Int J Clin Exp Pathol 2021;14(8):902-907
www.ijcep.com /ISSN:1936-2625/IJCEP0135160

Original Article

A new approach using a numerical diagnostic criterion for vitiligo diagnosis with HMB-45 and Melan-A staining

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Received May 11, 2021; Accepted July 19, 2021; Epub August 15, 2021; Published August 30, 2021

Abstract: This study aims to investigate the usefulness of the Melan-A and homatropine methyl bromide-45 (HMB-45) markers in identifying melanocytes and to evaluate cut-off values for the diagnosis of vitiligo. We also aim to identify the role of remaining melanocytes when using HMB-45. We counted and confirmed melanocytes and melanin in the samples using a high-magnification microscope. The Melan-A, HMB-45, and Fontana-Masson staining methods were utilized. Descriptive statistical analysis of quantitative traits was performed. For the comparison of the two diagnostic tools, receiver operating characteristic curve and the area under the curve were evaluated. We found out that there was no significant difference observed between the two methods. The cut-off value is <27 for HMB-45 and <15 for Melan-A per 100 cells in the basal cell layer. Thus, HMB-45 is as useful as Melan-A in the diagnosis of vitiligo.

Keywords: Depigmented diseases, HMB-45, Melan-A, melanocytes, vitiligo

Introduction

Vitiligo is a disease in which melanocytes of the epidermis are destroyed, causing decolorization with a clear boundary [1]. Depending on the progress of various clinical stages and clinical manifestations, vitiligo is sometimes difficult to differentiate from other depigmentation disorders [2] such as chemical leukoderma, tinea versicolor, piebaldism, tuberous sclerosis, post-inflammatory hypopigmentation, nevus depigmentosus, and idiopathic guttate hypomelanosis, that all require different courses of treatment and differ from vitiligo in their prognoses. Clinical findings and hematoxylin and eosin (H & E) staining are insufficient to differentiate between the disorders. Therefore, additional immunohistochemical staining is essential for diagnosis.

Moreover, immunohistochemical research on various melanocytic antigen expression in the skin provides important information about the status of melanocytes in vitiligo lesions. Thus, several different markers have been suggested for melanocyte identification in vitiligo: S-100, melanoma antigen recognized by T cell (MART-1), homatropine methyl bromide-45 (HMB-45), and 1-dihydroxy-phenylalanine (DOPA); the Fontana-Masson staining procedure is performed to detect melanin pigment. However, none of them show absolute specificity and sensitivity [3]. In addition, even with immunochemical staining, melanocytes and melanin are partially present. There is no clear interpretation and numerical diagnostic criterion for vitiligo. Various etiologies of vitiligo have been suggested, but the role of the remaining melanocytes has not yet been studied.

Melan-A/Melanoma antigen, recognized by T cells (MART-1), is a well-established melanocytic differentiation marker; it is regarded as one of the most important markers. In a number of studies, the Melan-A marker was shown to be more specific and sensitive than HMB-45 and S-100 melanocytic markers [4-8].

HMB-45 is a monoclonal antibody that was initially generated from an extract of metastatic melanoma. This antibody reacts with a melanosomal protein, GP-100, which tends to be expressed in immature or proliferating cells [9]. Therefore, HMB-45 is positive for fetal melano-

Table 1. Positive rates of vitiligo and normal by staining

Subjects	HMB-45+	Melan-A+	Fontana-Masson+
Vitiligo (n=40)	11 (27.5%)	7 (17.5%)	16 (40%)
Normal (n=20)	18 (90%)	16 (80%)	12 (60%)

cytes and activated melanocytes, thus helping to confirm the activity of melanocytes.

In this study, H & E, HMB-45, Melan-A, and Fontana-Masson staining were performed in vitiligo specimens. The aim of this study was to compare the markers Melan-A and HMB-45 for melanocyte detection in vitiligo and controls with normal epidermis. In addition, the cut-off value required for the diagnosis of vitiligo was obtained when the two types of staining were performed.

Materials and methods

This study was approved by the Institutional Review Board (IRB) of Daegu Catholic University Medical Center and conducted in accordance with the principles of the Declaration of Helsinki.

