Promotion of IL-17/NF-κB signaling in autoimmune thyroid diseases

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Abstract. IL-17 and other cytokines have a number of immunomodulatory effects on thyroid cells. The present study investigated the changes and correlations amongst IL-17, NF-κB, IL-6, IL-10, interferon-γ (IFN-γ), TNF-α, IL-2 and IL-4 in patients with different autoimmune thyroid diseases in order to further clarify the pathogenesis of autoimmune thyroid disease. A total of 82 patients with autoimmune thyroid diseases (41 with Graves' disease and 41 with Hashimoto's thyroiditis) and 53 healthy controls were enrolled. All relevant thyroid hormones were detected by electrochemiluminescence analyzer. The serum levels of IL-17 and other cytokines were detected using flow cytometry, NF-κB was detected by ELISA, reverse transcription-quantitative PCR was used to detect the protein expression of various mRNAs, and the correlations between IL-17 and these factors were analyzed. Significant differences occurred amongst all groups. NF-κB, TNF-α, IL-6, IL-17 and their mRNA levels were significantly higher in the healthy controls compared with those in the patients; whereas IFN-γ and IL-10 levels were significantly lower in the healthy controls compared with those in the patients . Correlation analysis showed that the expression levels of IL-17 and its mRNA were significantly positively correlated with the expression levels of NF-κB, IL-6, thyroid peroxidase antibody, thyroid gland globulin, thyroglobulin antibody, TNF-α and IFN-γ, and were also significantly negatively correlated with IL-10 . These findings suggested that IL-17 was elevated in patients with autoimmune thyroid disease and that IL-17 could activate the NF-κB signaling pathway, stimulate the production and release of inflammatory factors such as TNF-α, IL-6 and IFN-γ and participate in the pathogenesis of autoimmune thyroid injury.

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Introduction

Autoimmune thyroid diseases (AITDs) involve multiple factors such as heritage, environment and infection and include Graves' disease (GD) and Hashimoto's thyroiditis (HT) as the most common clinical diseases (1). Autoimmune thyroid disease patients are prone to fat metabolism disorder, and the serum thyroid hormone level has a close correlation with blood lipid metabolism, insulin metabolism and inflammatory factors (2). Autoimmune thyroid illnesses are a category of disorders characterized by aberrant lymphocyte activity directed against self-tissues, which affect 2-3% of the population, with a female predominance (3). NF-κB is a key nuclear transcription factor expressed in nearly all cell and tissue types. The transcription factor NF-κB, regulates multiple aspects of innate and adaptive immune functions and serves as a pivotal mediator of the inflammatory response (4). NF-κB is a central mediator of inflammation in response to DNA damage and oxidative stress. Because of its central role in numerous cellular processes, NF-κB dysregulation is implicated in the pathology of numerous important human diseases (5). NF-κB activation causes inappropriate inflammatory responses in diseases including rheumatoid arthritis, multiple sclerosis, coronavirus disease-2019 and severe acute respiratory syndrome-coronavirus-2 cases of pneumonia (6).

The present study investigated the differences in the expression levels of IL-17, NF-κB, IFN-γ, TNF-α, IL-2, IL-4, IL-6 and IL-10 in GD and HT, and the differences in the changes of NF-κB signaling pathway and the pathogenesis induced by different inflammatory stimuli. To investigate this and further clarify the pathogenesis of AITDs, the present study analyzed the relationships between IL-17, NF-κB, IL-6, IL-10 proteins and their mRNAs, thyroid-stimulating hormone (TSH), free triiodothyronine (FT3), free thyroxine (FT4), thyroid peroxidase antibody (ATPO), thyroid gland globulin (TG), thyroglobulin antibody (TGAb) and thyroid-stimulating receptor antibody (TRAb).

Materials and methods

Patients. The present study selected 82 patients with AITDs (41 with GD and 41 with HT) and 53 age-matched healthy controls who were outpatients and inpatients of Hebei General Hospital (Shijiazhuang, China) from June 2020 to November 2020. The GD group was 19-63 years old (mean, 38.49±8.81 years). The HT group was 17-66 years old (mean, 40.71±13.08 years). The controls were 15-67 years old (mean, 43.06±12.57 years). Sex and age did not significantly differ among the three groups. Basic parameters for all patients are presented in Table I.

