Decreased VDR Expression in Cutaneous Melanomas as Marker of Tumor Progression: New Data and Analyses

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Abstract. *Background: Vitamin D_3, acting via vitamin D receptor (VDR) affects a wide range of biological activities, including inhibition of proliferation and angiogenesis, with net antitumor effects. VDR expression is disturbed in many tumors, including melanomas. Aim: To find correlation between VDR expression in melanomas and prognostic biomarkers. Materials and Methods: VDR was analyzed immunohistochemically in 69 cutaneous melanomas in relation to prognostic factors. Results: Less advanced melanomas showed significantly higher VDR expression than the advanced stages. The presence of other markers such as ulceration and lack or non-brisk tumor infiltrating lymphocytes (TILs) was accompanied by significantly lower VDR expression. VDR expression also affected overall survival (OS) with most noticeable effect in the cases without ulceration. Conclusion: High VDR expression determines a less malignant phenotype and is related to better prognosis. Loss of VDR expression affects melanoma tumor behavior, allowing for progression of disease. VDR expression can also serve as a prognostic marker in routine histopathology evaluation.*

Ultraviolet radiation (UVR) is considered as a one of the most important environmental factors responsible for development of melanoma, an aggressive skin tumor with a relatively high mortality rate (1). Melanoma originates from melanocytes, cells responsible for production of melanin that protect skin against the damaging effect of UVR (2, 3). UVB photoconverts cutaneous 7-dehydrocholesterol into vitamin D, which is further hydroxylated in the liver at position 25 [by D-25-hydroxylase (CYP27A1)] and in the kidney at positions 1 [by 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1)] to the biologically-active form 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$; calcitriol) (4, 5). Calcitriol is also produced locally in a number of tissues, including skin (4, 5), where it is involved in regulation of cell proliferation, differentiation or apoptosis in autocrine or paracrine manners (5-7). Alternative pathways of vitamin D activation to biologically active novel vitamin D derivatives were also described recently (8-11).

Calcitriol, in addition to regulation of calcium-phosphate homeostasis, has wide spectrum of pleiotropic effects such as regulation of the adaptive and innate immune activity, endocrine activities and acts as a tumorostatic factor and protector against oxidative stress (4, 12). It also inhibits angiogenesis and regulates expression of adhesion molecules (13, 14). These effects are mediated through an interaction with vitamin D receptor (VDR) that is ubiquitously expressed in the body, including by melanoma cells (5, 15). However, alternative nuclear receptors for vitamin D derivatives were also identified (16). Novel vitamin D_3 analogs that are non-calcemic also have similar antitumor and anti-melanoma activities (17-21).

Our previous studies showed an inverse correlation between VDR expression and progression of skin pigmented lesions (22). Similar correlation was observed for melanomas stratified according Breslow's thickness and Clarks's level, with a decrease of VDR expression in advanced lesions (22). VDR expression affected survival of patients with melanoma (22). Lack of or reduced VDR expression in melanomas localized to the skin lesions or metastases was related to shorter overall survival. The level of VDR was reduced in strongly-melanized melanomas in comparison to amelanotic or slightly-pigmented melanomas (22). A high level of

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melanoma melanization was related to shorter overall survival and disease-free survival (DFS) in metastasizing melanomas (23). Our subsequent research (24) revealed that, similarly to VDR immunostaining, expression of CYP27B1 was inversely correlated with melanoma progression, marked by significantly reduced CYP27B1 expression in most advanced tumors (24). There was no correlation between CYP27B1 expression and ulceration or presence of tumor infiltrating lymphocytes (TILs). However, ulceration and lack of, or a low rate of, TILs were more frequent in melanomas with low or no expression of CYP27B. CYP27B1 expression also affected prognosis, *e.g.* its lack or a low level was related to significantly shorter overall and disease-free survival (24).

In the present study, we analyzed VDR expression in the same cohort of patients with melanoma (22) in relation to newly-collected pathomorphological data as follows: the American Joint Committee on Cancer (AJCC) pTNM classification, overall stage, and other prognostic markers such as ulceration and presence of TILs, to verify the involvement of VDR in melanoma behavior and its potential role as a prognostic and predictive marker.

