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STAT6 PROBABLY INHIBITS TRANSCRIPTION FACTOR PAX-5 VIA ID2 AND E2A IN THE PATHOGENESIS OF ALLERGIC BRONCHIAL ASTHMA

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ABSTRACT

Background. Asthma pathogenesis is known to be based on cytokine and transcription factors activities. Putative interactions between two B-lymphocyte transcription factors STAT6 and PAX-5 was analyzed.

Methods. Peripheral blood lymphocytes from allergic, non-allergic asthma patients and healthy persons were used. RT-PCR was performed to evaluate PAX-5 mRNA levels. STAT6 and pSTAT6 levels were analyzed using western blotting.

Result. PAX-5 mRNA expression in ABA with normal IgE levels correlated with STAT6 mRNA levels. In ABA, effects of IL-4 caused a more significant decrease of PAX-5 mRNA compared with healthy persons. Negative correlation between PAX-5 mRNA expression and STAT6 levels shown in high levels of mRNA PAX-5 and low levels of STAT6 groups.

Conclusion. The discovered associations between STAT6 levels and PAX-5 mRNA expression levels suggest that STAT6 may negatively influence PAX-5 transcription in ABA patients, thus, probably, promoting differentiation of B-cells into plasma cells.

KEYWORDS : PAX-5, STAT6, Id2, bronchial asthma

Introduction. Intracellular signaling system of lymphocytes are known to be key factors in the development of various pathological processes: tumors, allergic inflammation, immune deficiencies. We have recently reviewed the role of the signaling system of the JAK-STAT (Janus-kinase - signal transductor and activator of transcription) in the development and course of bronchial asthma (BA) [1, 2, 3]. We have also analyzed the role of transcription factor PAX-5 in allergic BA [4]; as far as we know, PAX-5 participates in the processes of immunoglobulin E synthesis and receptor FcεR II (CD23) in lymphocytes as well as a factor STAT6 [6, 7]. The participation of these two proteins in the crucial processes for BA pathogenesis may suggest the interference of these transcription factors, so the purpose of the current work was to identify possible interactions of PAX-5 and STAT6 in the mechanisms of asthma development.

Materials and methods. 39 healthy control persons, 91 patients with allergic bronchial asthma (ABA) and 69 with nonallergic bronchial asthma BA (NABA) were examined in the Department of Hospital Therapy by academician M.V. Chernorutsky of Pavlov First Saint Petersburg State Medical University, St. Petersburg. The diagnosis was established according to the GINA (Global Initiative for Asthma, 2010) classification.

In the study, we used lymphocytes from peripheral blood of healthy individuals and patients with asthma isolated on density gradient Medium Lymphoseparation ("ICN") using standard methods for isolation of mononuclear cells followed by removal of monocytes with deposition on plastic in the conditions of incubation in IMDM medium with 37ºC for 40 min.

24-hour incubation of lymphocytes was carried out at 37°C in medium IMDM in the presence of IL-4 at a concentration of 10 ng/ml; IMDM was also added in samples incubated without IL-4.

Western blotting. After incubation lymphocytes were placed on the ice and all further procedures were carried out at 4ºC. Cells were washed once in cold phosphate buffer saline solution. Total lysates were obtained by adding to the samples 0.1 ml of lysing buffer containing protease inhibitors. After incubation for 10 min at 4ºC, the cell lysates were centrifuged at 10000 rpm.

¼ of the buffer solution for electrophoresis samples was added to supernatant, and incubated for 5 min at 100ºC. Electrophoretic separation of proteins was performed in polyacrylamide gel at 30 mA for 2 hours. Separated proteins were transferred onto nitrocellulose membrane. Immunoblotting was performed in accordance with the method ECL Western blotting protocols (Amersham). The chemiluminescent emission was detected by exposure on x-ray film. STAT6 polyclonal antibody (Cell Signaling Technology, USA) were used. Secondary antibodies were goat antibodies developed against rabbit immunoglobulins conjugated with horseradish peroxidase (GAM-HRP, Sigma, USA).