This case-control study examined 60 specimens (40 vitiligo and 20 normal epidermis) that were collected from patients who visited the Department of Dermatology, Daegu Catholic University Hospital of Daegu, Daegu, Korea, between January 2013 and July 2018. Of the patients diagnosed with vitiligo by histopathological examination of the depigmentation site, 40 had the disease for at least 6 months, with no other underlying disease; non-segmental type was also included. The control group had no underlying disease and histological examination revealed a normal epidermis, with H & E and immunochemical staining. Demographic factors such as race, sex ratio, and average age were unified to reduce bias and to compare the two diagnostic methods. The tissue samples were fixed with 10% buffered formalin and were processed by the conventional histopathological method using immersion in paraffin, and H & E staining. In addition, immunohistochemical tests were performed using the following antibodies: Melan-A (A103 clone, Leica Biosystems, US) and HMB-45 (monoclonal, Dako, US). Deparaffinization of the sections with xylene was followed by the demasking of antigens by boiling in a citrate buffer (pH 6.0) using a microwave oven for 3 cycles, 5 minutes each cycle, with a 1-minute break between each cycle. After cooling, the slides were washed in two changes of TRIS-buffer (pH 5.54). In order to prevent endogenic peroxidase activity, a solution of 0.3% hydrogen peroxide in methanol was applied to the sections. Melanin granules in the samples were detected through Fontana-Masson histochemical staining of the sections.

The prepared specimens were studied using light microscope. The HMB-45+ cells and Melan-A+ cells were detected in the basal layer of the epidermis, and the melanin-containing cells were detected in the basal and suprabasal layers; the calculation was done per 100 basal keratinocytes. These cells were counted in five fields of vision, and following this, the average value was calculated for each specimen.

Statistical analysis was performed using MedCalc (USA). Descriptive statistics of quantitative traits are presented in the Results. For comparison of both the diagnostic tools, receiver operating characteristic (ROC) curve and the area under the curve (AUC) were evaluated.

Results

Of the 40 vitiligo specimens, 11 (27.5%) were positive for HMB-45, 7 (17.5%) were positive for Melan-A, and 16 (40%) for Fontana-Masson. In the control group, there were 18 (90%) positive specimens for HMB-45, 16 for Melan-A (80%), and 12 for Fontana-Masson (60%) (**Table 1**).

The general histologic features revealed more basal hypopigmentation and dermal inflammation in skin with vitiligo lesions than in normal skin. Of the 40 cases with vitiligo, complete disappearance of melanin and melanocytes was observed in 11 cases. The rest of the patients showed decreased melanin expression, as seen in Fontana-Masson test. HMB-45 and Melan-A showed melanocytes were reduced to less than 10 per 100 basal cells (**Figure 1**). In some cases, lymphocytes and histiocytes were observed in the papillary dermis. Degenerative changes have been found in keratinocytes and melanocytes.

The mean number of melanocytes in the basal layer with HMB-45 and Melan-A staining in vit-

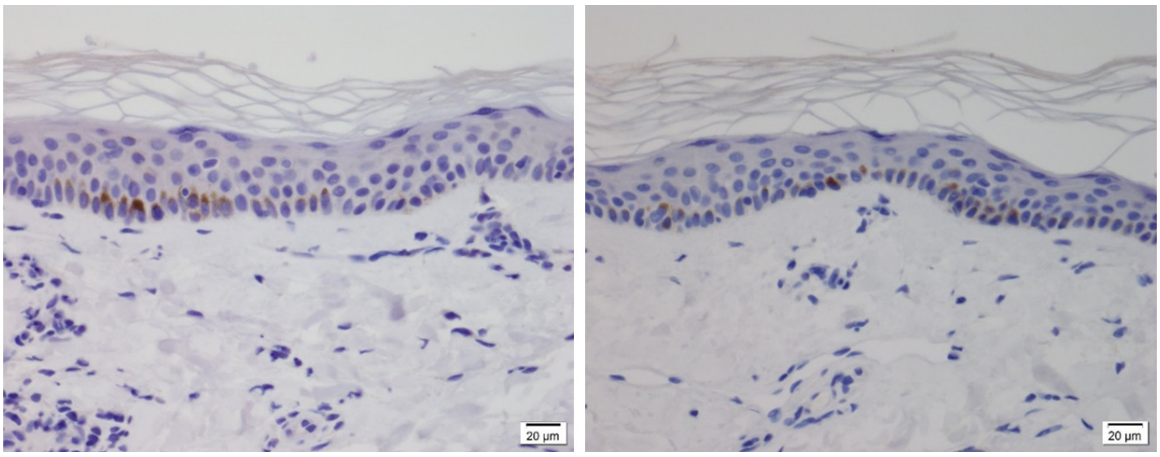

Figure 1. Microscopic findings showing partial disappearance of melanocytes (×400). There are some residual melanocytes in the basal layer in vitiligo. HMB-45, ×400 (Left), Melan-A, ×400 (Right).