Materials and Methods

Patients and pathomorphological assessment. The study was approved by the Committee of Ethics of Scientific Research of Collegium Medicum of Nicolaus Copernicus University, Poland (approval number KB 448/2009). Samples of melanomas were obtained from 69 patients, who underwent surgical excision of skin lesions in the Oncology Centre, Prof. Franciszek Łukaszczyk Memorial Hospital, Bydgoszcz, Poland, from 2003 to 2009. Clinicopathological data were obtained from database of the Oncology Center and date of deaths from Department of Registry Office in Bydgoszcz, Poland, and have been updated for 24 months from the end of experiments. The analyzed cohort comprised of 30 superficial-spreading, 37 nodular and 2 acral lentiginous melanomas. Standard hematoxylin and eosin-stained sections of melanomas included in this study were assessed, classified and described by a board-certified pathologist (WJ). The following features were evaluated: 2009 AJCC pTNM classification and stage, ulceration and TILs. The term TILs was used for lymphocytes disrupting or surrounding tumor cells of the vertical growth phase (VGP) (25, 26). Brisk TILs corresponded to lymphocytes infiltrating the entire invasive component of melanoma or the entire base of the VGP. Non-brisk TILs represented only focal lymphocytic infiltrate of the tumor or lymphocytes infiltrating less than the entire base of the VGP. The term absent TILs was used when lymphocytes were absent or were present but did not infiltrate the tumor. The clinicopathomorphological characteristics of melanomas are presented in Table I.

Table I. *Clinicopathological characteristics of patients with melanoma.*

Features	No. of patients (%)
Age, years	
<25	1 (1.5%)
26-35	3 (4.4%)
36-45	6 (8.7%)
46-55	19 (27.5%)
56-65	17 (24.6%)
66-75	13 (18.8%)
>75	10 (14.5%)
pT	
pT1	15 (27.7%)
pT2	10 (14.5%)
pT3	15 (27.7%)
pT4	29 (42.0%)
pN*	
pN0	34 (49.3%)
pN1	15 (21.7%)
pN2	11 (15.9%)
pN3	8 (11.6%)
pM	
pM0	64 (92.6%)
pM1	5 (7.6%)
Overall stage	
1	17 (24.6%)
2	17 (24.6%)
3	28 (40.6%)
4	7 (10.1%)
Ulceration	
Absent	31 (44.9%)
Present	38 (55.1%)
TILs	
Absent	16 (23.1%)
Non-brisk	32 (46.4%)
Brisk	21 (30.4%)

*There is lack of pN status for one patient.

VDR immunohistochemical staining protocol and evaluation. VDR expression was assayed using immunohistochemistry as previously described (22, 27). Briefly, standard formalin-fixed, paraffin-embedded 4-μm sections were stained overnight using rat antibody to VDR at a dilution of 1:75 (clone 97A; Abcam Inc., Cambridge, MA, USA), followed by incubation with anti-rat secondary antibody, conjugated with alkaline phosphatase and red alkaline phosphatase substrate (Vector Laboratories Inc., Burlingame, CA, USA).

Immunohistochemical evaluation was performed by two observers (WJ and AAB) in a blind manner, without knowing the detailed histopathological diagnosis, the newly-evaluated features mentioned above and the other clinical data. The intensity of VDR immunostaining of melanomas was scored semiquantitatively as previously described. Briefly, the staining intensity was scored from 0 to 3 arbitrary units (AU), evaluated in relation to VDR immunostaining in epidermis of normal skin. For statistical analysis purposes, moderate and strong VDR immunostaining was classified as high staining intensity, weak as low staining intensity, and negative as no staining intensity.

Statistical analyses. Statistical analysis was performed with the Prism 5.00 (GraphPad Software, San Diego, CA, USA). Results were considered as statistically significant when the *p*-value was less than 0.05. Data are presented as the mean±SD.

Table II. *Cytoplasmic and nuclear immunolocalization of vitamin D receptor (VDR) in melanomas stratified according to American Joint Committee on Cancer pTNM classification, overall stage, presence or absence of ulceration and of tumor infiltrating lymphocytes (TILs).*

