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Original Article

Expression of immune-related cytokine IL-1β in non-neoplastic epithelial disorders of the vulvar tissue and its clinical significance

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Abstract: Objective: To investigate the expression of immune-related cytokines interleukin (IL)-1β, IL-2 and IL-10 in non-neoplastic epithelial disorders of the vulva (NNEDV) tissues, and to analyze their relationship with the tumor necrosis factor receptor associated factor 5 (TRAF5)-mediated nuclear factor kappa B (NF-κB) pathway, and their clinical significance. Methods: This study was designed to detect the expression of immune-related cytokines IL-1β, IL-2 and IL-10, p65, c-Rel and TRAF5 protein in 11 cases of normal vulvar skin and 73 cases of NNEDV tissues by immunohistochemical SP method, and to analyze their relationship with clinicopathological features of patients with NNEDV and whether the patients suffer from other autoimmune diseases at the same time. Conclusions: NNEDV may be an autoimmune disease, which is associated with a significant decrease in the expression of immune-related cytokine IL-1β caused by abnormalities in NF-κB pathway activity. TRAF5, p65, c-Rel and IL-1β may serve as diagnostic markers and immunotherapy targets in NNEDV.

Keywords: NNEDV, IL-1β, p65, c-Rel, TRAF5, NF-κB pathway

Introduction

NNEDV is a common chronic disease manifested as pigmentation and degeneration of the female vulvar skin and mucosa, with vulvar itching and depigmentation as the main feature. The condition is often accompanied by genital atrophy, vaginal stenosis and pain during sexual intercourse at advanced stages, seriously affecting the health and quality of life of patients. Currently, there is no cure for NNEDV. The etiology and pathogenesis of NNEDV are not very clear, and may be related to genetic factors, infection, changes in sex hormones, and autoimmune disorders, among which autoimmune factors have attracted most attention. IL-1β is a lymphocyte-stimulating factor, IL-2 is a T cell growth factor, and IL-10 is an immunosuppressive factor, all of which play important roles in the regulation of *in vivo* immune responses. It has been found that IL-1β, IL-2 and IL-10 all play important roles in various autoimmune diseases [1-10]. P65 and c-Rel are two subunits of the NF-κB transcription factor protein family. After activation, they activate the transcription of multiple genes, regulate the expression of various cytokines and immune receptors, and participate in the inflammatory and immune responses [11-14].

Research has shown that IL-1β, IL-2 and IL-10 are all involved in regulation of the NF-κB signal transduction pathway during the immune response. Activation of the NF-κB pathway increases IL-1β and IL-2 production and release, and IL-1β and IL-2 can act as stimulants for further activation of the NF-κB pathway [15-20]. In contrast, IL-10 inhibits the activation of the NF-κB pathway [21-23]. TRAF5, a member of a family of tumor necrosis factor receptor associated factors, acts as the upstream mediator of the signal transduction pathway of NF-κB; it alters its activation and regulates the immune system [24-26].

Therefore, this study was designed to investigate the expression of immune-related cytokines IL-1β, IL-2 and IL-10, p65, c-Rel and TRAF5

protein in NNEDV tissues by immunohistochemical method, to investigate whether the occurrence of NNEDV is associated with changes in the expression of immune-related cytokines IL-1β, IL-2 and IL-10, and analyze the relationship of these changes with the TRAF5-mediated NF-κB pathway, thus providing a theoretical basis for immune targeted therapy of NNEDV.

Materials and methods

Specimen collection

This study recruited 73 patients with NNEDV, and 11 controls with a normal vulva. All specimens were confirmed by histopathological examination of paraffin-embedded tissue samples collected during gynecological surgery and outpatient biopsy in Shengjing Hospital of China Medical University from 2010 to 2014. None of the patients received any physical, chemical or drug treatment before specimen collection.

Reagents

Rabbit anti-human IL-1β polyclonal antibody, rabbit anti-human IL-10 polyclonal antibody, rabbit anti-human p65 (RelA) polyclonal antibody, and rabbit anti-human TRAF5 polyclonal antibody were purchased from Proteintech Group, Inc. (Rosemount, USA). Rabbit anti-human IL-2 polyclonal antibody and rabbit anti-human c-Rel polyclonal antibody were purchased from Wuhan Boster Bio-engineering Co. Ltd (Hubei, China). The Rabbit Biotin-Streptavidin (SP) link Detection Kits and DAB (3,3'-diaminobenzidine tetrahydrochloride) Detection Kit was purchased from Beijing Zhongshan Golden Bridge Biotechnology Co. Ltd (Beijing, China).

Immunohistochemical SP method

The paraffin-embedded tissue specimens were prepared to give 4 μm slices, followed by xylene dewaxing, gradient ethanol hydration, and thermal antigen retrieval with citric acid buffer. The sections were stained by the immunohistochemical SP method: addition of 3% hydrogen peroxide deionized water, incubation at 37°C for 30 min; addition of normal goat serum for blocking, following by 37°C incubation for another 30 min. Subsequently, 1:400 diluted IL-1β primary antibody, 1:200 diluted IL-2 primary antibody, 1:50 diluted IL-10 primary antibody, 1:600 diluted p65 primary antibody, 1:600 diluted c-Rel primary antibody and 1:300 diluted TRAF5 primary antibody were added, respectively, followed by incubation at 4°C overnight. Biotinylated secondary antibody working fluid was then added, and the samples were incubated at 37°C for 30 min. Following this, horseradish peroxidase labeled streptavidin working fluid was added before incubation at 37°C for 30 min. DAB working fluid was added for color development under the microscope, followed by thorough rinsing in running tap water. Counterstaining by hematoxylin was then conducted for 2 min, following by a 10 min rinse in running tap water. After dehydration, the sections were sealed and preserved.

