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Original Article

Expression of HMB-45, NKI/beteb and c-kit in amelanotic melanocyte in patients with follicles of vitiligo

Zhi-Wei Shang, Hai-Xia Feng, Wei-Ping Wang, Hong-Wei Fu

Department of Dermatology, Frist Affiliated Hospital of Henan University of Science and Technology, Luoyang, China

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Abstract: Objective: This study aims to observe the effect of HMB-45, NKI/beteb and c-kit on amelanotic melanocyte (AMMC) in follicles of vitiligo patients, and to analyze the role of hair follicle AMMCs and SCF/c-kit signaling pathways in the pathogenesis of vitiligo. Method: We collected 31 cases of vitiligo scalp skin and 30 cases of normal scalp skin from the head. Scalp hair follicles in these two groups were stained by H&E and three antibodies (HMB-45, NKI/beteb and c-kit) by immunohistochemistry. Morphological distribution characteristics and the expression of HMB-45, NKI/beteb and c-kit in hair follicle AMMCs were observed. The HPIAS-1000 color pathological graphic analysis system was employed to semi-quantitatively analyze the strength of these three specific markers, and observe differences in positive cell area and positive cell integrated optical density between these two groups. Differences in measurement data were compared by *t*-test, and $P<0.05$ was considered statistically significant. Results: There was no positive expression in HMB-45 immunohistochemical staining in the outer root sheath of hair follicles between the two groups. Scalp hair follicle AMMC NKI/beteb staining revealed that there was no obvious difference in mean optical density value in the positive staining area between the vitiligo group and normal group ($P>0.05$). However, the average positive rate of scalp hair follicle AMMC c-kit and the average optical density value in the positive staining area in the vitiligo group was lower than in the normal group, and the difference was statistically significant ($P<0.05$). Conclusion: Vitiligo scalp hair follicle AMMCs that exist in SCF/c-kit signaling pathways are abnormal, and is likely to be involved in the pathogenesis of vitiligo. This may be avenues for the repigmentation of vitiligo in the future.

Keywords: Vitiligo, Amelanotic melanocytes, HMB-45, NKI/beteb, C-kit

Introduction

Amelanotic melanocytes (AMMCs) are precursor cells of skin melanocytes, and are also the most important repositories. AMMCs play a very important role in the process of vitiligo healing [1-4]. Stem cell factor (SCF) and its receptor c-kit (SCF/c-kit signaling pathway) play an important role in regulating melanocyte growth, proliferation, differentiation and migration [5]. It remains unclear whether SCF/c-kit signaling pathways are abnormal in hair follicle AMMCs in vitiligo lesions. This study applied immunohistochemical methods to stain specific antibodies (HMB-45, NKI/beteb and c-kit) in scalp hair follicles in vitiligo patients and normal individuals, observed the morphologic distribution characteristics of hair follicle AMMCs, and preliminarily discussed whether the SCF/c-kit signaling pathway was abnormal.

Materials and methods

Sample source and preparation

Thirty-one vitiligo patients and 30 normal individuals were included in this study. Patients were diagnosed with vitiligo on their head in our department from September 2010 to September 2014, and had no other organic and autoimmune diseases. These patients did not use systemic glucocorticoids, immunosuppressants, photosensitizer, or UV treatment in the previous months. The 31 vitiligo patients (20 male, 11 female) were 18-61 years old (mean age, 32.3), and had a disease duration between