RT-PCR. The mRNA expression of PAX-5 was assessed by carrying out RT-PCR (reverse transcription-PCR – reverse transcription – polymerase chain reaction (PCR)) of nucleic acids isolated from peripheral blood lymphocytes. PCR was carried out in the amplifier "iCycler" (BIORAD) as follows: initiation at 95°C for 4 minutes, 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and polymerization at 70°C for 30 s. The final polymerization was carried out at 72°C within 7 minutes. Amplification products were subjected to electrophoresis in 1.5% agarose gel and colouring with ethidium bromide. The result of electrophoresis after photographing under UV light were analyzed by the software Gel-Pro 3.1. The level of mRNA expression of PAX-5 was evaluated relative to the level of β-actin.

The primers for PAX-5 and β-actin were developed on the basis of the known sequences (GenBank). (PAX-5 5': 5'- ACT GGA TGG AGA GGG AGC TT-3' and PAX-5 3': 5'- GGC TCT ACC TGG CTG TTC TG-3'; β-actin 5': 5'-TCC TGT GGC ATC CAC GAA ACT-3' and β-actin 3': 5'-GAA GCA TTT GCG GTG GAC GAT-3').

Statistical processing of research results was performed using the program SPSS 13.0 using methods and criteria nonparametric statistics for a small number of observations: criteria of Wilcoxon, Mann-Whitney, Pearson correlation coefficients (r), Spearman correlation coefficient (ρ). To assess the normality of the distribution Kolmogorov-Smirnov test was used.

Written informed consent was obtained from all subjects, and the medical ethics committee of First Pavlov State Medical University approved the study.

Results. To identify possible transcription factors PAX-5 and STAT6 interactions in the pathogenesis of allergic asthma the study was based on a step-by-step analysis.

The first step of the study was the establishment of correlation between the expression levels of the investigated molecules in healthy persons and in the two variants of the disease (allergic (ABA) and non-allergic (NABA)) (table 1).

Table 1. Correlation (r) of mRNA PAX-5 expression with STAT6 and pSTAT6 levels (integrated density relative to β-actin) in healthy and BA

Groups of patients	STAT6	pSTAT6
Healthy controls	r=-0,342; p=0,173; n=20	r=-0,456; p=0,043; n=21
ABA	r=-0,412; p=0,005; n=51	r=-0,352; p=0,023; n=52
NABA	r=-0,237; p=0,105; n=44	r=-0,165; p=0,255; n=46

Table 1 shows that the correlation between the investigated molecules was negative both in the control group, and in the two BA variants. In the control group, the most strong and significant correlation was found between mRNA PAX-5 and pSTAT6 but in the group of ABA patients it was found between mRNA PAX-5, STAT6 and pSTAT6. In the group of NABA patients revealed negative correlation had no statistical significance.

The second step of the study was the analysis of correlations between the expression levels of the investigated molecules in the groups of ABA patients of different severity (table 2).

Table 2. Correlation (r and ρ) of mRNA PAX-5 expression with STAT6 and pSTAT6 levels in BA different severity groups

BA severity	BA	STAT6	pSTAT6
Mild	ABA	r=-0,464; p=0,041; n=23	r=-0,216; p=0,308; n=23
	NABA	ρ=-0,500; p=0,667; n=3	ρ=0,500; p=0,667; n=3
Moderate	ABA	r=-0,598; p=0,011; n=19	r=-0,540; p=0,017; n=21
	NABA	ρ=-0,087; p=0,770; n=14	ρ=-0,682; p=0,019; n=15
Severe	ABA	r=-0,225; p=0,533; n=11	r=-0,416; p=0,266; n=11
	NABA	ρ=-0,413; p=0,293; n=9	ρ=-0,124; p=0,773; n=8

Table 2 shows that significant correlations between mRNA PAX-5 and STAT6 and phosphorylated form of STAT6 (pSTAT6) were detected in moderate ABA patients.

Significant correlation between mRNA PAX-5 and STAT6 was only detected in mild ABA patients.

The correlation between the investigated molecules was not significant in NABA patients with different severity of the disease.

The third step in factors PAX-5 and STAT6 interaction in the pathogenesis of allergic asthma analysis (taking into consideration their importance in switching B-lymphocytes to IgE synthesis) was to evaluate correlations in the expression levels of these factors in groups of ABA patients with normal and increased levels of serum IgE (table 3).