	Cytoplasmic VDR immunostaining (n/%)			Nuclear VDR immunostaining (n/%)		
	None	Low	High	None	Low	High
pT1 (n=15)	2/13.3	7/46.7	6/40.0	1/6.7	10/66.7	4/26.7
pT2 (n=10)	2/20.0	7/70.0	1/10.0	2/20.0	5/50.0	3/30.0
pT3 (n=15)	8/53.3	6/40.0	1/6.7	2/13.3	11/73.3	2/13.3
pT4 (n=29)	11/37.9	18/62.1	0/0.0	6/20.7	20/69.0	3/10.3
pN0 (n=34)	11/32.3	15/44.1	8/23.5	7/20.6	18/52.9	9/26.5
pN1 (n=15)	6/40.0	9/60.0	0/0.0	1/6.7	12/80.0	2/13.3
pN2 (n=11)	0/0.0	11/100.0	0/0.0	0/0.0	10/90.9	1/9.1
pN3 (n=8)	5/62.5	3/37.5	0/0.0	2/25.0	6/75.0	0/0.0
M0 (n=64)	20/31.2	36/56.3	8/12.5	12/18.8	43/67.2	9/14.1
M1 (n=5)	3/60.0	2/40.0	0/0.0	2/40.0	3/60.0	0/0.0
Stage 1 (n=17)	2/11.8	8/47.1	7/41.2	1/5.9	10/58.8	6/35.3
Stage 2 (n=17)	9/52.9	7/41.2	1/5.9	6/35.3	8/47.1	3/17.6
Stage 3 (n=28)	8/28.6	20/71.4	0/0.0	1/3.6	24/85.7	3/10.7
Stage 4 (n=7)	4/57.1	3/42.9	0/0.0	3/42.9	4/57.1	0/0.0
Without ulceration (n=31)	6/19.6	17/54.8	8/25.8	4/12.9	19/61.3	8/25.8
With ulceration (n=38)	17/44.7	21/55.3	0/0.0	7/18.4	27/71.1	4/10.5
TILs absent (n=16)	5/31.3	10/62.5	1/6.3	2/12.5	10/62.5	4/25.0
Non-brisk TILs (n=32)	11/34.4	20/62.5	1/3.1	6/18.8	24/75.0	2/6.3
Brisk TILs (n=21)	7/33.3	8/38.1	6/28.6	3/14.3	12/57.1	6/28.6

Results

VDR expression in relation to pTNM AJCC classification of melanomas. As we reported previously (22), VDR expression was localized in both the cytoplasm and nuclei of melanoma cells. Cytoplasmic VDR was observed in 46 cases (66.7%) and nuclear in 58 cases (84.1%). High VDR expression was seen in the cytoplasm of only eight cases (11.6%), and in the nucleus of 12 cases (17.4%). In the majority of melanomas, a low staining intensity of VDR was observed in the cytoplasm and nucleus in 38 cases (55.1%) and 46 cases (66.7%), respectively) (Figure 1). Table II summarizes the association between VDR expression and pTNM features, overall stage, presence of ulceration and TILs of analyzed melanomas.

VDR expression was inversely-correlated with melanoma progression. Less advanced melanomas exhibited higher VDR expression. Melanomas at stages pT1 and pT2 more frequently had higher VDR immunostaining than melanomas at stages pT3 and pT4 (Table II). On the contrary, melanomas without VDR expression were seen predominantly in advanced stages (pT3 and pT4, Table II). Significant differences were observed between less (pT1 and pT2) and more (pT3 and pT4) advanced stages ($p \leq 0.0001$ and $p=0.0161$ for cytoplasmic and nuclear staining, respectively). These differences were more visible in the cytoplasm (Figure 2A), because the mean VDR expression was significantly higher in pT1 *versus* pT3 and pT4 (ANOVA) and in pT2 *versus* pT3 and pT4 (*t*-test). For nuclear VDR immunostaining, the only significant difference observed was for pT1 *versus* pT4 stages (*t*-test) (Figure 2B).

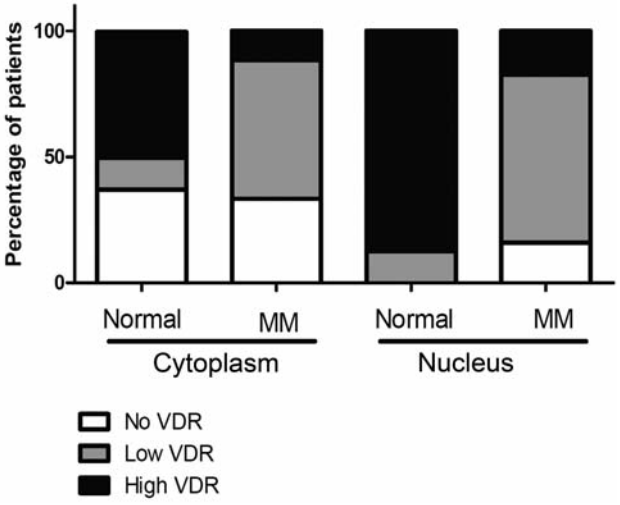

Figure 1. *Distribution of cytoplasmic and nuclear vitamin D receptor (VDR) levels in normal skin and malignant melanoma (MM).*

Melanomas with lymph node metastases exhibited a lack or low VDR expression. No single case at stage pN1-3 exhibited high cytoplasmic VDR immunostaining (Table II). High nuclear VDR staining was observed in two (13.3%) and

