Clinicopathological Features and Immunohistochemical Alterations of Keratinocyte Proliferation, Melanocyte Density, Smooth Muscle Hyperplasia and Nerve Fiber Distribution in Becker’s Nevus

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Background: Although Becker’s nevus (BN) is a relatively common disease, the systematic studies of clinicopathological and immunohistochemical results are poorly reported. Objective: To investigate the clinicopathological features and immunohistochemical alterations of keratinocyte proliferation, melanocyte density, smooth muscle hyperplasia and nerve fiber distribution in BN. Methods: Clinical and pathological data were collected in 60 newly-diagnosed BN cases. Immunohistochemical stain of Ki-67, Melan-A, keratin 15, smooth muscle actin and protein gene product 9.5 was performed in 21 cases. Results: The median diagnostic and onset age was 17 and 12 years, respectively. Skin lesions usually appeared on the upper trunk and upper limbs. The pathological features included the rete ridge elongation and fusion and basal hyperpigmentation. Epidermal Ki-67, Melan-A and keratin 15 expression and dermal nerve fiber length were significantly higher in lesional and perilesional skin than in normal skin (p<0.05 ∼ 0.01), while smooth muscle actin expression was upregulated only in skin lesion (p<0.05). Conclusion: Although the clinical diagnosis of BN is often straightforward, histopathology is helpful to differentiate from other pigmented disorders. The hyperproliferation of keratinocytes, melanocytes, arrector pili muscle and dermal nerve fibers could be involved in the pathogenesis of BN. (Ann Dermatol 28(6) 697 ∼ 703, 2016)

-Keywords- Becker’s nevus, Keratinocytes, Keratins, Melanocytes

INTRODUCTION

Becker’s nevus (BN) is a relatively common disease characterized clinically by unilateral distribution of irregularly-bordered brown patches and pathologically acanthosis, basal hyperpigmentation, and rete ridge fusion and elongation1,2. BN generally begins during the second decade of life and preferentially involves the upper trunk3. It can be concomitant with various cutaneous anomalies. Although the etiopathogenesis of BN is still unknown, it may be an androgen-dependent and paradigmatically inherited1. The detailed data of histopathology and immunohistochemistry could be helpful for the diagnosis and pathogenetic study of BN. However, there are only a few systematic studies of its clinicopathologic features2,4. The immunohistochemical observations of other indexes are nearly unavailable in BN except androgen receptor and melanocyte2,5. Thus, in order to clarify the changes of epidermal and follicular keratinocytes, melanocytes, arrector pili muscle (APM) and nerve fibers, we summarized the
clinicopathologic characteristics of 60 newly-diagnosed BN cases and investigated the immunohistochemical alterations of Ki-67, Melan-A, keratin 15 (K15), smooth muscle actin (SMA) and protein gene product 9.5 (PGP 9.5) in the lesional and perilesional normal-appearing skin of 21 BN patients.

MATERIALS AND METHODS

Patients

After approved by the Institutional Review Board of Affiliated Hospital of Guangdong Medical University (IRB no. PJ2013121), 35 patients with BN were prospectively collected during January 2014 and June 2015. Twenty-five archived cases of BN between January 2012 and December 2013 were also reviewed and followed up. The following data were collected: gender; diagnostic and onset age; morphology, distribution, and hairiness of lesions; subjective symptoms; associated diseases; and family history. All patients underwent the biopsies.

Histopathology

The biopsied samples were fixed in 10% neutral formalin and embedded in paraffin. The 3-μm sections were stained with hematoxylin and eosin (H&E).

Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded sections according to the manufacturers’ instructions. The panel of primary antibodies included: mouse anti-K15 (clone LHK15, SC-47697, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-Melan-A (MAB-0275) and SMA (KIT-0006) (Fuzhou Maixin Biotechnology Co. Ltd., Fuzhou, China); and mouse anti-Ki-67 (ZM-0166) monoclonal and rabbit anti-PGP 9.5 (ZA-0263) polyclonal antibodies (Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd., Beijing, China). Citrate buffer (MVS-0066), EDTA buffer (MVS-0098), Elivision Plus kit (KIT-9901) and diaminobenzidine kit (DAB-2031) were purchased from Fuzhou Maixin Biotechnology Co. Ltd.

