Original Article
A clinicopathological study of TRAIL expression in halo nevi

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Abstract: Pathogenesis of the halo phenomenon in halo nevus (HN) is not completely understood. Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is implicated in the causation of immune and inflammatory cutaneous disorders such as vitiligo. To evaluate the clinicopathological characteristics and the expression of TRAIL in HN, we analyzed clinicopathological data of 32 patients with HN and performed immunohistochemistry for TRAIL and CD8 on HN (n = 32) and melanocytic nevi of healthy controls (n = 10). Correlation of TRAIL expression with clinicopathological features was assessed. We also performed double immunofluorescence staining for TRAIL and CD8 to confirm their co-localization. A variable degree of inflammatory cell-infiltrate around the melanocytic nevus was observed in all cases. Immunohistochemical staining showed a significantly higher expression of TRAIL in HN lesions as compared to that in skin of healthy controls. The shorter the evolution time of HN, the stronger was the TRAIL expression. TRAIL had a positive association with the CD8^{+} T cells infiltrate. Co-localization of TRAIL in the CD8^{+} cells in HN was observed on double immunofluorescence. According to its abundant expression in HN lesions and close association with the disease course and predominant CD8^{+} inflammatory cells, it seems that TRAIL involves in the pathogenesis of HN.

Keywords: Halo nevus, leukoderma acquisitum centrifugum, Sutton's nevus, vitiligo, TNF-related apoptosis-inducing ligand, tumor necrosis factor, T cells, histopathology, inflammation

Introduction

Halo nevus (HN) (also known as Sutton's nevus or acquired centrifugal leukoderma) clinically presents as a leucodermic halo around a melanocytic nevus that usually undergoes involution associated with infiltration of inflammatory cells [1, 2]. The halo tends to persist for years before its gradual disappearance [1, 3]. Most commonly, the central lesion is an acquired nevi; however, this phenomenon may occur with congenital nevi, cellular nevi, melanoma, blue nevi, Spitz nevi and dysplastic melanocytic nevi [4]. The prevalence of HN in the general population is approximately 1%. The condition is more commonly seen in children and young adults, but shows no association with gender [5, 6].

Halo nevus may be the first manifestation of vitiligo, which is also the common associated entity [7, 8]. Several studies have highlighted similarities in the cellular mechanisms responsible for vitiligo and HN. Both conditions are characterized by an acquired loss of functional melanocytes, which manifests as depigmentation macules and predominance of CD8^{+} T-lymphocytes (T-cell) in the infiltrate [7, 9-12].

The pathogenesis of HN is not fully understood. It has been considered as a postinflammatory or immunological event [13]. CD8^{+} T-cells are thought to play a role in the immune-mediated degeneration of the central nevus and in the development of the lesional halo [12, 14]. One study detected apoptosis on nevi cells [15]. We speculate that apoptosis, rather than necrosis, may be involved in the regression of central nevus.

Apoptosis, is a highly regulated process which may be triggered by stress, ischemia, hypoxia and immune factors. Dysregulated apoptosis may be associated with inflammation and skin cancer [16]. Various inflammation-associated pro-apoptotic factors such as perforin, gran-

zymes, Fas ligand (FasL) and tumor necrosis factor (TNF) were reported to be involved in the development of HN [15, 17, 18]. However, the role of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) in the pathogenesis of HN has not been investigated.

TRAIL, another member of the TNF-superfamily, is a type-II membrane protein that exerts its functions by binding to certain cell surface TRAIL receptors. TRAIL is expressed by several cell types such as activated T lymphocytes, natural killer cells and activated macrophages. Its primary function is to induce apoptosis via activation of the cell death receptors and the mitochondria-mediated apoptotic pathway [19]. Although TRAIL was originally identified as a potent apoptosis-inducing protein, more recent evidence appears to implicate TRAIL in inflammation, immunomodulation and immune homeostasis [19, 20].

High expression of TRAIL was demonstrated in perilesional skin in vitiligo, a condition which shares many clinical, histological and immunological principles with HN [21]. *Type I interferon*, a strong inducer of TRAIL expression [19], which is also known to augment Th1-biased immune responses via recruitment of cytotoxic lymphocytes, has been demonstrated in HN lesions [17].