Results determination

Positive staining of IL-1β, IL-2, p65 and TRAF5 protein was present in the cytoplasm. Positive staining of IL-10 and c-Rel protein was found in the cytoplasm and/or nucleus. Positively stained cells showed brown or tan spots, which were mostly distributed in the epidermal layer. The tissue slices were placed under the Eclipse E800 fluorescence microscope (Nikon, Japan) and observed using a blinded method. All of the immunostained sections were first examined under low power field (;×100) to identify regions containing vulvar skin cells, with five high power fields (;×400) selected randomly. According to the intensity of staining of positive cells and the ratio of positive cells, a 13-point scoring method was utilized to perform a semi-quantitative analysis of the immunohistochemical results. The 13-point scoring method used score 0-4 to determine the percentage of positive cells among the total cell number: 0 points suggests no positive cells; 1 point indicates that the percentage of positive cells was less than 10%; 2 indicates that the percentage of positive cells was greater than 10% but less than 50%; 3 indicates that the percentage of positive cells was greater than 50% but less than 80%; 4 indicates that the percentage of positive cells was greater than 80% [27]. The intensity of staining was determined as negative (-), weak (+), medium (++), or strong (+++), which were scored as 0, 1, 2, 3, respectively. The final score was the product of these two indicators.

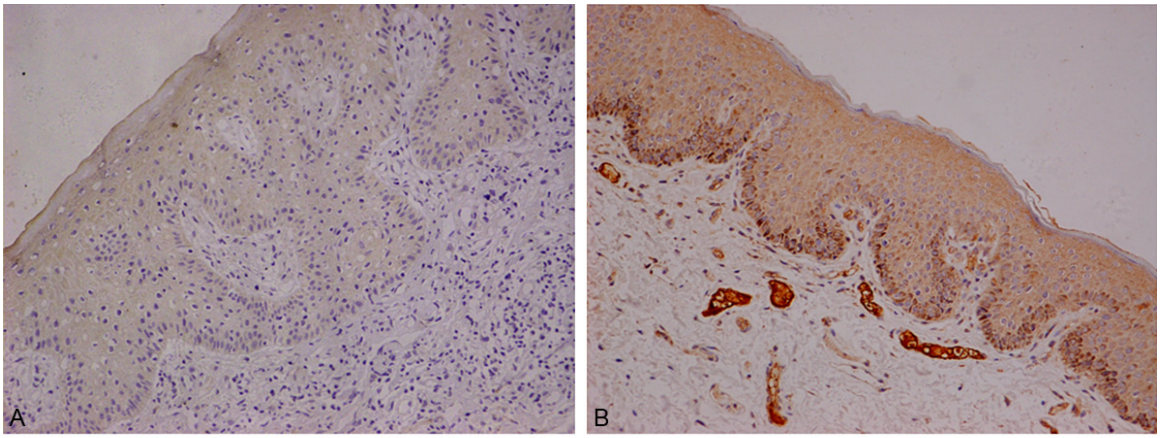

Figure 1. The expression of IL-1β in NNEDV and normal vulvar skin tissues. Observed under ×200 fields, positive IL-1β staining was located in the cytoplasm, with the positive staining cells mainly distributed in the epidermal basal layer, prickle cell layer and granular cell layer. A. The level of IL-1β expression in NNEDV tissues was significantly lower than that in normal vulvar skin tissues. B. The level of IL-1β expression in normal vulvar skin tissues was relatively high.

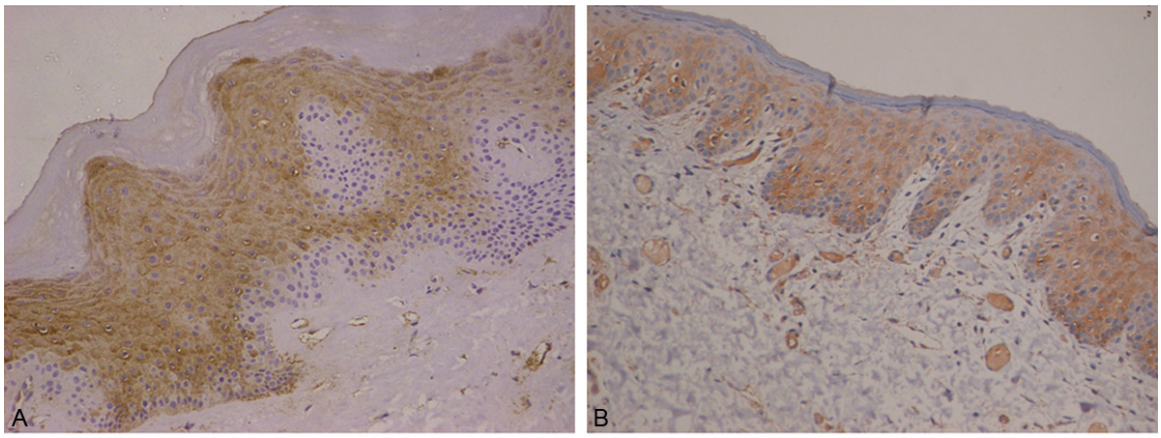

Figure 2. The expression of IL-2 in NNEDV and normal vulvar skin tissues. Observed under ×200 fields, positive IL-2 staining was located in the cytoplasm, with the positive staining cells mainly distributed in the epidermal prickle cell layer, partly distributed in the basal layer and granular cell layer. A. The level of IL-2 expression in NNEDV tissues was slightly higher than that in normal vulvar skin tissues. B. The level of IL-2 expression in normal vulvar skin tissues was moderate.