Diagnostic criteria for HT. HT was diagnosed per the following: i) Thyroid peroxidase antibody (TPOAb)-positive or thyroglobulin antibody (TGAb)-positive in serum autothyroid antibodies; ii) thyroid gland exhibited toughness or diffuse enlargement or non-obvious enlargement; iii) transient hyperthyroidism or permanent hypothyroidism; iv) ultrasound showed goiter or no obvious enlargement, diffuse changes in the thyroid gland, decreased echo, or nodules; v) cytological examination by fine needle puncture of the thyroid gland indicated lymphocyte infiltration and eosinophilic changes of follicular cells; and vi) normal or low thyroid uptake (7).

Diagnostic criteria for GD. GD was diagnosed per the following: i) Hypermetabolic symptoms and signs caused by thyrotoxicosis, which were confirmed by clinical manifestations; ii) diffuse thyroid enlargement confirmed by physical examination and imaging; iii) serum thyroid-stimulating hormone (TSH) level decreased and thyroid hormone level increased; iv) no obvious exophthalmia or other invasive eye signs; v) anterior tibial myxedema; vi) both TRAb-positive (>1.75 IU/l); vii) increased thyroid iodine uptake or enhanced thyroid uptake on thyroid nuclide imaging; and viii) pathological examination showed hyperplasia of thyroid cells, cells in cubic or columnar shape and visible papilla. Subclinical hyperthyroidism with reduced serum TSH levels and normal thyroid hormone levels may be caused by GD, but patients with this condition may be asymptomatic (7).

Exclusion criteria. Exclusion criteria were other acute and chronic inflammatory diseases, liver and kidney dysfunction, severe respiratory diseases, tumor radiotherapy and chemotherapy, severe cardiovascular diseases, pregnancy or lactation, severe mental diseases and infectious diseases. The Ethics Committee of Hebei General Hospital approved informed consent and voluntary participation [approval no. 2020(204)].

Electrochemiluminescence analyzer. TSH, FT3, FT4, ATPO, TG, TGAb, TRAb were detected using an electrochemiluminescence analyzer (Cobas e602; Roche Diagnostics).

Flow cytometry. There were seven types of capture microspheres with different fluorescence intensity in the capture microsphere mixture in the Human Th1/Th2/Th17 subpopulation detection kit (cat. no. P010002; Jiangxi Saiji Biological Co., Ltd.). The surface of the capture beads provided by this aforementioned kit were coated with IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ specific antibodies, and diluted with twice the capture bead buffer and incubate at 37˚C away from light for 30 min. The capture beads were specifically bound to IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α, IFN-γ in the samples to be tested, and then bound to the provided fluorescent detection antibody labeled by PE to form a double-antibody sandwich complex (capture bead + sample to be tested + antibody to be detected). After incubation for 2.5 h at room temperature and away from light, the fluorescence intensity of the double-antibody sandwich complex was analyzed. The contents of IL-2, IL-4, IL-6, IL-10, IL-17, TNF-α and IFN-γ in the samples to be tested were obtained. Multiprotein quantitative assay (CBA) was performed (FACS Canto, BD Biosciences), according to the kit Instruction Manual Software (8). FCAP Array v3 was used to analyze the results (Cellgene Biotech Co. Ltd).

ELISA. Serum NF-κB was detected using ELISA (cat. no. NBP2-29661; Novus Biologicals, LLC).

Cell collection. In the morning, 5 ml of venous blood was drawn from all participants on an empty stomach and treated as below.

Preparation of monocytes. Peripheral venous blood (1-2 ml) was drawn from all subjects on an empty stomach in the morning, the samples were treated with EDTA anticoagulant and added with 1.5X phosphate-buffered saline (PBS), and the treatment was completed within 4 h. Lymphocyte isolation solution was added within 1 h (Tiangen Biotech Co., Ltd.). The diluted blood cells were evenly vortexed, then the vortexed blood was added to the 1.5X lymphocyte separation solution (Tiangen Biotech Co., Ltd.) to ensure that the blood was in the upper layer and centrifuged at 1,220 x g for 20 min at 4˚C. The supernatant was removed and added to another glass test tube with 1.5x PBS. The mixture was centrifuged at 1,220 x g for 20 min at 4˚C, then the precipitate was removed from the discarded solution. Next, the 1.5x PBS was added, mixed and centrifuged at 1,220 x g for 20 min at 4˚C. The precipitate was removed from the discarded solution, and 1 ml of PBS was added, homogenized and transferred to a 1.5-ml centrifuge tube and centrifuged at 12 x g for 5 min at 4˚C. The precipitate was removed from the discarded solution to form mononuclear cells. The SYBR Green I (Tiangen Biotech Co., Ltd.) chimeric fluorescence method was used for quantitative detection (7500 Real-Time PCR System; Thermo Fisher Scientific, Inc.).

