

Figure 7. Proliferative activity in melanomas in RGP (A) and VGP (B) shows inverse correlation with expression of CYP27B1. Only one case of melanoma in VGP had high CYP27B1 immunostaining. Statistically significant differences are denoted by asterisks as $**P < .01$ with t test.

Figure 8.

Correlation of overall survival time (A) and diseases-free survival time (B and C) with relative expression of CYP27B1. A and B represents RGP, and C represents VGP.

Figure 9.

Expression of *CYP27B1* mRNA in melanoma lines in comparison with normal human keratinocytes (HEKn) (A) or melanocytes (HEMn) (B) measured by real-time RT PCR. Data are presented as mean $\pm$ SD (n = 3). Statistically significant differences are denoted with asterisks as * P < .05, ** P < .01, and *** P < .001 with t test.

Figure 10.

CYP27B1 expression decreases in highly melanized melanoma cells. CYP27B1 expression in melanoma samples measured by immunocytochemistry. Statistically significant differences are denoted with *P* values after ANOVA and with asterisks as $*P < .05$ with *t* test (A). Induction of melanin synthesis in cultured SKMel-188 melanoma cells reduced the CYP27B1 as measured by Western blotting (B and C). Whole extracts from nonpigmented (NP) and pigmented (P) cells were subjected to immunoblotting with anti-CYP27B1 and anti-β-actin (internal control)(B). Isolated nuclear and cytoplasmic extracts were subjected to immunoblotting with anti-CYP27B1 and anti-β-actin (internal control)(C). Representative CYP27B1 immunostaining of RGP (D) and VGP (E) pigmented melanoma. Arrows mark CYP27B1 staining; asterisks indicate melanin. Scale bar: 50 μm.

Table 1

Patient characteristics

Clinicopathologic features	n
Type of lesions	
All samples	154
Nevi	29
junctional	7
intradermal	9
compound	8
dysplastic	5
Primary melanomas	75
superficial spreading	36
nodular	37
lentiginous melanomas	2
Melanoma metastases	40
Recurrent melanomas	4
Normal skin	6
Age (y)	
Patients with nevi, mean (range, median)	39.9 ± 16.7 (21–85, 35)
<20	2
21–40	16
41–60	8
61–80	2
>80	1
Patients with melanomas, mean (range, median)	59.7 ± 15.1 (22–100, 59)
<20	0
21–40	7
41–60	32
61–80	30
>80	6
Male/female ratio	
All patients	49/43
Nevi	8/21
Melanomas	39/36
Anatomical Site Nevi	
Acral	0
Anogenital	0
Extremity	7
Head and neck	5
Trunk	17
Melanomas	
Acral	7

Clinicopathologic features	n
Anogenital	2
Extremity	24
Head and neck	15
Trunk	27
Metastases	
All cases	44
Lymph nodes	40
Liver	1
Skin	1
Parotid gland	2
Clark level	
I	11
II	6
III	22
IV	24
V	12
Breslow thickness (mm)	
0 to 1	23
1.1 to 2	10
2.1 to 3	8
3.1–4	4
>4.0	30

Table 2

Characteristic of melanomas according to AJCC classification and staging [22]

Pathologic features	n	Follow-up (d)		
		mean	median	range
pT				
is	6	976.1	960.0	128–2534
1	16	1147	1181	127–1807
2	10	818.0	1018	43–1821
3	13	718.7	527.5	99–2520
4	30			
pN^a				
0	39	938.5	964.0	127–2534
1	15	912.4	810.0	467–1821
2	11	560.5	471.0	69–1348
3	8	761.9	472.0	43–2297
pM				
0	71	876.4	912.0	43–2534
1	4	677	682.0	194–1303
Pathologic stage				
0	6	908.2	892.5	588–1252
1	17	1041	964.0	128–2534
2	16	841.2	1032	127–2520
3	31	798.2	748.0	43–2297
4	4	677.0	682.0	194–1303

^aFor 2 cases, information concerning lymph node metastasis is not available.

Table 3

Survival time in patients with melanoma in relation to CYP27B1 expression

CYP27B1 expression	Total cases, n (%)	Deaths, n (%)	OS, mean/median (d)	<i>p</i> ^a	Metastases, n (%)	DFST, mean/median (d)	<i>p</i> ^a
RGP							
Absent	30 (40.0)	18 (60.0)	810/766.9		19 (47.5)	114/206.2	
Low	36 (48.0)	16 (44.4)	982/912.6	>.05	13 (27.1)	339/477.9	<.01
High	9 (12.0)	3 (33.3)	1151/1244.0	<.001	2 (16.7)	737/841	<.001
VGP							
Absent	53 (80.3)	32 (60.4)	810/795.7		29 (54.7)	127/277.0	
Low	12 (18.2)	6 (50.0)	1166/1211.0	<.001	3 (25.0)	307/622.2	<.05
High	1 (1.5)	0 (0.0)	1151/1151.1	-	0 (0.0)	737/737.0	-
Metastases							
Absent	35 (87.5)	27 (77.1)	682/784.3		-	21/84.6	
Low	5 (12.5)	5 (100.0)	460/759.8	>.05	-	190/403.8	<.01
High	0 (0.0)	0 (0.0)	0 (0.0 %)	-	-	-	-

Abbreviations: OS, overall survival time; DFS, disease-free survival time; RGP, cells in radial growth phase; VGP, cells in vertical growth phase.

^aThe statistical analyses of differences vs absent CYP27B1 expression were performed using student *t* test.