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Down-regulation of TIPE2 expression in peripheral blood mononuclear cells from patients with asthma in childhood

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Abstract The breakdown of immune homeostasis contributes to the pathogenesis of asthma. Tumor necrosis factor α induced protein-8 like-2 (TIPE2) is an immune homeostasis maintaining gene identified recently. Wondering whether TIPE2 is associated with asthma, we examined the TIPE2 (in mRNA and protein levels) and FCER1A (in mRNA levels) in peripheral blood mononuclear cells (PBMC) from 37 asthma patients and 34 healthy controls by real-time reverse transcription-polymerase chain reaction analysis (RT-PCR) and Western blot analysis. The TIPE2 mRNA expression levels were significantly down-regulated in asthma patients compared with healthy controls ($P<0.001$), while the FCER1A mRNA expression levels was increased in asthma patients compared with healthy controls ($P=0.008$). Furthermore, the TIPE2 mRNA expression levels correlate with IgE negatively ($r=-0.433$, $P=0.007$), the FCER1A mRNA expression levels correlate with IgE positively ($r=0.658$, $P<0.001$) and the TIPE2 mRNA expression levels correlate with FCER1A mRNA expression levels negatively ($r=-0.423$, $P=0.009$) in all asthma patients. The TIPE2 protein expression levels were also down-regulated significantly in asthma patients compared with healthy controls ($P=0.019$). These results indicate that the decreased TIPE2 is associated with asthma closely and suggest TIPE2 is an important gene in the pathogenesis of asthma.

Key words:

Asthma; Childhood; Tumor necrosis factor α induced protein-8 like-2; Real-time reverse transcription-polymerase chain reaction analysis; Western blot analysis

Introduction

Asthma in Childhood is a heterogeneous disease comprising different phenotypes characterized by the symptoms of breathlessness, cough, and wheezing [1]. It shows as a chronic inflammatory condition of the airways and can wax and wane over time. Approximately 8.2% of the general population is affected [2].

In the past 20 years, we have known about the pathogenesis of asthma more and more, and it was improved with the publication of clinical guidelines from the National Asthma Education

and Prevention Program in 1991, 1997 and 2002 [3;4;5]. Since the 1980s, the allergy mechanism and helper T cells (Th1 / Th2 balance disorders) theory occupied the leading position to the pathogenesis [6], but the two theories can't explain all the asthma clinical manifestations and treatment response. Recent research suggests T regulatory cells (Tregs) might involve in the pathogenesis of the bronchial asthma. Tumor necrosis factor α induced protein-8 like-2 (TIPE2) was also confirmed to be a critical immuno-regulatory molecule involved in the immunosuppressive function of CD4+CD25+Tregs [7]. Although the pathogenesis of asthma is incompletely understood, dysfunction of immune regulation mechanisms, such as the abnormality of immune homeostasis maintaining genes, is an important contributor to the development of asthma, especially in children.

So far, the two classes of immune homeostasis maintaining genes are discovered. The first class genes have the functions limiting the strength of immune cell activation and expansion. These include interleukin [IL]-10, genes encoding inhibitory cytokines (such as transforming growth factor [TGF]- β , negative regulation genes of the antigen receptor), Toll-like receptor (TLR) signaling (such as cytotoxic T lymphocyte antigen [CTLA]-4, suppressor of tumor necrosis factor alpha-induced protein 3 [TNFAIP3]), cytokine signaling (such as SOCS-1) and genes encoding repressive transcription factors (such as Foxp3). The second class controls the death of cell, which includes Fas, Bax, Bim, the caspases-8 and caspases-10 [8]. Severe inflammatory diseases ensue, when the expression of these genes is down-regulated or absent, and immune homeostasis fails. TGF- β is an immunosuppressive cytokine and its expression level decreases in asthma patients [9]. CTLA-4, which is considered as a susceptibility gene for asthma, is a negative regulator of T cell activation. The serum level of soluble CTLA-4 elevates in asthma patients [10]. SOCS-1 can regulate both TLR signaling and antigen receptor negatively, and its dysfunction is considered as a pathogenic factor of asthma [11]. The TNFAIP3 restricts nuclear factor κ B (NF- κ B) dependent signaling and prevents inflammation. Multiple regions of polymorphisms in the TNFAIP3 gene are associated with asthma in childhood [12]. Foxp3 also plays an important role in asthma condition because it is the master control gene for the development and function of CD4+CD25+ Tregs [13]. Most of genes which maintaining the

immune homeostasis are involved in the pathogenesis of asthma as well as controlling the death of cell [14].

TIPE2 is recently identified as a novel gene independently required by maintaining immune homeostasis, belonging to tumor necrosis factor- α -induced protein-8 (TNFAIP8) family [8]. TIPE2 is preferentially expressed in lymphoid tissues, and its deletion in mice leads to multi-organ inflammation. Experiments in vitro also demonstrate that TIPE2 can inhibit activating protein 1 and NF- κ B activation, and TIPE2 deficient cells are hyper-responsive to T cell receptor (TCR) and Toll-like receptor (TLR) activation [15]. Considering the normal expression of TIPE2 gene in the immune system is essential to prevent hyper-responsiveness and maintains immune homeostasis, we hypothesized that the expression of TIPE2 gene in asthma patients may be different from it in the normal individuals and involved in pathogenesis of asthma. Therefore, in the present study, we compared the TIPE2 mRNA and protein expression levels in peripheral blood mononuclear cells (PBMC) from asthma patients with that from the healthy controls. After we analyzed the correlations of TIPE2 mRNA expression levels with asthma activity and the mRNA expression levels of FCER1A (Fc fragment of IgE, high affinity I, receptor for alpha polypeptide, one of IgE search-evade antagonistic type 1 hypersensitivity reactions of the key genes)[16;17], We found down-regulation of TIPE2 mRNA expression in asthma patients and its close association with FCER1A levels and the level of IgE. TIPE2 protein expression levels in asthma patients are also down-regulation compared with healthy controls. These results suggest that TIPE2 may participate in the pathogenesis of asthma.