Reverse transcription-quantitative PCR (RT-qPCR). IL-6, IL-10, IL-17 and NF-κB mRNA expression levels were detected using RT-qPCR. Total RNA was extracted from the mononuclear cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), then amplified using a genomic DNA removal reaction. RT was performed using FastKing RT Kit (with gDNase) (Tiangen Biotech Co., Ltd.) according to the manufacturer's instructions. qPCR was performed as per the instructions of the SuperReal PreMix Plus (SYBR Green) kit (Tiangen Biotech Co., Ltd.). The amplification procedure was pre-denaturation at 95˚C for 10 min for one cycle and amplification at 95˚C for 15 sec, 60˚C for 15 sec and 72˚C for 30 sec for 40 cycles. The ΔCq was calculated by subtracting the Cq value of the reference β-actin gene from the Cq value of the target gene, and the relative mRNA expression of the target gene was calculated via $2^{-\Delta\Delta Cq}$ (9). The reference method comes from the specification of Tiangen Biotech Co., Ltd. Primer sequences are presented in Table II.

Statistical analysis. IBM SPSS 21.0 software (IBM Corp.) was used for statistical analysis. Measurement data are expressed as the mean ± standard deviation (X ± S) for normally distributed variables in each group, Homogeneity of variance test and

Table I. Patient demographics.

Characteristics	Autoimmune thyroid diseases		Control
	Graves' disease patients	Hashimoto's thyroiditis patients	
Numbers	41	41	53
Age, years	38.49±8.81	40.71±13.08	43.06±12.57
Sex, W:M	34:7	36:5	41:12

M, men; W, women.

Table II. Primer sequences used for reverse transcription-quantitative PCR.

Gene name	Upstream primer (5'-3')	Downstream primer (5'-3')
β-actin	ACGAGGCCCAGAGCAAGAGA	GGTCTTTGCGGATGTCCACG
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
IL-10	TGAATTCCCTGGGTGAGAAG	CTCTTCACCTCCTCCACTGC
IL-17	ACGATGACTCCTGGGAAGACC	GGGATTGTGATTCCTGCCTTC
NF-κB	TGGCGCAGAAATTAGGTCTGG	GATCACTTCAATTGCTTCGGTGTA

one-way analysis of variance were performed among multiple groups, after which multiple comparisons of LSD were performed. Non-normally distributed variables are presented as the median and interquartile range (M [Q1-Q3]). Pearson correlation analysis was used for correlation analysis between variables and Spearman correlation analysis was used for non-parametric variables. P<0.05 was considered to indicate a statistically significant difference.

Results

Comparison of the thyroid hormone, antibodies and NF-κB. Index levels differed significantly between the GD and HT groups and the controls (P<0.05 or P<0.01). The levels of TSH, TGAb and NF-κB were significantly higher in the HT group compared with the GD group and controls; while the levels FT3, FT4 and TRAb were significantly lower in the HT group compared with the GD group (P<0.05 or P<0.01; Table III).

Comparison of cytokine levels. Cytokine levels differed significantly between the GD and HT groups and the controls (P<0.05 or P<0.01). TNF-α, IL-6 and IL-17A were significantly higher in the HT group compared with the GD group and controls. IFN-γ and IL-10 were significantly lower in the HT group compared with the controls (P<0.05). These results suggest an immunoinflammatory mechanism, especially in the HT group, which might be related to severer inflammatory lymphocyte infiltration (Table IV). Representative flow cytometric view of each cytokine analyzed by flow cytometry is presented in (Fig. S1).

mRNA comparisons among groups. RT-qPCR was used to analyze the mRNA expression levels of NF-κB (Fig. 1A), IL-6 (Fig. 1B), IL-17 (Fig. 1C) and IL-10 (Fig. 1D). mRNA levels in the GD and HT groups differed significantly from those of the controls (P<0.01). The mRNA expression levels of NF-κB, IL-6 and IL-17 were significantly higher in the HT group compared with the GD group and controls (P<0.05 or P<0.01). While IL-10 mRNA was lower in the HT and GD groups compared with the controls (P<0.01) (Fig. 1).

