ORIGINAL PAPERS

Adv Clin Exp Med 2016, **25**, 2, 273–278
DOI: 10.17219/acem/32211

ISSN 1899–5276

Sandra Dragicevic[1, A–F], Natasa Petrovic-Stanojevic[2, A–F], Aleksandra Nikolic[1, A–F]

TGFB1 Gene Promoter Polymorphisms in Serbian Asthmatics

[1] Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Serbia
[2] Department of Pulmonology, Zvezdara University Medical Center, Belgrade, Serbia

A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Asthma is a chronic respiratory disease caused by a combination of genetic and environmental factors. Transforming growth factor beta 1 (TGFB1) is a multifunctional cytokine that plays an important role in airway remodeling in asthma.
Objectives. The aim of this study was to analyze common TGFB1 gene promoter polymorphisms C-509T and G-800A in Serbian asthmatics and to investigate their association with exacerbations.
Material and Methods. The study involved 102 asthmatics and 58 healthy individuals from Serbia, age and gender matched. An analysis of the TGFB1 promoter was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).
Results. For polymorphism C-509T a significant difference in the allele frequency was observed between the patients and the controls ($p = 0.011$), while the genotype distribution was similar in the analyzed groups, with statistical significance near the borderline ($p = 0.061$). For the polymorphism G-800A no difference was observed between the groups. The frequency of the -509TT genotype was higher in patients with exacerbations compared to patients without exacerbations (36.4% vs. 17.0%), with statistical significance near the borderline ($p = 0.080$).
Conclusions. The results suggest that polymorphism C-509T may be associated with asthma and disease exacerbations, while G-800A is not significant for the etiology and clinical course of the disease. These findings should be confirmed in a larger study group, and since the TGFB1 promoter is highly complex and very responsive to environmental factors, future studies should also take other genetic and non-genetic factors into consideration (**Adv Clin Exp Med 2016, 25, 2, 273–278**).

Key words: asthma, gene polymorphism, transforming growth factor beta 1.

Asthma is a chronic inflammatory disease of the lungs characterized by airflow obstruction and airway remodelling [1, 2]. Previous studies have indicated that asthma is a complex disorder resulting from a combination of genetic and environmental factors [2, 3]. It has been proposed that a number of genes may be implicated in the pathogenesis of the disease, as well as in modulation of disease expression and response to therapy [4, 5]. Each patient with asthma is treated with a specific combination of medications, selected on the basis of clinical evaluation and disease history [6]. Interindividual differences in asthma susceptibility, disease severity and response to therapy can be partly attributed to genetic polymorphisms.

Transforming growth factor beta 1 (TGFB1) is a multifunctional cytokine that plays a pivotal role in normal cellular processes and disease [2, 7]. It is a key regulator of cell growth, cell differentiation, immune modulation, embryogenesis and apoptosis. Among many diverse effects, TGFB1 promotes accumulation of the extracellular matrix by increasing the

* This work was supported by grant 173008 from the Ministry of Education, Science and Technological Development of the Republic of Serbia.

synthesis of extracellular matrix components and by reducing matrix degradation. It has been suggested that TGFB1 may be the major mediator of progressive fibrosis in airways [1, 2, 8]. TGFB1 is expressed and released by structural cells in the airways and inflammatory cells that have infiltrated the bronchial mucosa [9, 10]. The levels of TGFB1 are known to be affected by many non-genetic factors, including corticosteroids and leukotriene modifiers, both of which are used routinely for asthma control [11].

In humans, TGFB1 gene is located at chromosome 19q13.2 and contains seven exons and very large introns. Polymorphisms in the gene regulatory sequences can impact TGFB1 expression levels and can potentially play a role in the etiology of asthma [8, 9]. Several polymorphisms have already been described in the promoter region of the TGFB1 gene, among which C-509T and G-800A have been suggested to be associated with altered transcriptional activity [12].

A C to G transition at position -509 in the TGFB1 gene promoter has been widely studied as a risk factor for asthma and other inflammatory diseases [13, 14]. This polymorphism is located in the silencer region of the TGFB1 gene in a Yin Yang 1 (YY1) consensus binding site, and the presence of the T allele increases basal promoter activity by approximately 30% [15]. It has been reported that the C-509T polymorphism is associated with a higher plasma concentration of TGFB1 [16, 17].

