

ORIGINAL ARTICLE

Evaluation of proteasomal gene polymorphisms in Lithuanian patients with asthma

Zivile Zemeckiene¹, Brigita Sitkauskienė, MD, PhD², Edita Gasiuniene, MD², Natalija Paramonova³, Laura Tamasauskiene, MD², Astra Vitkauskienė, MD, PhD¹, Tatjana Sjakste, PhD^{3*}, and Raimundas Sakalauskas, MD, PhD^{2*}

¹Department of Laboratory Medicine, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania, ²Department of Pulmonology and Immunology, Medical Academy, Lithuanian University of Health Sciences, Kaunas, Lithuania, and ³Department of Genomics and Bioinformatics, Institute of Biology, University of Latvia, Salaspils, Latvia

Abstract

Objective: To investigate polymorphisms of proteasomal genes *PSMA6* (rs1048990 and rs2277460), *PSMC6* (rs2295826 and rs2295827) and *PSMA3* (rs2348071) in Lithuanian patients with asthma. **Methods:** One-hundred forty-six asthma patients and 150 control subjects were studied. DNA was extracted from peripheral blood samples. Five single nucleotide polymorphisms (SNP's) of the three proteasomal genes were analyzed using allele-specific amplification or the cleaved amplified polymorphic sequence method. **Results:** While certain alleles and genotypes of *PSMA6* rs2277460 and rs1048990 and *PSMA3* rs2348071 SNP's occurred more frequently in asthma patients than in controls, no statistically significant differences in alleles or genotypes of *PSMA6*, *PSMC6* or *PSMA3* were observed between asthma patients and control subjects. However, when male and female study subjects were considered separately, we found that the CG genotype of *PSMA6* rs1048990 is significantly more frequent in male asthma patients compared to male control subjects. **Conclusions:** We found no significant differences in frequencies of selected five proteasomal gene *PSMA6*, *PSMC6* and *PSMA3* SNP's between asthma patients and control subjects overall. Among male subjects, however, the CG *PSMA6* rs1048990 genotype was significantly more frequent in asthma patients compared with control subjects.

Keywords

PSMA6, PSMC6, PSMA3

History

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Introduction

Asthma is a common chronic inflammatory airway disease affecting about 30 million people in Europe and about 300 million people in the world [1]. Although the pathogenesis of asthma is widely studied, its development still remains unclear. Many studies have focused on the genetics of asthma, and more than 100 loci have been implicated in the development of asthma and allergic disease [2,3]. Early studies showed a possible linkage of chromosome 14 to asthma [4,5]. Chromosome 14 contains several genes encoding proteasome subunits, including *PSMA6* gene, which encode alpha type 6 proteasome subunit, *PSMC6* gene encoding 26S proteasome ATPase subunit 6 and *PSMA3*, which encode alpha type 3 proteasome subunit. Polymorphisms in these and other proteasome encoding genes are associated with a variety of inflammatory diseases [6]. Chromosome 14, indicating location of studied single

nucleotide polymorphisms (SNP's) in three proteasome genes, is shown in Figure 1.

The proteasome is a multicatalytic protein complex. It is a key component of the ubiquitin-proteasome proteolytic pathway in all eukaryotic cells. This pathway is responsible for the degradation of approximately 80% of intracellular proteins [7]. Degradation of a protein *via* the ubiquitin–proteasome system occurs in two steps: covalent attachment of multiple ubiquitin molecules to the substrate and degradation by proteasome. The 26S proteasome is composed of a core 20S particle and two 19S regulatory particles on both ends [6,7]. Proteasomes play an important role in many cell processes and are essential to the normal functioning of cells [8].

In asthma, pathological factors are regulated by inflammatory cells, predominantly eosinophils, lymphocytes and neutrophils, which infiltrate the airways and release a myriad of pro-inflammatory substances, which trigger the development of disease [9]. Most of these inflammatory mediators are regulated by the nuclear factor kappa B (NF-κB) signaling pathway. Therefore NF-κB plays a very important role in the development and progression of inflammatory diseases, including asthma. Proteasomes within the ubiquitin–proteasome system are one of the main regulators of NF-κB [10].