Correlation analysis. Pearson and Spearman correlation analyses revealed that IL-17A was significantly positively correlated with ATPO, NF-κB, TGAb, TNF-α, IL-6 and IFN-γ (r=0.321, 0.42, 0.334, 0.554, 0.659 and 0.318, respectively; P<0.05 or P<0.01) and significant correlation with IL-10, TG (r=-0.242, 0.268; P<0.05). IL-17 mRNA was positively correlated with NF-κB and IL-6 mRNA (r=0.47 and 0.666, respectively; P<0.01) and significant correlation with IL-10 mRNA (r=-0.251; P<0.05). These data suggested that IL-17 activated the NF-κB signaling pathway to produce inflammatory factors, and the high expression difference may be caused by abnormal NF-κB expression (Fig. 2).

Discussion

IL-17, also known as IL-17A (10), is an important inflammatory factor that promotes recruitment, chemotaxis and amplification of neutrophils. It also recruits monocytes and neutrophils to gather at inflammatory sites by increasing production of C-X-C motif ligand (CXCL)1 and CXCL2 chemokines in tissues, thus causing chronic inflammation (11).

Studies have revealed that IL-17 levels in thyroid tissues are highly expressed in patients with both HT and GD (12,13) and can be used as a novel marker and potential prognostic indicator for diagnosing HT deterioration. Such high expression stimulates Th17 cells under the synergistic effect of IL-23 and IL-6 and activates relevant signaling pathways to induce

Table III. Comparison of indexes between the patients and controls.

Groups	NC (n=53)	GD (n=41)	HT (n=41)
TSH (uIU/ml)	1.5 (1.16-2.57)	0.015 (0.005-0.145)[a]	3.72 (2.24-5.16)[a,b]
FT3 (pmol/l)	4.69±0.77	12.96±7.37[a]	4.5±1.2[b]
FT4 (pmol/l)	16.19±1.80	33.64±19.81[a]	15.87±5.15[b]
ATPO (IU/ml)	13.09±6.39	148.15±108.41[a]	189.28±111.32[a]
TG (ng/ml)	17.81±2.45	44.63±34.73[a]	56.67±42.02[a]
TGAb (IU/ml)	13.66(10.53-26.25)	116.7(48.20-232.60)[a]	285.8(171.89-476.90)[a,c]
TRAb (IU/l)	0.64±0.26	14.83±11.91[a]	0.94±0.39[a,b]
NF-κB (ng/ml)	1.45±0.59	2.81±1.28[a]	3.62±1.67[a,c]

[a]P<0.01 compared with the control group; [b]P<0.01 and [c]P<0.05 compared with the HT and GD groups. NC, negative control; GD, Graves' disease; HT, Hashimoto's thyroiditis; TSH, thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; ATPO, thyroid peroxidase antibody; TG, thyroid gland globulin; TGAb, thyroglobulin antibody; TRAb, thyroid-stimulating receptor antibody.

Table IV. Comparison of cytokine levels between patients and controls.

Cytokines	NC (n=53)	GD (n=41)	HT (n=41)
IFN-γ (pg/ml)	4.43±2.78	3.66±2.29[a]	3.21±1.86[b]
TNF-α (pg/ml)	2.24±1.03	3.42±1.54[b]	4.33±1.88[b,c]
IL-2 (pg/ml)	3.45±1.99	4.14±2.28[a]	3.91±1.89
IL-4 (pg/ml)	1.95±1.02	1.72±0.98	1.69±0.94
IL-6 (pg/ml)	2.76±1.55	3.61±1.82[b]	4.94±2.04[b,d]
IL-10 (pg/ml)	2.49±1.43	1.89±0.94[a]	1.79±0.95[a]
IL-17A (pg/ml)	4.76±1.71	7.79±1.12[b]	8.59±1.74[b,c]

[a]P<0.05 and [b]P<0.01 compared with control group; [c]P<0.05 and [d]P<0.01 compared with the HT and GD groups. TNF-α, IL-6 and IL-17A were significantly higher in the HT group than in the GD group and controls. IFN-γ and IL-10 were significantly lower in the HT group than in the controls (P<0.05). NC, negative control; GD, Graves' disease; HT, Hashimoto's thyroiditis; IFN-γ, interferon-γ.