In this study, we sought to investigate the potential involvement of TRAIL in the pathogenesis of HN. The association between TRAIL and the clinicopathological characteristics is examined.

Materials and methods

Subjects and materials

The study was conducted at the Department of Dermatology, the First Affiliated Hospital of Sun Yat-sen University, from 2009 to 2015, with the approval of the hospital ethics committee (2013-188).

A total of 32 patients with HN and 10 control subjects were enrolled. Diagnosis of halo nevus was based on the history, the typical clinical features and confirmed on histopathology. The central nevi of HN patients and healthy controls were <1 cm in diameter. Biopsy specimens were obtained from the central area of the halo nevus.

Exclusion criteria included history of any systemic or local therapy in the 4-week period immediately preceding the start of the study. Control nevi were obtained from 10 age- and sex-matched healthy subjects who underwent surgical excision of HN on head, neck or trunk for cosmetic purposes.

Clinical characteristics

Data pertaining to age, gender, location and duration of the lesion, the number of lesions, clearance time of the central melanocytic nevus, personal and family history of autoimmune diseases (including vitiligo, thyroid diseases, connective tissue diseases, alopecia areata and psoriasis), allergic disorders (allergic rhinitis, atopic dermatitis, chronic urticaria and asthma) were compared between HN and control groups.

Histopathological and immunohistochemical analysis

4-μm thick tissue sections were prepared from formalin-fixed, paraffin-embedded excisional biopsy specimens and examined using hematoxylin-eosin (H/E) and immunohistochemical staining.

Immunohistochemistry procedure: Deparaffinization was followed by optimized antigen retrieval techniques (incubated for 30 minutes at 95°C in 0.01 mol/L sodium citrate buffer [pH 6.0]), and serial sections were incubated with monoclonal antibodies specific for TRAIL (clone C92B9; Rabbit mAb, Cell signaling technology 1:100, USA) for 1 h at room temperature, followed by the secondary labeling using the Dako EnVision TM Detection kit (Dako K5007) with 3',9'-diaminobenzidine (DAB) as chromogen. Sections were then counterstained with Mayer hematoxylin. The stained slides were independently examined by two researchers. Cells were counted in five high-power fields (HPF) per slide from target in proximity to central nevus, and the mean number was calculated. Mean counts from both observers were used for quantitative analysis.

The presence of cytoplasmic brown stain confirmed positivity for TRAIL.

Double immunofluorescence staining

Co-localization of TRAIL with CD8^{+} immune cells was assessed by double immunofluores-

Figure 1. Halo nevus (HN). Melanocytic nevus surrounded by a depigmented halo located in the left infraorbitary region.

cence. Sections were subjected to deparaffinization, rehydratation and subsequent antigen retrieval as described above. After incubating in 1% bovine serum albumin (BSA), sections were re-incubated overnight at 4°C with monoclonal antibodies specific for -TRAIL (clone C92B9; Cell signaling technology, USA), which was diluted (1:20) with 1% BSA. The sections were washed three times with PBS, blocked with 1% BSA and incubated with goat anti-rabbit Alexa Fluor® 594-Conjugated antibodies (Chinese in Jinqiao ZF-0516) diluted (1:200) in 1% BSA. The sections were then washed three times with PBS, and further incubated for 1 h with a monoclonal mouse anti-CD8 (Clone C8/144B, Dako, Denmark) diluted (1:20) in 1% BSA.

After washing three times with PBS, the samples were incubated with goat anti-mouse Fluor® 488-Conjugated antibodies (Chinese in Jinqiao ZF-0512) diluted (1:200) in 1% BSA. Finally, the samples were labeled with the nucleic acid stain, ProLong Anti-Fade DAPI (Invitrogen, USA), and observed under a ZEISS Axio Imager Z1 immunofluorescence microscope (Zeiss, Germany). Images were captured using a Carl Zeiss AxioCam.

Statistical analysis

Data analyses were performed using the statistical package SPSS 16.00 (SPSS, Chicago, IL, U.S.A.). Data are expressed as mean ± standard deviation (SD). Data pertaining to quantitative variables were analyzed using the independent samples t test; Mann-Whitney test was used for non-normally distributed variables. Between-group differences with respect to categorical variables were assessed using the Pearson's χ^2 test. $P<0.05$ was considered statistically significant.