A base substitution of G to A at position -800 contributes to lowering the production of circulating TGFB1 [18, 19]. This polymorphism is located in the enhancer region of the TGFB1 gene promoter in a consensus cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) half site [20]. The A allele is associated with reduced TGFB1 gene expression and a lower circulating concentration of TGFB1 [18].

The aim of this study was to analyze common TGFB1 gene promoter polymorphisms C-509T and G-800A in Serbian asthmatics and to investigate their association with exacerbations in order to explore their role in asthma and their potential application as molecular markers for predicting the course of the disease and the response to therapy.

Material and Methods

The Patients

The study included 102 adult asthmatics being treated at the Clinical Department of Pulmonology, Allergology and Clinical Immunology of the University Hospital in Belgrade (Serbia) in the period from April 2009 to November 2011. The criteria for inclusion in the study were a diagnosis of asthma and age older than 18 years. Patients were excluded from the study based on the following criteria: non-compliance, inadequate cooperation, pregnancy and serious concomitant diseases. Out of 102 patients with asthma, 97 were available for follow-up for a period of at least three months. The patients were physically evaluated and answered questions about the quality of their life (Asthma Control Test – ACT). Spirometry testing (using a SpiroJet PowerCube LF8.5F device, Ganshorn, Waltham, MA, USA) was performed and each patient's forced expiratory volume in 1 sec (FEV1) and forced vital capacity (FVC) were registered. Asthma control and the severity of the disease were evaluated according to the applicable Guidelines of the Global Initiative for Asthma (GINA) based on the results of spirometry testing and ACT scores [6]. Each patient was treated depending on the clinical evaluation and severity of asthma. The long-term asthma control medications taken daily included inhaled corticosteroids (ciclesonid [Alvesco, Nycomed GmbH, Constance, Germany]; budesonide [Pulmicort, AstraZeneca AB, Sodertalje, Sweden]; fluticasone [Flixotide, GlaxoSmithKline Pharmaceuticals SA, Poznań, Poland]; beclomethasone [Becloforte, GlaxoSmithKline, Brentford, UK]), leukotriene modifiers (montelukast [Singulair, Merck Sharp & Dohme, Haarlem, Netherlands]), long-acting beta agonists (salbutamol [Ventolin, GlaxoSmithKline Pharmaceuticals S.A., Poznań, Poland]), combination inhalers (Salmeterol with fluticasone [Seretide, Glaxo Wellcome Operations, Ware, UK], or formoterol with budesonide [Symbicort, AstraZeneca AB, Sodertalje, Sweden]), and theophylline (Euphylong, Altana AG, Wesel, Germany).

The control group consisted of 58 healthy subjects who were recruited during the regular annual health assessment carried out by Occupational Medicine Services. All the control subjects underwent regular physical examination and laboratory testing, and participants with any chronic disease and medical therapy were excluded. All the study participants filled out a questionnaire consisting of basic information (date of birth, anthropometric data, use of medications) and smoking habits.

The study was approved by the Ethics Committee of the University Hospital and all participants gave informed consent to participate in the study.

Analysis of TGFB1 Promoter Polymorphisms

Peripheral blood samples were taken from the patients with asthma and the control subjects. Genomic deoxyribonucleic acid (DNA) was extracted

from the whole blood using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

Amplification of the 5' regulatory region of the TGFB1 gene was performed by polymerase chain reaction (PCR) for all samples using the following program: 95°C for 10 min; 35 cycles at 95°C for 45 sec, 60°C for 60 sec and 72°C for 30 sec; and 72°C for 10 min. The region containing polymorphism C–509T was amplified using forward primer 5'-GGGAAGCTTGCTTAGC-CACATGGGAGGTGC-3' and reverse primer 5'-GGGCCATGGGCTCAGCCGGGGGG GT-GC-3'. The region containing polymorphism G-800A was amplified using forward primer 5'-GGG CTC GAG CAC TGG GGA GCT ATG GAA GG-3' and reverse primer 5'-GGG AAG CTT CCC AGA ACG GAA GAG TC-3'.

The patients and control subjects were tested for both polymorphisms using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The PCR products for the C-509T polymorphism were digested with restriction endonuclease *DdeI* (ThermoScientific, Waltham, MA, USA). The reaction mixture was incubated at 37°C overnight, followed by an analysis of the obtained products by electrophoresis on 2% agarose gel. The fragment containing the C allele was not digested (640 bp), whereas the presence of the T allele resulted in two fragments (590 bp and 50 bp). The PCR products for the G-800A polymorphism were digested with restriction endonuclease *TaiI* (ThermoScientific). The reaction mixture was incubated at 60°C for 3 h, followed by an analysis of the obtained products by electrophoresis on 2% agarose gel. The presence of the G allele resulted in three fragments (400 bp, 370 bp and 210 bp), whereas the presence of the A allele resulted in two fragments (610 bp and 370 bp).