Statistical analysis

The data were analyzed using statistical software SPSS Statistics 17.0. The experimental data were expressed as the mean ± standard deviation ($\bar{x} \pm s$). The immunohistochemical scores of each index were analyzed using independent sample t test to determine significant differences between NNEDV group and normal vulvar skin group, and $P<0.05$ was considered statistically significant. GraphPad Prism® version 5.01 software was used to build statistical histograms. Respectively with the median values (M) of immunohistochemical scores of TRAF5, p65, cytoplasmic and nuclear c-Rel as well as IL-1β expression in NNEDV tissues as the boundary, 73 samples were divided into high expression group and low expression group. The median values were TRAF5 (M=8), p65 (M=4), cytoplasmic c-Rel (M=8), nuclear c-Rel (M=9) and IL-1β (M=3) respectively. The relationships among the relative expression of

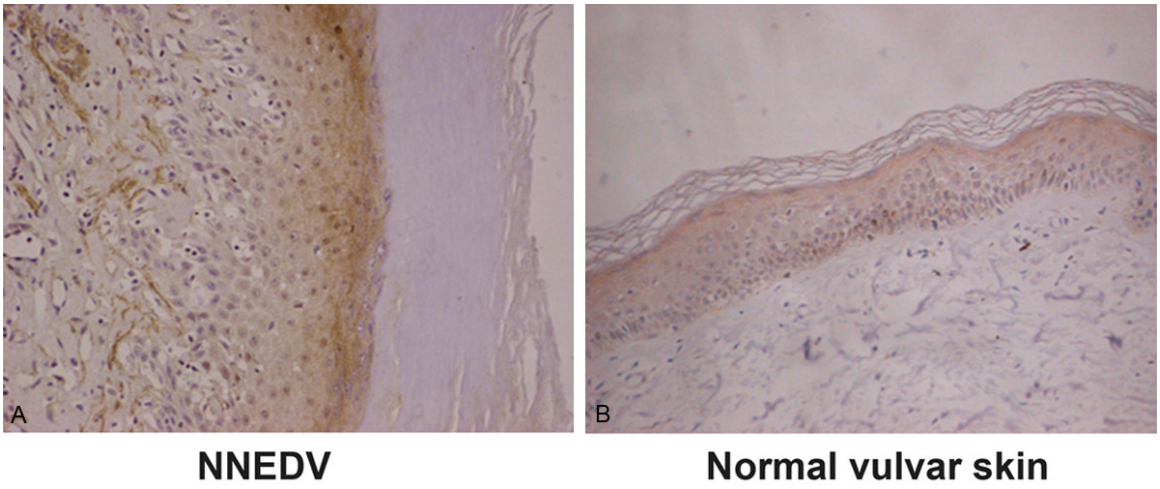

Figure 3. The expression of IL-10 in NNEDV and normal vulvar skin tissues. Observed under ×200 fields, positive IL-10 staining was located in the cytoplasm and/or nucleus, with the cytoplasm-staining positive cells mainly distributed in the epidermal basal layer, prickle cell layer and granular cell layer. Among these cells, the intensity of staining in some cytoplasm-staining positive cells distributed in the granule cell layer was strong, while cells with positive staining of the nucleus were mainly distributed in the prickle cell layer. A. The levels of IL-10 expression in the cytoplasm and nucleus of NNEDV tissues were both slightly lower than those in normal vulvar skin tissues. B. The level of IL-10 expression in the cytoplasm of normal vulvar skin tissues was moderate, whereas that in the cell nucleus was relatively low.

the indicators were analyzed using Pearson linear correlation analysis. $|R|<0.4$ indicates weak correlation; $0.4\leq|R|<0.7$ indicates moderate correlation; $0.7\leq|R|<1$ indicates strong correlation. $P<0.05$ was considered statistically significant. The chi-square test or a corrected χ^2 test was used to assess the association of the level of expression of each index with clinicopathological features of patients suffering from NNEDV and the presence of concomitant other autoimmune diseases. $P<0.05$ was considered statistically significant.

Results

Expression of IL-1β, IL-2 and IL-10, as well as p65, c-Rel and TRAF5 protein in NNEDV and normal vulvar skin tissues

The level of IL-1β expression in NNEDV tissues (**Figure 1A**) was significantly lower than that in normal vulvar skin tissues (**Figure 1B**). The quantitative score results for IL-1β expression were 3.03±3.08 in NNEDV tissues, and 8.82±3.49 in normal vulvar skin tissues, and the difference was statistically significant ($P<0.001$) (**Figure 7**). The level of IL-2 expression in NNEDV tissues (**Figure 2A**) was slightly higher than that in normal vulvar skin tissues (**Figure 2B**). The quantitative score results for IL-2 expression were 7.03±3.61 in NNEDV tissues, and 6.55±4.08 in normal vulvar skin tissues, but the difference was not statistically significant ($P=0.686$) (**Figure 7**). The levels of IL-10 expression in the cytoplasm and nucleus of NNEDV tissues (**Figure 3A**) were both slightly lower than those in normal vulvar skin (**Figure 3B**). The quantitative score results for IL-10 expression in the cytoplasm were 5.00±3.83 in NNEDV tissues, and 5.36±4.27 in normal vulvar skin tissues. The quantitative score results for IL-10 expression in the nucleus were 1.74±2.17 in NNEDV tissues, and 1.91±2.98 in normal vulvar skin tissues, but the differences were both not statistically significant ($P=0.773$ and 0.819 respectively) (**Figure 7**). The level of expression of p65 protein in NNEDV tissues (**Figure 4A**) was lower than that in normal vulvar skin tissues (**Figure 4B**). The quantitative score results for p65 protein expression were 5.19±3.82 in NNEDV tissues, and 8.73±3.35 in normal vulvar skin tissues, and the difference was statistically significant ($P=0.005$) (**Figure 7**). The levels of expression of c-Rel protein in the cytoplasm and nucleus of NNEDV tissues (**Figure 5A**) were both higher than those in normal vulvar skin tissues (**Figure 5B**). The quantitative score results for c-Rel protein expression in the cytoplasm were 7.21±4.49 in NNEDV tissues, and 4.27±1.95 in normal vulvar skin tissues.