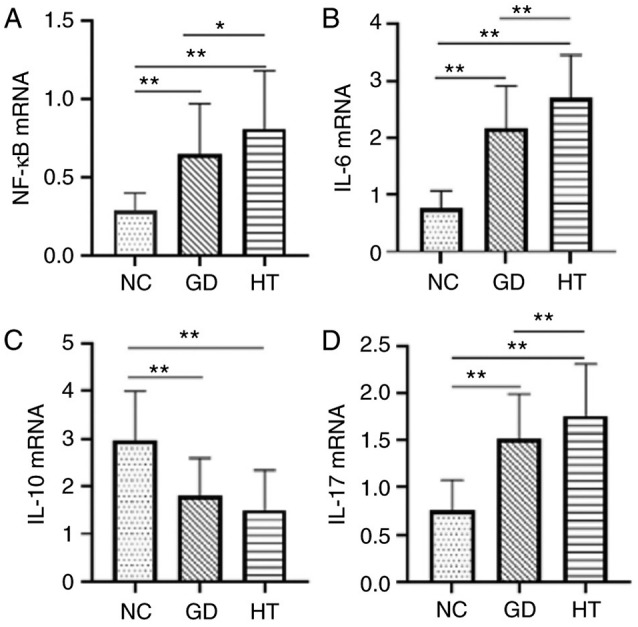

Figure 1. Comparison of mRNA levels among GD, HT and NC groups. RT-qPCR was used to analyze the mRNA expression levels of (A) NF-κB, (B) IL-6, (C) IL-17 and (D) IL-10. *P<0.05, **P<0.01. GD, Graves' disease; HT, Hashimoto's thyroiditis; NC, negative control.

pathogenic phenotypes to induce AITDs (14). IL-17 further aggravates the local inflammatory response of HT thyroid tissue by promoting interstitial fibrosis, which leads to local fibrosis of thyroid tissue and accelerates disease progression (15). Studies have shown that IL-17 is closely associated with thyroid hormone levels and has different correlations with various thyroid disease inflammatory factors, such as IL-6, IL-23 and IL-10, and thyroid antibody titers, indicating that Th17 cells have an independent stimulating effect on thyroid cells (16).

In the present study, the thyroid hormone levels were much higher in the GD group compared with the HT group, but the ATPO, TGAb and TRAb levels were significantly higher in the HT group, indicating that NF-κB levels also differ significantly under different stimuli. IL-17A, TNF-α and IL-6 levels were significantly higher in the patients compared with the controls, whereas IFN-γ and IL-4 levels were lower, indicating the existence of an immunoinflammatory mechanism. The HT group demonstrated the most significant inflammation, possibly owing to the more severe degree of inflammatory lymphocyte infiltration.

Inflammation destroys thyroid follicular cells and lymphocytes and activates lymphocyte chemokine expression (17), resulting in disorder of the thyroid hormones, increased antibody levels and toxic effects. Additionally, IL-17 significantly inhibits the anti-inflammatory and antitumor effects of IFN-γ and further upregulates the expression of protein inhibitor of activated STAT1, a negative feedback regulator of the JAK/STAT1 pathway, by enhancing NF-κB activation, thereby accelerating tumor development (18). Autoantigen TG induces B cells and CD4 T cells to secrete IL-10 and IL-6 and induce Th17 differentiation biased by HT to further produce IL-17, thus resulting in a vicious cycle (19). Long-term inflammation destroys the immune microenvironment of the thyroid