*These authors contributed equally to this work.

Correspondence: Raimundas Sakalauskas, MD, PhD, Prof., Department of Pulmonology and Immunology, Lithuanian University of Health Sciences, Eiveniu str. 2, LT-50009 Kaunas, Lithuania. E-mail: raimundas.sakalauskas@kaunoklinikos.lt

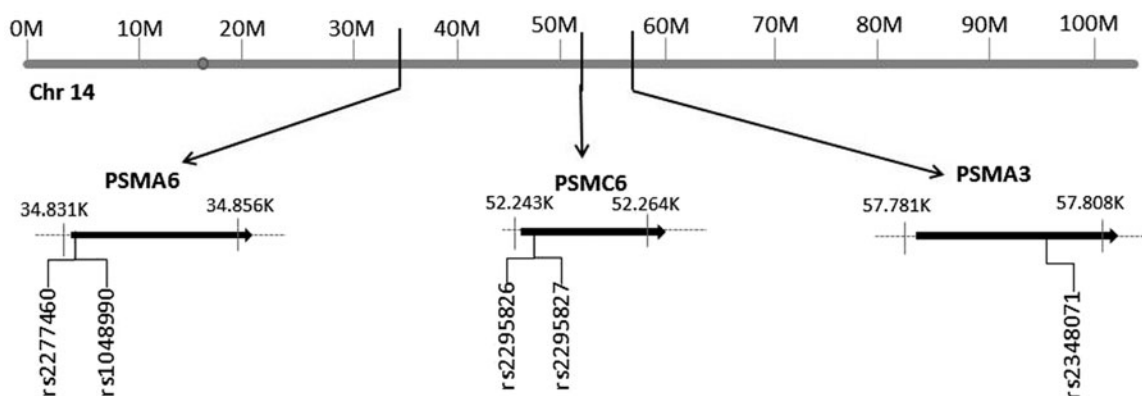


Figure 1. Scheme of the chromosome 14, indicating location of studied SNP's in three proteasome genes.

Thus proteasomes may participate in the regulation of inflammation and pathogenesis of asthma.

The proteins of the ubiquitin–proteasome system are encoded by many genes, some of which have been shown to be involved in the pathogenesis of various diseases [8,11,12]. Possible links between proteasomal gene polymorphism and human pathologies were first reported in 1994 [13] and association between *LMP2* (*PSMB9*) gene, which encode beta type 9 subunit of proteasome, and autoimmune diseases was reported in 1995 [14]. In 2006, Ozaki et al. published evidence of a possible association between *PSMA6* gene polymorphism and myocardial infarction [15]. Since then many studies have shown association of proteasome encoding genes, particularly *PSMA6*, with various diseases, including ischemic heart disease, myocardial infarction and other cardiovascular diseases, type 2 diabetes mellitus, neurodegenerative, hematological and autoimmune diseases and other chronic inflammatory disorders [6,16–18]. No studies investigating the relationship between proteasomal gene polymorphisms and asthma have been published, however. In this study, we investigate polymorphisms of proteasomal genes *PSMA6* (rs1048990 and rs2277460), *PSMC6* (rs2295827 and rs2295826) and *PSMA3* (rs2348071) in Lithuanian patients with asthma.

Methods

Study population

The study group consisted of 296 individuals, 146 diagnosed as having asthma (asthma group) and 150 without asthma (control group). Asthma was recruited and diagnosed by allergologist-clinical immunologist or pulmonologist based on clinical history (with duration of symptoms more than two years), lung function measurements and methacholine provocation test according to the guidelines of the Global Initiative for Asthma [1].