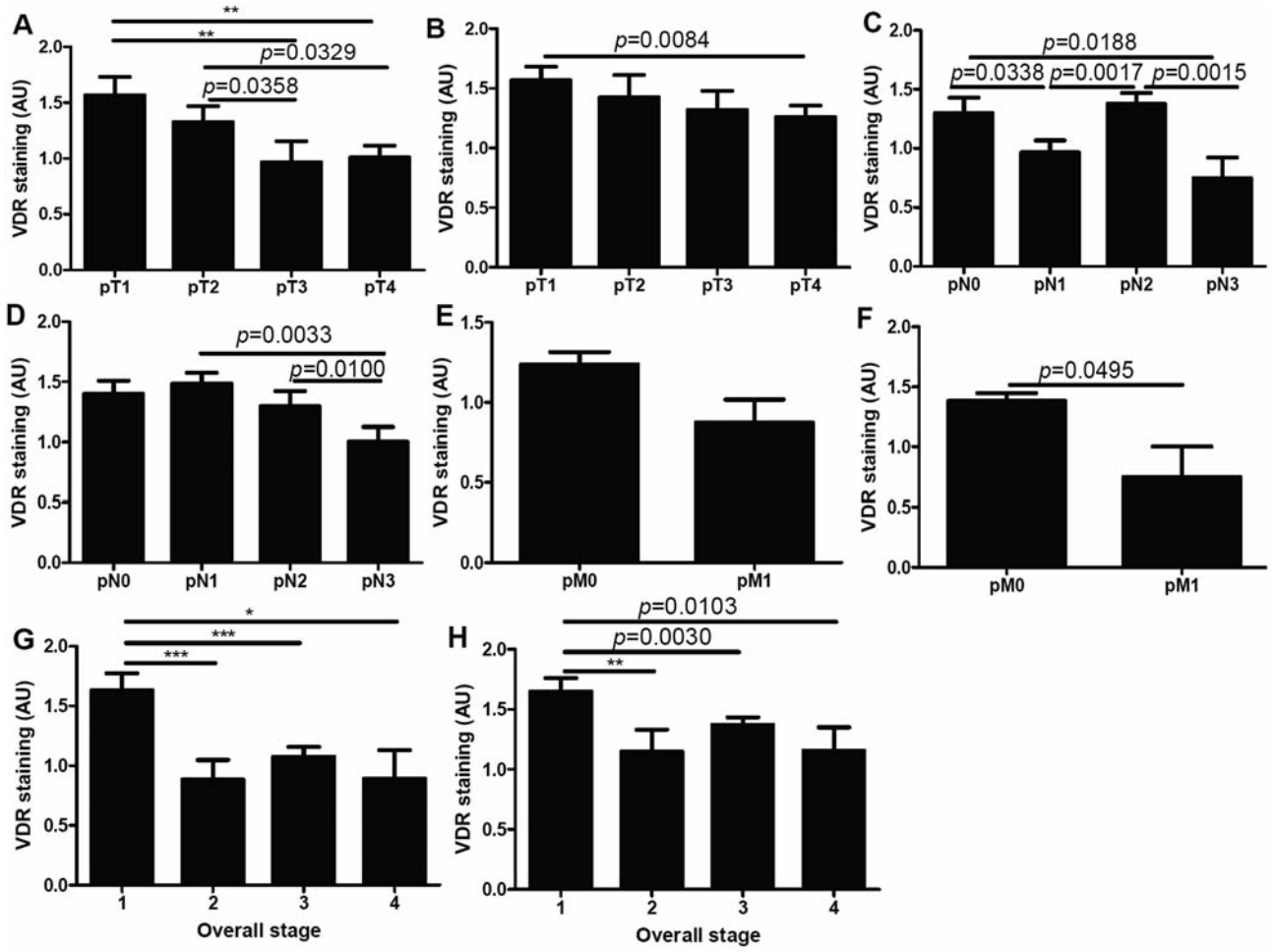

Figure 2. *Mean cytoplasmic (A, C, E, G) and nuclear (B, D, F, H) expression of vitamin D receptor (VDR) in melanomas of different pT (A, B), pN (C, D), pM (E, F) and overall (G, H) stage. Statistically significant differences are denoted with p-values as determined by t-test, and with asterisks as *p<0.05, **p<0.01 and ***p<0.001 by ANOVA.*

one (9.1%) stage pN1 and pN2 cases, respectively. Melanomas at stage pN3 exhibited low or no VDR expression (Table II). In melanomas with four or more lymph node metastases (pN3), nuclear VDR expression was significantly lower than in melanomas without metastases or with less advanced pN stage. This relationship was observed only for nuclear VDR staining (Figure 2 C and D).

