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## GERIATRICS

# PDCD1 PD-1.3 polymorphism and allergic bronchial asthma in Russian and Buryat patients

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### ABSTRACT

**Objective:** The programmed death-1 receptor, PD-1, is a negative regulator of T-cell activation. The PD-1.3 polymorphism of the PD-1 gene (*PDCD1*) has been previously shown to be associated with several autoimmune and inflammatory disorders including systemic lupus erythematosus and multiple sclerosis. We examined for the first time PD-1.3 association with another inflammatory disease with strong immune component, IgE-mediated bronchial asthma, its severity and its biochemical markers (total serum IgE and IL-4). **Methods:** PD-1.3 G/A was genotyped by PCR-RFLP analysis using two different populations: Caucasian (492 Russian individuals) and Asian (276 Buryat individuals). **Results:** We found a significant association of the PD-1.3 polymorphism with IgE-mediated bronchial asthma and total serum IgE level in the Russian population. Combined genotype AA+AG was correlated with risk of developing allergic bronchial asthma ( $OR = 1.78$ , 95% CI 1.13–2.78,  $p = 0.011$ ) and lower concentrations of total serum IgE ( $p = 0.001$ ) compared with the wild-type genotype GG. However, PD-1.3 was not polymorphic in the Buryat population. **Conclusions:** PD-1.3 polymorphism of the PD-1 gene (*PDCD1*) may contribute to the development of allergic asthma in the Russians but not in the Buryats. Our results could be helpful for a better understanding of the effect of this polymorphism on the development of diseases with strong immune components.

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## Introduction

The programmed death-1 receptor, PD-1, has been characterized as a negative regulator of T cell activation. PD-1 is expressed on the surface of activated CD4+ and CD8+ T cells, B cells and some myeloid cells [1]. The PD-1 receptor and its two specific ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) belong to the costimulatory molecule B7:CD28 superfamily [2]. PD-L1 is expressed broadly on various hematopoietic and non-hematopoietic cell types and PD-L2 is expressed only on dendritic cells, macrophages, mast cells and the B1 subset of B cells [3–5].

PD-1 and its ligands play an important role in the downregulation of the immune system by preventing the activation of T cells, which in turn reduces autoimmunity and promotes self-tolerance [6,7]. Binding of PD-1 to the ligands together with TCR or BCR cross-linking decreases T cell activation by inducing phosphatases and dephosphorylation of effector molecules involved in TCR and BCR signaling [8]. PD-L2 has a crucial role in mucosal immune responses to environmental antigens, while PD-L1 protects self-tissue from T cell-mediated attack. The breakdown of peripheral tolerance or self-tolerance leads

to autoimmunity and allergic diseases, suggesting critical roles for PD-1 ligands in each case [7].

Allergic bronchial asthma is a chronic heterogeneous disease characterized by Th2-driven airway inflammation, mucus secretion, airway hyperreactivity, remodeling and reversible airway obstruction. T helper 2 cells orchestrate the asthmatic inflammation through the secretion of interleukin 4 (IL-4), IL-13, IL-5 and IL-9 by CD4+ T cells, which in turn enhance the growth, differentiation and recruitment of eosinophils, basophils, mast cells and IgE-secreting B cells. Several studies indicate an essential role of PD-1, PD-L1 and PD-L2 in allergic asthma [2, 9–11]. Despite the fact that, in general, PD-1 has inhibitory effect on CD4+ T cell activation, the role of the PD-1/PD-L axis in allergic asthma is still unclear.

The *PDCD1* gene located on chromosome 2q37.3 encodes the programmed death-1 receptor. Complete sequencing of the *PDCD1* gene revealed the 7146G>A regulatory polymorphism known as PD-1.3 (rs11568821) [12]. Case-control studies provided a significant evidence for association of the PD-1.3 polymorphism with inflammatory and autoimmune disorders: systemic lupus erythematosus, rheumatoid arthritis, lupus nephritis,

multiple sclerosis, ankylosing spondylitis, neuromyelitis optica and type 1 diabetes [12–19]. However, a few articles reported absence of association with lupus erythematosus, ankylosing spondylitis and type 1 diabetes [20–24].

The aim of our study was to assess, for the first time, a role of PD-1.3 for another inflammatory disorder with strong immune component, allergic bronchial asthma. We used case-control groups of 492 Russians and 276 Buryats to detect association with allergic bronchial asthma and its severity in different populations.