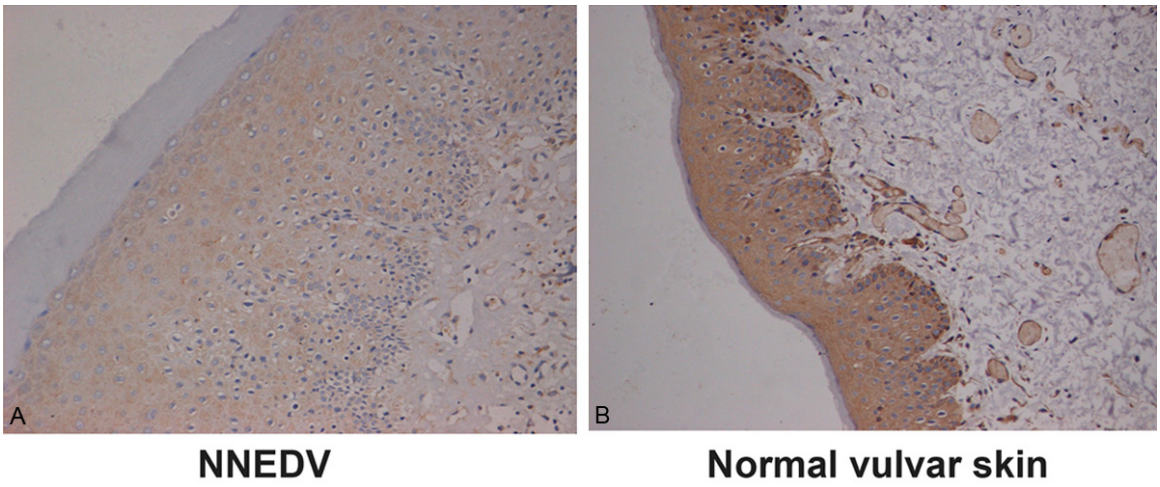

Figure 4. The expression of p65 protein in NNEDV and normal vulvar skin tissues. Observed under ×200 fields, positive staining of p65 protein was located in the cytoplasm, with the positive staining cells mainly distributed in the epidermal basal layer, prickle cell layer and granular cell layer. A. The level of expression of p65 protein in NNEDV tissues was lower than that in normal vulvar skin tissues. B. The level of expression of p65 protein in normal vulvar skin was relatively high.

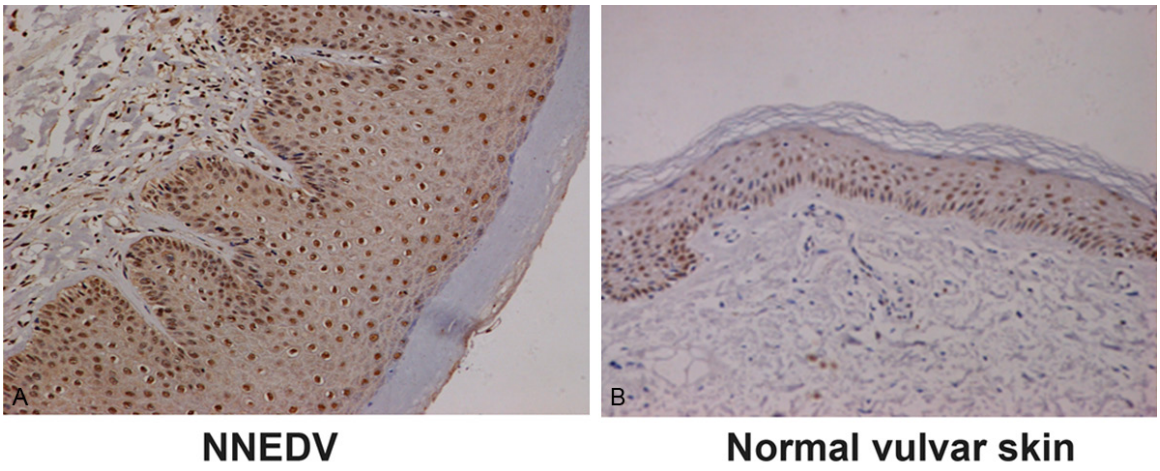

Figure 5. The expression of c-Rel protein in NNEDV and normal vulvar skin tissues. Observed under ×200 fields, positive staining of c-Rel protein was located in the cytoplasm and/or nucleus. Cells with positive staining in the cytoplasm and nucleus were distributed in the epidermal basal layer, prickle cell layer and granular cell layer, therein nucleus-positive cells were mainly concentrated in the prickle cell layer. A. The levels of expression of c-Rel protein in the cytoplasm and nucleus of NNEDV tissues were both higher than those in normal vulvar skin tissues. B. The levels of expression of c-Rel protein in the cytoplasm and nucleus of normal vulvar skin were both relatively low.

The quantitative score results for c-Rel protein expression in the nucleus were 7.66±4.49 in NNEDV tissues, and 4.55±3.75 in normal vulvar skin tissues, and the differences were both statistically significant (P=0.001 and 0.032 respectively) (**Figure 7**). The level of expression of TRAF5 protein in NNEDV tissues (**Figure 6A**) was significantly lower than that in normal vulvar skin tissues (**Figure 5A**). The quantitative score results for TRAF5 protein expression were 6.68±3.71 in NNEDV tissues, and 11.73±0.90 in normal vulvar skin tissues, and the difference was statistically significant (P<0.001) (**Figure 7**).

Correlation analysis of the expression of TRAF5, p65, c-Rel protein and IL-1β in NNEDV tissues

As shown in **Table 1**, in NNEDV tissues, both the expression of p65 protein and that of nuclear c-Rel protein were weakly linearly correlated