The same correlation was seen for pM feature (Figure 2 E and F). In contrast to melanomas at stage pM0, melanomas at stage pM1 exhibited lack of high VDR immunostaining both in the cytoplasm and nucleus. In patients with distant metastases (pM1), a lower mean VDR expression was seen in both the cytoplasm and nucleus when compared to pM0 melanomas. However, these differences were statistically significant only for nuclear immunostaining. It should be noted that only five cases with pM1 stage were included in this study.

Overall melanoma stage was also related to VDR expression. More than 80% of melanomas at a less advanced overall stage (Stage 1) exhibited VDR expression, with 41.2% and 35.3% of cases showing strong cytoplasmic and nuclear immunostaining, respectively. On the contrary, none of the melanomas at stage 4 had high VDR staining (Table II). Melanomas at less advanced stage 1 were characterized by higher mean VDR immunostaining when compared to more advanced melanomas (Stages 2-4). These differences were seen both for cytoplasmic and nuclear VDR expression (Figure 2 G and H).

VDR expression is related to pathological prognostic markers. The relationship between VDR expression and the presence of ulceration was also observed. No single case with high cytoplasmic VDR immunostaining and ulceration was found (Table II). Lack of cytoplasmic VDR expression

was more frequent in melanomas with ulceration: 44.7% of cases of ulcerated melanomas *versus* 19.6% of cases without ulceration (Table II). High nuclear staining was seen in melanomas both with and without ulceration, but the frequency of such staining was less common in the former tumors: 10.5% *versus* 25.8% (Table II). Cases without ulceration had a significantly higher mean cytoplasmic and nuclear VDR expression than ulcerated melanomas (Figure 3 A, B, G and H).

In our population of patients with melanoma, the presence of ulceration was related to a shorter OS [log-rank (Mantel-Cox) test: χ^2=7.180, p=0.0074; Gehan-Breslow-Wilcoxon Test: χ^2=6.876, p=0.0087] (data not shown).

In melanomas without cytoplasmic VDR, the presence of ulceration had no impact on OS, but in melanomas expressing cytoplasmic VDR the ulceration was related to shorter OS [cytoplasmic VDR and no ulceration *versus* VDR and ulcer: 2297 *versus* 525 days (median) of OS, respectively; log-rank (Mantel-Cox) test: χ^2=9.447, p=0.0021; Gehan-Breslow-Wilcoxon test: χ^2=8.542, p=0.0032] (Figure 3 C). In melanomas without nuclear VDR, the presence of ulceration had no impact on OS, but in melanomas expressing nuclear VDR, ulceration correlated with a shorter OS [VDR present and no ulceration *versus* VDR present and ulceration: 2297 *versus* 663 days (median) OS, respectively; log-rank (Mantel-Cox) test: χ^2=7.158, p=0.0075; Gehan-Breslow-Wilcoxon test: χ^2=7.555, p=0.0060] (Figure 3D).

The relationship between lack or low-level of both cytoplasmic and nuclear VDR with shorter survival was more obvious in the subgroup of patients with melanomas without ulceration [log-rank (Mantel-Cox) test: χ^2=6.125, p=0.0468 for cytoplasmic VDR staining and log-rank (Mantel-Cox) test: χ^2=8.632, p=0.0134; Gehan-Breslow-Wilcoxon test: χ^2=6.974, p=0.0083 for nuclear VDR staining] (Figure 3E and F). More detailed analysis demonstrated statistically significant differences between survival curves for patients with lack of VDR *versus* high nuclear VDR immunostaining [log-rank (Mantel-Cox) test χ^2=11.250, p=0.0008; Gehan-Breslow-Wilcoxon test: χ^2=10.500, p=0.0012] (Figure 3F). Survival curve comparison also showed statistically significant differences for cases with a lack of *versus* low cytoplasmic VDR staining [log-rank (Mantel-Cox) test: χ^2=4.564, p=0.0327; Gehan-Breslow-Wilcoxon test: χ^2=4.301 p=0.0381] and low *versus* high VDR staining [log-rank (Mantel-Cox) test: χ^2=4.695, p=0.0302; Gehan-Breslow-Wilcoxon test: χ^2=4.219 p=0.0400] (Figure 3 E).

In patients with melanoma included in this study, the presence of TILs was related to a better prognosis, and OS of patients with TILs was significantly longer when compared to patients with non-brisk TILs [log-rank (Mantel-Cox) test: χ^2=8.387, p=0.0038; Gehan-Breslow-Wilcoxon test: χ^2=7.181, p=0.0074] and to patients without TILs [log-rank (Mantel-Cox) test: χ^2=6.970, p=0.0083; Gehan-Breslow-Wilcoxon test: χ^2=7.266, p=0.0070] (data not shown).