Materials and methods

Human subjects

We enrolled 37 patients fulfilling the criteria of the PRACTALL consensus report [18], which is made by Europe allergy clinical immunology and the American of allergy, asthma and clinical immunology (EAACI/ AACI/AAAAI) in 2008 for this study. It is suitable for the children's asthma diagnosis and treatment guidelines. 34 healthy controls were also recruited considering

the sex- and age-matched, all of whom did not have any asthma conditions. Each child was taken blood samples anti-coagulated in Qilu Children's Hospital (from January to February in 2011). In order to exclude the influence of drug on TIPE2 expression, peripheral bloods were sampled from all the patients before they took any drug. All patients signed an informed consent form prior to the initiation of the study. The characteristics of the patients and healthy subjects are summarized in Table 1.

Laboratory assessments

For asthma patients, laboratory assessments included serum levels of eosinophilic granulocyte, IL-4, IL-10, IL-12, IgE and INF- γ . We also detected food allergy test in the serum from all the members using immune-fluorescence assay (IFA) according to the instruction of the manufacturer (ImmunoCAP100 System, Pharmacia, Sweden).

RNA and cDNA preparation from PBMC

Peripheral blood was sampled in sodium citrate-containing cell preparation tubes. PBMC were separated by density gradient centrifugation. Total RNA was extracted from PBMC (6×10^5) using TRIzol (Invitrogen, California, USA) and treated with RNase-free DNase (Qiagen, Hamburg, Germany) to remove genomic DNA contamination. RNA ($1 \mu\text{g}$) was reverse transcribed to cDNA using reverse transcription system kit (Promega, WI, USA).

Quantitative real-time PCR

The expression of TIPE2 and FCER1A gene were evaluated by quantitative real-time PCR. The level of β -actin mRNA was also detected as an internal control for each sample. The forward primer for TIPE2 was 5'-GGAACATCCAAGGCAAGACTG-3', the reverse primer for TIPE2 was 5'-AGCACCTCACTGCTTGTCTCATC-3'. The forward primer for FCER1A was 5'-GGTGGGATAGGGAGTGGAGT-3' and the reverse 5'-TGGTGCTTACTGTGCTGGAG-3'. For β -actin, we used the following primers: the forward 5'-GACTACCTCATGAAGATCCTCACC3' and the reverse

5'TCTCCTTAATGTCACGCACGATT3'. Real-time PCR was performed using the SYBR Green I real-time PCR kit in accordance to the instructions of the manufacture (Takara, Dalian, China) in an ABI PRISM 7000 Sequence Detection System (Perkin-Elmer, Norwalk, CT, USA). Amplification conditions were as follows: 95°C for 10s, followed by 35 cycles of 95°C for 5 s and 59°C for 41 s. Each sample was run in triplicate. The PCR products were run in an agarose gel and were confined to a single band of the expected size in all cases. A melting-curve analysis was also performed to ensure specificity of the products. The relative mRNA expression of TIPE2 and FCER1A was determined using the comparative ($2^{-\Delta\Delta C_t}$) method.

Western blot

Western blot was performed to determine the protein expression level of TIPE2 in PBMC. Cells were washed with ice-cold PBS, suspended in the lysate buffer containing 1% protease inhibitor, and then centrifuged at 12,000×g for 15 min at 4°C. Protein levels were quantified by the BCA method (Sangon, Shanghai, China), and equal amounts (45µg) of protein were separated in SDS–PAGE and the products were electro transferred onto nitrocellulose membranes. Membranes were then blocked with 1% BSA in Tris Buffer Saline +Tween (TBST) containing 0.1% Tween 20 for 1 h, and was incubated with rabbit anti-human TIPE2 antibody (1:250) or human β-actin antibody (1:1000) at 4°C overnight, followed by secondary antibodies (goat anti-rabbit IgG) conjugated with peroxidase for 1 h at room temperature. The protein bands were developed using the enhanced chemiluminescence method according to the manufacturer's instructions (ECL, Amersham Biosciences, and Buckinghamshire, UK) after the membrane was washed three times. Western blots were repeated at least three times for each sample.

Statistical analysis

Data were presented as mean ±standard deviation. With the number of respective experiment run in triplicate, the difference between TIPE2 and FCER1A mRNA levels was analyzed by Mann–Whitney test. Correlations analysis was carried out using Spearman's rank test. P values

less than 0.05 were considered statistically significant. All analyses were performed with the Graph Pad Prism software, version 11.0.

RESULT

Patient characteristics

The demographic characteristics, clinical manifestations and laboratory measurements were shown in Table 1. The asthma is the major indicator of morbidity and mortality of in asthma patients, which was found in 36 of 37 patients. The anaphylactic rhinitis, food allergy test and eczema history disease were found in 22, 29 and 34 patients, respectively. The P value of IL-4, IL-10, IL-12 and INF- γ in asthma patients compared with healthy controls is 0.121, 0.172, 0.425, 0.232 respectively, there were no statistically significant differences between them. Eosinophilic granulocyte and IgE were significantly increased compared with the healthy controls ($P < 0.01$).