Semiquantitative analysis of immunohistochemistry

Histomorphometry was performed using Image-Pro® Plus v6.0 (Media Cybernetics Inc., Silver Spring, MD, USA). Epidermal Ki-67+ and Melan-A+ cells were counted in 5 different fields at ×200 magnification and expressed as per mm of the basement membrane zone. K15 expression was assessed semi-quantitatively in 5 different fields at ×200 magnification based on the percentage of stained cells (0%~1% = 0; 2%~25% = 1; 26%~50% = 2; 51%~75% = 3; 76%~100% = 4). SMA+ APM were measured within the whole dermis at ×40 magnification (excluding microvessels, hair follicles, and eccrine glands), and expressed as per square millimeter of dermal tissue. Dermal nerve fiber morphometry of PGP 9.5 staining was referred to the previous report6. Five different fields were chosen from each section at ×200 magnification. After delineating the dermal-epidermal junction, multiple orthogonal lines were marked at a distance of 200 μm below every rete ridge and dermal papilla, and their distal ends were joined. The dermal nerve fiber length (DNFL) was obtained (DNFL/mm2) after validating the dermal area of interest. Nerve fibers running into the dermal annexes, APM and nerve plexus were not included.

Statistical analysis

Because all immunoreactive results showed non-normal distribution, Ki-67, Melan-A, SMA and PGP 9.5 results were analyzed using Welch test with Dunnett T3 posthoc test, while K15 data were calculated using Kruskal-Wallis test with Mann-Whitney U test. Statistical analysis was performed using SPSS ver. 17.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Demographic and clinical data

Of 60 cases, 49 (81.7%) were male and 11 (18.3%) were female. The diagnostic age ranged from 11 to 38 years (median 17 years) and onset age ranged from birth to 36 years (median 12 years). The adolescent (10~20 years), childhood (<10 years), congenital and late (>20 years) onset accounted for 70%, 20%, 6.7%, and 3.3%, respectively. The lesions manifested as light to dark brown patches with irregular borders, and their sizes varied from 5×1.5 cm to 56×25 cm (Fig. 1A). Scapular region, four limbs and chest were the predilection sites, while head, neck, abdomen, waist and buttock were uncommon (Table 1). Single lesion (93.3%) and unilateral distribution (95.0%) were the most common. Hypertrichosis was observed in 33 males and 8 females, of which there was no significant difference in gender (χ²=0.12, p=0.729) and diagnostic age (t=0.77, p=0.939) in patients with or without hypertrichosis. Multiple papules were seen within BN lesions in 20% cases, but acneform eruptions were absent. Acne vulgaris, neurofibromatosis type 1 and ovarian teratoma were concomitant in 17, 2, and 1 cases, respectively. There were no symptoms, mucosal involvement and family history of BN.

Histopathologic findings

The rete ridge elongation (100.0%) and fusion (91.7%),
Fig. 1. Clinicopathologic images of Becker’s nevus in an 18-year-old man. (A) A brown hypertrichotic patches with multiple papules on the right chest. (B) Keratotic plugging, acanthosis, mild rete ridge elongation and fusion, basal hyperpigmentation, as well as smooth muscle and sebaceous hyperplasia and superficial perivascular lymphohistiocytic infiltration in the dermis (H&E, ×40).