Study subjects were enrolled according to the following criteria: older than 18 years, without any congenital disease and/or any known family predisposition to congenital disease (except asthma), without acute or chronic infections and without oncological, autoimmune or any other chronic disease/condition that might have a negative influence on the study parameters. Only patients with asthma who have clinical symptoms two or more years and no exacerbation of

this disease for three months were involved in this study. Subjects with and without asthma who underwent prophylactic evaluation at Kaunas Family Medicine Centers and Hospital of Lithuanian University of Health Sciences and fulfilled inclusion/exclusion criteria were enrolled in the study. Informed consent was obtained from all the participants. The study was approved by The Kaunas Regional Biomedical Research Ethics Committee (no. BE-2-31). This study is a part of international project ‘‘Proteasomal genes alleles as risk factors for bronchial asthma in Latvian, Lithuanian and Taiwanese populations’’.

DNA extraction and genotyping

DNA from peripheral blood samples was extracted using QIAamp DNA blood mini kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Five SNP's of three proteasomal genes on chromosome 14 were analyzed in this study using allele specific amplification and cleaved amplified polymorphic sequence methods. SNP's and genotyping details are listed in Table 1. Primer sequences and genotyping methods were previously described [19,20]. PCR was performed in a total volume of 30 μ l using the DreamTaq polymerase or DNA Taq polymerase (recombinant) (Fermentas, Vilnius, Lithuania) under the following conditions: denaturation at 94 $^{\circ}$ C for 5 min followed by 35–40 cycles of 94 $^{\circ}$ C for 45 s, appropriate annealing temperature (55–61 $^{\circ}$ C) for 45 s, 72 $^{\circ}$ C for 45 s and a final extension at step at 72 $^{\circ}$ C for 7 min. DNA digestion by restriction enzymes was performed according to the manufacturer's protocols (Fermentas). *RsaI* digestion was used to determine the rs1048990 genotype, *TspRI* digestion was used to determine the rs2348071 genotype and *DdeI* (*HpyF3I*) digestion was used to determine the rs2295826 and rs2295827 genotypes. All amplified and digested PCR products were analyzed by electrophoresis in 2–4% agarose gel.

For quality control, 16 randomly chosen samples per each marker were genotyped in duplicate in different experiments. Furthermore, 20 randomly chosen samples were verified by direct sequencing. The concordance was 100%.

Statistical analysis

Allele and genotype frequencies were estimated by direct gene counting. The χ^2 test was performed to compare the

Table 1. SNP information and genotyping details.

Gene	SNP ID	Motif (common allele is first indicated)	Genotyping method	Primer sequences	Annealing temperature/ cycle number
<i>PSMA6</i>	rs2277460	c.-110C > A	Allele specific amplification	F ^C -5'-ATGCAAGAGCGGAAGAAAC-3' F ^A -5'-ATGCAAGAGCGGAAGAAA-3' R-5'-TACCATGACAGGGCAATTCAG-3'	55 °C/35
	rs1048990	c.-8C > G	Cleaved amplified polymorphic sequence		
<i>PSMC6</i>	rs2295826	c.86-104A > G	Cleaved amplified polymorphic sequence	F-5'-GCTTAAACAAGTATTGCCGATCA-3' R-5'-AATTGTTCCCTTACTGAAATAATACAA-3'	61 °C/40
	rs2295827	c.86-46C > T			
<i>PSMA3</i>	rs2348071	c.543 + 138G > A	Cleaved amplified polymorphic sequence	F-5'-GTCTAAGGCAGGGATGTCCA-3' R-5'-ACCAGCTTTCCTTCAGTG-3'	61 °C/35

F^C - forward primer for *PSMA6* gene to identify nucleotide C at -110 position. F^A - forward primer to identify nucleotide A

Table 2. Study group characteristics.