The level of VDR expression was also related to the presence of TILs (Figure 4A-D). In melanomas with brisk TILs, VDR expression was higher, and for cytoplasmic VDR immunostaining, these differences were statistically significant (p<0.0133). In melanomas without cytoplasmic VDR, there was a lack of correlation between brisk TILs and OS. However, in melanomas with cytoplasmic VDR, the presence of brisk TILs correlated with significantly better prognosis (median OS: 2297 days for melanomas with brisk TILs *versus* 715 days for melanomas without or with non-brisk TILs, log-rank (Mantel-Cox) test: χ^2=8.809, p=0.0030; Gehan-Breslow-Wilcoxon test: χ^2=7.536, p=0.0060]. A similar trend was observed for nuclear VDR (median OS: 2520 days for melanomas with brisk TILs *versus* 748 days for melanomas without or with non-brisk TILs, log-rank (Mantel-Cox) test: χ^2=9.521, p=0.0020; Gehan-Breslow-Wilcoxon test: χ^2=7.835, p=0.0051) (data not shown).

There was no relationship between melanin level and the presence of ulceration or TILs. However, a slight decrease of melanin level in melanomas with brisk TILs was observed (data not shown).

Discussion

In the present study, we found significant associations between VDR expression, melanoma progression and prognostic factors in melanoma. In melanomas at lower stages (pT1-2, overall stage 1), statistically significant higher VDR expression was observed. Similarly, melanomas with fewer than three lymph node metastases and melanomas without distant metastases had the strongest VDR expression. Moreover, ulcerated melanomas without TILs or with non-brisk TILs were characterized by lower VDR expression. Furthermore, survival of patients in sub-groups of melanomas without ulcerations was affected by VDR expression, with better prognosis in patients with melanomas expressing VDR. These results are consistent with our previous studies demonstrating decreased expression of VDR during progression of pigmented lesions, including melanoma progression (22). Analysis of OS and well-established factors of unfavorable prognosis showed that the presence of ulceration and lack of TILs or presence of non-brisk TILs correlated with significantly shorter OS.

These results are also consistent with experiments showing inhibition of melanoma proliferation in cell culture by vitamin D_3 (21, 28), which was seen only in cells expressing VDR (29). Similar anti-melanoma activity was shown for other active vitamin D_3 derivatives in human and rodent melanomas grown in monolayer or soft agar (20, 21, 27). In addition, $1,25(OH)_2D_3$ inhibited metastases of B16 melanoma in mice (30).