Quantification of TIPE2 mRNA and protein expression in PBMC from asthma patients and healthy controls by real-time RT-PCR and Western blot analysis

We examined the expression of TIPE2 mRNA and protein express levels in PBMC from 37 asthma patients and 34 sex- and age-matched healthy controls using quantitative real-time RT-PCR and Western blot analysis. The results showed that the mean of TIPE2 mRNA expression levels in asthma patients (3.7414) were significantly lower than that in healthy controls (10.1791) ($P < 0.001$) (Fig. 1A and 1C, Fig.3). TIPE2 protein expression levels in asthma patients were significantly lower than that in healthy controls ($P = 0.019$) (Fig. 1B and 1C). We analyzed the association of TIPE2 expression with demographic characteristics, clinical manifestations and laboratory parameters. The results revealed that the TIPE2 mRNA expression levels correlated with IgE negatively ($r = -0.433$, $P = 0.007$) (Fig. 2).

Quantification comparison of TIPE2 and FCER1A mRNA expression in PBMC from asthma patients and healthy controls by real-time RT-PCR

FCER1A is the gene encoding for IgE binding α -subunit of the Fc ϵ RI maps to chromosome 1q21-23, the region implicated in susceptibility to multiple autoimmune disorders and/or allergy

[19]. Interestingly, altered expression of FCER1A has been observed in asthma patients [20].

Measuring the expression of FCER1A genes by real-time PCR can estimate the total IgE production [21]. We examined TIPE2 and FCER1A mRNA expression levels in PBMC samples from 37 asthma patients and 34 healthy controls using quantitative real-time RT-PCR. We found that the mean of FCER1A mRNA expression levels in asthma patients (42.6892) was higher than that in healthy controls (15.5568) ($P=0.008$) (Fig. 2), and a positive correlation between FCER1A mRNA expression and IgE ($r=0.658$, $P<0.001$) (Fig. 4).

The correlation between TIPE2 mRNA expression and FCER1A mRNA expression in asthma patients

Since IgE plays a central role in asthma pathogenesis and the FCER1A mRNA level in PBMC represents the serum level of IgE, we investigated the association of TIPE2 expression with FCER1A expression. Our results showed a negative correlation between TIPE2 and FCER1A mRNA expression levels ($r=-0.423$, $P=0.009$) (Fig. 5). This indicates that TIPE2 mRNA expression levels negatively correlate with serum levels of IgE in asthma patients.

Discussion

Immune homeostasis is maintained by multiple immune regulation genes, which cannot compensate for each other. The breakdown of immune homeostasis caused by dysfunction of these genes contributes to the pathogenesis of asthma. TIPE2 is a novel gene maintaining immune homeostasis and negatively regulating innate and adaptive immunity [8]. In this study, we first reported the down-regulation of TIPE2 mRNA and protein expression, and the negative correlation between TIPE2 and the disease activity in asthma patients.

The decreased expression of TIPE2 gene may contribute to the pathogenesis of asthma through regulating T cell activation, because hyper-activation of auto-reactive T cell is one of hallmarks of asthma [22]. TIPE2 is a negative regulator of T cell activation. Both CD4⁺ and CD8⁺ T cells immune responses are significantly augmented in TIPE2 gene knockout mice as compared with their littermate controls in infected condition [8]. Down-regulation of TIPE2 expression in childhood asthma patients may cause hyper-reactivity of CD8⁺ T cells. Down-regulation of

TIPE2 expression in asthma patients may also make auto-reactive T cells hypersensitive to autoantigens [23]. Recently research suggests that Tregs might be involved in the pathogenesis of the bronchial asthma. TIPE2 was also confirmed to be a critical immune-regulatory molecule involved in immunosuppressive function of CD4⁺CD25⁺ Tregs [7].

The continual activation of lymphocytes due to the down-regulation of TIPE2 expression may result in the increased levels of apoptotic lymphocytes and elevated the expression of Fas in asthma patients, which is a characteristic feature of asthma [24]. The direct role of TIPE2 in apoptosis is still a puzzle. TIPE2 was thought to contain a DED-like domain or death effector domain (DED), but its structure and topology differ from that of DED domains contained in caspase-8 or c-FLIP [25]. The reduced apoptosis effects on the activation of cell in TIPE2 knockdown and knockout cells indirectly. In addition, FasL-induced apoptosis was significantly blocked in TIPE2 knockdown cells [8]. The decreased native expression of Fas on lymphocytes may be related to airway inflammation in the patients with asthma. The increased expression of Fas induced by dexamethasone may be one mechanism of lymphocytes apoptosis [26]. TIPE2 has been proved to promote Fas-induced apoptosis through inhibiting the combination of NF- κ B with caspase-8. Therefore, the down-regulation of TIPE2 expression in PBMC from asthma patients may be responsible or partially responsible for the decreased levels of Fas in asthma patients. The direct relationship between the decreased TIPE2 expression and lymphocyte apoptosis needs further investigation in childhood asthma patients.