Statistical Analysis

The statistical analysis was performed using Statistical Package for Social Sciences 20.0 (SPSS Inc., Chicago, Illinois, USA). The demographic characteristics of the patients and the controls were compared using an independent sample *t*-test and the χ^2 test. The data were expressed as percentages and means ± standard deviation (SD). Differences between the patients and controls in terms of the genotype and allele frequencies for each polymorphic site were tested by the χ^2 analysis. A p-value less than 0.05 was considered statistically significant.

Results

In this study, TGFB1 gene promoter polymorphisms were analyzed in 102 asthmatic patients and 58 healthy controls from Serbia. The characteristics of the two study groups are presented in Table 1. There were no significant differences between the patients and the healthy controls in terms of age, sex and body mass index (BMI), but there were significantly more smokers in the control group than in the patients' group (62.5% vs. 17.5%, $p < 0.001$).

Samples from the patients and healthy individuals were genotyped for TGFB1 gene promoter polymorphisms by PCR-RFLP (Fig. 1). The distribution of alleles and genotypes obtained for both polymorphisms in the patients and controls is presented in Table 2. There was a statistically significant difference between the patients and controls in the distribution of C-509T alleles ($p = 0.011$), but no significant difference was observed for genotype distribution. Also, there was no significant difference between the patients and controls in the distribution of G-800A alleles and genotypes.

Table 1. Description of the study population

	Asthma patients (n = 102)	**Control subjects (n = 58)**	**p-value**
Age, years (mean ± SD)	43.7 ± 14.3	46.4 ± 10.8	0.179
Males/females, %	46/54	50/50	0.633
BMI, kg/m^2	27.2 ± 4.8	26.8 ± 4.6	0.620
Smokers, %	17.5	62.8	< 0.001*
FEV1, %	79.1 ± 21.4	–	–
FVC, %	93.5 ± 17.1	–	–
ACT, %	19.4 ± 4.6	–	–

*statistically significant; SD – standard deviation; BMI – body mass index; FEV1 – forced expiratory volume in 1 sec; FVC – forced vital capacity; ACT – asthma control test.

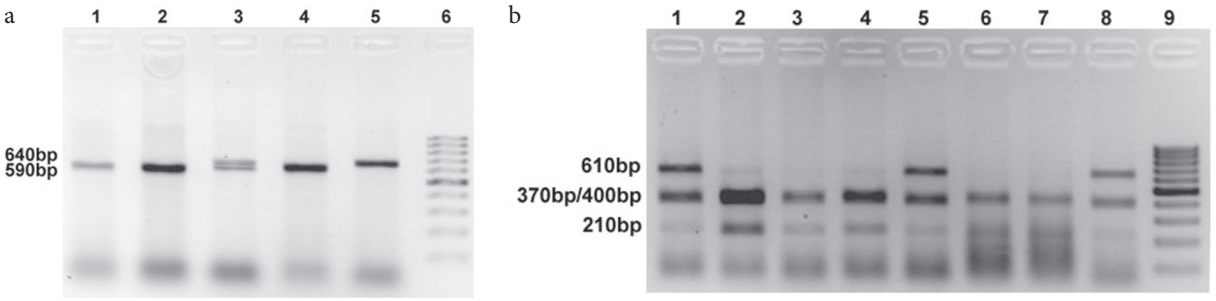

Fig. 1. Analysis of TGFB1 polymorphisms C-509T (a) and G-800A (b) by PCR-RFLP on agarose gels: a. C-509T genotypes: 1, 2, 4 – TT; 3 – CT; 5 – CC (6 – 100 bp DNA ladder); b. G-800A genotypes: 1, 5, 8 – GA, 2, 3, 4, 6, 7 – GG (9 – 100 bp DNA ladder)

Table 2. Distribution of allele and genotype frequencies in asthmatics and controls

	Patients (n, %)	Controls (n, %)	p-value
Alleles			
-509C	113 (55.4)	47 (40.5)	0.011*
-509T	91 (44.6)	69 (59.5)	
-800G	191 (93.6)	104 (89.7)	0.203
-800A	13 (6.4)	12 (10.3)	
Genotypes			
-509 CC	36 (35.3)	13 (22.4)	0.061
-509 CT	41 (40.2)	21 (36.2)	
-509 TT	25 (24.5)	24 (41.4)	
-800 GG	91 (89.2)	48 (82.8)	0.506
-800 GA	9 (8.8)	8 (13.8)	
-800 AA	2 (2)	2 (3.4)	

*statistically significant.