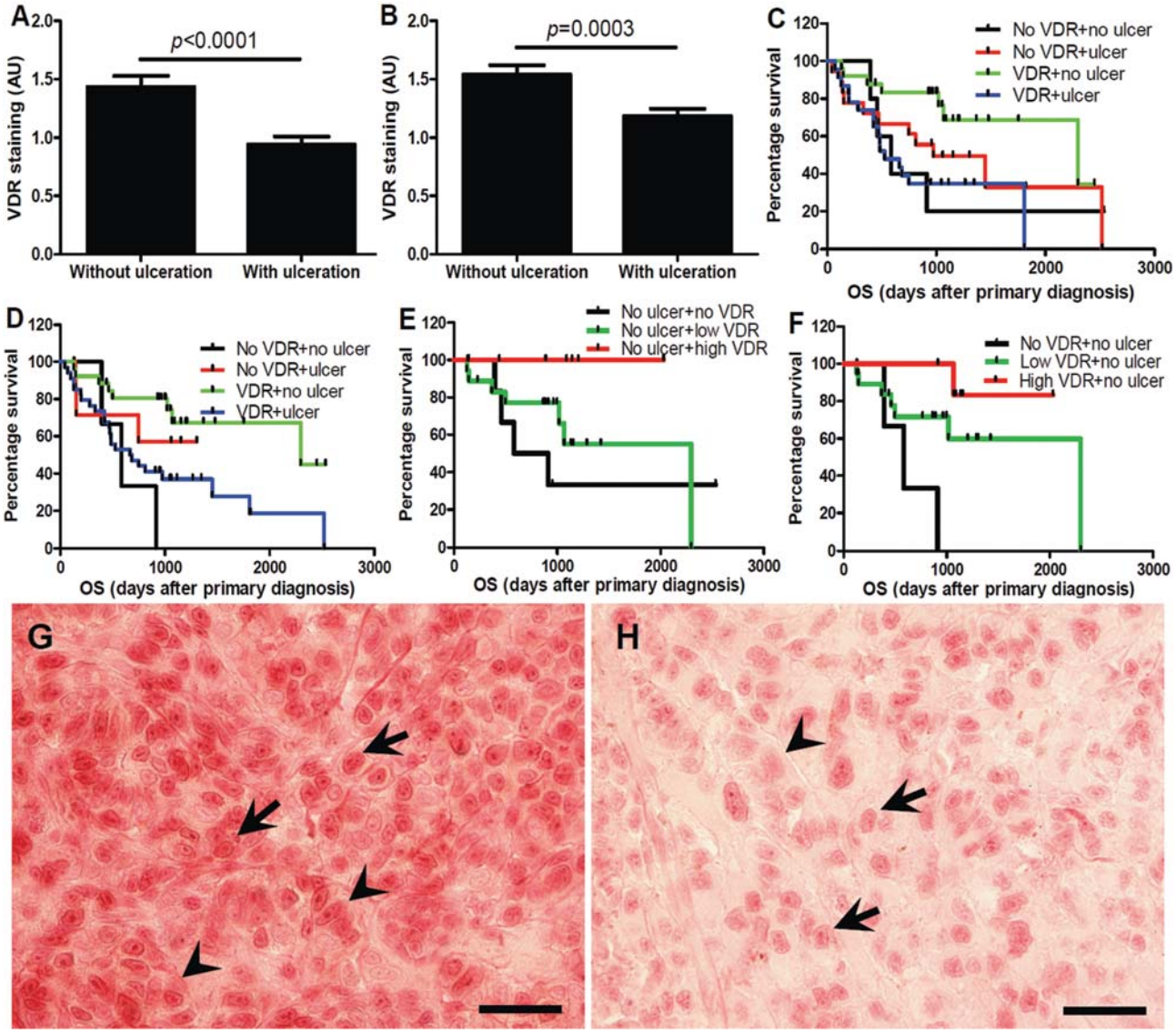

Figure 3. *Mean cytoplasmic (A) and nuclear (B) expression of vitamin D receptor (VDR) in melanomas with and without ulceration. Statistically significant differences are denoted with p-values, as determined by the t-test. Dependence of overall survival (OS) time on ulceration and VDR expression in the cytoplasm (C) and nuclei (D) of melanoma cells. Dependence of overall survival time on VDR expression in the nuclei (E) and cytoplasm (F) of melanoma cells in cases without ulceration. Representative VDR immunostaining in melanoma without (G) and with (H) ulceration. Arrows indicate nuclear VDR immunostaining, arrowhead indicates cytoplasmic VDR immunostaining; scale bar: 50 μm.*

In patients with melanoma, both systemic and local vitamin D_3 synthesis may also affect biology of malignant cells (31). Population-based study showed that a low serum level of 25(OH)D3 was related to more advanced disease (overall stage and Breslow thickness), poor prognosis and higher mortality risk (32, 33). Population-based studies also revealed that *VDR* gene polymorphisms can alter susceptibility and prognosis of patients with melanoma (34).

TILs have been found in many types of human cancer (*e.g.* colon, ovarian, and laryngological cancer) (35-37). Their presence is also related to better prognosis and longer OS. In melanomas, the presence of brisk TILs is considered a good prognostic factor. A weak immune response in patients with melanomas, determined as a lack of brisk TILs is considered a significant predictor of sentinel lymph node metastasis and shorter DFS (25, 26). As was mentioned above, immune responses can be modulated by calcitriol, and immune cells express VDR and enzymes activating vitamin D_3 (4, 9, 38). In our previous study, we observed a high expression of VDR in TILs and lymph node lymphocytes