TIPE2 is preferentially expressed in lymphoid tissues, and its deletion in mice leads to multi-organ inflammation. TIPE2-deficient animals are hypersensitive to septic shock, and TIPE2-deficient cells are hyper-responsive to TLR (innate immunity) and TCR (adaptive immunity) activation. Importantly, TIPE2 inhibits the activation of activating protein-1 (AP-1) and NF- κ B by binding to caspase-8. The inhibited caspase-8 can significantly block the hyper-responsiveness of TIPE2-deficient cells. These results show that TIPE2 is an essential negative regulator of TLR and TCR function, and its selective expression in the immune system prevents hyper-responsiveness and maintains immune homeostasis. [8,15]. The decreased expression of TIPE2 gene may raise the appearance of hyper-responsiveness and the elevation

of pro-inflammatory cytokines, which are the characteristic in children asthma. The serum levels of hyper-responsiveness molecule and inflammatory cytokines, such as IgE, IL-4, IL-6, IL-12 and IFN- γ , increase in asthma patients significantly with incompletely understood mechanisms [27]. The major source of the pro-inflammatory cytokines is from macrophage and mononuclear cell [28]. The expression of TIPE2 in mouse macrophages regulates Nuclear Factor (NF)- κ B signaling negatively, and then inhibits the secretion of some inflammatory cytokines. TIPE2 knockdown and knockout macrophages in mice is hypersensitive to TLR stimulation, producing more IL-6 and IL-12 than the wild-type macrophages [8;29]. Therefore, the down-regulation of TIPE2 expression in PBMC from asthma patients may be responsible or partially responsible for the elevated levels of inflammatory cytokines.

In recent years, a number of studies indicate that IgE plays a central role in the pathogenesis of asthma. Allergic sensitization is defined by the production of IgE against environmental antigens which can lead to diseases that include asthma, rhinitis and atopic dermatitis [30]. FCER1A is an important immunity-related gene that encodes the ligand-binding subunit of the high-affinity IgE receptor [17;31]. It is the gene encoding for IgE binding α -subunit of the Fc ϵ RI maps to chromosome 1q21-23. In a recent population-based GWAS, they demonstrated that the gene encoding the α chain of the high-affinity IgE receptor (FCER1A) are strongly associated with total IgE levels^[32]. The high-affinity receptor for IgE (Fc ϵ RI), which is a tetramer contained a ligand-binding alpha chain (FCER1A), a signal-augmenting beta subunit (FCER1B), and a signal-transduction gamma chain dimer (FCER1G), plays a key role in the induction and maintenance of allergic responses. In addition to participate in IgE-mediated antigen presentation, it also controls the activation of mast cells and basophils through IgE, [33]. The combination of IgE and high affinity receptor (Fc ϵ RI) is the first link of allergic disease. The activation signaling of from the IgE and Fc ϵ RI trigger the activation signal-transduction to combine beta and gamma chain [34]. Fc ϵ RI, which is the interaction link between Fc ϵ R and IgE, is the key protein who induces IgE type 1 hypersensitivity reactions. There have been many research reports about the

interaction between IgE and FcεRI. The combination of IgE and FcεRI on the surface of allergic reaction cells is a bidirectional effect, It can not only inhibit the degradation of FcεRI in order to increase the quantity of the cell surface expression, but also extend the IgE half-life to adjust to the serum level of total IgE [35]. Its effect on the secretion of cell factors in the interaction mechanism of allergic diseases has been revealed clearly, but the cell surface FcεRI receptor expression regulation mechanism need a further discussion. We found that FCER1A mRNA levels significantly increased compared with the healthy controls. There was a positive correlation between the FCER1A mRNA expression and disease activity in asthma patients compared with healthy controls. These results are in accordance with the previous studies indicating an important role of FCER1A in the pathogenesis of asthma [17]. We further analyzed the correlation between the TIPE2 expression and FCER1A expression, and found a significant negative correlation between them. This result indicates that there is a close association between TIPE2 expression and the production of FCER1A. Just as we hypothesized before, our result is concordant with H. Sun et al; the decreased TIPE2 can cause the disorder of immune function and the hyper-inflammatory cytokines. Our data has confirmed that the decreased TIPE2 causes the significantly increased of IgE in children asthma, FCER1A is merely a gene encoding for IgE binding α -subunit of the FcεRI, the increased FCER1A is a normal reaction to the increased IgE. It is very natural to speculate that the increased FCER1A may be induced by the decreased TIPE2. It is worth to investigate the signaling pathway further that how TIPE2 influence the producing of FCER1A.

Immunosuppressive drug treatments, such as glucocorticoids(such as dexamethasone), are usually used to release the symptoms of asthma, though it can wax and wane over time [36]. The mRNA and protein expression of TIPE2 in PBMC from asthma patients was analyzed before treatments by using any glucocorticoids drug. Investigating potential influence of glucocorticoids drug on TIPE2 expression, assessing the stability of TIPE2 expression in

individual patients and healthy controls will be the emphases of our work being process, which has a preliminary result, and need to be confirmed.

In conclusion, we first report that the mRNA and protein expression levels of TIPE2 are decreased in PBMC from asthma patients in childhood compared with the healthy controls, which is negatively correlated with FCER1A and serum levels of IgE. These findings suggest that TIPE2 is an important gene in the pathogenesis of asthma.

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Figure legends

Figure 1 TIPE2 mRNA and protein expression levels in PBMC from asthma patients and healthy controls. Real-time RT-PCR and Western blot analysis were performed to quantify the expression of TIPE2 in PBMC from asthma patients (n=37) and healthy controls (n=34). (A) TIPE2 mRNA levels in PBMC from asthma patients and healthy controls as determined by Real-time RT-PCR. β -actin was used as a loading control, whereas H₂O was used as the background control. (B) TIPE2 protein levels in PBMC from asthma patients and healthy controls was determined by Western blot analysis. β -actin was used as a loading control. (C) The light density ratio of purpose strip compared with β -actin. There is a significant decrease in TIPE2 expression both in mRNA and protein levels in asthma patients compared with that of healthy controls ($P<0.001$ and $P=0.018$, respectively).