In addition, assessment of the correlation of expression levels of these factors in groups of ABA patients with such signs as eosinophilia of blood and sputum was performed (table 3).

Table 3. Correlation (r, ρ) of mRNA PAX-5 expression with STAT6 and pSTAT6 levels in ABA patients with total IgE levels, blood eosinophilia

Signs	STAT6	pSTAT6
IgE≤150 IU/ml	r=-0,486; p=0,023; n=23	r=-0,273; p=0,266; n=24
IgE>150 IU/ml	r=-0,269; p=0,151; n=28	r=-0,406; p=0,036; n=30
Blood eosinophils count (>2%)	r=-0,464; p=0,002 n=50	r=-0,339; p=0,027 n=52
Blood eosinophils count (≤ 2%)	r=-0,225; p=0,175 n=56	r=-0,225; p=0,113 n=56
Sputum eosinophils count (12-20%)	ρ=-0,326; p=0,144 n=27	ρ=-0,591; p=0,003 n=27
Sputum eosinophils count (>20%)	ρ=-0,437; p=0,131 n=17	ρ=-0,276; p=0,348 n=19

As it follows from a table 3, expression of STAT6 was significantly correlated with mRNA PAX- 5 in patients with the normal IgE level. On the other hand, levels of expression of the active form of transcription factor pSTAT6 correlate with expression of mRNA PAX-5 in the group of patients with high level of serum IgE. Both cross-correlations are negative.

Besides negative correlation of mRNA expression of PAX-5 with the expression of STAT6 protein and pSTAT6 was revealed, and this correlation was observed at higher blood eosinophilia only.

As for analogous correlation in the investigation of sputum eosinophilia the highly significant negative correlation between mRNA PAX-5 and pSTAT6 was only detected in patients with the sputum eosinophils content 12-20%. In more severe sputum eosinophilia (>20%) patients this correlation is not significant.

IL-4 is considered to be the key cytokine in BA pathogenesis, which is mediated by transcription factor STAT6. We evaluated the expression of mRNA PAX-5 in lymphocytes incubated in the medium with 10 ng/ml of IL-4 and without IL-4.

In ABA patients there was the significant decrease of expression mRNA PAX-5 under the influence of IL-4 (p = 0,042; n = 19, t = 2,466). In the group of healthy controls this decrease was not significant (p = 0,840; n = 9, t = 0,071) (t-test). For the Wilcoxon-Mann-Whitney test the result was similar: Z = -2,198; p = 0,026 for the group of ABA patients and Z = -0,140; p = 0,889 (Figure 1) for control group.

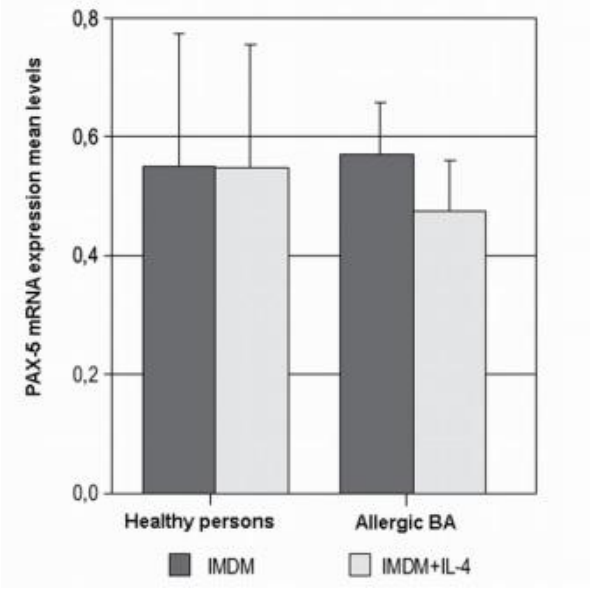

Figure 1. Lymphocyte PAX-5 mRNA expression mean levels in healthy persons and bronchial asthma patients (relatively to beta-actin mRNA).

The mRNA PAX-5 expression (relatively to beta-actin mRNA) in lymphocytes of patients with allergic asthma and healthy persons under 24-hour incubation in IMDM medium without IL-4 (IMDM) and with IL-4 (IMDM + IL-4) (M±σ). In allergic BA under the influence of IL-4 a significant decrease in the mRNA PAX-5 expression in lymphocytes was observed. In healthy persons this expression did not change significantly.