Table 2. Mean number of HMB-45+melanocytes and Melan-A+melanocytes in the epidermis of vitiligo and normal groups (number of cells per 100 basal keratinocytes, median values ± standard deviation and quartiles)

Subjects	HMB-45+melanocytes	Melan-A+melanocytes
Vitiligo (n=40)	14.55 ± 13.17 (0; 54)	10.58 ± 14.88 (0; 58)
Normal (n=20)	36 ± 18.42 (5; 62)	38.65 ± 22.56 (2; 65)

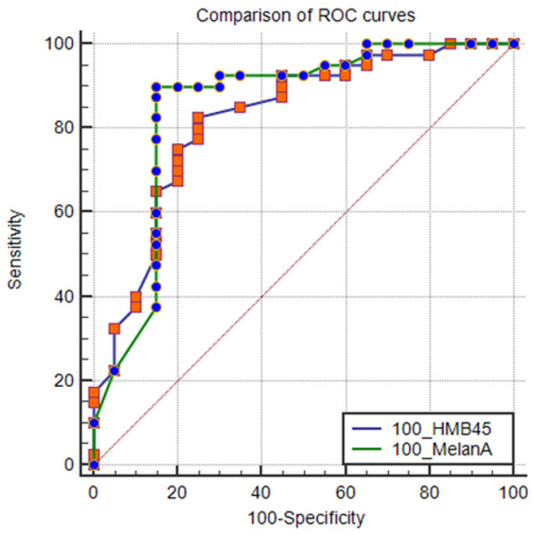

Figure 2. Comparison of ROC curves between HMB-45 and Melan-A stains.

iligo and normal epidermis groups were calculated. In the vitiligo group, 14.55 ± 13.17 of melanocytes were positive in HMB-45 and 10.58 ± 14.88 in Melan-A. In the normal group, it was 36 ± 18.42 in HMB-45 and 38.65 ± 22.56 in Melan-A (**Table 2**).

To compare the two diagnostic tools, HMB-45 and Melan-A, the number of cells per 100 basal keratinocytes were used to analyse the ROC curve for 100-HMB-45 and 100-Melan-A (**Figure 2**; **Table 3**). In both graphs, the AUC was 0.5 or greater, with HMB-45 being 0.823 and Melan-A 0.851 (P<0.001). The sensitivity of HMB-45 was 82.50% and the specificity was 75.00%; the sensitivity of Melan-A was 90.00% and the specificity was 85.00%. AUC, sensitivity, and specificity were higher with Melan-A stain. Comparison of the two ROC curves was performed. The AUC difference between the two tests was 0.0275. When the significance level (*P*-value) was measured, it showed no significant difference between the two tests (P=0.3114).

The cut-off value for each test was obtained from the graph. The cut-off value for 100-HMB-45 was measured as 73 and for 100-Melan-A as 85. These represent the number of melanocytes per 100 basal keratinocytes. Thus, HMB-45 can be confirmed as vitiligo when 27 or fewer melanocytes are present per 100 basal keratinocytes; for Melan-A, that number is 15 or less.

Discussion

The world-wide prevalence of vitiligo is estimated to be between 0.2 and 1.8% [10] and the disease has a great personal and healthcare cost [11, 12]. Furthermore, it is negatively affects a patient's self-esteem and quality of

Table 3. Comparison of ROC curves between HMB-45 and Melan-A stains

Subjects	AUC	Significance level P (AUC=0.5)	Sensitivity (%)	Specificity (%)	Cut-off value
100-HMB-45	0.823	<0.001	82.50	75.00	>73 (HMB-45 <27)
100-Melan-A	0.851	<0.001	90.00	85.00	>85 (Melan-A <15)

AUC, area under the curve.

life [12, 13]. Therefore, it is important for physicians to diagnose vitiligo accurately and determine the treatment direction for patients.