Figure 2 Negative correlations between TIPE2 mRNA expression levels and IgE for all asthma patients (n=37).

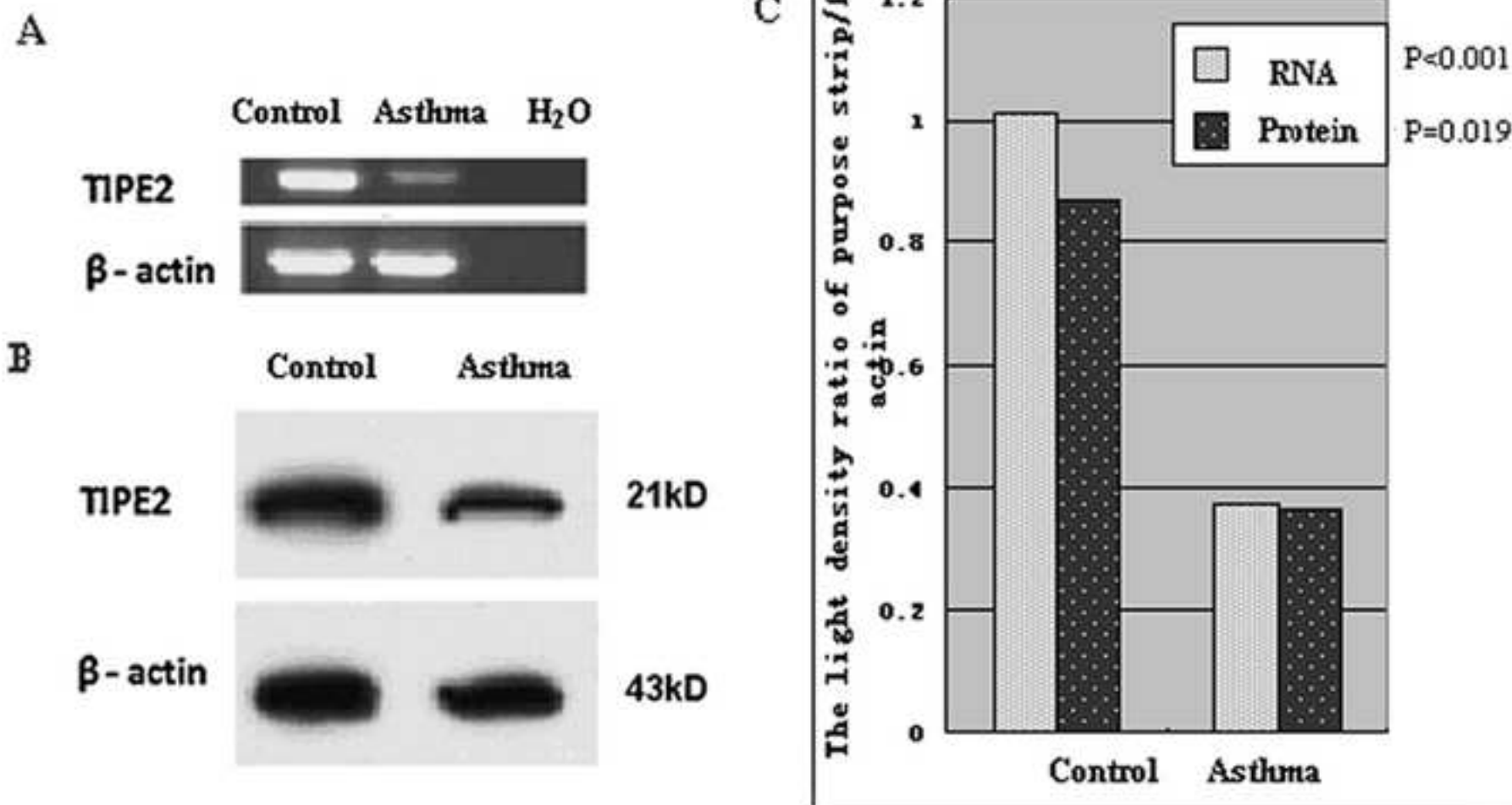
Figure 3 Comparison of TIPE2 and FCER1A mRNA expression levels in asthma patients and healthy controls. Real-time RT-PCR was performed to qualify the expression of TIPE2 and FCER1A gene in PBMC from asthma patients (n=37) and healthy controls (n=34). There is a significant decrease TIPE2 expression in asthma patients compared with healthy controls ($P<0.001$). The medians is 3.7414 in asthma patients, compared with 10.1791 in healthy controls. There is a significant increase in FCER1A expression in asthma patients compared with healthy controls ($P=0.008$). The median is 42.6892 in asthma patients, compared with 15.5568 in healthy controls.