## Materials and methods

### Participants

Groups of 282 allergic bronchial asthma patients (BA+ group) and 210 control individuals (BA– group) of Russian origin were collected in clinical department of Institute of Immunology (Moscow, Russian Federation). In addition, 124 patients with allergic bronchial asthma and 152 healthy individuals of Buryats were collected in Department of Gerontology and Geriatrics of Irkutsk State Institute of Advanced Training of Medical Doctors (Ulan-Ude, The Republic of Buryatia, Russian Federation). Healthy individuals without asthma and allergic, autoimmune and bronchopulmonary diseases were included in both control groups.

Inclusion criteria for ethnic groups: samples were taken from unrelated individuals of Russian and Buryat descent, whose parents and grandparents considered themselves as Russians and Buryats, respectively (i.e., at least three generations belong to those ethnic groups). Among these, the Russians originate from Moscow region (in the third generation) and Buryats originate from Ulan-Ude region (in the third generation). Exclusion criteria: at least one of parents or grandparents belongs to other ethnic group or originates from regions other than those specified.

A standardized questionnaire was used to obtain the patient's medical history and demographic information. The data collected were gender, age at onset of disease, asthma duration, asthma severity, antigen sensitization (domestic, pollen, drug, food), smoking and allergic comorbid conditions. Atopy status was defined by the presence of elevated total serum IgE titers and at least one positive skin prick test result with a common allergen and confirmed clinical symptoms of allergy.

The sensitivity to six common aeroallergens (*Dermatophagoides pteronyssimus*, *Dermatophagoides farinae*, *Mucor*, cat dander, grass and tree pollen) was assessed by using highly standardized extracts and lancets, according to the ISAAC II protocol. Patients were diagnosed as being atopic if their skin prick test reaction was

**Table 1.** Russian and Buryat patients skin prick test results.

| Aeroallergen                          | Positive (Russians)<br>n = 282 | Positive (Buryats)<br>n = 124 |
|---------------------------------------|--------------------------------|-------------------------------|
| Tree pollen                           | 120 (42.6%)                    | 64 (51.8%)                    |
| Cat dander                            | 98 (34.7%)                     | 37 (29.4%)                    |
| <i>Dermatophagoides farinae</i>       | 154 (54.5%)                    | 70 (56.1%)                    |
| <i>Dermatophagoides pteronyssimus</i> | 153 (54.4%)                    | 69 (55.8%)                    |
| <i>Mucor</i>                          | 14 (4.9%)                      | 9 (7.00%)                     |
| Grass pollen                          | 109 (38.6%)                    | 56 (45.5%)                    |

≥ 3 mm or ≥ 50% of the histamine positive control response (Table 1). Total serum IgE and IL-4 were measured using enzyme-linked immunosorbent assay (ELISA) test kits (IgE-ELISA and IL4-ELISA, CCPP).

Basic clinical characteristics of Russian and Buryat patients in BA+ and BA– groups are shown in Table 2. BA+ group of Russians was divided into two subgroups of 135 moderate/severe asthma (SA) and 147 mild asthma (MA) patients. Asthma severity was assessed on the base of treatment required to control symptoms and exacerbations according to Global Initiative for Asthma (GINA) criteria [25].

The local ethical and medical committee approved the study, and all patients gave written informed consent.

### Genotyping

Genomic DNA was extracted from peripheral blood leukocytes of all the participants using a standard protocol of proteinase K digestion and phenol/chloroform extraction. PD-1.3 genotyping was performed using PCR-restriction fragment length polymorphism method