Results

Clinical data of HN patients

The male/female ratio was 1.13:1. Median age of patients was 18.5 years (mean ± SD: 23.25 ± 13.00; range: 5-57). Median age at onset was 17.5 years (mean ± SD: 22.06 ± 13.35; range: 4-57). Median duration of halo was 12 months (mean ± SD: 15.19 ± 14.36; range: 1-60). Lesions were most commonly located on head and neck (62.5%) (**Figure 1**), followed by trunk (34.4%) and limbs (4.2%). No lesions were found on hands, feet or genitals. The average number of nevi per patient was 1.4. A single lesion was found in 25 patients (78.1%); multiple lesions (≥2) were present in seven patients (21.9%). The maximum number of HN in one patient was five. With respect to autoimmune diseases, 13 cases (40.6%) had vitiligo, of which 2 cases were complicated by thyroid diseases, and one case was complicated by alopecia areata. Eleven cases (34.4%) had an allergic disorder. There were no patients with just thyroid diseases or alopecia areata.

Family history of autoimmune diseases (6 cases vitiligo, 2 cases thyroid diseases, 1 case alopecia areata and 2 cases connective tissue diseases) and allergic disorders was associated in 9 and 8 cases respectively. One patient had more than one autoimmune disease. Four cases had family history of both autoimmune and allergic disorder.

Four patients were lost to follow up. Twenty eight patients were followed-up 6 months to 89 months [Median duration of follow-up was 35.5 months (range, 6-89)]. Persistence of halo was observed in 19 cases (67.9%).

Clinical data of control group

Out of 10 controls, four were men and six were women. Median age was 20.5 years (mean ± SD: 21.7 ± 9.71; range, 8-45). Seven subjects had nevi in the head and neck area, two on the trunk and one on the limb. No significant difference was found in the distribution of the cen-
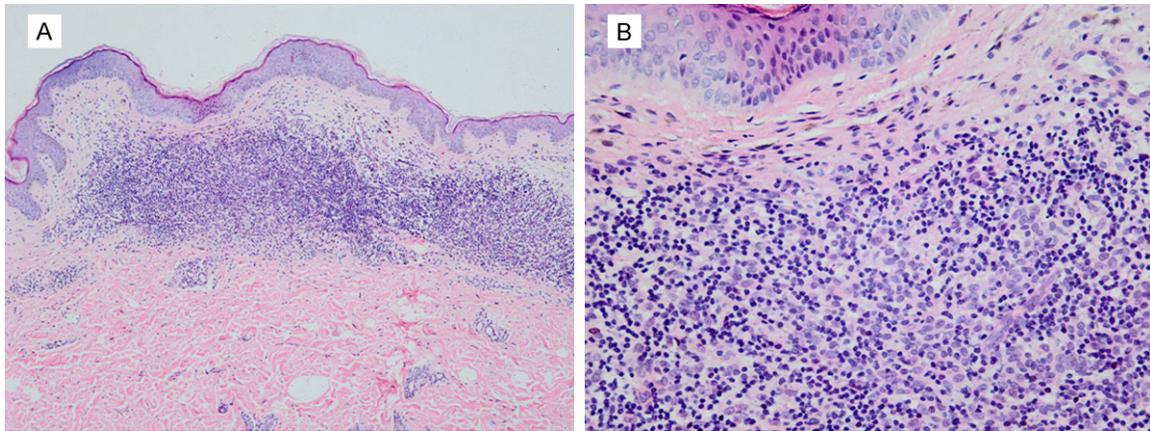
Figure 2. HE-stained section of halo nevus (HN) (A) and an intradermal nevus (B) showing extensive inflammatory-cell infiltrate [magnification: (A) ×10; (B) ×40]. HE, hematoxylin and eosin.
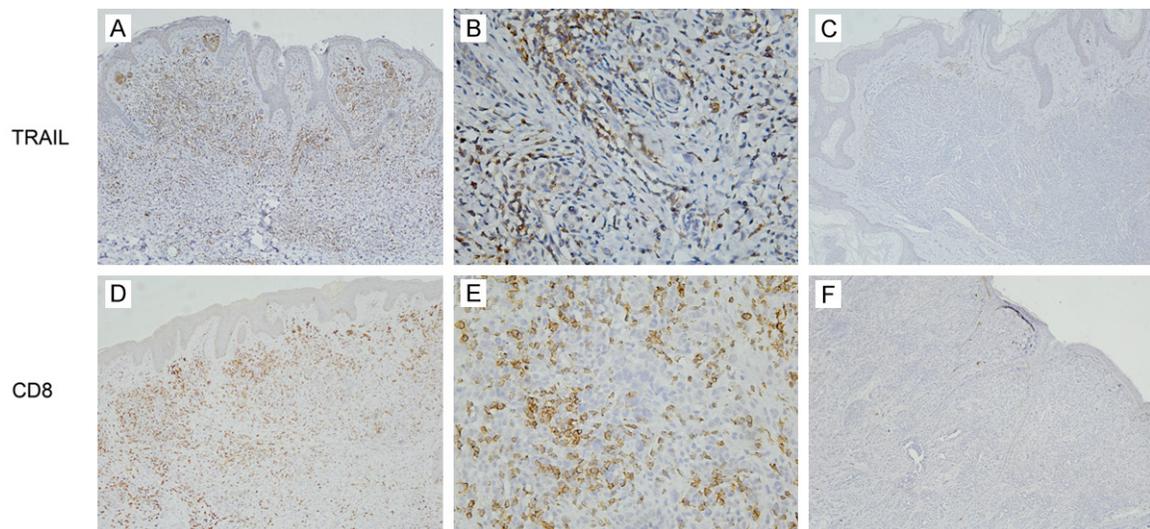

