The comparison between the effect of *Glycyrrhizae uralensis* (Gan-Cao) and Montelukast on the expression of T-bet and GATA-3 genes in children with allergic asthma

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ARTICLE INFO

ABSTRACT

Pediatric allergic asthma is a chronic disease that affects the lungs and airways. If a child is exposed to certain stimulants such as pollen inhalation, colds, or respiratory infections, the lungs become inflamed and if left untreated can lead to dangerous asthma attacks. One of the most important treatments for this disease is the use of leukotriene modulators, such as montelukast. But recently, due to easier access, cheaper prices and fewer side effects, attention has shifted to non-chemical treatments. Gan-Cao (*Glycyrrhizae uralensis*), as traditional Chinese medicine, has been proved to have a good therapeutic effect on experimental allergic asthma. But its anti-asthma mechanism is currently unclear. Therefore, the study aimed the comparison between the effect of Gan-Cao and montelukast on the expression of T-bet and GATA-3 genes in children with allergic asthma. For this purpose, fifty children with allergic asthma were divided into two groups. The first group was treated with montelukast for one month. The second group was treated with Gan-Cao root extract. Then the peripheral blood mononuclear cells were isolated, their RNA was extracted, and the relative expression of T-bet and GATA3 transcription factors was evaluated by Real-time PCR. The relationship between them and risk factors for asthma was assessed by relevant statistical tests. The result showed the expression of the GATA3 gene ($P = 0.102$), T-bet gene ($P = 0.888$), and the expression ratio of T-bet/GATA-3 genes ($P = 0.061$) was not significantly different between the two groups. It showed that Gan-Cao can affect the expression of these genes just as much as montelukast. Therefore, this Chinese herb can be used as an alternative or supplement medicine to treat allergic asthma in children.

Introduction

Allergic asthma is a chronic inflammatory disease characterized by increased airway response to various stimuli and increased mucus secretion and is associated with symptoms such as sneezing, coughing, and shortness of breath (1). Asthma is one of the most common chronic diseases affecting approximately 350 million people worldwide and will reach 400 million by 2025 (2). Among different races, Africans and Americans have slightly higher rates of asthma hospitalization and mortality than other races (3). Allergens such as pollen, house dust, pet skin, and hair are the causes of this disease (4).

Most cells and cell mediators, such as mast cells, eosinophils, T lymphocytes, macrophages, neutrophils, and epithelial cells, play an essential role in this disease. TCD4 cells are primary immune response factors in the health and inflammatory diseases divided into Th1, Th2, Th17, and Treg cells (5, 6). Asthma is more of a TH2-dependent illness characterized by the secretion of IL-9, IL-5, IL-4, and IL-13 interleukins. These cytokines, especially IL-5, IL-4, and IL-13, play an essential role in developing asthma (7). IL-4 is the cytokine that produces IgE immunoglobulin, and IL-5 is responsible for the final differentiation, activation, and recall of eosinophils. IL-13 also increases goblet cell proliferation and increases mucus secretion (8, 9). The GATA3 transcription factor acts as a critical regulator of Th2 differentiation and increases the expression of its cytokine genes. In addition, GATA3 can inhibit Th1 cell differentiation by inhibiting the expression of the IL-12 receptor signal chain (10, 11). T-bet transcription factors are members of the T-box family

and are involved in the differentiation and effector functions of Th1 cells. The cytokines secreted by Th1 cells include IFN and IL-12, which play a role in cellular immunity and can effectively reduce the immune response of Th2 cells (12, 13). Recently, an imbalance between T-bet and GATA3 transcription factors has been reported in bronchial asthma, especially allergic asthma (14).

Leukotriene modulators such as montelukast are used to treat allergic asthma. But recently, due to easier access, cheaper prices and fewer side effects, attention has shifted to non-chemical treatments (15, 16). Chinese herbal medicine (CHM) can also help treat this disease (17). *Glycyrrhiza uralensis* (Gan-Cao), also known as Chinese liquorice is used in traditional Chinese medicine (11, 18). It is a flowering plant native to Asia and it has been proved to have a good therapeutic effect on experimental allergic asthma (19). However, its anti-asthma mechanism is currently unclear (20). In the current study, the effect of this medicine was considered on T-bet/GATA3 ratio compared to montelukast in children with allergic asthma.

Materials and methods
Study population

This case-control study was performed on 50 children aged 6-15 years with chronic allergic asthma who entered the study voluntarily after learning about the protocol and objectives of the study. These children were divided into groups. The first group (25 children) was treated with montelukast at a dose of 5 mg/day at night for one month. The second group was treated for one month with 100 mg/day of Gan-Cao root extract.