Taking into consideration putative interaction between STAT6 and PAX-5, we analyzed the correlation between the expression levels of these factors in the groups with high and low levels of these factors (Table 4).

Table 4. Correlation (r) of mRNA PAX-5 expression with STAT6 and pSTAT6 levels in individuals with its different levels

Expression of transcription factors	STAT6	pSTAT6
STAT6 < 0,6 (relative to β-actin)	r=-0,403; p=0,003; n=62	r=-0,371; p=0,006; n=61
STAT6 > 0,6 (relative to β-actin)	r=-0,136; p=0,285; n=57	r=-0,125; p=0,340; n=56

pSTAT6 < 0,15 (relative to β-actin)	r=-0,349; p=0,016; n=58	r=-0,144; p=0,284; n=57
pSTAT6 > 0,15 (relative to β-actin)	r=-0,165; p=0,184; n=55	r=-0,186; p=0,135; n=59
mRNA PAX-5 < 0,55 (relative to β-actin)	r=-0,206; p=0,166; n=51	r=-0,216; p=0,132; n=53
mRNA PAX-5 > 0,55 (relative to β-actin)	r=-0,327; p=0,018; n=67	r=-0,116; p=0,325; n=68

The analysis of the correlations in Table 4 suggests that the strongest and significant negative correlation of expression mRNA PAX-5 and factor STAT6 is determined in the patients groups with a high content of mRNA PAX-5 and low STAT6.

This fact suggests a complex regulatory mechanism of PAX-5 transcription. The STAT6 transcription in high PAX-5 - cells is probably slightly affected by the negative impact of this factor and its active form, thus expression of mRNA PAX 5 is increased. When production of PAX-5 becomes more intensive, its transcription becomes more sensitive to the negative effects of STAT6. At the same time, at low STAT6 concentrations the transcription of PAX-5 is likely under the stronger negative control of STAT6, but when the expression of mRNA PAX-5 is reduced the inhibitory effect of STAT6 weakens. We can suppose a somewhat different nature of the relationship between expression of pSTAT6 and mRNA PAX-5 in the groups is due to "delayed" reaction of pSTAT6 on changes in the expression of STAT6 and PAX-5 as pSTAT6 is a secondary form of activity against STAT6. These observations do not allow exclude feedback regulatory mechanism between STAT6 and PAX-5, possibly due to indirect inhibition of STAT6 activity by factor PAX-5.

Using cluster analysis we divided studied patients into three groups depending on the level of STAT6 (in relation to beta actin) in lymphocytes: less than 0.31, 0.31 - 0.72, 0.72 and more. To estimate the expected contribution of STAT6 to the mRNA PAX-5 synthesis we conducted dispersion analysis using a STAT6 cluster group value as a factor. With increasing of STAT6 value expression of mRNA PAX-5 was decreased: F = 5,463 and p = 0,004 for all investigated patients and F = 7,588 and p = 0,002 for allergic bronchial asthma patients. This association was not significant in groups of healthy controls and in NABA patients.

Discussion. The transcription factor PAX-5 has several functions. It does not only switch the B-lymphocytes to IgE production but also ensures the commitment of its B-cell lineage, preventing their apoptosis, and transformation into plasma cells due to the inhibition of plasma cell specific genes. STAT6 takes also part in the process of switching to IgE synthesis [6]. STAT6 and PAX-5 are involved in the regulation of expression of low affinity receptor to IgE - FcεRII (CD23) which is also involved in the in the regulation of the IgE action [6]. Taking in consideration the existence in lymphocytes the whole cascade of transcription factors that interact in regulation of expression of the same molecules [8], we may not exclude the interaction between STAT6 protein and PAX-5. The revealed negative significant correlation between the STAT6 protein and mRNA PAX-5 allows to suppose that STAT6 is able to inhibit the transcription factor PAX-5.