Figure 3. Immunohistochemical examination showing increased TRAIL-positive and CD8+ T cells in halo nevus (HN) lesions as compared to that in skin of controls. Increased TRAIL expression was found among the infiltrated inflammatory cells adjacent to nevus nests (A: ×10; B: ×40); Most of the inflammatory cells were CD8+ cells; (D: ×10; E: ×40); TRAIL-positive and CD8+ cells were occasionally detected in normal controls (C: ×10; F: ×10, respectively). TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

tral melanocytic nevus between HN patients and controls (P>0.05).

Histopathological analysis

The central lesion was found to be intradermal nevi (27 cases, 84.4%), compound nevi (4 cases, 12.5%) and junctional nevus (1 case, 3.1%). No significant difference was found in histological type of central nevi between HN patients and control group (P>0.05). In all HN cases, a variable degree of mononuclear cells were present in the vicinity of the melanocytic nevus cells, which mainly comprised of lympho-

histocytes (**Figure 2A**, **2B**) and sometimes mixed with melanophages. Nevus cells did not show cellular atypia. In the depigmented region, the number of epidermal melanocytes and melanin was significantly decreased. No apparent inflammatory infiltration was observed in control nevus.

Immunohistochemical staining

Immunohistochemical staining of TRAIL and CD8+ T cells in HN lesion: TRAIL-positive and CD8+ T cells predominantly found among the infiltrated inflammatory cells adjacent to nevus

Table 1. Comparison between HN and control group regarding TRAIL and CD8 expression (positive cells per HPF ×40)

Group	TRAIL [min, max (median)/HPF]	CD8 [min, max (median)/HPF]
Control (n = 10)	0.40, 5.80 (2.20)	5.75, 22.75 (11.63)
HN (n = 32)	4.8, 375 (43.8)	19.8, 535 (131.30)
HNO (n = 19)	11.8, 375.0 (75.0)	19.8, 535.00 (166.10)
HNV (n = 13)	4.8, 176.0 (21.4)	25.6, 400.38 (82.20)

HN, halo nevi; HPF, high-power fields; HNV, halo nevus associated with vitiligo; HNO, halo nevus only; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

nests or around the blood vessels in the upper dermis (**Figure 3A**, **3B**, **3D**, **3E**). Two independent observers counted and averaged the number of TRAIL-positive cells on microscopy. The total number of TRAIL cells in halo nevi ranged from 4.8 to 375.0/high-power field (HPF), with a median value of 43.8/HPF (**Table 1**).

Immunohistochemical staining of TRAIL and CD8+ in control nevus

TRAIL-positive and CD8+ T cells were occasionally detected around the blood vessel in the upper dermis (**Figure 3C**, **3F**).

