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Association of Nuclear Vitamin D Receptor (VDR) Immunoexpression with Breslow's Thickness in Acral Melanoma

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Abstract

Background: Acral melanoma (AM) is a subtype of malignant melanoma (MM) that has a predilection for glabrous skin and is the most common type of melanoma among Asians. Depth of invasion is one of the prognostic factors in AM. Vitamin D receptor (VDR) may have an association with depth of invasion in AM. However, reports from Asian patients are still lacking. The aim of this study was to explore the association between VDR immunoexpression and depth of invasion in AM patients in Bandung, Indonesia.

Methods: This study was a retrospective cross-sectional study that analyzed the nuclear and cytoplasm VDR expression of melanoma cells and its association with Breslow's thickness in patients with AM in Dr. Hasan Sadikin General Hospital Bandung. VDR immunostaining was performed on paraffin blocks. The Breslow's thickness was measured using dot slide software. Chi square statistical analysis was done to determine the results.

Results: Of the 30 patients, 73% had a Breslow's thickness of >4 mm. There was a significant inverse association between nuclear VDR immunoexpression and Breslow thickness of AM ($p<0.001$), but not for cytoplasmic VDR immunoexpression ($p=0.914$).

Conclusion: Low nuclear VDR immunoexpression is associated with the depth of invasion in AM. Nuclear VDR immunoexpression possibly affect progression and should be considered before administration of vitamin D therapy in AM.

Keywords: Acral melanoma, Breslow's thickness, VDR

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Introduction

Malignant melanoma (MM) is the most common, potentially fatal skin neoplasm , and its incidence has continued to increase over the past few decades.[1] The MM has a relatively high incidence rate, with an estimated 96,480 new cases and 7,230 deaths in 2019 in the United States (US).[2] The mortality rates from MM reaches 80% worldwide.[3] Acral melanoma (AM) is a subtype of MM that has a predilection for glabrous skin (palms, soles, and nails).[4] This disease represents only 2–10% of all melanoma cases over the world and is an uncommon type of melanoma in Caucasian patients, with an annual incidence varying from 0.04 to 0.25 per 100,000 per year.[5] However, AM is the most common type of melanoma in non-white populations such as Asian, Hispanic, and African. The AM is the most frequent subtype of melanoma in Asian populations, accounting for 41.8% of melanoma patients in China, 86.6% in Korea, 50.8% in Hong Kong, and 50% in Singapore.[6] The AM constitutes 58% of all cutaneous melanoma in Asians in general.[1] There is no exact data on the AM incidence rate in Indonesia. At Dr. Hasan Sadikin General Hospital, Bandung, Indonesia, AM is the most common subtype of MM and represents 32.07% of all melanoma cases in the last 5 years (2017–2021).

It is known that AM has a worse prognosis and might be related to delayed diagnosis due to misdiagnosis, as well as more aggressive intrinsic tumor such as more advanced clinical stage, advanced Breslow's thickness, vertical growth phase, and ulceration.[5,7] These features could be related to the lower

serological levels of vitamin D observed in AM patients.[8] Melanoma thickness with Breslow depth measurement on AM is one of the most significant factors associated with poor survival outcomes.[9]

Several factors have been identified as possible inducers for AM patients including ultraviolet rays, chemical exposure, and traumatic risk factors.[10] The pathogenesis of AM differs from other types of melanoma and involves c-KIT mutation.[10] Recently, there have been several studies that showed a correlation between the serological level of vitamin D and the evaluation of vitamin D receptor (VDR) in melanoma including AM as a prognostic factor and vitamin D administration as one of the promising supplementary therapeutic modalities in MM.[11] Vitamin D can affect pathways and processes essential for the maintenance of cell integrity. It also inhibits growth and induces differentiation of cultured normal and malignant cells by arresting the cell cycle at the G0/G1 and/or G2/M phases.[12] The ability of vitamin D to regulate these processes depends on the presence of the VDR, which belongs to a large superfamily of nuclear receptors with highly conserved nuclear and ligand-binding domains.[13] The decrease in VDR expressions with advanced melanocytic lesions correlates with the Breslow's thickness, which is a method that measures the depth of the melanoma from the top of the granular layer of the skin to the deepest point of the tumour, and the Clark's level, which describes the level of anatomical invasion of the melanoma in the skin.[14,15]

To date, VDR expression in MM has not yet been standardized, currently most studies of VDR in MM including AM have been done in Caucasian patients. The aim of this study was to explore the relationship between VDR and depth of AM invasion .