**Table 2.** Clinical characteristics of patients and controls.

| Russians                               | Bronchial asthma group (n = 282) | Control group (n = 210) |
|----------------------------------------|----------------------------------|-------------------------|
| Gender (m/f)                           | 114/168                          | 105/105                 |
| Age (yrs)                              | 38.1 (13.7)                      | 38.9 (10.2)             |
| Asthma duration (yrs)                  | 11.7 (8.6)                       | —                       |
| Age onset (yrs)                        | 26.3 (5.2)                       | —                       |
| Total serum IgE (kU/L)                 | 255 (80, 635)                    | 45 (23, 89)             |
| Total serum IL-4 (pg/ml)               | 12 (6, 56)                       | 2 (1, 5)                |
| Asthma severity (mild/moderate-severe) | 147/135                          | —                       |
| FEV <sub>1</sub> (%)                   | 78.3 (9.9)                       | 95.6 (3.2)              |
| Co-morbid allergic pathology (%)       | 78                               | 0                       |
| Buryats                                | Bronchial asthma group (n = 124) | Control group (n = 152) |
| Gender (m/f)                           | 45/79                            | 47/105                  |
| Age (yrs)                              | 37.6 (15.1)                      | 28.4 (12.8)             |
| Asthma duration (yrs)                  | 8.2 (5.2)                        | —                       |
| Age onset (yrs)                        | 29.3 (10)                        | —                       |
| Total serum IgE (kU/L)                 | 713 (112, 854)                   | 53 (28, 81)             |
| Total serum IL-4 (pg/ml)               | 18 (11, 87)                      | 4 (2, 7)                |
| FEV <sub>1</sub> (%)                   | 83.5 (5.8)                       | 93.7 (4.6)              |
| Co-morbid allergic pathology (%)       | 73                               | 0                       |

Note. Age and asthma duration are shown as mean (SD, standard deviation); IgE and IL-4 are shown as median (25%;75%).

as followed. PCR was performed using 50 ng of each DNA and primer pair (PD1.3 F-5'-GCAGCAACCTCAAATC CCTAA-3' and PD1.3-R-5'-CATTGGAGACAGGAG AGCTTG-3') with a programme of 2' at 95°C, 35 cycles of 15'' at 95°C, 15'' at 56°C and 20'' at 72°C, and an additional 4' at 72°C. The products were digested with PstI and identified using 2% agarose gel as G with 330 bp band, A with 280+50 bp bands and AG with 330, 280 and 50 bp bands.

### Statistical analysis

*PDCD1* PD-1.3 allele and genotype distributions in asthmatic and control individuals, the significance of association ( $p$ -value), the odds ratios (OR) and 95% CIs were calculated using SPSS 10.0 software (SPSS Inc, USA). Fisher's exact two-tailed test was applied for  $p$ -value calculation. The difference between groups was considered statistically significant when  $p < 0.05$ . Differences in levels of total serum IgE and IL-4 between groups were analyzed using the nonparametric Mann-Whitney test. Hardy-Weinberg equilibrium was tested using Arlequin software (University of Geneva, Switzerland).

## Results

### Association with allergic asthma and asthma severity

In all 276 individuals of Buryat origin, we were unable to identify the PD-1.3 polymorphism at the *PDCD1* gene. This study indicated that all genotyped samples had the GG genotype. It appeared that the allele frequencies of mutation at this position are rare (lower than 1%). Thus, PD-1.3 is not polymorphic in Buryats that corresponds with absence of this polymorphism in Chinese populations [26–28]. However, the minor A allele frequency in healthy Russians was 8.3% that correlate with those in European ethnic groups: 5% in Nordic families, 4% in Swedish families, 5% in European-American families, 6.8% in Danish population and 13% in continental Spanish [12,19,29].

Before association studies the PD-1.3 genotyping data in both case and control groups of Russians were tested for Hardy-Weinberg equilibrium and successfully passed the test ( $p = 0.120$  for asthma patients and  $p = 0.419$  for control individuals).

Association with allergic bronchial asthma was assessed by comparing allele and genotype frequencies between two groups: asthma patients (BA+) and healthy individuals (BA–). The calculated distributions of allele and genotype frequencies of Russian individuals

**Table 3.** Distribution of allele and genotype frequencies of PD-1.3 of *PDCD1* gene in asthma patients (BA+) and healthy individuals (BA–) in Russians.

| Alleles and genotypes | Frequency         |                   | $p$   | OR (95% CI)      |
|-----------------------|-------------------|-------------------|-------|------------------|
|                       | BA+ ( $n = 282$ ) | BA– ( $n = 210$ ) |       |                  |
| G                     | 0.867             | 0.917             | 0.975 | —                |
| A                     | 0.133             | 0.083             | 0.975 | —                |
| GG                    | 0.737 (208)       | 0.833 (175)       | 0.011 | 0.56 (0.35–0.88) |
| AA+AG                 | 0.263 (74)        | 0.167 (35)        | 0.011 | 1.78 (1.13–2.78) |

are shown in Table 3. The minor A allele tends to appear more frequently in asthmatic patients (13.3% vs. 8.3%). No significant difference in allele frequencies was found between BA+ and BA– groups of Russians. However, we found a statistically significant difference in genotype distribution. The AG and AA genotypes were treated together because the AA genotype was found in only one asthma patient and was not found in control group. Carriers of the combined genotype AA+AG had an increased risk of asthma (OR = 1.78, 95% CI 1.13–2.78,  $p = 0.011$ ). Individuals with GG genotype had reduced susceptibility to allergic bronchial asthma (OR = 0.56, 95% CI 0.35–0.88,  $p = 0.011$ ) (Table 3).