Allergic asthma was diagnosed according to the latest diagnostic criteria of the American Asthma Association (GINA, 2015) and by asthma and allergy specialist. The patients had inclusion criteria including clinical and paraclinical findings (spirometry, IgE assay) of asthma. Patients had no underlying disease, including diabetes, high blood pressure, obstructive airways diseases such as COPD, viral or bacterial infections and did not take any medication other than the test medicine.

Sampling and isolation of peripheral blood mononuclear cells (PBMCs)

After obtaining the consent of the subject's parents, 5ml of peripheral blood was collected from the patient in a relaxed state (no asthma attack). It was poured into a test tube containing EDTA anticoagulant (50mM) and was transferred to the laboratory. Then a concentration gradient of Ficoll 1.077 (Sigma-Aldrich, USA) was used to separate peripheral blood mononuclear cells (PBMC). 2.5 ml Ficoll was added to an 18 ml Falcon tube, and whole blood was gently added via Pipette Pasteur. Then centrifuge at 400°C for 20 minutes at room temperature to gently remove PBMC. The cells were washed three times with PBS and centrifuged at 250°C for 10 minutes. Then, to fit the number of cell populations in each sample, the separated PBMC was added to 1 ml with RPMI1640, and the number of cells was counted using a Neobar slide and adjusted to 4 million cells.

RNA extraction and quantity and quality evaluations

In this study, a column kit (Sigma-Aldrich, USA) was used to extract RNA. The basis of this kit for extracting RNA is to attach RNA to the silica-gel membrane of the filter in the extraction column under high salt concentration. Also under low salt conditions (RNase-free deionized water), the attached RNA is separate from the filter. According to the manufacturer's instructions, inserting 4 to 6 million cells in each column will give good results in terms of RNA quantity.

After extraction, RNA was released from the column by adding 100μl of release buffer and collected in sterile micro-tubes containing RNase-free and DNase-free. Given the importance of maintaining RNA integrity in later stages, in addition to estimating the quantity of RNA, the quality and purity of the obtained RNA should also be evaluated. The amount of RNA was measured using a nano-spectrophotometer and photometer at 260nm and 280 nm, and after calculating the ratio of light absorption at 260nm to 280nm, it was obtained using the following formula:

$$\text{RNA value} = OD_{260} / OD_{280} \times 40 \times \text{dilution coefficient}$$

The electrophoresis technique was used to evaluate the quality of the extracted RNA. A small amount of RNA sample was used on 2% agarose gel, which showed two bands of 28 s and 18 s in the form of a gel containing the cyber safe.

The cDNA synthesis

The cDNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific, USA) containing oligo (dT) RT and Random Hexamer. The RT solution contains the enzyme MMLV RTase, which increases cDNA synthesis and its stability. According to the kit protocol, the compounds required to synthesize cDNA were added to the RNA under completely sterile conditions and at 25°C for 10 minutes, 47°C for 60 minutes, and 70°C for 10 minutes. BioRad performed complete steps of cDNA synthesis for 10 minutes. Electrophoresis and 2% agarose gel were used to evaluate the quality of the synthesized cDNA.

Real-Time PCR

In the present study, the Real-Time PCR (Bio-Rad, USA) reaction was performed according to the temperature protocol in Table 1. After performing the amplification reaction by Relative Quantitative Real-time PCR (21), the raw data were extracted as a Ct (Threshold cycle). In this study, the EF-1 reference gene was used to normalize the expression of target genes. For the relative analysis of mRNA of target genes, the innovative method of Livak was used (22). In this method, instead of Ct, which are obtained numbers from the logarithmic-linear graph of gene amplification $2^{-\Delta\Delta Ct}$ is used, which has a linear amplitude.

Also, to obtain the amplification efficiency, the genes of the amplification curve were obtained using 6 dilutions based on 10 cDNAs, and the slope of the curve was obtained using the Ct of each dilution in the logarithm of the cDNA concentration. The replication efficiency of each gene was also obtained. Because the efficiency of target genes (GATA-3 and T-bet) was very close to EF-1, the $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of target genes. The primer for GATA-3 was designed by AlleleID software version 7.5 (Premier Biosoft). T-bet (23) and

EF-1 (24) gene sequences were also obtained from previous studies (Table 1).