	Control group	Asthma group
Subjects (n)	150	146
Men/women (n)	53/97	52/94
Age, years ^a	39.1 ± 14.9	46.4 ± 15.3
Positive skin prick test, %	0	67.3
	Asthma group	
	Allergic asthma	Non-allergic asthma
FEV1, l ^a	3.2 ± 0.1	2.3 ± 0.1
FEV1, % ^a	95.1 ± 2.3	91.6 ± 4.3
FVC, l ^a	4.1 ± 0.2	3.2 ± 0.2
FVC, % ^a	104.7 ± 2.0	104.3 ± 4.2
Eosinophil count, % ^{a*}	5.9 ± 0.5	4.2 ± 0.5
IgE level, IU/ml ^{a,**}	616.9 ± 116.0	110.2 ± 35.1

^aValues are expressed as mean ± SEM.

***p* < 0.01.

**p* < 0.05.

results between two groups, and the binary logistic regression was carried out to evaluate the expectancy of asthma having different genotypes. A *p* value of <0.05 was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 13, Kaunas, Lithuania).

Deviation from Hardy–Weinberg equilibrium was tested for each genetic marker by the χ^2 test, using an online calculator [21]. Online software Cubex (from <http://www.oeg.org/software/cubex/>) was used to evaluate the pairwise linkage disequilibrium (LD) between the different SNP's (*D'* and *r*²) [22]. Statistical power for the χ^2 test and binary logistic regression varies from 30% to 50%.

Results

Characteristics of the study subjects are listed in Table 2. The number of men and women and the mean age did not differ between asthma and control groups. Eosinophil count and IgE level were significantly more common in patients with allergic asthma than in those with non-allergic. Lung function parameters did not differ significantly between these groups.

The observed genotypes and allele distributions were in Hardy–Weinberg equilibrium (*p* > 0.05). We found that two SNP's – rs2295826 and rs2295827 – were in complete LD (*D'* = 1, *r*² = 1).

The genotyping results are listed in Table 3. We did not find significant differences in genotypes or alleles of *PSMA6* (rs1048990, rs2277460), *PSMC6* (rs2295827, rs2295827) or *PSMA3* (rs2348071) genes between the asthma group and the control group. The A allele and CA genotype of rs2277460 (*PSMA6*), the G allele of rs1048990 (*PSMA6*) and the GA genotype of rs2348071 (*PSMA3*) occurred more frequently in the asthma group than the control group, although not to the level of statistical significance. In addition, the GG genotype of rs2348071 (*PSMA3*) is less common in the asthma group than in the control group, but again, this difference was not statistically significant.

We investigated proteasomal genes diversity between patients with different asthma phenotypes and found no significant results. We also investigated the frequencies of *PSMA6*, *PSMC6* and *PSMA3* SNP's according to subject gender (Table 4). We found no significant differences between men and women in the asthma group, but we did find that the CG genotype of rs1048990 (*PSMA6*) is significantly more common in women than in men in the control group. In addition, we found that the CG rs1048990 genotype occurred significantly more frequently in men from the asthma group than in men from the control group. The frequency of this genotype did not differ significantly between women from the asthma and control groups.

We used binary logistic regression to find out the expectancy of asthma having different genotypes, and we found no statistically significant results (Table 5). We also used this test to evaluate the impact of other factors such as age and gender on asthma development and found that these criteria did not play important role in our study.

Discussion

This study investigates five SNP's of three proteasomal genes in Lithuanian individuals with and without asthma. We have chosen the current proteasomal genes after precise analysis of scientific literature regarding proteasomes and genes that might be involved in the pathogenesis of chronic inflammatory disease. No significant differences in frequencies of proteasomal gene polymorphisms were observed between the asthma group and the control group, although some polymorphisms tended to be more prevalent in the asthma group.

Table 3. Comparison of genotypes and alleles of *PSMA6* (rs1048990 and rs2277460), *PSMC6* (rs2295827 and rs2295827) and *PSMA3* (rs2348071) between asthma group and control group.