Table 3. Comparison of FEV1, FVC and ACT values and genotype distribution in patients with and without exacerbations

	With exacerbations (n = 44)	Without exacerbations (n = 53)	p-value
FEV1, %	68.4 ± 21.6	89.0 ± 16.2	< 0.001*
FVC, %	87.4 ± 18.3	98.6 ± 14.4	< 0.001*
ACT, %	16.3 ± 4.9	21.9 ± 2.3	< 0.001*
Genotypes, n (%)			
-509 CC	12 (27.3)	22 (41.5)	0.080
-509CT	16 (36.4)	22 (41.5)	
-509 TT	16 (36.4)	9 (17.0)	
-800 GG	40 (90.9)	46 (86.8)	0.745
-800 GA	3 (6.8)	6 (11.3)	
-800 AA	1 (2.3)	1 (1.9)	

* statistically significant; FEV1 – forced expiratory volume in 1 s; FVC – forced vital capacity; ACT – asthma control test.

The Hardy-Weinberg equilibrium was tested for both polymoprhisms in the patients and the control subjects. In both groups the distribution of the observed genotypes for the G-800A polymorphism was significantly different from what was expected (p = 0.01 for the patients' group and p = 0.05 for the control group), while the distribution of the C-509T polymorphism was consistent with the Hardy-Weinberg equilibrium (p = 0.06).

The patients who were followed up for at least three months were divided into two subgroups according to their asthma exacerbations and clinical characteristics, and the distribution of the TGFB1 polymorphism genotypes was analyzed (Table 3). There were significant differences in FEV1, FVC and ACT values between patients with and without exacerbations, but the distribution of genotypes for the polymorphisms under investigation was similar in both subgroups. The frequency of the -509TT genotype was higher in patients with exacerbations compared to patients without exacerbations (36.4% vs. 17.0%), with statistical significance near the borderline (p = 0.080).

Discussion

Since TGFB1 is associated with fibrosis and accumulation of the extracellular matrix after lung injury or inflammation, it has an essential role in airway remodeling in asthma [21]. It is postulated that variants in the TGFB1 gene could increase the profibrotic and anti-inflammatory effects of TGFB1. Also, the TGFB1 promoter is highly responsive to various stimuli and its levels may be significantly affected by corticosteroids and leu-

kotriene modifiers, which are used routinely for asthma control [11]. It is therefore expected that the functional TGFB1 polymorphisms C-509T and G-800A affect asthma susceptibility, disease severity and response to therapy.

In the Serbian asthmatics and healthy controls analyzed in this study a statistically significant difference was observed in the distribution of the C-509T alleles, with the C allele being more frequent in the patients than in the controls (55.4% vs. 40.5%, p = 0.011). The distribution of C-509T genotypes also differed in the two groups, with lower frequencies of -509CT and -509TT genotypes among the patients than in the controls, with statistical significance near the borderline (p = 0.061). The role of the polymorphism C-509T in asthma remains unclear, since it has been associated with asthma and asthma severity in some populations, but those results have not been replicated in others [15, 17, 22–29]. The findings of the present study are consistent with reports on Czech and German populations. In general, the polymorphism C-509T as a risk factor in asthma appears to be more evident in Asian than in Caucasian subjects. These contradictory data may be due to different genetic backgrounds and varying environmental exposure.

No significant difference between the patients and the controls was found in genotype and allele frequency distributions for the G-800A polymorphism, indicating that it is not involved in asthma susceptibility. A small number of studies have investigated the role of the G-800A polymorphism in asthma [17, 19, 26], none of which found a positive correlation between this polymorphism and asthma. This was confirmed by the present study. In the present study, the distribution of observed genotypes for the G-800A polymorphism deviated from the Hardy-Weinberg equilibrium among both the patients (p = 0.01) and the controls (p = 0.05). There was an excess of GG homozygotes, while heterozygotes were much less frequent than expected. This deviation is probably due to the small number of analyzed samples.