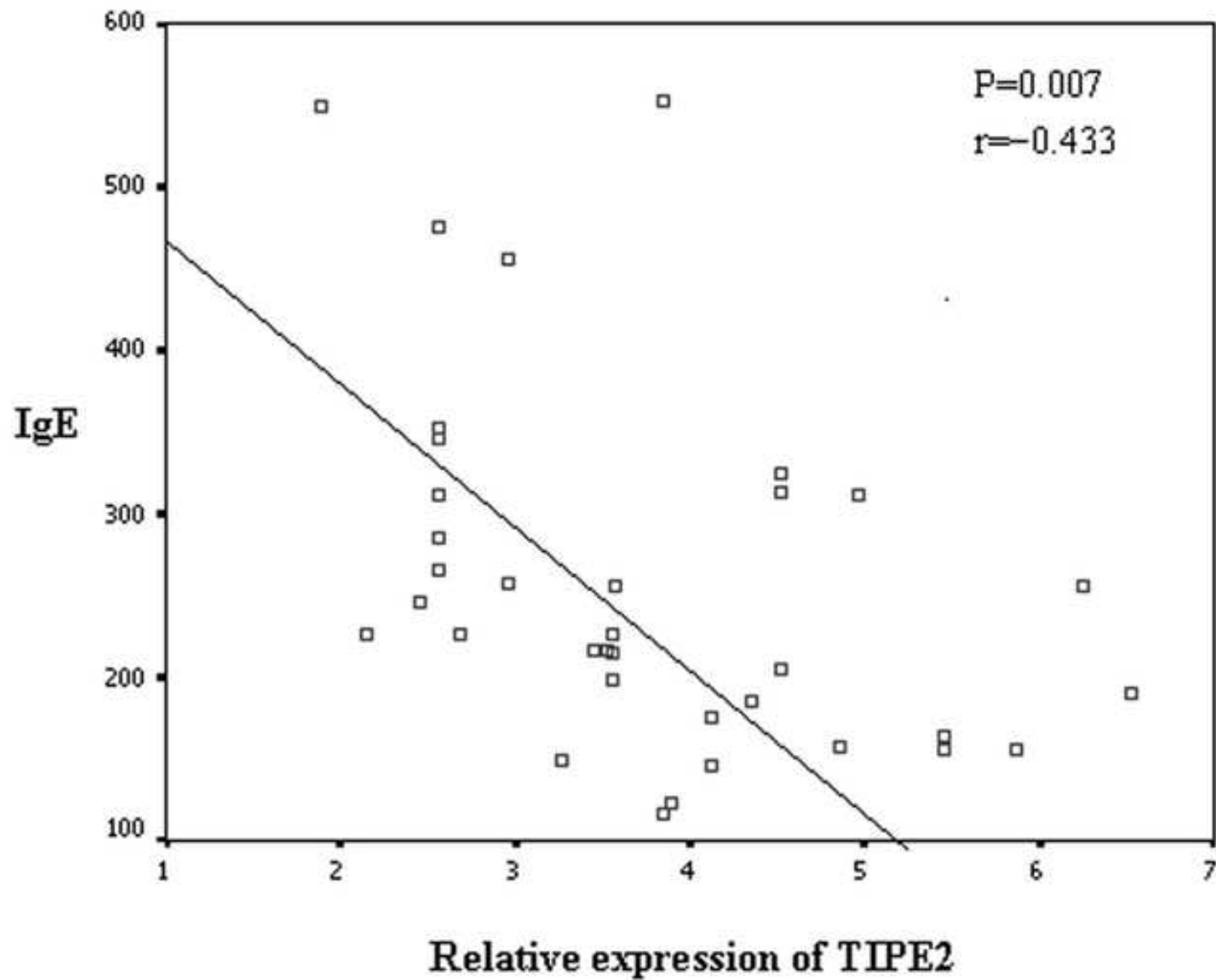
Figure 4 Positive correlations between the FCER1A mRNA expression levels and IgE for all asthma patients (n=37).

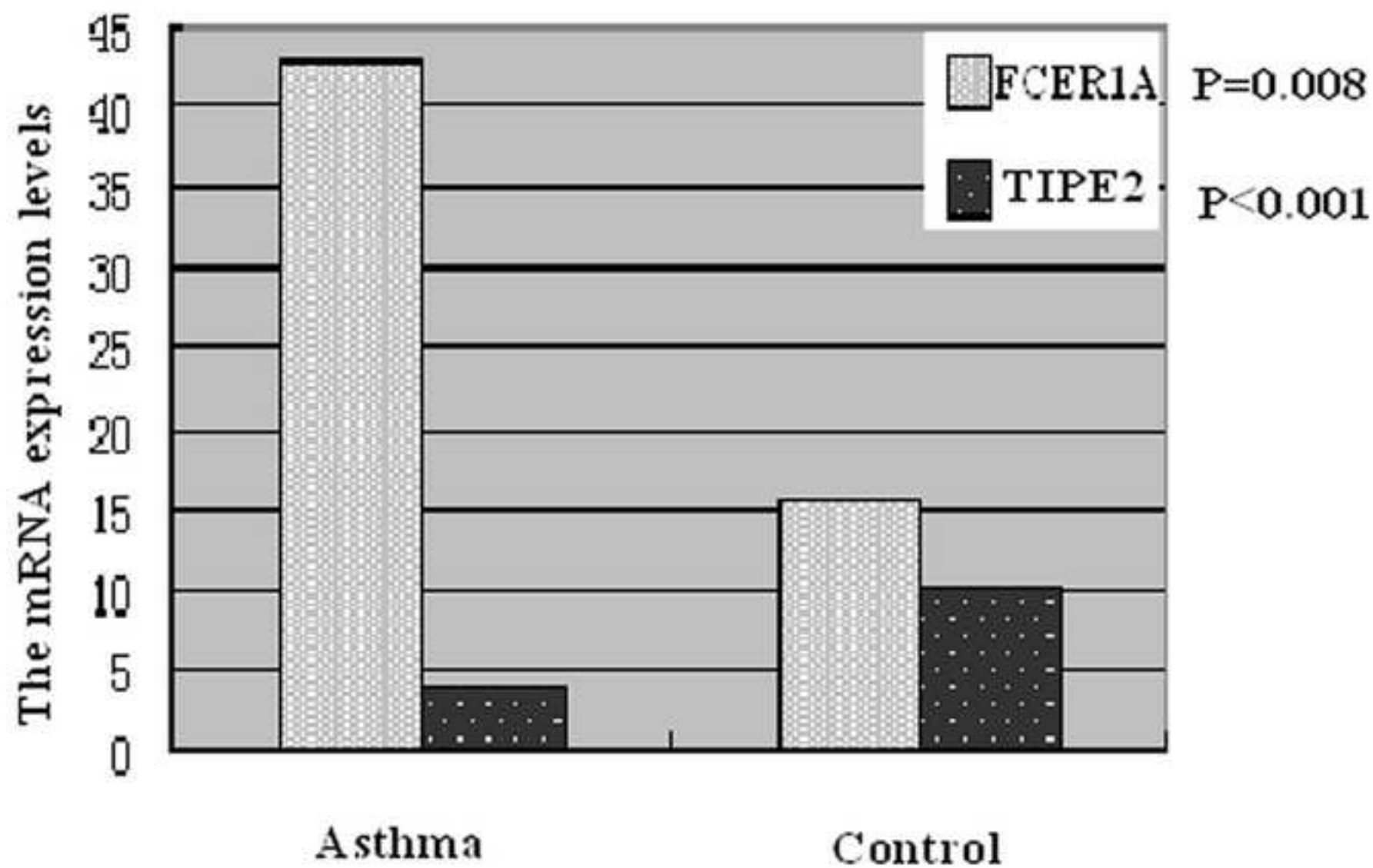
Figure 5 Negative correlations between the TIPE2 mRNA expression levels and FCER1A for all asthma patients (n=37).