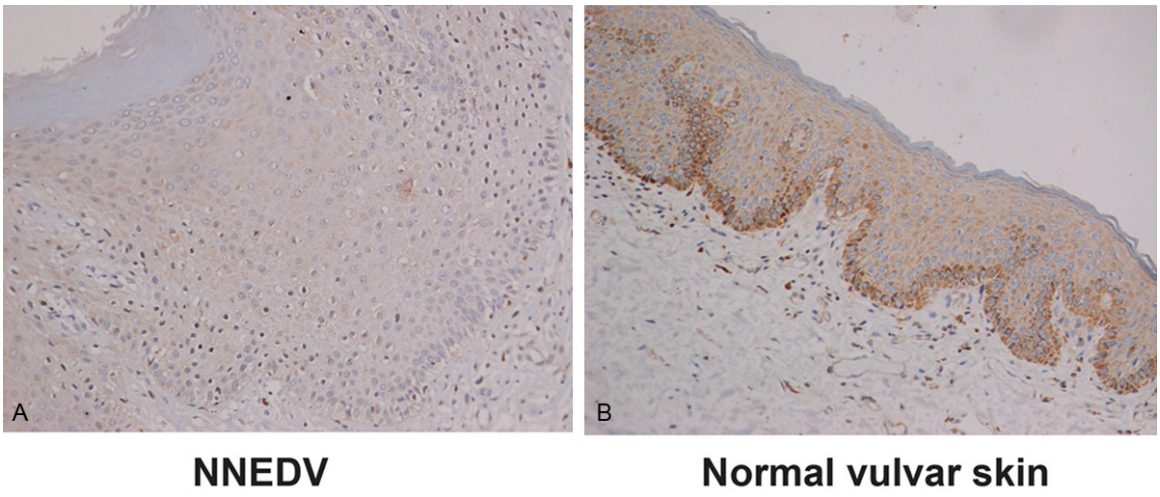

Figure 6. The expression of TRAF5 protein in NNEDV and normal vulvar skin tissues. Observed under ×200 fields, positive staining of TRAF5 protein was located in the cytoplasm, with the positive staining cells mainly distributed in the epidermal basal layer, prickle cell layer and granular cell layer. Among these cells, the intensity of staining in some positive cells distributed in the basal layer was stronger than that distributed in the prickle cell layer and granular cell layer. A. The level of expression of TRAF5 protein in NNEDV tissues was significantly lower than that in normal vulvar skin tissues. B. The level of expression of TRAF5 protein in normal vulvar skin tissues was relatively high.

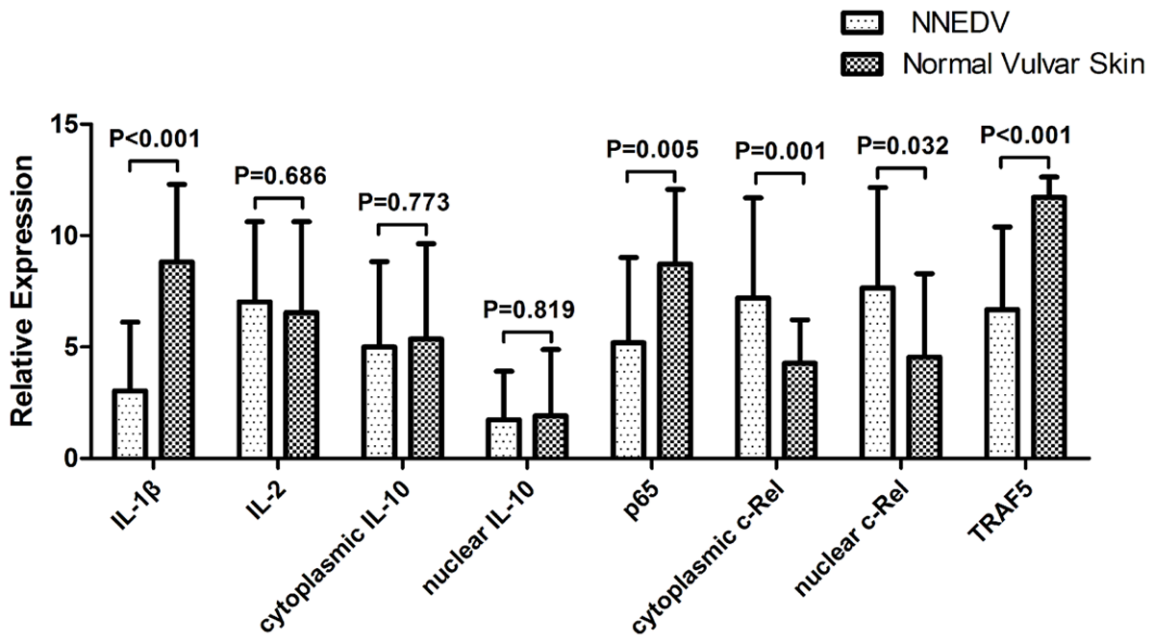

Figure 7. The expression of IL-1β, IL-2, IL-10 and p65, c-Rel, TRAF5 protein in NNEDV and normal vulvar skin tissues were counted and compared. The level of IL-1β expression in NNEDV tissues was significantly lower than that in normal vulvar skin tissues (P<0.001). The level of IL-2 expression in NNEDV tissues was slightly higher than that in normal vulvar skin tissues, but the difference was not statistically significant (P=0.686). The levels of IL-10 expression in the cytoplasm and nucleus of NNEDV tissues were both slightly lower than those in normal vulvar skin tissues, but the differences were both not statistically significant (P=0.773 and 0.819 respectively). The level of expression of p65 protein in NNEDV tissues was significantly lower than that in normal vulvar skin tissues (P=0.005). The levels of expression of c-Rel protein in the cytoplasm and nucleus of NNEDV tissues were both significantly higher than those in normal vulvar skin tissues (P=0.001 and 0.032 respectively). The level of expression of TRAF5 protein in NNEDV tissues was significantly lower than that in normal vulvar skin tissues (P<0.001).

Table 1. Pearson correlation analysis of TRAF5, p65, c-Rel protein and IL-1β expression in NNEDV tissues

Pearson correlation analysis	TRAF5		Cytoplasmic c-Rel	IL-1β		
	p65	Cytoplasmic c-Rel	Nuclear c-Rel	Nuclear c-Rel	p65	Cytoplasmic c-Rel	Nuclear c-Rel
Correlation coefficient (R)	0.319**	0.490**	0.377**	0.816**	0.219	0.379**	0.255*
P value	0.006	<0.001	0.001	<0.001	0.063	0.001	0.029

**P<0.01, *P<0.05.

Table 2. The relationship between TRAF5, p65, c-Rel, IL-1β expression and clinicopathological features of the patients with NNEDV