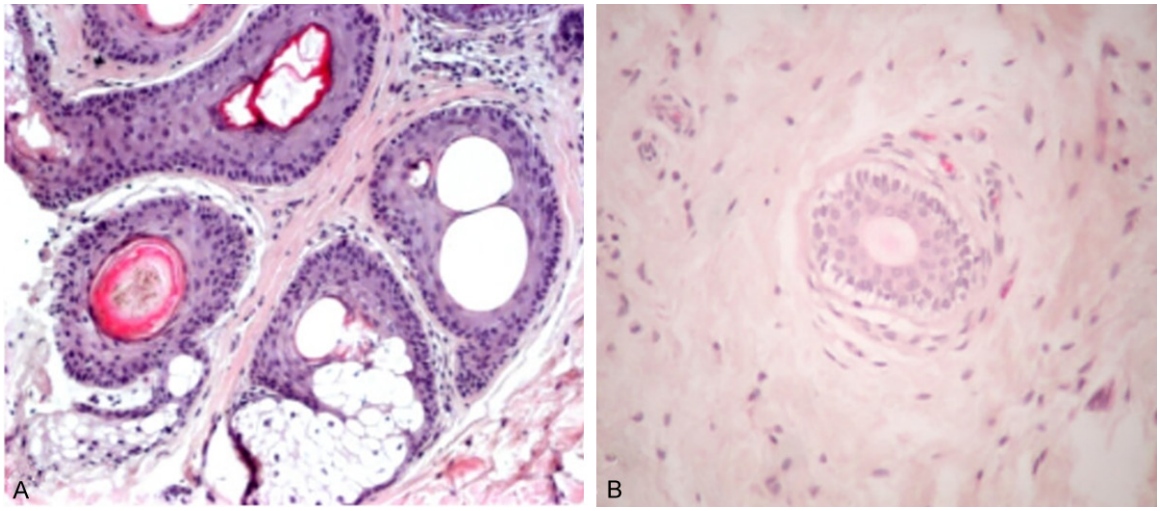

Figure 1. Normal hair follicles and vitiligo lesions after HE staining, under the microscope (× 200) sebaceous gland structure. A. Normal hair follicle HE staining (× 200). B. Vitiligo lesions after HE staining (× 200).

1 week and 12 months (mean duration, 2.5 months). Normal individuals were patients who had an emergency surgical operation on their head in the Department of Neurosurgery of our hospital. These patients had normal hair, did not have systemic or autoimmune diseases, and did not receive systemic glucocorticoids, immunosuppressants, photosensitizer, or UV treatment in the previous month. Patients (20 male, 11 female) were 17-63 years old (mean age, 28.5). There was no significant difference in gender and age between these two groups.

For pathological materials from the vitiligo scalp, the entire layer of the scalp was cut off at the central area of the vitiligo by routine biopsy. For pathological materials from normal scalp, the entire layer of the scalp was cut off from patients who had emergency surgery for head injuries and cerebrovascular accidents in Neurosurgery. The draw materials of the two groups were both 0.5 × 0.3 cm. Excess subcutaneous fat was removed and fixed for 24 hours with 10% neutral formalin, conventional dehydration, paraffin embedded, and cut into 4-μm white pieces.

The slices were conventionally dewaxed, placed into water, and H&E staining was applied for 10 minutes. Then, color separation was performed with 1% hydrochloric acid and ethyl alcohol, eosin staining was applied for one minute, and gradient alcohol 80%, 95%, 95% II, 100% I and 100% II were applied for three, three, three, 10 and five minutes, respectively. Next, slices were immersed into two bottles of TO biological transparent agent for 10 minutes each bottle, neutral gum sealing slices were used, and observed under a microscope.

Immumohistochemical staining

The streptomycin avidin-peroxidase connection (SP) method was used for staining. Primary antibodies were: HMB-45 monoclonal antibody (mouse anti-human; dilution at 1:100; Zymed, USA), NKI/beteb monoclonal antibody (mouse anti-human; dilution at 1:5; Abcam, USA), c-kit polyclonal antibody (rabbit anti-human, dilution at 1:100; Thermo Scientific, USA), and Ultra sensitive SP kits (ready-to-use reagent; Fujian Maixin Biotechnology Co. Ltd., China). The secondary antibody was biotin labeled DAB color solution. This experiment selected the HMB-45, NKI/beteb and c-kit positive expression of nevus cell as a positive control; and used immunohistochemical staining slices, in which primary antibodies was replaced by PBS as a negative control.