We have previously shown that serum IgE was not increased in those with a high mRNA PAX-5 in lymphocytes [4], supposing that STAT6 contributes more to the increase of IgE production. STAT6 possibly suppresses PAX-5 and thus contributes to B cells differentiation into plasma cells that are the main IgE producers. This assumption can be confirmed by observation indicating a decrease of mRNA PAX-5 expression in lymphocytes stimulated with IL-4 for 24 hours as the main effect of this cytokine is carried out by protein STAT6 protein.

Canonical DNA sequence that recognizes STAT6 is considered as oligonucleotide portion 5′ -TTC- (N_4) -GAA-3′ where N is any nucleotide. STAT6 binding site may also contain 2 and 3 Ns and not only 4 Ns [9]. In the known sequence of the PAX-5 gene (NCBI Reference Sequence: NM_016734.1) there are no similar sites. However the possible sites of STAT6 binding may be found in the non-coding region of the molecule Id2 gene (NCBI Reference Sequence: NM_002166) and in the untranslated region of the transcription factor E2A (NCBI Reference Sequence: NM_003200.3).

Cofactor Id2 (Inhibitor of DNA binding 2) inhibits DNA binding with several transcription factors, including E2A binding with regulatory regions of PAX-5 gene [10].

Thus, it can be assumed that the increase in STAT6 expression leads to an increase in the cofactor Id2 concentration, which inhibits binding transcription factor E2A to promoter PAX-5 gene, which ultimately leads to decrease of the PAX-5 expression (Figure 2).

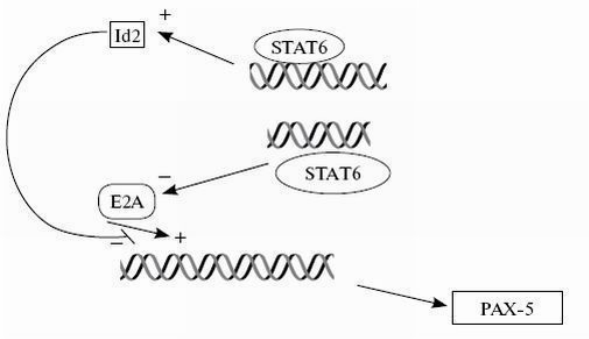

Figure 2. Putative STAT6 – PAX-5 interaction pathway.

The proposed scheme of the STAT6 – PAX-5 interaction pathway. STAT6 probably may inhibit (-) E2A transcription and may induce (+) Id2 transcription (as both genes of these molecules contain potential STAT6-binding sites). Id2 prevents E2A DNA-binding. Both these ways of STAT6 activity inhibit PAX-5 transcription.

E. Cavalcanti et al. [11] analyzing the state of CD8+ T lymphocytes in renal cell carcinoma, showed that in these cells there is a decrease both in concentration of a factor STAT6 and cofactor Id2, so, the expression of these molecules changes unidirectional. On the other hand we can assume that STAT6 regulates transcription factor E2A as well. According to some researchers STAT6 can not only activate transcription, but also to inhibit it [12, 13].

These assumed interactions and the fact that in bronchial asthma on the one hand the strength and significance of correlation between STAT6 and mRNA PAX-5 compared with healthy controls reduces, and on the other hand this correlation is increased with an increase in blood and sputum eosinophilia, indicate apparently at the imbalance of interactions of transcription factors in asthma. We have previously identified more severe course of asthma in patients with high levels of STAT6 expression [4]. Perhaps this phenomenon is partly mediated by inhibition of PAX-5 with activation of Id2 transcription and repression of E2A.

In conclusion, it should be noted that in B-lymphocytes there is undoubtedly a ramified network of interacting transcription factors and cofactors that regulate the immunoglobulins production and the antigen-presenting activities of these cells. The imbalance in this network may underlie the development of asthma.

In particular, increased expression of STAT6 causes a decrease in PAX-5 production, perhaps via inhibition of synthesis of factors E2A and activation of Id2 transcription. At the same time, it is known that STAT6 together with the PAX-5 is involved in class switching of B-lymphocytes to IgE synthesis. Inhibition of the expression of PAX-5 may prevent excessive switching to IgE, and thus it may be a feedback mechanism for B-cells in differentiating into plasma cells.

Further examination and testing of suspected interactions will allow to analyze the allergic asthma pathogenesis at a new level, which could result in the development of new target drugs.

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