Statistical analysis

Qualitative data were expressed in absolute and relative frequency, and the chi-square test was used for comparison between groups. Quantitative data was displayed as mean + SD. Data distribution was first examined using the Kolmogorov-Smirnov test to compare quantitative data. T-Student test or ANOVA was used to compare quantitative data if they had a normal distribution. For data with nonnormal distribution, non-parametric Mann-Whitney U test and Kruskal-Wallis H test were used. In all statistical analyzes, a significance level of less than 0.05 was considered. Data analysis was performed with SPSS 19.

Table 1. Primer sequences and temperature protocol in Real-Time PCR; Genes (A), Primer Sequence (B), Product Size (C), Temperature Protocol (D), forward (F), reveres (R)

A		B	C	
GATA-3	F	5′-GTCCTGTGCGAACTGTCA-3′	139 bp	60°C (30 second)
	R	5′-GATGCCTTCCTTCTTCATAGTCA-3′		
T-bet	F	5′-GATGCGCCAGGAAGTTTCAT-3′	83 bp	95°C (15 second)
	R	5′-GCACAATCATCTGGGTCACATT-3′		
EF-1	F	5′-CTGAACCATCCAGGCCAAAT-3′	59 bp	95°C (30 second)
	R	5′-GCCGTGTGGCAATCCAAT-3		

Results and discussion
Demographic and clinical characteristics

In terms of age and gender, no significant differences were observed between the two groups (Table 2). In addition, to assess the patient's allergic status, the peripheral blood eosinophil counts of the two groups were evaluated, but no significant difference was observed between the two groups ($P = 0.421$). Given that family history of asthma may influence subsequent evaluation; this condition was evaluated between the two groups and the results showed that there was no significant difference between the two groups ($P = 0.071$). The pulmonary

function assessment of the two groups was also performed using spirometry. The results showed no significant difference in the percentages of FEV1 and FVC in asthma patient.

Table 2. Comparison of demographic and clinical characteristics of the two groups (Patients treated with montelukast and Patients treated with Gan-Cao); Characteristics (A), Patients treated with montelukast (B), Patients treated with Gan-Cao (C), and P-value (D)

A	B	C	D
Age (year)	14.8 ± 6.2	13.67 ± 7.3	0.421
Gender			
Male (number %)	15 (60%)	13 (52%)	0.341
Female (number %)	10 (40%)	12 (48%)	0.372
Eosinophils (number/µl)	824.3 ± 48.9	831.7 ± 39.2	0.421
Family history (number)	2 (8%)	4 (16%)	0.071
FEV1 (%)	(46-81) 61.37%	(43-87) 60.88%	0.123
FVC (%)	(55-97) 76%	(52-94) 72%	0.093

Table 3. Relationship between disease risk factors and expression of T-bet (#) and GATA3 genes (##); Variable (A), 12 years old> (B), 12 years old< (C), *P*-value (*P*), Male (E), Female (F), Having (G), Not Having (H)

A	Age			Gender			Family history		
	B	C	*P*	E	F	*P*	G	H	*P*
##	17.0 ± 4.4	17.72 ± 2.7	0.663	16.95 ± 2.4	17.65 ± 3.5	0.668	16.65 ± 3.05	17.5 ± 2.7	0.628
#	3.31 ± 0.7	3.38 ± 0.9	0.968	3.99 ± 1.01	4.22 ± 0.35	0.436	4.42 ± 0.73	4.01 ± 0.3	0.217

Genetic Evaluations

In order to evaluate the pattern of specific immune responses in the study population, two key transcription factors that reflect the type of TH1 (Tbet) and TH2 (GATA3) responses were examined. As Figure 1 shows, the expression of the GATA3 gene was increased in patients of the second group (patients treated with Gan-Cao), but this increase was not significant compared to the first group (patients treated with montelukast) (*P* = 0.102). Evaluation of Tbet gene expression in PBMC of the two groups showed that the difference was not statistically significant (*P* = 0.888).

Also, to evaluate the effect of different risk factors of this disease to give immune responses in patients with asthma, we examined the expression status of T-bet and GATA3 transcription factors. Since age can play an essential role in the incidence of the disease,

the age variable was divided into two categories: less than 12 years (children) and equal to or more than 12 years (adolescents). As shown in Table 3, there was no significant difference in the expression of T-bet and GATA3 transcription factors based on the age of the patients (p> 0.05). Gender also had little effect on the expression of these transcription factors.