Result determination

Three slices were observed from each specimen, and five microscope fields were observed for each slice (200 ×). HMB-45, NKI/beteb and c-kit positive staining was confirmed when light yellow to brown granules appeared in the cytomembrane and cytoplasm. A HPIAS-1000 high-definition color pathological image analysis system was used to measure the positive cell area

Table 1. The immunohistochemical of NKI/beteb and c-kit of the vitiligo scalp group and the normal group

		Mean positive cell area rate		MOD	
Group	Cases	NKI/beteb	c-kit	NKI/beteb	c-kit
Vitiligo	31	0.1183±0.0136	0.0912±0.0116	0.3521±0.0319	0.3149±0.0203
Normal	30	0.1241±0.0132	0.1173±0.0143	0.3604±0.0257	0.3501±0.0194
t		1.376	7.841	1.117	6.936
P		0.174	0.000	0.269	0.000

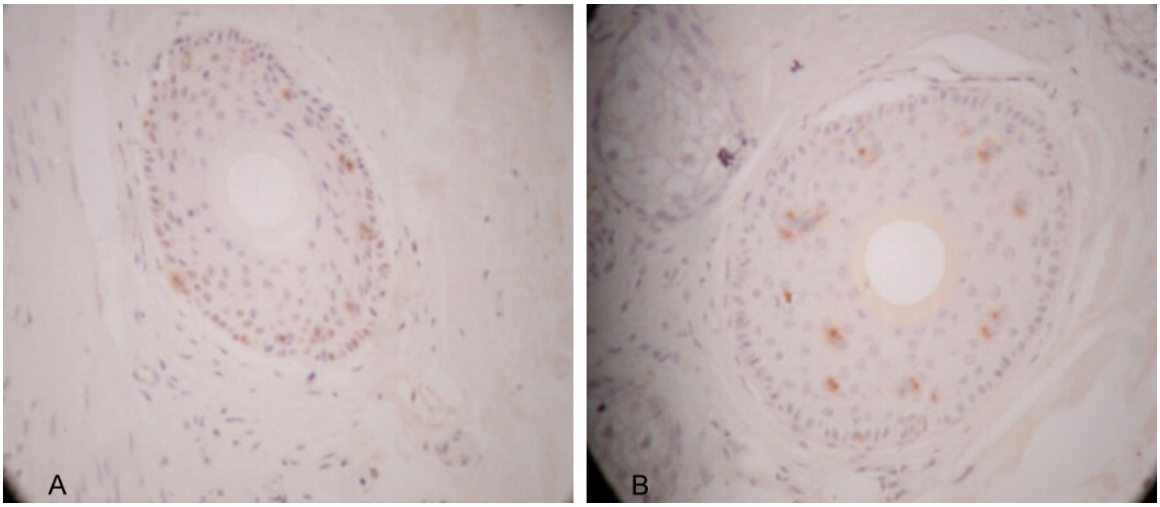

Figure 2. Normal hair follicles and vitiligo lesions of the hair follicles NKI/beteb staining, under the microscope (× 200) to observe the positive melanocytes. A. Normal hair follicle NKI/beteb staining (× 200). B. NKI/beteb staining of hair follicles in vitiligo (× 200).

and positive cell integrated optical density (IOD) of HMB-45, NKI/beteb and c-kit. Then, the positive cell area rate (positive cell area rate = positive cell area/unit cell area × 100%) and mean optical density (MOD = positive cell integrated optical density/positive cell area) were calculated; and the mean value of the positive cell area rate and MOD were used as the measured value of this sample for statistical analysis.

Statistical approach

The SPSS 13.0 statistical software was used to analyze all data. Differences in measurement data were compared by *t*-test. $P<0.05$ was considered statistically significant.

Results

H&E staining

The scabbard of the hair follicle was clearly visible and intact in the two groups, including the inner root sheath, outer root sheath (ORS) and dermal sheath. ORS was the thickest layer, which consisted of approximately 3-9 layers of cells; and the structure of the sebaceous glands were partly visible (**Figure 1**).

Immumohistochemical staining

The positive expression of HMB-45 immunohistochemical staining was not found in the ORS of hair follicles in the two groups.