Gene	Allele/genotype	Control group		Asthma group		χ^2
		Frequency (n)	Frequency (%)	Frequency (n)	Frequency (%)	
<i>PSMA6</i> rs2277460	A	28	9.33	36	12.33	1.38
	CC	122	81.33	110	75.34	1.57
	CA	28	18.67	36	24.66	1.57
<i>PSMA6</i> rs1048990	G	46	15.33	50	17.12	0.35
	CC	106	70.67	100	68.49	0.17
	CG	42	28.00	42	28.77	0.02
<i>PSMC6</i> rs2295826	GG	2	1.33	4	2.74	0.74
	G	51	17.00	40	13.70	1.24
	AA	105	70.00	109	74.66	0.80
<i>PSMC6</i> rs2295827	AG	39	26.00	34	23.29	0.30
	GG	6	4.00	3	2.05	0.95
	T	51	17.00	40	13.70	1.24
<i>PSMA3</i> rs2348071	CC	105	70.00	109	74.66	0.80
	CT	39	26.00	34	23.29	0.30
	TT	6	4.00	3	2.05	0.95
<i>PSMA6</i> rs1048990	A	80	26.67	90	30.82	1.25
	GG	81	54.00	67	45.89	1.95
	GA	58	38.67	68	46.58	1.90
<i>PSMA6</i> rs2277460	AA	11	7.33	11	7.53	0.004

Significance level of all studied parameters between asthma and control groups was >0.05 .

Table 4. Comparison of genotypes and alleles of *PSMA6* (rs1048990 and rs2277460), *PSMC6* (rs2295827 and rs2295827) and *PSMA3* (rs2348071) between men and women.

Gene	Allele/genotype	Control group		χ^2	Asthma group		χ^2
		Men (%)	Women (%)		Men (%)	Women (%)	
<i>PSMA6</i> rs2277460	A	11.3	8.25	0.77	11.8	12.2	0.01
	CC	77.4	83.5	0.86	76.5	75.5	0.02
	CA	22.6	16.5	0.86	23.5	24.5	0.02
<i>PSMA6</i> rs1048990	G	12.3	17.0	1.19	18.6	16.5	0.21
	CC	79.2	65.9	2.91	62.7	71.3	1.11
	CG	17.0 [#]	34.1	4.94 [*]	37.3 [#]	24.5	2.63
<i>PSMC6</i> rs2295826	GG	3.8	0.0	3.71	0.0	4.2	2.23
	G	15.1	18.0	0.42	17.6	11.2	2.38
	AA	73.6	68.0	0.50	68.6	78.7	1.81
<i>PSMC6</i> rs2295827	AG	22.6	27.8	0.48	27.5	20.2	0.99
	GG	3.8	4.2	0.01	3.9	1.1	1.33
	T	15.1	18.0	0.42	18.6	11.2	3.68
<i>PSMA3</i> rs2348071	CC	73.6	68.0	0.50	66.7	78.7	2.53
	CT	22.6	27.8	0.48	29.4	20.2	1.56
	TT	3.8	4.2	0.01	3.9	1.1	1.33
<i>PSMA6</i> rs1048990	A	26.4	26.7	0.01	27.5	31.4	0.49
	GG	56.6	52.6	0.22	51.0	44.7	0.53
	GA	34.0	41.2	0.77	43.1	47.9	0.30
<i>PSMA6</i> rs2277460	AA	9.4	6.2	0.53	5.9	7.4	0.13

* $p < 0.05$.

[#] $p < 0.05$ comparing men from asthma group and men from control group.

When the data was analyzed according to subject gender, we found that the CG allele of rs1048990 (*PSMA6*) was significantly more common in men from the asthma group than in men from the control group.

While the link between proteasomal gene polymorphisms and asthma has not been investigated until now, we could not find any data about mechanisms of proteasomal genes in asthma. However, links between these polymorphisms, mainly SNPs of *PSMA6* gene, and other diseases, particularly diseases involving chronic inflammatory processes, have been investigated [18,20,23–25]. Some studies provide evidence that *PSMA6* polymorphism is associated with diabetes mellitus [18]. Ozaki et al. reported that the G allele of

rs1048990 (*PSMA6*) was significantly associated with myocardial infarction in Japanese population [15]. They showed that G allele, which is considered to be a risk allele, might increase the expression of *PSMA6* protein *in vivo* and *in vitro*, leading to enhanced inflammation through the activation of the NF- κ B protein [15]. Free NF- κ B moves to the nucleus where it binds to target DNA elements and activate transcription of genes encoding proteins involved in immune responses, inflammation or cell proliferation. NF- κ B could be considered as a coordinating element in the body's responses to situations of stress, infection or inflammation [26,27]. Thus, this polymorphism might be important for the development of chronic inflammation. Despite the

Table 5. Expectancy of asthma having different genotypes.