Clinical and histopathological relationship with TRAIL and CD8+

On immunohistochemical analysis, expression of TRAIL in HN patients was significantly higher than that in control nevi (Z = -4.007, *P<0.001*). With respect to the correlation between the number of TRAIL-expressing cells and halo duration, TRAIL expression increased early with the regression of the HN (r = -0.389, *P = 0.028*). Similarly, a negative correlation was observed between the number of CD8+ T cells and HN duration (r = -0.352, *P = 0.048*). A positive correlation was observed between the number of TRAIL-expressing cells and CD8+ T cell infiltration in lesions of HN (r = 0.907, *P<0.001*).

In HN patients, no correlation of TRAIL expression with sex, age at onset, or with number of lesions was observed (*P>0.05*). No significant difference in the number of TRAIL-positive cells was observed between patients with or without personal or family history of autoimmune or atopic disorders (*P>0.05*). There was no statistically significant difference in the expression of TRAIL and CD8+ T cells between patients

with HN-only and HN with vitiligo (Z = -1.707, *P = 0.088*; Z = -1.702, *P = 0.089*, respectively).

Co-localization of TRAIL and CD8+ cells in HN lesions

TRAIL was found to be expressed by the CD8+ cells of the inflammatory infiltrate in HN lesions, but not limited to this phenotype cells. Representative staining photomicrographs for co-localization of TRAIL and CD8+ cells are shown in **Figure 4**.

Discussion

The gender and age distribution of HN in our study is comparable to that reported elsewhere [1, 7]. The most commonly affected sites were the head and neck (62.5%), followed by trunk (34.4%), which is comparable to that reported from South Korea [9], but not with that reported from Europe and America [1, 7]. This discrepancy may be attributable to the racial differences in skin color. Lesions are more readily noticeable in a backdrop of dark colored skin, and thus are more likely to lead to medical consultation.

Classical clinical progression of HN could be correlated between the appearance of the halo and the onset of nevus regression followed by complete disappearance of the central nevus, which leaves behind a depigmented macule and ultimately, the halo repigments [1, 3]. However, few studies have explored the time interval between disappearance of the central nevus and re-pigmentation of the halo. In our study, persistence of halo was observed in 19 cases (67.9%) over a median duration of 35.5 months, which indicates that re-pigmentation may be delayed even after the disappearance of the central nevus. This finding is consistent with the results of a recently published study [9] and supports the notion of HN as being a chronic entity.