Methods

This study used 30 paraffin blocks that fulfilled the inclusion and exclusion criteria, from patients who had undergone excisional and surgical biopsies and were diagnosed with AM histopathologically at Dr. Hasan Sadikin General Hospital from 2014 to 2021. The paraffin blocks were then made into 4 mm thick histopathological preparation slides and selected for VDR immunostaining with following procedure: after dewaxing, rehydration and quenching of endogenous peroxidase with 0.3% H_2O_2 in methanol for 15 minutes, antigen retrieval in Tris EDTA buffer at 96^0C in decloaking chamber for 20 minutes, used of background sniper as blocking serum, application and incubation of primary antibody (VDR rabbit anti-human polyclonal antibody; Gene-Tex VDR C1C2; GTX104615) at a dilution of 1:3500 in 1 hour at room temperature. Immunolabeled sections were viewed under Olympus CX21 microscope and semi-quantitative evaluation was performed to determine the VDR expression in nuclear and cytoplasm of malignant melanoma cells by two blind observers, without knowing the histopathological detail especially the Breslow's thickness. Epidermis tissue was stained with VDR and used as a positive control and showed strong and diffuse positivity in the nucleus. Intensity was determined through four stages, namely negative (0), weak (1), moderate (2), and strong (3) compared with positive controls. Distribution of VDR stained nucleus and cytoplasm in melanoma cells was described as negative (0), <10% (1), 10–50% (2), and >50% (3). Histoscore was counted by multiplying intensity and distribution, resulting in four tiers, namely negative (0), low (1), moderate (2), and high (3). Breslow's thickness of tumor cells was measured form Hematoxylin and Eosin-stained slides by digital imaging using an Olympus XC10 camera, Olympus BX51 microscope, and dot slide software at 100x magnification. Breslow's thickness cut-off points of ≤4 mm and >4 mm were taken because of their clinical implication as prognostic factor. Chi-square statistical analysis was performed to analyze results which were considered significant if the p-value <0.05.

Ethical clearance was approved by the Health Research Ethics Committee of Universitas Padjadjaran with number 133/UN6.KEP/EC/2022.

Results

A total of 30 paraffin blocks from AM patients were included in this study, with the mean age of the patients being 64 years. Eighteen patients (60%) were male, and 12 patients (40%) were female. The mean Breslow's thickness was 8.1 mm. Most patients (73%) had a Breslow's thickness of >4 mm, and 8 patients (27%) had a Breslow's thickness of ≤4 mm. Fifteen patients (50%) had moderate nuclear VDR expression on melanoma cells, meanwhile low nuclear VDR expressions was found in 7 patients (23%) and high nuclear VDR expressions was found in 8 patients (27%). Cytoplasmic VDR expression was 10%

Table 1 Characteristics of Patients

Variable	N = 30 n(%)
Age	
Mean ± SD	64±11
Gender	
Male	18 (60)
Female	12 (40)
Breslow's thickness (mm)	
Mean ± SD	8.1±5.4
≤4 mm	8 (27)
>4 mm	22 (73)
Nuclear VDR imunoexpressions	
Low	7 (23)
Moderate	15 (50)
High	8 (27)
Cytoplasmic VDR imunoexpressions	
Low	3 (10)
Moderate	13 (43)
High	14 (47)

Note: SD= Standard deviation

at low criteria, 43% at moderate criteria, and 47% at high criteria.

In Table 2, the results of nuclear VDR expressions with a Breslow thickness of ≤4 mm showed low (0%), moderate (12%), and high (88%), whereas those with a depth of invasion >4 mm showed a decreased with low expression (32%), moderate (64%) and high (4%). The results of statistical tests showed that there was a significant correlation between nuclear VDR immunoexpression and Breslow's thickness in AM (p<0.001), meaning that nuclear VDR expression that appeared at Breslow's thickness ≤4 mm had more expression with high criteria, meanwhile at Breslow's thickness >4 mm, had moderate and low nuclear VDR immunoexpression. The results of cytoplasmic VDR expression with a Breslow's thickness of ≤4 mm showed results with low (12%), medium (38%) and high expression (50%), meanwhile those with a Breslow's thickness of >4 mm showed low (10%), moderate (45%), and high expression (45%). The statistical test results showed that there was no significant relationship between cytoplasmic VDR expressions and depth of invasion or Breslow's thickness in AM (p=0.914), meaning that cytoplasmic VDR expressions appeared in low, moderate, and high proportions of AM with Breslow's thickness ≤4 mm which was not different from lesions with a depth of invasion >4 mm.