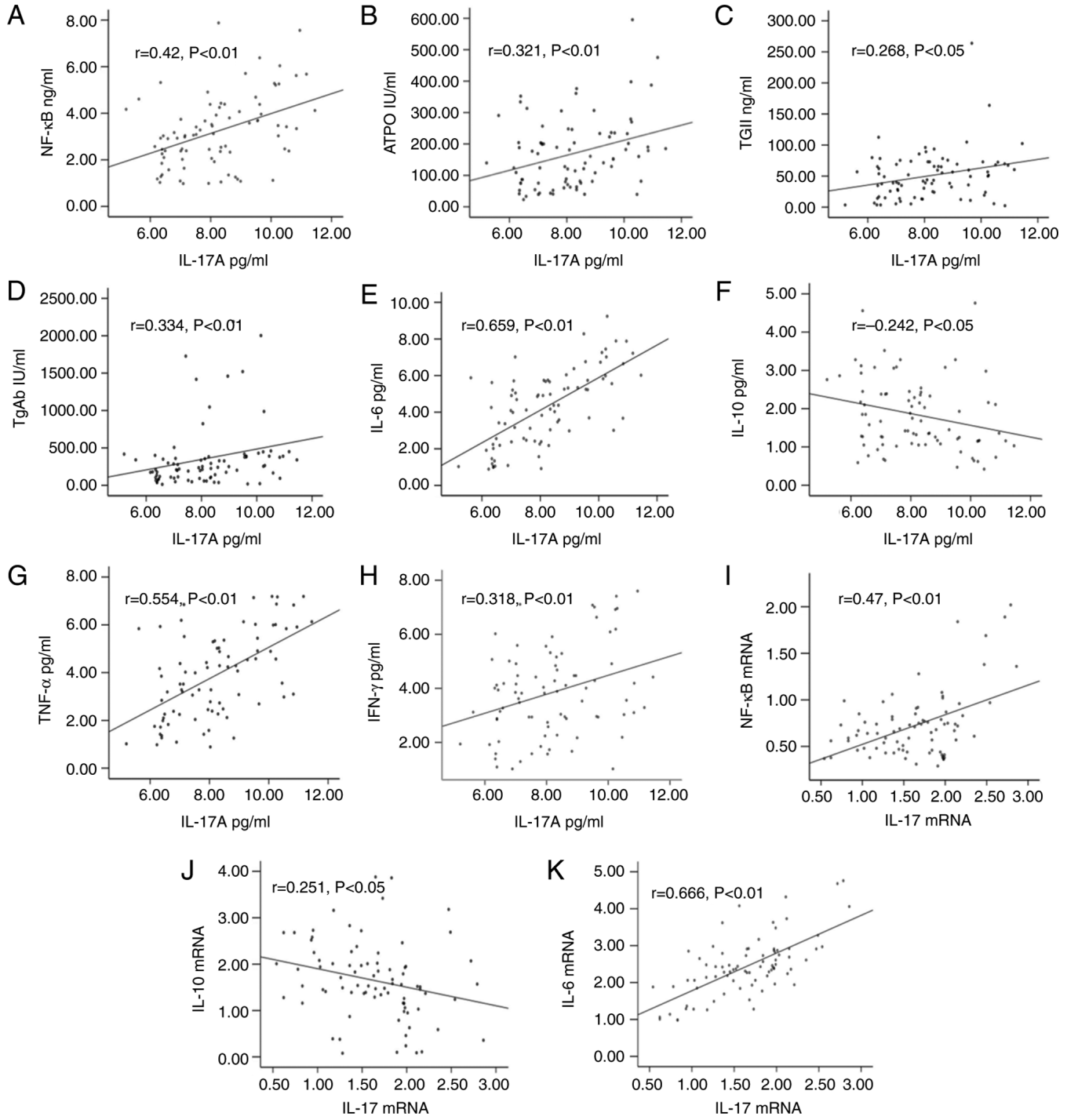

Figure 2. Correlation analysis of interleukin-17 and its protein with each index. Pearson and Spearman correlation analyses revealed that IL-17A was significantly positively correlated with (A) NF-κB, (B) ATPO, (C) TG, (D) TgAb, (E) IL-6, (F) TNF-α and (G) IFN-γ and significantly negatively correlated with (H) IL-10. IL-17 mRNA was positively correlated with (I) NF-κB and (J) IL-6 mRNA and negatively correlated with (K) IL-10 mRNA. ATPO, thyroid peroxidase antibody; TG, thyroid gland globulin; TgAb, thyroglobulin antibody; IFN-γ, interferon-γ.

tissue, rendering the immune self-stabilizing mechanism ineffective (20), further causing carcinogenesis and significantly increasing TG and cytokines Bcl-2, IL-8 and TNF-α.