The cytoplasm and cell membrane of AMMCs were dyed with tan granules in the ORS of hair follicles in both groups and were few in number. Furthermore, the cell body was smaller and rounder, had a hyperchromatic nucleus, was surrounded by perinuclear halos, had no obvious dendrites, and its surrounding positive melanin granules could not be seen. There was no significant difference in HMB-45 and NKI/beteb between this two groups ($P>0.05$) (**Table 1** and **Figure 2**).

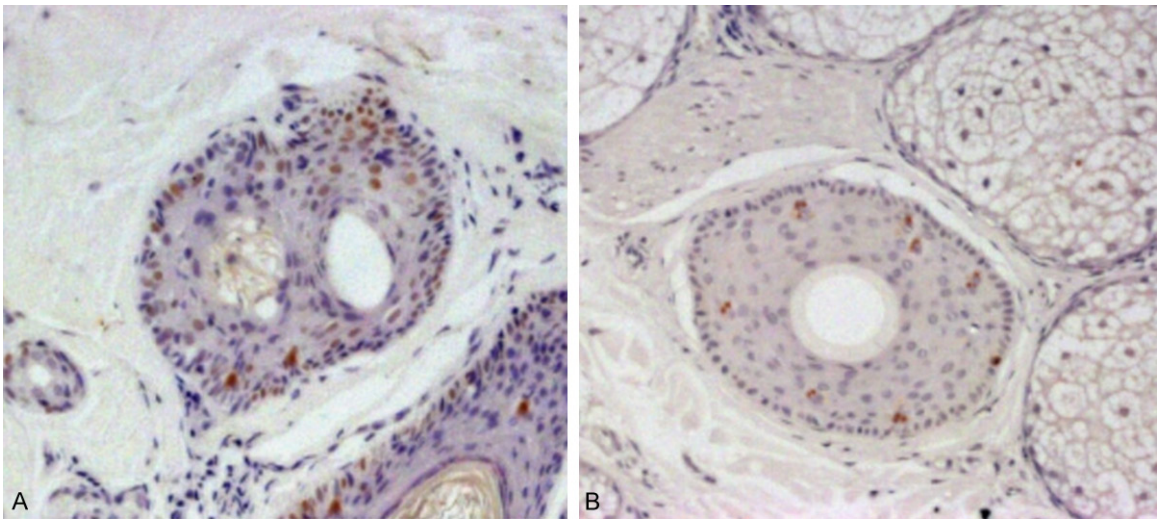

Figure 3. Normal hair follicles and vitiligo lesions after NKI/beteb staining (*200). The average positive rate of MOD. A. c-kit normal expression in follicles (× 200). B. c-kit expression in vitiligo follicles (× 200).

C-kit positive staining cells were sparse in the ORS of scalp hair follicles in the vitiligo group, distribution was diffused, staining intensity was weak, and mean positive cell area rate and MOD were lower than the normal group. The difference was statistically significant ($P<0.05$) (**Table 1** and **Figure 3**).

Discussion

When pigment recovery occurred in most vitiligo patients, it formed a pigment island that started from the orifice of the hair follicle and gradually expanded outwards. The deepest pigment was found near the orifice of the hair follicle and the lightest pigment was found at the edges, suggesting that melanocytes, which were either active melanocytes or inactive melanocytes and may include melanocyte stem cells (MelSC), were not totally absent from the vitiliginous hair follicles [6]. MelSCs form a stem cell system within individual hair follicles and provide a 'hair pigmentary unit' for each cycle of hair pigmentation [7]. Melanocytic stem cells and their immediate proliferative progenies are presumed as AMMCs in the ORS [8-11], and pigments are derived from AMMCs in the ORS of hair follicles [12]. Early work suggests that AMMCs in HFs proliferate, differentiate and migrate into the interfollicular epidermis, leading to "follicular repigmentation". At present, it has been considered that AMMCs can be activated by specificity factors, and that pigments derived from AMMCs are transferred along the bottom of the hair follicle to the epidermis and to the decolored areas in order for the pigment to regenerate. AMMCs are the most important pigment cell providers and storage pools in the process of vitiligo acquiring pigments. The development of pigment cells went through three periods: neural crest cells, melanoblasts and melanocytes; and only neural crest cells expressing c-kit can differentiate into melanocytes, namely AMMCs [13]. Tyrosinase is fairly specific for melanocytic differentiation, and its expression is very similar to HMB-45 labeling; which is considered a marker for melanocyte activity. Due to the developmental immaturity of melanosome in AMMCs, enzymatic molecular expression did not correlate with melanogenesis; thus, HMB-45 could not display the melanocyte by itself [14, 15]. NKI/beteb can identify the gp-100 protein of immature melanocytes; therefore, NKI/beteb monoclonal antibody and HMB-45 can be used to identify AMMCs [16]. In skin hair follicle tissues of the whole body, the hair follicle of the head skin is the thickest and complete, and it is easy to observe and analyze; therefore, this study was carried out to observe head skin lesions of vitiligo and normal scalp tissues. We used the average positive rate and average positive light density value of two indicators to analyze the semi-quantitative analysis of three kinds of specific markers in the ORS of hair follicle AMMC expression, which was more objective and accurate, and minimized deviations caused by the artificial counting