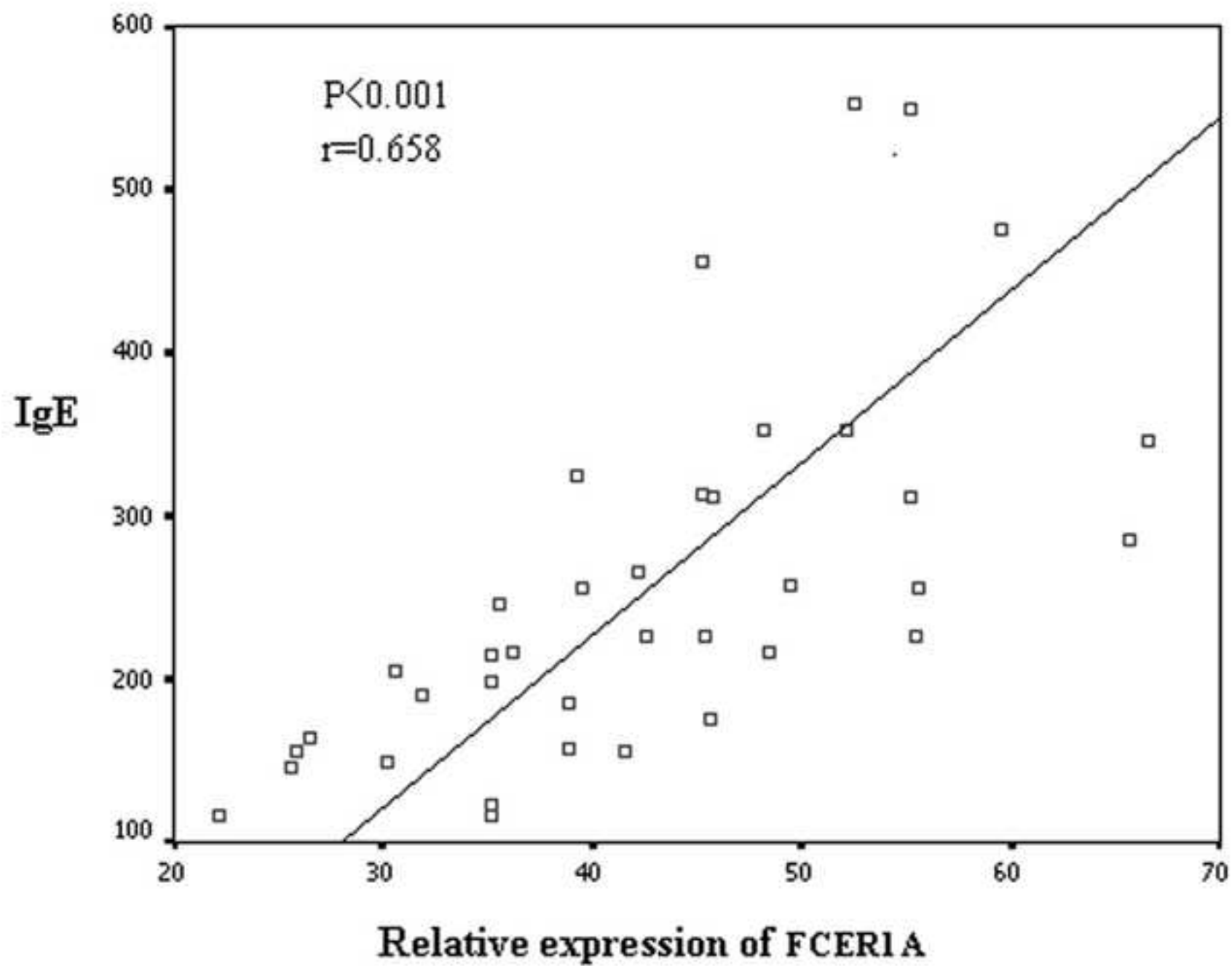
Highlights

- > TIPE2 is down-regulated in asthma significantly compared with healthy controls both in mRNA and protein levels.
- > TIPE2 mRNA levels correlate with IgE and the FCER1A in asthma patients negatively.
- > The decreased expression of TIPE2 gene is closely associated with asthma.
- >The result suggest an important role for TIPE2 gene in the pathogenesis of asthma.