However, it is challenging to differentiate vitiligo only by observing the history or by microscopic examination, from diseases such as idiopathic guttate hypomelanosis, post-inflammatory hypopigmentation, and pityriasis alba.

To understand the histopathological findings of vitiligo, it is important to detect melanocytes or melanin pigmentation in lesions to discriminate between various low-pigment diseases of the skin. Changes in melanin pigment can be observed by Fontana-Masson staining, which is positive when silver-rich granules of melanin are observed, or by electron microscopy to identify melanin or melanocytes in keratinocytes [14]. Melanocytes can be observed using S-100 protein, but the method has a disadvantage of non-specific positive reaction to melanocytes, Langerhans cells, neurons, tissue, muscle cells, and apocrine glands [15]. More recently,an antibody specific for melanocytes such as MART-1 or NKI-beteb [13, 14] has been used to observe melanocytes. MART-1, an antibody (molecular weight, 20-22 kDa) specific to melanocytes, is a protein originating from malignant melanoma cells. It is recognized by T cells. It can be stained in both frozen tissue specimens and paraffin-embedded tissue, and is specific for normal melanocytes and melanomas.

The antibody HMB-45 has been designated as a marker for melanoma, even though some benign lesions have been noted to show positive staining reactions with this reagent [16]. The specific antigen recognized by HMB-45 is now known as Pmel [17]. HMB-45 correlates with melanosome production and, thus, a melanocytic origin of HMB-45-positive cells. HMB-45 may correlate best with factors that stimulate melanocytic proliferation and production of melanosomes. Active studies of HMB-45 in melanoma or nevus disease have been done; however, the usefulness of HMB-45 in vitiligo has not been established. In this study, we confirmed that both tests were at par through the pairwise comparison of ROC curves between HMB-45 and Melan-A, which proved that they are effective tests for vitiligo with little difference in their usefulness. This suggests that HMB-45 can detect not only the positive effect of melanocytic proliferation and melanosome production but also the negative effect of contrast.

Moreover, most authors of previous studies concluded that long-standing vitiligo lesions show a complete absence of melanin and loss of melanocytes in the epidermis [2, 17, 18]. However, there are some sporadic reports indicating that vitiligo lesions are not fully devoid of melanocytes. Tobin et al [19] showed that melanocytes could be isolated and established in vitro, from all samples of lesional and normal skin, independent of disease duration and treatment. Husain et al [20] also found epidermal melanocytes in vitiligo and showed enzymatic hydroxylation of tyrosine to DOPA. Non-negligible amounts of melanin were detected in basal keratinocytes in 1- to 3-year-old vitiligo lesions. Therefore, the possibility of intact melanogenesis or melanin transfer in vitiligo was suggested [21, 22].

Currently, several etiologies of vitiligo have been presented, among which the hypothesis of melanocyte destruction by autoimmune reaction is considered the most potent [23-25]. We performed an HMB-45 test that was positive for premelanocytes, suggesting that the activity of the remaining melanocytes could be estimated. Thus, it can be inferred that fetal melanocytes seen in the epidermis compensate for the reduced number of melanocytes and try to maintain normal pigmentation through melanogenesis but may eventually lead to complete absence due to the accelerated or uncontrolled destruction of melanocytes.

Therefore, in order to diagnose vitiligo, immunohistochemical staining requires specific quantitative criteria rather than positive or negative results. Both melanocytes and melanosomes were also detected in some samples that were diagnosed with vitiligo in this study, although the number was lower than normal. Therefore, the cut-off values at the peak of specificity and sensitivity from ROC curves were obtained. The limitations of this study include that it is a single institution study, and that normal controls had fewer samples than experimental groups. However, no studies are published to date that quantitatively evaluate immunohistochemical staining for vitiligo diagnosis. This study indicates that the HMB-45 stain and the Melan-A stain are useful diagnostic tests forvitiligo that is difficult to distinguish clinically and histologically. In addition, we present a diagnostic tool that can diagnose vitiligo easily and quickly by presenting the numerical cut-off value necessary for diagnosis of vitiligo with partial absence of melanocytes or melanosomes.

Acknowledgements

This study was submitted as a poster to the 24th World Congress of Dermatology Milan 2019.

Disclosure of conflict of interest

None.

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