To analyze influence of TGFB1 gene promoter polymorphisms on the clinical course of the disease, the patients were subgrouped according to exacerbations of their asthmatic symptoms. Exacerbations of asthma are acute or subacute episodes of progressive increase in shortness of breath, cough, wheezing or chest tightness, or combinations of these symptoms, and they occur when asthma control is poor. The results of both objective (spirometry test FEV1 and FVC values) and subjective (ACT values) measures of asthma control were significantly decreased in patients with exacerbations compared to patients without exacerbations. Genotype distribution failed to exhibit statistically significant differences for the studied polymorphisms between patients with and without exacerbations. However, the statistical significance for the distribution of the C-509T genotypes was near the borderline, with the -509TT genotype more common in patients with exacerbations (36.4% vs. 17.0%, p = 0.080). Considering that all the subjects in this study were followed up for at least three months after administration of the therapy, this finding indicates that response to therapy may be better in asthmatics who are carriers of genotypes other than -509TT and is worth further investigation.

The results of the present study suggest that polymorphism C-509T may play a role in asthma and predict exacerbations in asthmatic patients, and indicate that G-800A is not significant for either disease development or the clinical course. These findings should be confirmed in a larger study group, and the potential role of polymorphism C-509T in response to therapy should be further investigated. The impact of other polymorphisms in the TGFB1 gene or other genes suspected to be involved in inflammation or airway remodeling has not been ruled out. Since the TGFB1 promoter is highly complex and very responsive to environmental factors, future studies should also take other genetic and non-genetic factors into consideration. Identification of drug responders and non-responders, as well as patients most susceptible to adverse effects, may prove valuable in clinical practice and may lead to personalized treatments. This is of particular importance in asthma, due to the heterogeneity of the disease and the variety of available medications.

References

[1] **Postma DS, Timens W:** Remodeling in asthma and chronic obstructive pulmonary disease. Proc Am Thorac Soc 2006, 3, 434–439.
[2] **Bartram U, Speer CP:** The role of transforming growth factor beta in lung development and disease. Chest 2004, 125, 754–765.
[3] **Lv J, Liu Q, Hua L, Dong X, Bao Y:** Association of five single nucleotide polymorphism loci with asthma in children of Chinese Han nationality. J Asthma 2009, 46, 582–585.
[4] **Weiss ST, Raby BA, Rogers A:** Asthma genetics and genomics. Curr Opin Genet Dev 2009, 19, 279–282.
[5] **Kumar A, Ghosh B:** Genetics of asthma: A molecular biologist perspective. Clin Mol Allergy 2009, 7, 7.
[6] Global Strategy for Asthma Management and Prevention. Global Initiative for Asthma 2014.