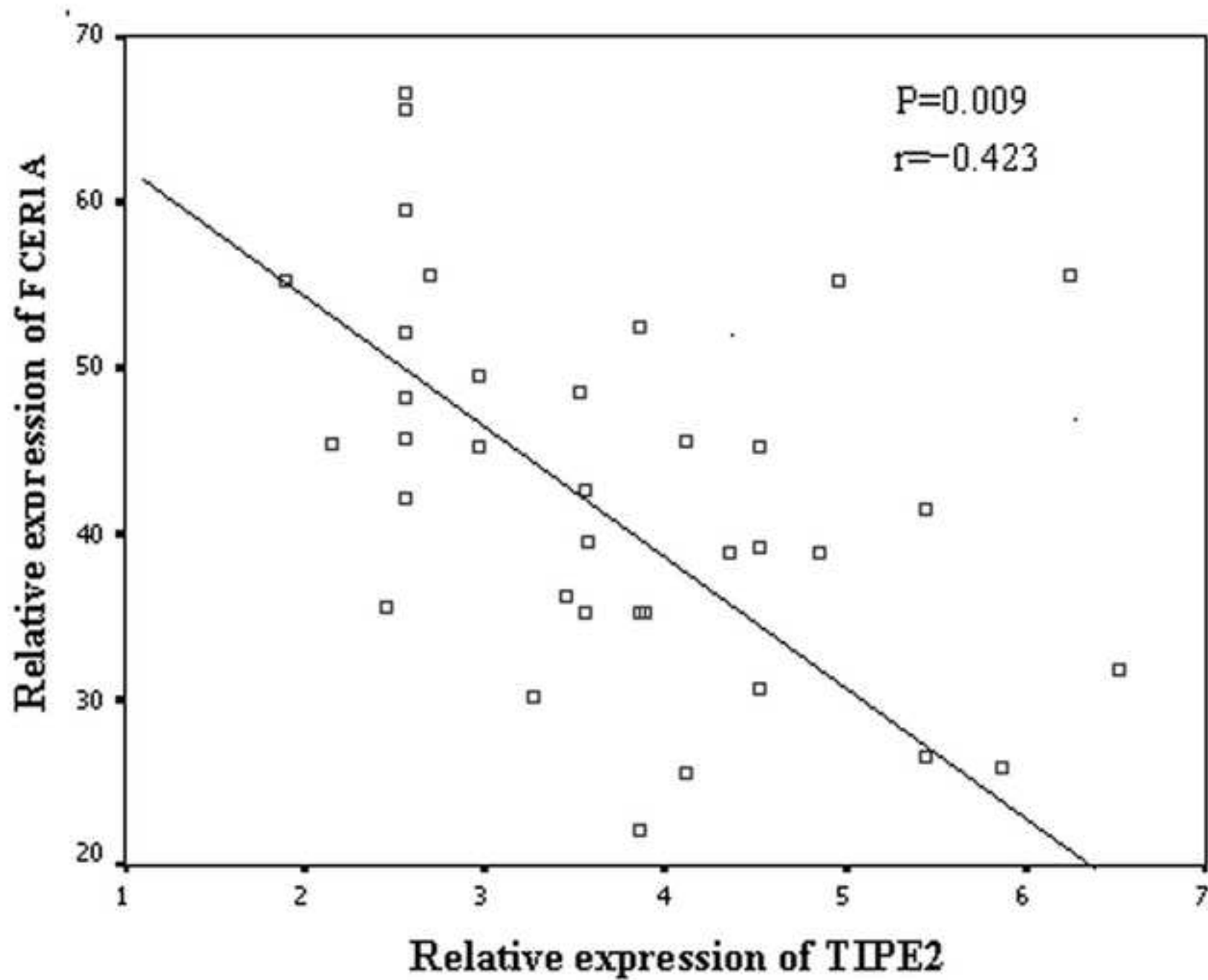


Table 1 Characteristics of the studied subjects.

Characteristics	Asthma patients (n=37)	Healthy controls (n=34)
Female	18	15
Male	19	19
Age, years (range)	3.52 (1.2-8)	3.85 (2.2-7.5)
Asthma	36/37	0
Anaphylactic rhinitis	22/39	0
Eczema history	34/37	0
Food allergy test	29/37	0
Eosinophilic granulocyte (10 ⁹ /L)	0.75±0.45	0.12±0.16
IgE (IU/ML)	255.95±62.16	45.25±22.16
IL-4(pg/ml)	12.6±1.86	16.4±2.16
IL-10(pg/ml)	3.5±0.25	4.5±0.20
IL-12(pg/ml)	42±4.57	59.0±5.89
INF- γ (pg/ml)	11.2±2.19	17.9±2.59

Values are mean $\pm$ standard deviation, unless stated.