In addition to the association with bronchial asthma itself, we tested for association of the PD-1.3 polymorphism with asthma severity. The allele and genotype frequencies were compared in two subgroups: mild (MA) and moderate/severe (SA) asthma patients. No significant association was found but the GG genotype tends to be associated with moderate/severe asthma (79.2% vs 69.4% in MA group;  $p = 0.076$ ) and the combined genotype AA+AG– with mild asthma (30.6% vs 20.8% in SA group;  $p = 0.076$ ) (Table 4).

### Association with inflammation markers

The concentration of atopic markers in the blood of asthma patients is an important factor of disease severity. In this study we assessed a correlation between two main atopic markers, total serum immunoglobulin E (IgE) and interleukin-4 (IL-4), and the *PDCD1* PD-1.3 genotypes.

**Table 4.** Distribution of allele and genotype frequencies of PD-1.3 of *PDCD1* gene in Russian patients with mild (MA) and intermediate/severe bronchial asthma (SA).

| Alleles and genotypes | Frequency        |                  | $p$   | OR (95% CI) |
|-----------------------|------------------|------------------|-------|-------------|
|                       | MA ( $n = 147$ ) | SA ( $n = 135$ ) |       |             |
| G                     | 0.847            | 0.918            | 0.102 | —           |
| A                     | 0.153            | 0.072            | 0.102 | —           |
| GG                    | 0.694 (102)      | 0.792 (107)      | 0.076 | —           |
| AA+AG                 | 0.306 (25)       | 0.208 (28)       | 0.076 | —           |

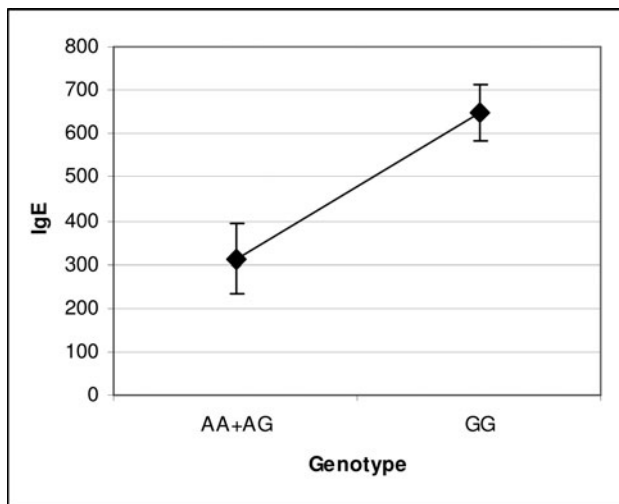


**Figure 1.** Analysis of total serum IgE concentration variance between GG and AA+AG genotypes carriers of Russian origin. Vertical bars denote 95% confidence intervals.

Significant differences between the GG and AA+AG genotypes carriers were achieved for total IgE concentration (Figure 1), suggesting the role of *PDCD1* in the progression of allergic inflammation. The AA+AG genotype was correlated with lower concentration of total serum IgE ( $313 \pm 79.4$  kU/L vs.  $649 \pm 65.3$  kU/L,  $p = 0.001$ ) comparing to the wild-type GG genotype. Lower total serum IgE level in the AA+AG genotype is consistent with higher frequency of these genotypes in group with mild asthma but not with moderate/severe asthma. We did not find any differences in total serum IL-4 levels between the GG and AA+AG genotype carriers.

## Discussion

It is currently known the following studies investigating the role of PD-1 and its ligands in allergic bronchial asthma. Deppong et al. showed that expression of PD-1 on lymphocytes was crucial for the termination of the allergic inflammatory response, as PD-1-deficient mice had persistent inflammation following allergen challenge compared with wild-type mice [30]. McGee et al. confirmed that PD-1 was the key player for Tregs-mediated modulation of AHR and airway inflammation in asthma, and anti-PD1-agonist lead to the increasing of Th2 cytokines, IL-4, IL-5 and IL-13 [31]. It was also demonstrated that the mouse model of allergic asthma highly expressed PD-1 on lung CD4+ T cells and PD-L1 and PD-L2 on pulmonary dendritic cells and macrophages in OVA-sensitized mice [9, 32].