[7] **Wu L, Chau J, Young RP, Pokorny V, Mills GD, Hopkins R:** Transforming growth factor-beta1 genotype and susceptibility to chronic obstructive pulmonary disease. Thorax 2004, 59, 126–129.
[8] **Howell JE, McAnulty RJ:** TGF-beta: its role in asthma and therapeutic potential. Curr Drug Targets 2006, 7, 547–565.
[9] **Bossé Y, Rola-Pleszczynski M:** Controversy surrounding the increased expression of TGF beta 1 in asthma. Respir Res 2007, 8, 66.
[10] **Duvernelle C, Freund V, Frossard N:** Transforming growth factor-beta and its role in asthma. Pulm Pharmacol Ther 2003, 16, 181–196.
[11] **Eap R, Jacques E, Semlali A, Plante S, Chakir J:** Cysteinyl leukotrienes regulate TGF-β(1) and collagen production by bronchial fibroblasts obtained from asthmatic subjects. Prostaglandins Leukot Essent Fatty Acids 2012, 86, 127–133.
[12] **Shah R, Rahaman B, Hurley CK, Posch PE:** Allelic diversity in the TGFB1 regulatory region: Characterization of novel functional single nucleotide polymorphisms. Hum Genet 2006, 119, 61–74.
[13] **Tamizifar B, Lankarani KB, Naeimi S, Rismankar Zadeh M, Taghavi A, Ghaderi A:** Promoter polymorphism of transforming growth factor-beta1 gene and ulcerative colitis. World J Gastroenterol 2008, 14, 243–247.
[14] **Schulte CM, Goebell H, Röher HD, Schulte KM:** C-509T polymorphism in the TGFB1 gene promoter: Impact on Crohn's disease susceptibility and clinical course? Immunogenetics 2001, 53, 178–182.
[15] **Silverman ES, Palmer LJ, Subramaniam V, Hallock A, Mathew S, Vallone J:** Transforming growth factor-beta1 promoter polymorphism C-509T is associated with asthma. Am J Respir Crit Care Med 2004, 169, 214–219.
[16] **Ueda T, Niimi A, Matsumoto H, Takemura M, Yamaguchi M, Matsuoka H:** TGFB1 promoter polymorphism C-509T and pathophysiology of asthma. J Allergy Clin Immunol 2008, 121, 659–664.
[17] **Nagpal K, Sharma S, B-Rao C, Nahid S, Niphadkar PV, Sharma SK:** TGFbeta1 haplotypes and asthma in Indian populations. J Allergy Clin Immunol 2005, 115, 527–533.
[18] **Syrris P, Carter ND, Metcalfe JC, Kemp PR, Grainger DJ, Kaski JC:** Transforming growth factor-beta1 gene polymorphisms and coronary artery disease. Clin Sci (Lond) 1998, 95, 659–667.
[19] **Wiśniewski A, Obojski A, Pawlik A, Jasek M, Luszczek W, Majorczyk E:** Polymorphism of the TGFB1 gene is not associated with bronchial allergic asthma in a Polish population. Hum Immunol 2009, 70, 134–138.
[20] **Saltzman BS, Yamamoto JF, Decker R, Yokochi L, Theriault AG, Vogt TM:** Association of genetic variation in the transforming growth factor beta-1 gene with serum levels and risk of colorectal neoplasia. Cancer Res 2008, 68, 1236–1244.
[21] **Yang YC, Zhang N, Van Crombruggen K, Hu GH, Hong SL, Bachert C:** Transforming growth factor-beta1 in inflammatory airway disease: a key for understanding inflammation and remodeling. Allergy 2012, 67, 1193–1202.
[22] **Li H, Romieu I, Wu H, Sienra-Monge JJ, Ramírez-Aguilar M, del Río-Navarro BE:** Genetic polymorphisms in transforming growth factor beta-1 (TGFB1) and childhood asthma and atopy. Hum Genet 2007, 121, 529–538.
[23] **Pulleyn LJ, Newton R, Adcock IM, Barnes PJ:** TGFbeta1 allele association with asthma severity. Hum Genet 2001, 109, 623–627.
[24] **Salam MT, Gauderman WJ, McConnell R, Lin PC, Gilliland FD:** Transforming growth factor-1 C-509T polymorphism, oxidant stress, and early-onset childhood asthma. Am J Respir Crit Care Med 2007, 176, 1192–1199.
[25] **Hobbs K, Negri J, Klinnert M, Rosenwasser LJ, Borish L:** Interleukin-10 and transforming growth factor-beta promoter polymorphisms in allergies and asthma. Am J Respir Crit Care Med 1998, 158, 1958–1962.
[26] **Buckova D, Izakovicová Hollá L, Benes P, Znojil V, Vácha J:** TGF-beta1 gene polymorphisms. Allergy 2001, 56, 1236–1237.
[27] **Heinzmann A, Bauer E, Ganter K, Kurz T, Deichmann KA:** Polymorphisms of the TGF-beta1 gene are not associated with bronchial asthma in Caucasian children. Pediatr Allergy Immunol 2005, 16, 310–314.
[28] **Mak JC, Leung HC, Ho SP, Law BK, Ho AS, Lam WK:** Analysis of TGF-beta(1) gene polymorphisms in Hong Kong Chinese patients with asthma. J Allergy Clin Immunol 2006, 117, 92–96.
[29] **Acevedo N, Vergara C, Gusmão L, Jiménez S, Martínez B, Mercado D:** The C-509T promoter polymorphism of the transforming growth factor beta-1 gene is associated with levels of total and specific IgE in a Colombian population. Int Arch Allergy Immunol 2010, 151, 237–246.

Address for correspondence:

Sandra Dragicevic
Institute of Molecular Genetics and Genetic Engineering
Vojvode Stepe 444A
PO Box 23
11010 Belgrade
Serbia
Tel.: +381 11 39 76 658
E-mail: sandra.d@imgge.bg.ac.rs

Conflict of interest: None declared

Received: 6.10.2014
Revised: 5.11.2014
Accepted: 11.12.2014