Table 1. Lesional distribution of Becker’s nevus (BN) (n=60)

| Site                   | n (%) |
|------------------------|-------|
| Head                   | 4 (6.7) |
| Neck                   | 2 (3.3) |
| Scapular region*       | 19 (31.7) |
| Chest                  | 6 (10.0) |
| Waist and abdomen      | 5 (8.3) |
| Buttock†               | 4 (6.7) |
| Upper limbs‡           | 7 (11.7) |
| Lower limbs            | 13 (21.7) |

*BN extended to the ipsilateral chest in 2 cases and arms in 4 cases. †BN extended to the ipsilateral thigh in 1 case. ‡Concomitant with BN on the contralateral thigh in 1 case.

keratotic plugging (85.0%), basal pigmentation (98.3%), and mild perivascular lymphohistiocytic infiltration in the superficial dermis (100.0%) were the most common, followed by hyperkeratosis (61.7%), acanthosis (58.3%), bottom flattening of rete ridge (78.3%), dermal fibrosis (78.3%), and smooth muscle (75.0%) and sebaceous hyperplasia (70.0%), while papillomatosis (6.7%) and dermal melanophages (35.0%) were uncommon (Fig. 1B).

**Immunohistochemical evaluation**

Skin lesions and perilesional samples were collected in 21 patients (18 males and 3 females; mean age 19.7±6.6 years, range 12~38 years). Normal skin samples were obtained from 10 healthy subjects (8 males and 2 females; mean age 20.4±4.9 years, range 13~30 years) who underwent aesthetic surgery.

**K15**

Cytoplasmic K15 immunostaining was seen in the basal layer of the epidermis and secretory cells of some eccrine glands, and its epidermal expression was higher in lesonal and perilesional skin than in normal skin (p<0.01; Table 2). In the hair follicles, K15 was expressed mainly in the outermost ORS layer at the isthmus level and sometimes in the bulbar and suprabulbar cells (Fig. 2E, F).

**SMA**

Cytoplasmic staining of SMA was observed in APM, vessel walls, dermal sheath of hair follicles, and myoepithelial cells of eccrine glands (Fig. 3). SMA⁺ APM area was higher in skin lesion than in normal skin (p<0.05; Table 2).

**PGP 9.5**

Intraepidermal nerve fibers were not counted due to non-specific staining of the basal keratinocytes and a small number of nerve fibers in the epidermis. PGP 9.5⁺ nerve fibers were abundantly distributed in the nerve plexus and APM and around eccrine ducts (Fig. 4). DNFL was higher in lesional and perilesional skin compared with normal skin (p<0.01; Table 2).
Fig. 2. Epidermal Ki-67, Melan-A, and keratin 15 (K15) expression in lesional skin (A, C, E) was higher than in perilesional skin (B, D, F). Follicular K15 expression was located in the outermost layer of outer root sheath at the isthmus level (E, F) (immunostaining, ×200).

**DISCUSSION**

BN lesions usually appeared on the upper trunk and upper limbs in this series, especially on scapular region, while the frequency of lower limb involvement was higher than the previous reports (21.7% vs. 3.2% ~ 12.8%)⁴,⁷. This discrepancy may be due to the case recruitment and mis-diagnosis and/or undiagnosis of atypical cases. In addition, the frequency of hypertrichosis ranged from 17% to 70%³, while it was prevalent (68.3%) in this study. Although the diverse age may be a credible explanation for this difference³,⁴, no statistical difference of gender and diagnostic age in this study seems not to support this hypothesis. Furthermore, multiple papules within the lesion might be
Table 2. Immunohistochemical results of Ki-67+, Melan-A+, keratin 15 (K15), smooth muscle actin (SMA) and dermal nerve fiber length (DNFL) in lesional, perilesional and normal skin

| Group          | n  | Ki-67+  | Melan-A+ | K15     | SMA+   | DNFL    |
|----------------|----|---------|----------|---------|--------|---------|
| Lesional skin  | 21 | 3.71±1.36| 1.85±0.90| 0.96±0.05| 1.25±1.35| 0.94±0.48|
| Perilesional skin | 21 | 2.77±0.96**| 1.54±0.81*| 0.88±0.09**| 0.56±0.59| 0.57±0.32**|
| Normal skin    | 10 | 1.15±0.50**,†† | 1.08±0.54*,†| 0.80±0.09**,†† | 0.33±0.42* | 0.28±0.22**,†† |

Values are presented as number only or mean±standard deviation. *p<0.05; **p<0.01, compared with lesional skin; †p<0.05; ††p<0.01, compared with perilesional skin.