Approximately half of all HN patients had a personal or family history of autoimmune and/or allergic disorders, both of which are characterized by chronic inflammation. Only one study has so far explored the association of HN with autoimmune diseases, in which 9.3% and 44.9% of HN patients had a personal history and family history of autoimmune diseases,
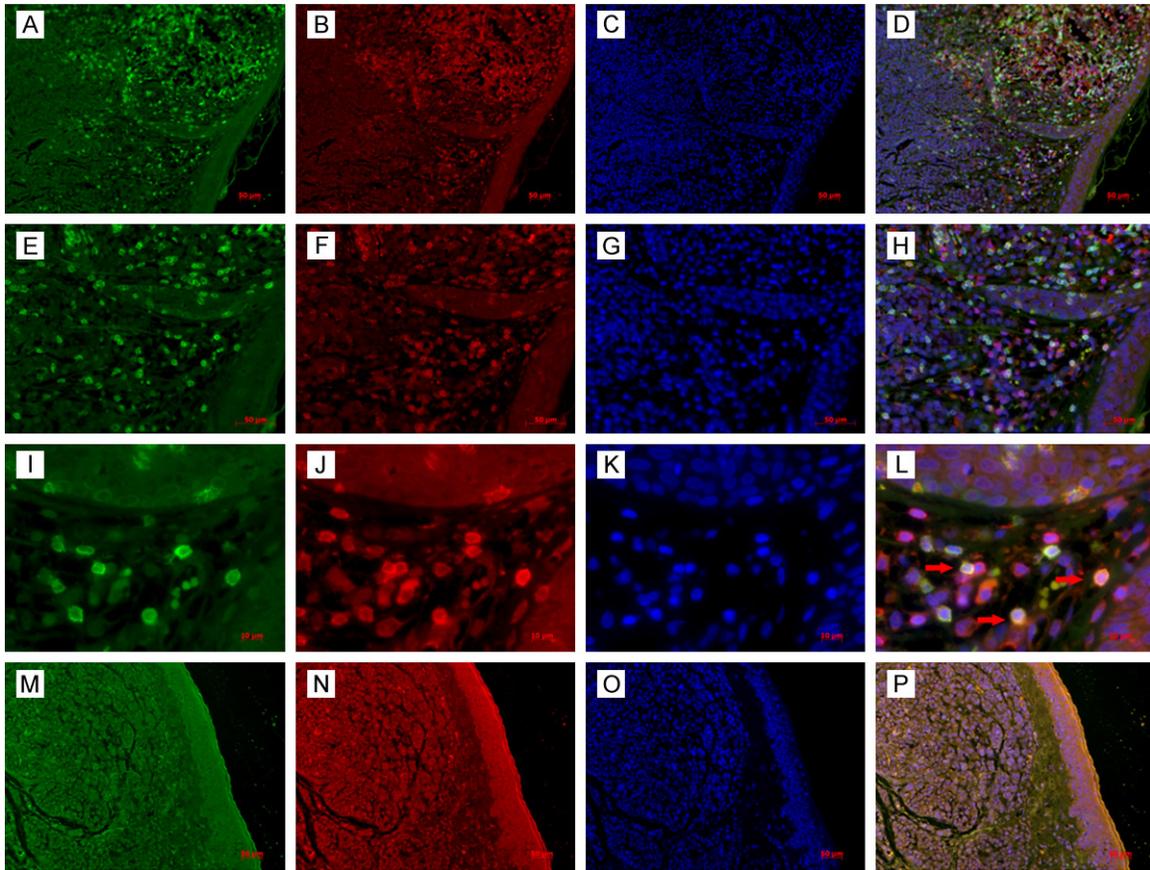
Figure 4. Representative double immunofluorescence images showing TRAIL expressing cells in red (Alexa Fluor® 594), CD8 positive cells in green (Fluor® 488); DNA was stained with Hoechst (blue). Double positive cells are shown in orange (fourth panel of each row) and indicated by arrow (A-D: Original magnification ×20; E-H: ×40; I-L: ×100). TRAIL-positive and CD8+ cells were rare in normal controls (M-P: ×20). TRAIL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand.

respectively [7]. It is conceivable that the proinflammatory state of the underlying autoimmune/allergic disorders may predispose individuals to destruction of melanocytes and nevus cells, possibly mediated via upregulation of local and/or systemic proinflammatory cytokines including TNF-α, which has been shown to inhibit melanocyte activity [22]. Indeed, this association may play an important role in onset and prolongation of the disease course.

Similar to the finding of Mooney *et al*. [4], intradermal or compound nevus were the predominant histological subtypes of central nevi; occurrence of junctional nevus was rare. Histological examination showed a variable degree of inflammatory cells infiltration around the melanocytic nevus, which indicates that the co-occurrence of halo formation and nevus disappearance is mediated through an inflammatory

response. We speculate that some molecules related to inflammatory infiltration may be involved in the pathogenesis of HN. These molecules may induce both apoptotic and proinflammatory signaling pathways.

TRAIL is a pleiotropic cytokine and the latest discovered apoptosis-inducing ligand of the three major death receptor pathways of apoptosis. This cytokine and its associated signal pathways were initially reported to lead to apoptosis and thus have been considered as a potential therapeutic agent in cancer therapy [23, 24]. TRAIL can be recognized by four membrane-bound receptors (TRAIL R1-R4) and it can also bind to the soluble receptor osteoprotegerin. Two of the membrane-bound receptors (TRAIL R1 and TRAIL R2) mediate the pro-apoptotic effects, while the other two (TRAIL R3 and TRAIL R4) suppress the TRAIL-induced apopto-

sis [19]. Therefore it may behave as a pro- or anti-apoptotic molecule depending on the tissue. Some studies suggest that it is related to a more aggressive phenotype such as in melanoma [25]; while others have reported an association between loss of TRAIL expression and poorly differentiated carcinoma, for example, in cervical squamous cell carcinoma [26]. Recently evidence suggests that TRAIL regulates inflammatory and immune diseases by exerting pro- or anti-inflammatory effects. Its' involvement in the pathogenesis of autoimmune/inflammatory diseases such as vitiligo [21], psoriasis [27, 28], atopic dermatitis [29], rheumatoid arthritis [30], scleroderma [31] , dermatitis herpetiformis [32], cutaneous lupus erythematosus (CLE) [33] and chronic infectious diseases (including leishmaniasis [34] and molluscum contagiosum [35]) has been reported. To the best of our knowledge, the association between TRAIL and HN has not been reported. Since melanocytes express TRAIL R1 [21], it is more likely that TRAIL would be involved in the pathogenesis of HN.