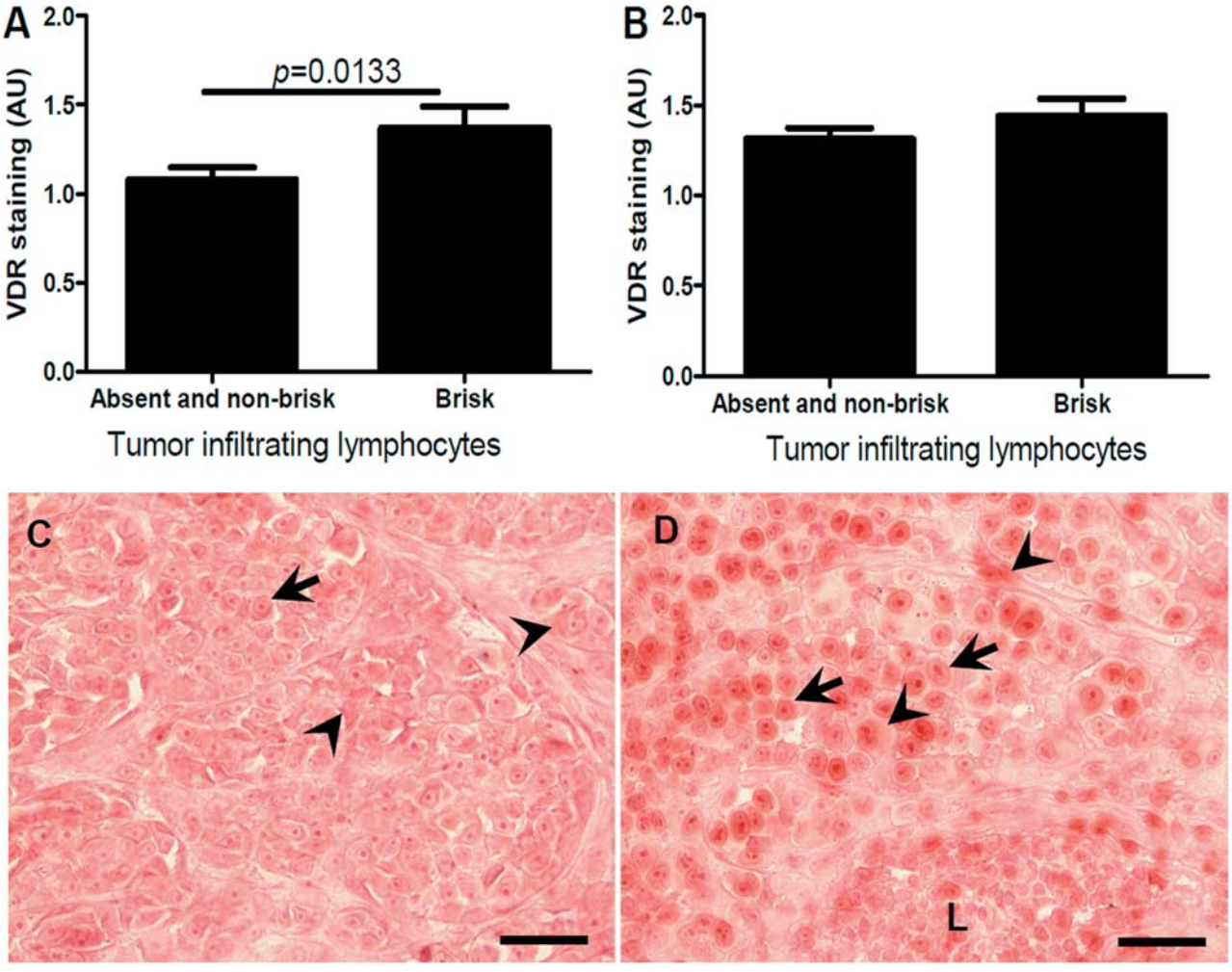

Figure 4. *Mean cytoplasmic (A) and nuclear (B) expression of vitamin D receptor (VDR) in melanomas with absent or non-brisk brisk tumor infiltrating lymphocytes (TILs). Statistically significant differences are denoted with p-values, as determined by the t-test. Representative VDR immunostaining in melanomas without (C) or with brisk (D) TILs. Arrows indicate nuclear VDR immunostaining, arrowhead indicates cytoplasmic VDR immunostaining, L marks lymphocytes; scale bar: 50 μm.*

(22). In the present study, VDR expression in melanoma cells was positively-related to the presence of brisk TILs, suggesting that activation of vitamin D_3 by lymphocytes could negatively-affect proliferation of melanoma cells, resulting in better prognosis and outcome. Activation of the vitamin D_3-mediated signaling pathway could represent an additional mechanism of tumor-specific action of T-cells.

Another prognostic marker in patients with melanoma is ulceration, and its presence is a hallmark of more aggressive tumor biology, related to shorter OS (39). In our study, we found a significantly lower level of VDR expression both in the cytoplasm and in the nucleus in ulcerated melanomas in comparison to melanomas without ulceration. Accordingly, in nonulcerated melanomas, high expression of VDR was related to a longer OS.

In conclusion, we propose that increased VDR expression allows for prediction of a better prognosis, as demonstrated by its correlation with prognostic factors such as TIL presence, ulceration, overall stage and pTNM classification. Accordingly, loss of VDR should affect melanoma behavior, allowing it to evade host surveillance, and releasing the break on cell proliferation that would lead to progression of disease.

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