	<i>p</i>	EXP (B)	95% CI for EXP(B)	
<i>PSMA6</i> rs2277460 (CC)	0.15	1.53	0.87	2.69
<i>PSMA6</i> rs1048990 (CC)	0.40	0.47	0.08	2.68
<i>PSMA6</i> rs1048990 (CG)	0.47	0.52	0.09	3.04
<i>PSMC6</i> rs2295826 (AA)	0.35	1.98	0.48	8.22
<i>PSMC6</i> rs2295826 (AG)	0.50	1.66	0.38	7.26
<i>PSMC6</i> rs2295827 (CC)	0.31	2.08	0.51	8.52
<i>PSMC6</i> rs2295827 (CT)	0.46	1.74	0.41	7.51
<i>PSMA</i> rs2348071 (GG)	0.70	1.20	0.48	2.96
<i>PSMA</i> rs2348071 (GA)	0.20	1.43	0.88	2.31

fact that data showing role of proteasomal genes in asthma is lacking, we can explain this in the context of other chronic inflammatory disease studies. Furthermore, that is why in this study, we aimed to evaluate this polymorphism in association with asthma in which inflammation is the predominant mechanism. We found that the rs1048990 G allele was slightly more frequent in the asthma group than in the control group, and that the heterozygous rs1048990 CG genotype was significantly more frequent in men from the asthma group compared to men from the control group. The inflammation-enhancing effect of the rs1048990 G allele could be stronger in men, leading to an increased probability of developing asthma. Gender differences have been previously demonstrated in asthma and inflammation [27,28]. However, asthma is more common in men than women only in their young age [1].

In our study, the A allele and CA genotype of *PSMA6* rs2277460 was found more frequently in the asthma group compared to the control group, although this was not a statistically significant difference. Since this SNP is in the promoter region, the minor A allele might increase the expression of *PSMA6* [23] and thereby increase inflammation. However, this polymorphism was not associated with myocardial infarction in other studies [23,24].

In this study, some *PSMA6* and *PSMA3* SNP's occurred less or more frequently in the asthma group than in the control group, but these differences were not statistically significant. One explanation for this lack of significance could be the relatively small number of subjects in this study. Other studies showing positive association between *PSMA6* gene polymorphism and various diseases (mainly myocardial infarction) had varied number of subjects (from 224 [23] to 4527 genotyped individuals [17]).

Taken all together, some proteasome gene polymorphisms might affect inflammation and asthma, and we found that some SNP's tended to be more prevalent in the asthma group. Although, the results showed no statistical significance, we cannot completely reject possible association. Analysis of haplotypes could add additional information; however, we need additional software that will be used in our future project. Since asthma is a very complex genetic disease, the influence of proteasomal gene polymorphism could be very small, and it could be difficult to establish its association with the disease. Further and larger studies will be needed to clarify the possible associations between proteasomal gene polymorphisms and asthma.

Conclusion/key findings

We found no significant differences in the frequencies of five selected proteasomal gene *PSMA6*, *PSMC6* or *PSMA3* SNP's between asthma patients and control subjects, but we did observe a trend toward higher frequency of certain *PSMA6* and *PSMA3* alleles and genotypes in the asthma group. The relatively small number of study subjects may have affected the results. Among male subjects, the CG *PSMA6* rs1048990 genotype is significantly more frequent in asthma patients compared to control subjects. Since the rs1048990 G allele is associated with other inflammatory diseases, further study is warranted to clarify the relationship between that polymorphism and asthma.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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