Discussion

VDR is localized in both nucleus and cytoplasm of melanoma cells.[11] VDR is a transcription factor that includes in the nuclear family receptor that binds to the active form of vitamin D (1α,25-dihidroxyvitamin D) or calcitrol with high affinity and specificity.[16,17] After binding to calcitrol, VDR translocates from the cytoplasm to the nucleus and forms a heterodimer with the retinoid x receptor (RXR). This VDR-RXR complex will then structurally bind to vitamin D responsive elements (VDREs), then cause modifications to either coactivator or cosupressor and upregulate or downregulate hundreds of genes that are directly controlled by vitamin D, including genes that play a role in progression of tumor cells.[18]

The population of patients in this study were Indonesian citizens with the majority of patients being male. This was confirmed by a study in the US which found that the incidence of ALM from 2006–2015 among all

Table 2 Association of Nuclear and Cytoplasmic VDR Immunoexpressions with Depth of Invasion (Breslow's Thickness) in Acral Melanoma

Variable	Depth of Invasion (Breslow's Thickness)		P-value
	≤4 mm n=8	>4 mm n=22	
Nuclear VDR expressions			
Low	0 (0)	7 (32)	
Moderate	1 (12)	14 (64)	<0.001*
High	7 (88)	1 (4)	
Cytoplasmic VDR expressions			
Low	1 (12)	2 (10)	
Moderate	3 (38)	10 (45)	0.914
High	4 (50)	10 (45)	

Note: significant if the p-value <0.05

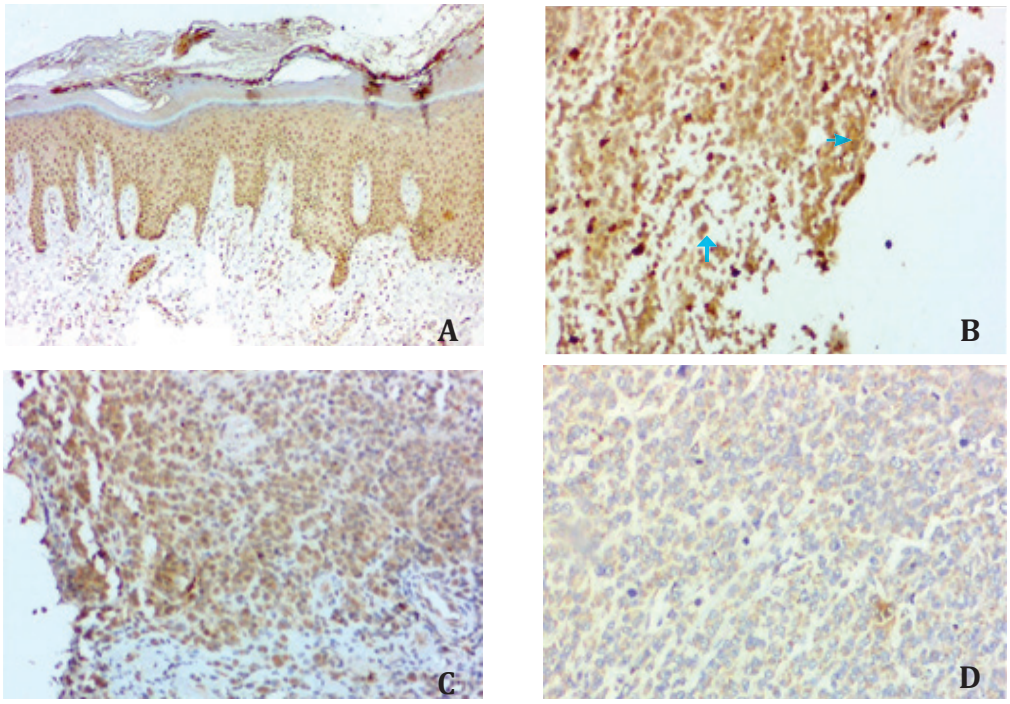

Figure 1 Immunoexpressions of VDR

(a) Positive control (skin epidermis) showed diffuse and strong nuclear VDR expression. (b) Immunostaining of VDR in melanoma cells showed strong nuclear (blue arrowhead) and cytoplasmic expression (blue arrow), note the melanin pigment in tumor cells (blue asterisk), (c) moderate nuclear immunoexpression (blue arrowhead), and (d) weak cytoplasmic immunoexpression (blue arrow)

races (Black American, Asian, and Hispanic) was higher in males.[19] However, in contrast to another study in Caucasians, the incidence of AM was slightly higher in females.[8]