Clinicopathological factor	All cases	TRAF5		*P* value	p65		*P* value	Cytoplasmic c-Rel		*P* value	Nuclear c-Rel		*P* value	IL-1β		*P* value
		High	Low		High	Low		High	Low		High	Low		High	Low	
Age/year				0.017			0.039			0.020			<0.001			0.020
≤40	22	17	5		12	10		18	4		19	3		16	6	
>40	51	24	27		40	11		27	24		20	31		22	29	
Duration/year				0.016			0.013			0.017			0.015			0.027
≤10	39	27	12		23	16		29	10		26	13		25	14	
>10	34	14	20		29	5		16	18		13	21		13	21	
Disease site				0.156			0.817			0.957			0.960			0.485
labia minora	35	17	18		26	9		21	14		19	16		16	19	
labia majora	24	13	11		16	8		15	9		13	11		13	11	
Others	14	11	3		10	4		9	5		7	7		9	5	
Itching Degree				0.018			0.083			0.030			0.026			0.646
Mild Itching	27	20	7		16	11		21	6		19	8		15	12	
Severe Itching	46	21	25		36	10		24	22		20	26		23	23	
Clinical manifestation				0.459			0.704			0.617			0.453			0.032
White Patches	38	23	15		28	10		22	16		19	19		14	24	
Rahagades	11	5	6		7	4		7	4		8	3		6	5	
Vaginal Stenosis	14	6	8		11	3		8	6		8	6		10	4	
Ulcer	10	7	3		6	4		8	2		4	6		8	2	
Recurrent vaginitis				0.029			0.033			0.957			0.103			0.683
Yes	31	22	9		18	13		19	12		20	11		17	14	
No	42	19	23		34	8		26	16		19	23		21	21	

with the expression of TRAF5 protein (R=0.319 and 0.377 respectively, P=0.006 and 0.001 respectively). The expression of TRAF5 protein also showed moderate linear correlation with the expression of cytoplasmic c-Rel protein (R=0.490, P<0.001). There was a high linear correlation observed between the expression of c-Rel in the cytoplasm and nuclei (R=0.816, P<0.001). No significant linear relationship was found between the expression of p65 and IL-1β (R=0.219, P=0.063). A weak linear correlation was found between expression of cytoplasmic c-Rel and IL-1β (R=0.379, P=0.001), as well as between expression of nuclear c-Rel and IL-1β (R=0.255, P=0.029).

Analysis of the relationship between the expression of TRAF5, p65, c-Rel protein, as well as IL-1β in NNEDV tissues and clinicopathological features of patients

As shown in **Table 2**, the level of expression of TRAF5 protein in NNEDV tissues was closely related to patients' age (P=0.017), duration (P=0.016), itching degree (P=0.018) and recurrent vaginitis (P=0.029), but was not correlated with disease site (P=0.156) and clinical manifestation (P=0.459). The level of expression of p65 protein was closely related with age (P=0.039), duration (P=0.013) and recurrent vaginitis (P=0.033), while was not relevant to disease site

Table 3. The relationship between TRAF5, p65, c-Rel, IL-1β expression and autoimmune diseases of the patients with NNEDV

Autoimmune disease	All cases	TRAF5		*P* value	p65		*P* value	Cytoplasmic c-Rel		*P* value	Nuclear c-Rel		*P* value	IL-1β		*P* value
		High	Low		High	Low		High	Low		High	Low		High	Low	
Autoimmune diabetes				0.023			0.023			0.006			0.031			0.019
Yes	29	21	8		16	12		12	17		11	18		20	9	
No	44	20	24		36	8		33	11		28	16		18	26	
Hyperthyroidism				0.832			1.000			0.027			0.011			0.858
Yes	11	7	4		8	3		3	8		2	9		6	5	
No	62	34	28		44	18		42	20		37	25		32	30	
SLE				0.793			0.691			0.971			1.000			0.667
Yes	4	3	1		2	2		3	1		2	2		3	1	
No	69	38	31		50	19		42	27		37	32		35	34	
Rheumatoid arthritis				0.013			0.811			0.045			0.030			0.524
Yes	17	14	3		13	4		14	3		13	4		10	7	
No	56	27	29		39	17		31	25		26	30		28	28	
Ulcerative colitis				0.447			0.869			1.000			1.000			0.046
Yes	8	6	2		5	3		5	3		4	4		1	7	
No	65	35	30		47	18		40	25		35	30		37	28	
Chronic atrophic gastritis				1.000			0.022			0.443			0.350			0.196
Yes	9	5	4		3	6		4	5		3	6		7	2	
No	64	36	28		49	15		41	23		36	28		31	33	
Chronic liver disease				0.453			1.000			0.227			0.357			0.212
Yes	8	3	5		6	2		7	1		6	2		2	6	
No	65	38	27		46	19		38	27		33	32		36	29	
Malignant tumors				0.023			0.011			0.760			0.518			0.010
Yes	13	11	2		5	8		9	4		8	5		11	2	
No	60	30	30		47	13		36	24		31	29		27	33	
ITP				1.000			0.407			1.000			1.000			1.000
Yes	3	2	1		1	2		2	1		2	1		2	1	
No	70	39	31		51	19		43	27		37	33		36	34	
Vitiligo				0.017			0.039			0.020			0.007			0.429
Yes	22	17	5		12	10		18	4		17	5		13	9	
No	51	24	27		40	11		27	24		22	29		25	26	

SLE: systemic lupus erythematosus, ITP: idiopathic thrombocytopenic purpura.

(P=0.817), itching degree (P=0.083) and clinical manifestation (P=0.704). The level of expression of c-Rel in the cytoplasm was significantly correlated with age (P=0.020), duration (P=0.017) and itching degree (P=0.030), however was not related with disease site (P=0.957), clinical manifestation (P=0.617) and recurrent vaginitis (P=0.957). The level of expression of nuclear c-Rel was obviously related to age (P<0.001), duration (P=0.015) and itching degree (P=0.026), whereas was not correlated with disease site (P=0.960), clinical manifestation (P=0.453) and recurrent vaginitis (P=0.103). The expression of IL-1β was closely relevant to age (P=0.020), duration (P=0.027) and clinical manifestation (P=0.032), but was not related with disease site (P=0.485), itching degree (P=0.646) and recurrent vaginitis (P=0.683).