method and subjective judgment of staining intensity. We found that AMMCs were mostly located in the middle lower part of the ORS of hair follicles, were small in number, were mostly scattered in distribution, had a smaller and rounder cell body, had hyperchromatic nucleuses, were surrounded by perinuclear halos, and had no obvious dendrites. Furthermore, its surrounding HMB-45 positive melanin granules could not seen, and the NKI/beteb positive cell area rate and MOD in the two groups was not significantly different. That is, compared with the normal group, AMMCs in the ORS of hair follicles in the vitiligo group had no significant reduction in number.

There is a possibility of cross-talk between Notch and c-Kit signaling in the regulation of the lineage of melanocytes [17], which is known to be critical for the activation and survival of melanocytes [18]. SCF/c-kit signaling pathways play an important role in regulating melanocyte growth, proliferation, differentiation and migration. Studies have found that c-kit expression in the vitiligo epidermis was reduced and that SCF/c-kit signaling pathways were abnormal [19]. Thus, whether AMMC SCF/c-kit signaling pathways are abnormal or not, it is significantly meaningful to understand the role of AMMC and SCF/c-kit signaling pathways in the pathogenesis of vitiligo and find novel treatments for vitiligo. This places AMMCs of hair follicles at the center and target of studies. Our study observed the expression of SCF/c-kit signaling pathways in vitiligo hair follicles using the immunohistochemical method. Results revealed that the mean positive cell area rate and MOD of c-kit in the ORS of hair follicle AMMCs in vitiligo lesions were significantly lower than in normal scalp, c-kit expression was significantly reduced, and there was damage in SCF/c-kit signaling pathways in the ORS of hair follicle AMMCs in vitiligo lesions. However, the mechanism of the reduction in c-kit expression remains unclear; and whether if this is due to gene mutation, the deletion and rearrangement in c-kit, or downstream factors such as expression defects on MITF acting on the E box of c-kit promoters that downregulate the expression of c-kit protein on the transcriptional level, or c-kit regulating factors such as changes in the expression of SCF and transfer factor AP-2, further studies are needed. In addition, further studies are also required for same changes that occur in hair follicles in vitiligo lesions in the trunk and limbs.

In the assumption that ORS AMMC SCF/c-kit signaling pathway damage is involved in the pathogenesis of vitiligo, it is possible to use a c-kit excitomotor or positive regulator such as SCF and transfer factor AP-2 to stimulate the proliferation and differentiation of hair follicle AMMCs to transfer into the basal layer of the epidermis, which would speed up the recovery of depigmentation areas; thus, the vitiligo is cured. We believe that with continuous development of related studies, the regulatory mechanisms and processes of AMMCs in the root sheath of hair follicles would be eventually elucidated. The application of hair follicle AMMCs as the center and target for vitiligo treatment would shed new light to the cure for vitiligo.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Zhi-Wei Shang, Department of Dermatology, Frist Affiliated Hospital of Henan University of Science and Technology, 24 Jinghua Street, Jianxi District, Luoyang 471003, China. Tel: +86 379 64830799; Fax: +86 379 64830799; E-mail: zhiweishangdoc@163.com

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