In this study, general median Breslow value was higher with an average depth of invasion of 8.1 mm, compared with another report with a general median Breslow value of 0.8 mm.[8] This could be because the serological vitamin D levels of Asians, including Indonesians, are lower than in Caucasians. Serological levels of vitamin D are lower in individuals with darker skin and living in areas exposed to sunlight.[3] This is because pigmentation in the skin can reduce the effectiveness of sunlight to produce vitamin D.[20,21] Lower vitamin D levels are related to greater progression of melanoma including Breslow's thickness, Clark level, and American Joint Committee on Cancer (AJCC) stage.[22] Serological levels of vitamin D have a correlation with VDR expression in peripheral blood mononuclear cells. Individuals with low serological levels of vitamin D tend to have lower VDR expression.[23]

This study found a correlation between VDR expression in the nucleus and Breslow's thickness in AM. VDR expression in nucleus of melanoma cells was higher in AM with deeper Breslow's thickness ($p<0.001$). These results are compatible with another study of VDR in MM.[11] Furthermore, the same study showed that there was a reduction in nuclear VDR expression as melanocytic lesions progressed. This can be caused by the nuclear localization of VDR in accordance with the VDREs in target genes, including genes that control cell proliferation, differentiation, and apoptosis.[11,14] Vitamin D and VDR complex are also implicated in the development and progressions of cancer.[16] There are several possible mechanisms by which the vitamin D-VDR complex can affect the depth of invasion in melanoma. Vitamin D mediated by VDR inhibits the proliferation of malignant cells by inducing cell cycle arrest by increasing the expression of p21 and p27, which will inhibit cyclin-dependent kinase (CDK) in the cell cycle. Lack of VDR in cells will downregulate the p21 and p27 expressions and upregulate CDK, then causing the progression of malignant cells.[16] Furthermore, VDR suppresses nuclear factor kappa b (NF-kB) through inhibiting

the translocation of the NF-kB p65 subunit in the nucleus of cells.[24] There is a correlation between immunoexpression of NF-kB with the depth of invasion in AM. The AM with positive expression of NF-kB tends to have deeper Breslow's thickness.[25] In the lungs of VDR-KO mice, there is increased activation of NF-kB and this leads to upregulation of several matrix metalloproteinases (MMPs). The MMPs are a group of human zinc-binding endopeptidases that regulate extracellular matrix (ECM) proteolysis and cellular migration and increase angiogenesis in a number of cancers.[26] Overexpression of MMP-1, MMP-2, and MMP-13 correlates with depth of invasion in nodular melanoma.[27]

Meanwhile, in this study, VDR immunoexpression in the cytoplasm did not have a significant association with Breslow's thickness (p=0.914) in AM. This result differs from another study that showed melanomas with the highest Breslow's thickness had significantly reduced cytoplasm VDR immunostaining compared with less advanced tumors in 69 primary cutaneous melanomas, of which only 2 were diagnosed as acral lentiginous melanoma.[14]

VDR is a central protein for vitamin D signaling.[28] VDR needs the active form of vitamin D to translocate from the cytoplasm to the nucleus.[18] In other words, the levels of serological vitamin D will affect the translocation process of VDR from the cytoplasm to the nucleus. Low levels of vitamin D may have correlation with low nuclear expression of VDR in melanoma cells. Meanwhile, the genomic effect of the vitamin D/VDR complex occur through gene modification in the nucleus of cells.

There are several limitations in this study, including the limited number of research subjects and the absence of vitamin D serological data on subject. This study also did not analyze the differences between various anatomical locations of lesions. Future research on VDR immunoexpression in AM accompanied by vitamin D serological and anatomical location data should be carried out with a larger number of samples.

In conclusion, low VDR immunoexpression in the nucleus but not in cytoplasm has an inverse association with Breslow's thickness in AM. This finding may have implications for consideration in vitamin D therapy on AM. Further studies are needed to explore the VDRbroles in the pathogenesis of AM in Asian patients.

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