Analysis of the relationship between the expression of TRAF5, p65, c-Rel protein, as well as IL-1β in NNEDV tissues and whether the patients suffer from other autoimmune diseases

As shown in **Table 3**, the level of expression of TRAF5 protein in NNEDV tissues was closely related to autoimmune diabetes (P=0.023), rheumatoid arthritis (P=0.013), malignant tumors (P=0.023) and vitiligo (P=0.017), but was not correlated with hyperthyroidism (P=0.832), systemic lupus erythematosus (SLE) (P=0.793),

ulcerative colitis (P=0.447), chronic atrophic gastritis (P=1.000), chronic liver disease (P=0.453) and idiopathic thrombocytopenic purpura (ITP) (P=1.000). The level of expression of p65 protein was closely related with autoimmune diabetes (P=0.023), chronic atrophic gastritis (P=0.022), malignant tumors (P=0.011) and vitiligo (P=0.039), while was not relevant to hyperthyroidism (P=1.000), SLE (P=0.691), rheumatoid arthritis (P=0.811), ulcerative colitis (P=0.869), chronic liver disease (P=1.000) and ITP (P=0.407). The level of expression of c-Rel in the cytoplasm is significantly correlated with autoimmune diabetes (P=0.006), hyperthyroidism (P=0.027), rheumatoid arthritis (P=0.045) and vitiligo (P=0.020), however was not related with SLE (P=0.971), ulcerative colitis (P=1.000), chronic atrophic gastritis (P=0.443), chronic liver disease (P=0.227), malignant tumors (P=0.760) and ITP (P=1.000). The level of expression of nuclear c-Rel was obviously related to autoimmune diabetes (P=0.031), hyperthyroidism (P=0.011), rheumatoid arthritis (P=0.030) and vitiligo (P=0.007), whereas was not correlated with SLE (P=1.000), ulcerative colitis (P=1.000), chronic atrophic gastritis (P=0.350), chronic liver disease (P=0.357), malignant tumors (P=0.518) and ITP (P=1.000). The expression of IL-1β was closely relevant to autoimmune diabetes (P=0.019), ulcerative colitis (P=0.046) and malignant tumors (P=0.010), but was not related with hyperthyroidism (P=0.585), SLE (P=0.667), rheumatoid arthritis (P=0.524), chronic atrophic gastritis (P=0.196), chronic liver disease (P=0.212), ITP (P=1.000) and vitiligo (P=0.429).

Discussion

Not only is the etiology of NNEDV unknown, but NNEDV also has a relatively high recurrence rate and cannot be cured. The occurrence of the disease is related to many factors, and autoimmune factors have attracted a great deal of attention. Studies have shown that many patients with NNEDV suffer concomitantly from other autoimmune diseases with specific autoantibodies [28], indicating that this disease may be associated with autoimmune dysfunction. The immune-related cytokines IL-1β, IL-2 and IL-10 are involved in the regulation of immune responses and play important roles in many autoimmune diseases [1-10, 29]. The NF-κB protein family consists of five subunits: c-Rel (Rel), p65 (RelA, NF-κB3), RelB, p50 (NF-κB1) and p52 (NF-κB2), which have Rel homology and can form homodimers or heterodimers. When activated, these dimers are translocated to the nucleus to regulate gene transcription by binding to fixed nucleotide sequences of the promoter region; this is implicated in the occurrence of several diseases [11, 12]. Many chronic autoimmune diseases, including rheumatoid arthritis, Crohn's disease, ankylosing spondylitis, and multiple sclerosis, are associated with the continued production of cytokines regulated by the NF-κB pathway [12]. Literature reports suggest that once the NF-κB pathway has been activated through the classical pathway, the p50/p65 dimer is activated, which enhances gene transcription of IL-1β and IL-2, and increases release of IL-1β and IL-2. The latter further activates the NF-κB pathway through a positive feedback effect, leading to continuous production of IL-1β and IL-2 [15-18]. However, IL-10 negatively regulates the NF-κB pathway (p50/p65) and inhibits the production of NF-κB pathway-dependent cytokines, thereby regulating autoimmune function [20, 21, 30].

The c-Rel subunit of the NF-κB family plays a role in the pathogenesis of several autoimmune diseases. Studies have shown that c-Rel promotes autoimmune response in autoimmune encephalomyelitis, psoriasis, psoriatic arthritis and rheumatoid arthritis [31-33]. Another study has shown that knocking down the c-Rel gene in nonobese diabetic (NOD) mice can accelerate the progression of autoimmune diabetes [34]. In addition, c-Rel has a regulatory role in maintaining epidermal homeostasis, and affects the viability and proliferation of epidermal keratinocytes [35]. Studies have shown that IL-1β activates the c-Rel subunit, while activation of the c-Rel subunit further promotes the expression of IL-1β, and c-Rel is essential for the normal immune function of IL-1β [16, 17]. Moreover, c-Rel regulates the production and release of IL-2, which plays an important role in autoimmune diseases [19, 20]. IL-10 inhibits the NF-κB/c-Rel pathway by inhibiting the degradation of inhibitor of kappa B alpha (IκBα) and subsequent translocation of c-Rel into the nucleus, resulting in a decrease in the production and release of IL-12, thereby further regulating autoimmune function [23]. TRAF5 is an important signal transduction protein in the cell; it mediates the activation of the NF-κB pathway and plays an important role in immune regulation [26]. The purpose of this study was

to investigate the relationship between the occurrence of NNEDV and the expression of immune-related cytokines IL-1β, IL-2 and IL-10, and to explore whether the TRAF5-mediated NF-κB pathway is involved.