Fig. 3. Smooth muscle actin expression in arrector pili muscle, vessel walls, dermal sheath of hair follicles in lesional (A) and perilesional (B) skin (immunostaining, ×100).

Fig. 4. Protein gene product 9.5+ nerve fibers in the arrector pili muscle and neuromplexus in lesional (A) and perilesional (B) skin (immunostaining, ×400).

a clue to the clinical diagnosis of BN based on the present and previous observations. On the other hand, we did not find positive family history, breast hypoplasia and increased prevalence of acne vulgaris, while the concomitance of neurofibromatosis type 1 in 2 cases may be interesting. Although neurofibromatosis may be a component of BN syndrome, the underlying mechanism is poorly understood.

The diagnosis of BN mainly hinges on the clinical features, but histopathology may be sometimes necessary to...
In line with previous report, we also found Ki-67 expression was significantly higher in lesional and perilesional skin compared with normal skin, especially in lesional skin. Immunohistochemical observation disclosed the increased counts of Melan-A+ melanocyte in BN, while Dopa and immunohistochemical stain displays a significant increment. We also found the incremental counts of Melan-A+ melanocyte in the lesional and perilesional skin compared with normal skin. Although the increment of melanocyte density may partly explain the cause of basal hyperpigmentation in BN, its pathogenetic role remains to be further investigated.

H&E stain demonstrates normal or increased number of melanocytes in BN, while Dopa and immunohistochemical stain displays a significant increment. We also found the incremental counts of Melan-A+ melanocyte in the lesional and perilesional skin compared with normal skin. Although the increment of melanocyte density may partly explain the cause of basal hyperpigmentation in BN, its pathogenetic role remains to be further investigated.

Since hypertrichosis and acanthosis are common features of BN, we examined the keratinocytic proliferation using Ki-67 and K15 immunostaining. Because the hair follicles had various growth cycles and profiles in each section, Ki-67+ and K15+ follicular cells were not compared in this study. The number of Ki-67+ and K15+ epidermal keratinocytes was significantly higher in lesional and perilesional skin compared with normal skin. Although the increment of melanocyte density may partly explain the cause of basal hyperpigmentation in BN, its pathogenetic role remains to be further investigated.

In conformity with the histopathological results, our immunohistochemical observation disclosed the increased number of SMA+ APM in skin lesion compared with normal skin. APM can protect the bulge stem cells and maintain the follicular integrity, while the bulge stem cells in turn may induce APM differentiation and anchorage to the bulge via producing a specialized basement membrane. The APM-bulge connection plays a crucial role in the follicular morphogenesis and renewal. Therefore, APM hyperplasia in BN might originate from bulge stem cells and then promote follicular renewal, which may partly explain the cause of the hairy presentation in BN. In addition, immunostaining pattern of SMA in the dermal sheath of hair follicles is coincident with the previous reports. SMA expression in the dermal sheath cells may play a role in the shortening and curly morphology of hair follicles, and suggest an epithelial-mesenchymal interaction for hair follicle development.

H&E stain demonstrates normal or increased number of melanocytes in BN, while Dopa and immunohistochemical stain displays a significant increment. We also found the incremental counts of Melan-A+ melanocyte in the lesional and perilesional skin compared with normal skin. Although the increment of melanocyte density may partly explain the cause of basal hyperpigmentation in BN, its pathogenetic role remains to be further investigated.

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In summary, this study demonstrates the hyperproliferation of keratinocytes, melanocytes, APM and dermal nerve fibers could be involved in the pathogenesis of BN. More research is needed to determine the relationship between their proliferation and sex hormone expression.

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