In the present study, IL-10 was lower in patients compared with the controls, suggesting that the effects of IL-17, IL-6 and TNF-α significantly weakened the anti-inflammatory effect and major histocompatibility complex-II expression on the down-regulated antigen-presenting cells, and the T-cell response was not effectively inhibited, thus promoting disease progression.

A number of cells express NF-κB, a member of the nuclear hormone receptor family and protein molecule with multidirectional regulatory effects, which can be activated by inflammatory factors, growth factors and chemokines to activate a cytokine cascade and produce proinflammatory mediators to regulate the inflammatory response (18). For example, activation of the NF-κB signaling pathway and an imbalance of Treg/Th17 may be involved in the GD pathogenesis (21). IL-17 directly activates NF-κB through the classic

pathway by promoting NF-κB p65 and other nuclear ectopic genes. Stimulation of IL-17-expressing cells induces MCP-1 expression dose- and time-dependently, resulting in the persistence and aggravation of chronic inflammatory processes (22). IL-17A signals intracellular responses by activating IL-17R and synergistically induces interstitial transformation, proliferation and inflammation of bronchial epithelial cells via IL17R/NF-κB signal transduction (23). IL-17 upregulates IL-6 expression by activating the NF-κB pathway, upregulates the expression of programmed cell death ligand 1 in thyroid cells, accelerates inflammatory infiltration and promotes tumor development (24).

TNF-α activates the NF-κB signaling pathway, and its expression is regulated by NF-κB (25). TNF-α contains specific NF-κB binding sites; thus, a positive feedback loop can be formed between TNF-α and the NF-κB signaling pathway, resulting in continuous activation of the NF-κB pathway and Bcl-2 and intercellular adhesion molecule-1 genes. This promotes proliferation and infiltration of inflammatory cells into thyroid tissues and occurrence and development of AITDs (26). Inhibiting the NF-κB pathway can regulate the release of inflammatory factors such as IL-6, IL-10, IL-12 and TNF-α (27) and improve autoimmune thyroid function. The present study demonstrated that IL-6, TNF-α and NF-κB increased successively in the three groups and were positively correlated with IL-17A. IL-17 mRNA was also positively correlated with IL-6 mRNA and NF-κB mRNA. These results suggested that IL-17 activated the NF-κB signaling pathway to produce a number of inflammatory factors such as IL-6 and TNF-α, forming a positive feedback effect that leads to the occurrence and development of AITDs.

In the present study, IFN-γ, IL-4, IL-2, IL-10 and IL-10 mRNA did not significantly differ between the HT and GD groups, but they showed a decreasing trend. IL-17A was negatively correlated with IL-10, and IL-17 mRNA was negatively correlated with IL-10 mRNA. The proinflammatory effects of IL-6 and TNF-α formation are likely much greater compared with their anti-inflammatory effects, and abnormal NF-κB expression may cause the large differences in their expressions. A previous study has shown that although IL-17 is highly expressed in thyroid cells in patients with HT and GD, the levels of IL-23 that induce Th17 differentiation differ, and the correlation between IL-17 and IL-23 also differs (28). IL-23 induces peripheral blood mononuclear cells to secrete prostaglandin E2, which further increases the proportion of $IL\text{-}23R^{+}CD4^{+}T$ cells, promotes IL-17A secretion, reduces secretion of anti-inflammatory factor IL-38 and increases inflammatory cell release (29). These factors induce the different pathogenic phenotypes among AITDs, including GD and HT, which requires further research.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YL, CX and DZ designed the study, collected data, analyzed relevant information, wrote the manuscript and approved the final submission. GL, FC, ZH and CZ performed the formal analysis, analyzed data, organized articles and checked papers YL and XL designed the investigation and wrote the original draft. CX and YL designed the methodology. YL, CX and XL were project administrators. YL and CX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Hebei General Hospital [approval no. 2020(204)], and all subjects signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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