The results of this study showed that, compared with normal vulvar skin, the expression of IL-1β, p65 and TRAF5 protein in the cytoplasm of epidermal cells in NNEDV tissues was significantly reduced, whereas the expression of c-Rel protein in the cytoplasm and the nucleus was significantly increased. The expression of IL-2 and IL-10 in NNEDV tissue was not significantly different from that in normal vulvar skin. The expression of TRAF5 was positively correlated with the expression of p65 protein, and the expression of c-Rel protein in the cytoplasm and nucleus. The expression of c-Rel protein in the cytoplasm and nucleus showed strong positive correlation. The expression of cytoplasmic c-Rel protein and the expression of nuclear c-Rel protein were also both positively correlated with the expression of IL-1β. The results suggest that the activation of NF-κB/p65 pathway mediated by TRAF5, and a certain intensity of activation of NF-κB/c-Rel pathway, may occur in normal vulvar skin. This co-regulates the transcription of IL-1β and IL-2 genes, leading to a certain level of expression of IL-1β and IL-2 in the local tissue, exerting normal immune function. There is also a certain level of IL-10 present in normal vulvar skin, inhibiting an excessive local immune response.

The expression of TRAF5 protein is decreased in NNEDV tissues. The NF-κB/p65 pathway, which is mediated by TRAF5, is relatively restrained, leading to decreased p65 protein in the cytoplasm, as well as decreased phosphorylated p65 translocation to the nucleus, reducing the gene transcription level of IL-1β and IL-2, as well as the production and release of IL-1β and IL-2. Meanwhile, excessive activation of the NF-κB/c-Rel pathway may also occur in NNEDV tissues. The expression of c-Rel protein in the cytoplasm increases, leading to a large number of c-Rel subunits being activated and translocated to the nucleus, so that the amount of c-Rel protein in the nucleus increases. After entering the nucleus, the c-Rel subunits bind to specific sequences in promoter regions of the IL-1β and IL-2 genes, regulating transcription of IL-1β and IL-2 genes and resulting in increased IL-1β and IL-2 production and release. According to the results of this study, compared with normal vulvar skin, IL-1β expression was significantly reduced in NNEDV, but the expression of IL-2 was not significantly different, indicating that the effects of NF-κB/p65 pathway inhibition and excessive NF-κB/c-Rel pathway activation in the regulation of IL-2 gene transcription are well-matched. However, the degree of inhibition of IL-1β gene transcription by the NF-κB/p65 pathway was greater than the activation by NF-κB/c-Rel pathway, resulting in significantly reduced expression of IL-1β. In NNEDV, the NF-κB/p65 pathway and NF-κB/c-Rel pathway are interrelated and mutually constrained, which may be because inhibition of the NF-κB/p65 pathway leads to excessive activation of the NF-κB/c-Rel pathway; the effects of excessive activation of the NF-κB/c-Rel pathway partially offset the changes in autoimmune function due to inhibition of the NF-κB/p65 pathway. The imbalance in their degree of activation leads to significantly reduced local expression of IL-1β, decreasing immune responses to external stimuli, which may result in the occurrence of NNEDV. However, the expression of IL-10 was not significantly changed in NNEDV, indicating that the local immune suppression function was normal. In summary, abnormality in the TRAF5-mediated NF-κB pathway in NNEDV leads to significantly reduced expression of IL-1β, resulting in local immune dysfunction, and subsequently, occurrence of NNEDV.

The study also found that the level of expression of TRAF5 protein in patients with NNEDV is closely related to their age, duration, itching degree and recurrent vaginitis. The level of expression of p65 protein is significantly related with age, duration and recurrent vaginitis. The level of expression of both cytoplasmic c-Rel and nuclear c-Rel is obviously correlated with age, duration and itching degree. The expression of IL-1β is closely related with age, duration and clinical manifestation. The results of this study suggest that the immune function of patients with NNEDV may change with the increase in age and the duration of the disease, and that the strength of local immune function affects the clinical manifestations of disease. In addition, decreased local immune function may lead to recurrent vaginitis.

This study also showed that the expression of TRAF5, p65, cytoplasmic and nuclear c-Rel protein, as well as IL-1β in NNEDV tissues were associated with several autoimmune diseases, among which autoimmune diabetes was closely related to the expression of TRAF5, p65, and cytoplasmic and nuclear c-Rel as well as IL-1β. Rheumatoid arthritis was closely related to the expression of TRAF5 and cytoplasmic and nuclear c-Rel, malignant tumors were closely related to the expression of TRAF5, p65 and IL-1β, and vitiligo was closely related to the expression of TRAF5, p65, cytoplasmic and nuclear c-Rel. The results show that NNEDV may be associated with systemic immune dysfunction, which may also be an autoimmune disease, and that the immune mechanisms of its pathogenesis may be related to the mechanisms of other autoimmune diseases, including autoimmune diabetes, rheumatoid arthritis, malignant tumors and vitiligo.

In summary, inhibition of the TRAF5-mediated NF-κB/p65 pathway and excessive activation of the NF-κB/c-Rel pathway were observed in NNEDV tissues, leading to significantly decreased production and release of IL-1β, which results in local immune response disorders, and occurrence of NNEDV. Local immune function is closely associated with the clinicopathological features of patients, who may suffer from other autoimmune diseases at the same time, indicating that NNEDV is an autoimmune disease, and TRAF5, p65, c-Rel and IL-1β may serve as diagnostic markers and immunotherapy targets in NNEDV. However, further exploration is needed for the following reasons: the results of this study show that there are two kinds of NF-κB pathway activation mechanism involved in the occurrence of NNEDV, but the relationship between these two activation mechanisms is not clear; only three immune-related cytokines, IL-1β, IL-2 and IL-10, were investigated in the study, but other immune-related cytokines may play a role in NNEDV; the research results only indicate that production and release of IL-1β at local lesions is significantly reduced, resulting in local immune response disorders, but the differentiation and function of which immune cells are specifically affected by the decrease in IL-1β, and the specific mechanism, remain unclear. In addition, a larger sample size is needed to verify the findings of the current study, and other experimental methods may be added to improve the accuracy of the relevant tests. The occurrence and development of NNEDV is a complex process influenced by many factors. This study is only a preliminary exploration from the perspective of the immune system, which lays a theoretical foundation for clinical immunotherapy of NNEDV.

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Disclosure of conflict of interest

None.

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