Because the number of cells expressing these transcription factors can be different between the study population, so to eliminate these interfering changes and better observe the changes in TH1/TH2 responses, the T-bet/GATA3 expression ratio was calculated and between the two groups. As Figure 2 shows, the T-bet/GATA ratio in the first group (1.46 ± 0.13) did not have differences significantly compared to the second group (1.33 ± 0.20) (*P* = 0.061).
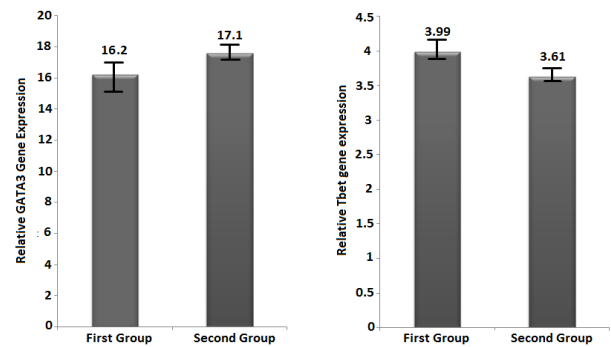

Figure 1. The Relative expression of GATA3 and Tbet genes in two groups; First group is treated with montelukast and the second group is treated with Gan-Cao
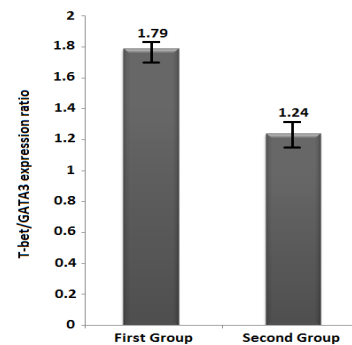

Figure 2. T-bet/GATA3 expression ratio between two groups; First group is treated with montelukast and second group is treated with Gan-Cao

Allergic asthma is a complex heterogeneous disease characterized by reversible inflammation and airway constriction, increased bronchial sensitivity, and infiltration of lymphocytes and eosinophils (2). T lymphocytes, eosinophils and mast cells play an

important role in this disease (25). Environmental and genetic factors affect the severity of asthma reactions, and factors such as viruses and allergens can change the course of the disease (26). A family history of asthma may be a risk factor for the disease (27). According to the study population, there was no significant relationship between the family history, age and gender of the two groups. This evaluation shows that there is no significant difference between the two groups, and the influence of these factors cannot affect the main results of the experiment (comparison between Gan-Cao and montelukast).

Also, in this study, there was no significant difference between the two groups in terms of eosinophil levels in patients' blood. Elevated eosinophil levels are not limited to allergic asthma, but can also occur in the non-allergic type (26). Studies have shown that Leukotriene modulators such as montelukast have the ability to reduce eosinophil and inflammatory cytokine levels in patients with allergic asthma (27). The results of this study showed that Gan-Cao acted like montelukast and reduced the levels of eosinophils and inflammatory cytokines.

The results of some studies show that eosinophilic asthma can almost be associated with decreased FEV1 levels (28). This decrease could be due to an increase in eosinophils in the disease. The activity of eosinophils, which is related to the depletion of granules, leads to the release of the significant alkaline protein, cationic protein, and peroxidase, which can damage airway epithelial cells and disrupt airflow (29). The present results showed that the level of spirometry indices (FVC, FAV1) which indicates the volume of respiratory discharge in patients with asthma was not significantly different between the two groups.

Liang *et al*. (30) using the RT-PCR technique in patients with asthma showed that IL-4 levels increased in these patients but IFN-γ levels and T-bet/GATA-3 ratio also decreased in these patients. Also in a study conducted by Yong *et al*. (31), patients with allergic asthma used the RT-PCR technique and their results showed that the T-bet/GATA-3 ratio in these patients was reduced. All of these studies confirm the important and undeniable role of the two transcription factors GATA-3 and T-bet and the ratio of the two in patients with asthma. Increasing or decreasing the expression of these two transcription

factors, which indicate the increase or decrease of Th1 and Th2 cells, changes the ratio of these cells in the body (32). These changes are associated with an increase in the cytokines IL-4, IL-13, as well as an increase in antibodies affecting Th-2 cells and asthma aggravators including IgE (33, 34). According to the results of the present study, the expression of the GATA3 gene, T-bet gene, and the expression ratio of T-bet / GATA-3 genes were not significantly different between the two groups. It showed that Gan-Cao can affect the expression of these genes just as much as montelukast. Therefore, this Chinese herb can be used as an alternative or supplement medicine to treat allergic asthma in children.

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