Besides that PD-L2, but not PD-L1, inhibited Th2 cytokine production, including IL-4, IL-5, and IL-13, by an interferon- $\gamma$ -dependent, but PD-1-independent mechanism [32]. In addition, an administration of the

PD-L2 cross-linking antibody to the mouse model of allergic asthma prevented the development of allergic airway inflammation [33]. Akbari and colleagues showed the important but opposite role of PD-L1 and PD-L2 in modulating and polarizing T cell functions in airway hyperactivity. PD-1/PD-L1 interaction initiated a Th2 cytokine response, but PD-1/PD-L2 - a Th1 type cytokine response by iNKT cells in a murine model of asthma [9,34]. These findings suggest that PD-L2 preferentially mediates the effector phase of the asthmatic response [32,34]. The inhibitory role of PD-L2 has already been reported in earlier studies [5,35].

However, Lewkowich and colleagues showed that PD-L2 expression in bronchial biopsies from human asthmatics was correlated with asthma severity. In mice, PD-L2 inhibited allergen-driven IL-12 production through a PD-1-independent mechanism, exacerbating airway hyperresponsiveness [10]. Recent reports have demonstrated the elevated Th1 and Th17 levels, but diminished Th2 cytokine levels in naive PD-1/- mice, using a mouse model of allergic asthma. PD-1/PD-L1 blockade enhances airway hyperactivity by the development of a Th17-type immune response, not by altering the magnitude of the Th2-type immune response [11].

Zhao and colleagues showed that Th17 cells induced airway remodeling, a prominent feature of human asthma, independent of the Th2 response [36]. Animal asthma models suggest that Th17 cells promote neutrophilic inflammation, and, together with Th2 cells, are important in the development of airway hyperresponsiveness. There were identified Th17/Th2 cells in allergic asthma, capable of producing both IL-4 and IL-17, which was in accordance with the observation that different clinical asthma phenotypes could coexist in one patient [37].

It is known several functional analysis reports of the *PDCD1* gene PD-1.3 polymorphism. PD-1.3 is located in an enhancer within the intron 4 of the *PDCD1* gene and affects the binding site for the runt-related transcription factor 1 (RUNX1, also called AML1) [12]. RUNX1 is a critical regulator of hematopoiesis and is essential for fetal development and cellular differentiation [19]. Functional relevance of the PD-1.3 polymorphism was confirmed using electrophoretic mobility shift assay (EMSA). It was demonstrated that RUNX1 bound oligonucleotide containing the PD-1.3 G allele and did not bind oligonucleotide with the PD-1.3 A allele.

Disruption of the RUNX1-binding site by the A allele could potentially alter the level of expression or the stability of PD-1 mRNA. Moreover, they found an association of the PD-1.3 A allele with the development of systemic lupus erythematosus [12]. Other report by Kristjansdottir and colleagues showed a significant correlation of the PD-1.3 A allele with lower PD-1 expression in

systemic lupus erythematosus patients [38]. Thus, it has been suggested that a G→A nucleotide substitution at PD-1.3 lead to the disturbance in the binding of the RUNX1, then to decreased PD-1 expression and the breakdown of self-tolerance [13].

Before discussing our results, we should say a few words about Buryat ethnic affiliation. The Buryats is the most numerous indigenous ethnic group of Siberia. Besides the Buryats live in Northern Mongolia and in northeast China. The Buryats exhibit pronounced anthropological features of Mongoloid type. They are generally can be considered as representatives of a Central Asian anthropological type and demonstrate strong genetic affinity with Mongols based on haplotype set and frequencies of all major haplotypes [39]. Thus they have almost completely Central Asian gene pool, whereas Russians from European part of Russia have Central European gene pool.

We suppose that such the difference in PD-1.3 frequencies between the Russians and the Buryats especially the total absence A-allele in the Buryats is due to interpopulation diversity. As regarding to Asian populations the PD1.3 is not polymorphic in several populations of Southeast Asia – Hong Kong Chinese, Han Chinese and Japanese. Those populations are partly different from Central Asian populations and unfortunately there is no PD1.3 data from Central Asian populations to compare with Buryats. The second point is the great difference in IgE levels between Buryat and Russian asthmatics. It is difficult to presume the reason for this difference at the coincidence of all other clinical factors. Perhaps the reason is due to genetic factors, or it may be associated with different level of medical care.