Our results demonstrate that TRAIL is strongly expressed by mononuclear immune cells, which infiltrate the HN and its positive correlation with the CD8^{+} T cells, supporting its participation in the involution of central nevus. Furthermore, positive expression of TRAIL in the CD8^{+} cells indicates that it may have a cytotoxic effect similar to that of perforin, granzymes and FasL released by CD8^{+} cells which have a pro-apoptotic and pro-inflammatory effect. The present study provides first evidence that TRAIL might also be involved in the pathogenesis of HN. However, the exact impact of TRAIL-positive cells in various chronic inflammatory dermatoses is still debated. A study related to vitiligo suggested that TRAIL is a major pro-apoptotic cytokine in dendritic cell-mediated cytotoxicity towards stressed melanocytes [21]; TRAIL derived from dendritic cells and keratinocytes was shown to participate in the pathogenesis of CLE by triggering abnormal apoptosis of keratinocytes, which led to an excessive production of autoantigens or by promoting inflammation via induction of interleukin (IL)-8 and intercellular adhesion molecule-1 (ICAM-1) [33, 36]. A series of studies on psoriasis have shown a proinflammatory effect of TRAIL mediated via induction of CCL20, ICAM-1 and other inflammatory cytokines, proliferation

of blood vessels, persistent inflammatory infiltration and epidermis proliferation [27, 28]. Increased TRAIL expression by T cells was shown to be associated with an increased expression of IL-1 receptor antagonist (IL-1Ra) by keratinocytes in atopic dermatitis. Furthermore, TRAIL was shown to induce the expression of IL-1Ra in keratinocytes *in vitro* [29]. Therefore, it is thought to exert its anti-inflammatory effect by inducing apoptosis of inflammatory cells and by blocking multiple inflammatory pathway through the induction of IL-1Ra [29, 37].

The high expression of TRAIL in HN, especially in the earlier stages, may have several functions such as: direct triggering of apoptosis of central nevus cells or indirectly by working synergistically with other melanocyte-cytotoxic cytokines such as IFN-γ or IFN-α. Since HN shares histological and immunological features with vitiligo, which is associated with high levels of proinflammatory cytokines including IL-6 and TNF-α [22], we speculate that TRAIL may be one of major proinflammatory cytokines released by the inflammatory cells. It cannot be totally ruled out that over-expression of TRAIL in HN may represent a physiological compensatory mechanism of the body, a negative feedback mechanism to induce apoptosis of overactive inflammatory cells and thus limit the development of autoimmune and inflammatory disease [20, 38, 39]. Also, we cannot rule out the possibility that TRAIL target cells can be implicated in HN more than melanocytes or inflammatory cells. For instance, TRAIL may induce indirect adverse effects on melanocytes in HN through inhibition of adjacent keratinocytes including apoptosis induction under inflammatory conditions leading to hyposecretion of major growth factors for melanocytes including stem cell factor, basic fibroblast growth factor, which result in functional inhibition or damage of melanocytes. The finding that TRAIL can induce apoptosis of keratinocyte (HaCaT) *in vitro* seems to support this possibility [40, 41].

Since the expression of TRAIL was not limited to CD8^{+} T cells, other inflammatory cells may also be involved in this condition.

In summary, our study indicates that TRAIL is strongly expressed in HN, especially in the earlier stages. TRAIL expression on CD8^{+} T lympho-

cytes as well as the other cells that predominate in the infiltrate around HN suggests a positive association between TRAIL and pathology of HN. Further research about TRAIL and its actual functional pathway is warranted to delineate its pathogenic role in the pathogenesis of HN. Understanding the *in vivo* mechanism of TRAIL in HN pathogenesis may lead to novel strategies to manage HN.

Disclosure of conflict of interest

None.

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