We demonstrated for the first time an effect of the *PDCD1* PD-1.3 polymorphism on allergic bronchial asthma susceptibility in Russian population. The combined genotype AA+AG was associated with an increased risk of developing allergic bronchial asthma. Two studies have shown that individuals with the PD-1.3 A allele presumably had a reduced level of PD-1 expression [12,38], suggesting a mechanism through which it could contribute to the development of asthma in humans. Our result is consistent with other studies that have shown that the disruption of PD-1 or its ligands might contribute to the development and maintenance of allergic inflammation, airway hyperreactivity, and hence to asthma [11,30,34].

Despite the asthma is mainly Th2-mediated disease, recent reports demonstrated that Th17 cells played an important role in the development and progression of allergic diseases. Th17 cells are able to recruit and activate neutrophil granulocytes. Neutrophil activity in asthma

can exist alongside IgE-mediated asthma [11,37,40,41]. In a mouse model of allergic asthma McAlees and colleagues showed that despite the fact that the decrease in PD-1 expression lead to a reduced Th2 response, airway hyperreactivity was enhanced in response to elevated Th17 response [11]. In turn it has explained the association of the PD-1.3 polymorphism with asthma development we have received. Thus, PD-1.3 may be indirectly associated with Th17 component activation of asthma immune response.

Correlation of the AA+AG genotype with asthma was confirmed in analysis of atopic markers. We found that the AA+AG combined genotype correlated with lower concentration of total serum IgE than the wild-type GG genotype. Generally, we might expect higher concentrations of atopic markers in mutant allele carriers, but in our group of Russian patients the results were opposite. The serum IgE level reflects the severity of bronchial asthma. No significant association was found between PD-1.3 and asthma severity.

The significant correlation of predisposing AA+AG genotype with low IgE plasma concentration is consistent with our observation of the combined genotype prevalence in mild asthma group but this prevalence was not significant. Based on the reports of Prokunina et al and McAleese et al, we suggest that the A allele reduces an expression of PD-1, which in turn leads to a decrease in Th2 response, and hence reduced production of IgE by B cells [11,12]. From the other side Bottema et al showed that PDCD-1 had no main effect on IgE level in children of different age groups [42].

As regarding to functional effect of different PD-1.3 genotypes in humans there is a study by Bertsias et al. that demonstrated that the heterozygous (AG) condition doesn't produce any alteration on PD-1 expression level, but in homozygous (AA) SLE patients, T cell proliferation at 48 hours was increased by  $5.0 \pm 8.0\%$ , while significantly reduced in heterozygous and wild-type (G/G) SLE patients and healthy controls [43]. By contrast, Prokunina and colleagues suggest that expression levels of PD-1 mRNA differ in peripheral blood mononuclear cells from individuals with the PD-1.3 GG genotype when compared with AG or AA genotypic individuals [12,39]. These data support our hypothesis regarding the indirect non-Th2 effect PDCD1 on atopic asthma.

Also PD-1.3 may be in strong linkage disequilibrium with etiological polymorphism. In Korean population, where PD-1.3 was non-polymorphic, it was in linkage disequilibrium with other polymorphisms, PD-1.5 and PD-1.9 associated with ankylosing spondylitis. In Brazils (both European and African origin) PD-1.3 was in linkage disequilibrium with PD-1.5 and partly with PD-1.6

(mostly for European then for African origin), and one of the haplotypes of these three polymorphisms was associated with pemphigus foliaceus [18, 44].

It should be noted that our study has some limitations. The sampling sizes were not large and there are some interpopulation differences in IgE levels and allele frequencies. These facts and contradictory data from other investigators indicate that further research is needed to define the effect of PDCD1 and its polymorphisms on atopic asthma.

## Conclusions

We detected a significant association of PD-1.3 polymorphism with allergic bronchial asthma and total serum IgE levels in case-control study of Russians. We suggest PD-1.3 may be implicitly correlated with Th17 component activation of asthma immune response. Our findings are consistent with a hypothesis of the general role of the *PDCD1* gene polymorphisms in susceptibility to diseases with immune inflammation. Alternatively, PD-1.3 may be in strong linkage disequilibrium with etiological polymorphism.

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## Declaration of interest

The authors have no conflicts of interest to declare. The work is done in the framework of the State Academy of Sciences fundamental research program for 2013–2020.

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