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ASTHMA

The Variants in the 3′ Untranslated Region of the Matrix Metalloproteinase 9 Gene as Modulators of Treatment Outcome in Children with Asthma

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Abstract

Purpose The maintaining of asthma control is difficult due to high variability in response to therapy among patients. Since matrix metalloproteinase 9 (MMP9) is implicated in inflammation and remodeling of asthmatic airways, it could be associated with adequate response to asthma therapy. The aim of this study was to investigate whether variants in 3′ end of the *MMP9* gene are associated with clinical phenotype and responsiveness to treatment in children with asthma.

Methods The study included 127 asthmatic children from Slovenia. Variants in the 3′ end of the *MMP9* gene were analyzed by direct DNA sequencing and the obtained results were correlated with clinical parameters.

Results Two variants were detected, rs13925 and rs20544. For the variant rs20544, statistically significant difference in airway hyperresponsiveness ($p = 0.011$) and asthma control ($p = 0.049$) between genotypes was found. Patients with TT genotype had lower airway sensitivity, and after 12 months of treatment showed significant improvement in Asthma Control Test (ACT) scores compared to CC and CT genotype. For the variant rs13925, the association with lung function was observed. The carriers of A allele showed noticeable improvement of lung function after the first 6 months of treatment in comparison to the carriers of G allele ($p = 0.046$).

Conclusion The main finding of our study is the association of MMP9 genotypes rs20544 TT and rs13925 AA and AG with better asthma control, and indirectly better response to treatment. Based on these results, MMP9 deserves further research as a potential predictive biomarker for asthma.

Keywords 3′ UTR · Genotype · Matrix metalloproteinase · Variant

Introduction

Asthma is one of the most common chronic diseases of childhood, affecting millions of children (1 in 10), with increasing prevalence in most countries [1]. It is a heterogeneous disease, usually characterized by chronic airway inflammation and symptoms such as wheeze, shortness of breath, chest tightness, cough, and variable expiratory airflow limitation [2]. One of the consequences of the chronic inflammation in asthma is remodeling of the airway extracellular matrix (ECM).

Matrix metalloproteinases (MMPs) are major players in tissue remodeling [3]. They are produced by many types of cells, such as fibroblasts, endothelial cells, and inflammatory cells (macrophages, neutrophils, and eosinophils). In order to prevent the damage that their action can induce in the tissue, the MMPs expression and function must be

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tightly regulated and controlled. The inadequate regulation of MMPs is implicated in many pathologies, including asthma [3].

Matrix metalloproteinase 9 (MMP9) is thought to be the major MMP in the airway of the asthmatics, affecting both airway remodeling and inflammation. Elevated MMP9 is considered a marker of the damage that inflammation causes to bronchial epithelium. Increased levels of MMP9 were found in bronchial biopsies, bronchoalveolar lavage fluid (BALF), and sputum of asthmatic patients when compared to healthy controls [4–6].

The gene encoding for MMP9 maps to the chromosome region 20q11.2–q13.1 [7]. Sequence analysis revealed several variants, some of which were shown to be functionally important. Regulation of *MMP9* gene expression has not been sufficiently studied. One functional variant in the 5′ untranslated region (UTR) that leads to increased protein expression has been reported (− 1562 C/T, rs3918242). This variant was associated with chronic obstructive pulmonary disease (COPD), asthma, myocardial infraction, and various types of cancer. Several variants in the 3′ UTR of the *MMP9* gene were identified (rs1056628, rs13925, and rs20544), and they have been associated with higher risk of developing several pathological conditions, but have not been studied in asthma [8, 9].

The most commonly used controller medications for asthma treatment are inhaled corticosteroids (ICS). They reduce inflammation and hyperresponsiveness of the airways by inhibiting inflammatory cell migration and activation, and by blocking late phase reaction to allergens. Leukotriene receptor antagonists (LTRA), such as montelukast, are used either alone as second-line therapy or in combination with ICS for the management of persistent asthma [2, 10]. There are evident differences in the response to therapy in asthmatic patients [11]. Studies have revealed that this diversity is mainly due to genetic factors, which can account for up to 60% of the interindividual variability in response to therapy [12]. There are 5–10% of asthmatic patients that do not respond to ICS treatment [13]. Treatment with ICS has been shown to decrease expression of MMP9 and increase expression of its inhibitor TIMP-1 in bronchial mucosa of asthmatics. Interindividual variability in response to montelukast in both children and adults with asthma is significant, with 35–78% of patients treated with montelukast being classified as non-responders [14–16]. Given the important role of MMP9 in asthma pathogenesis, it can be hypothesized that the variants in the regulatory regions of the *MMP9* gene can affect response to asthma treatment.

The aim of this study was to determine whether variants in the 3′ UTR of the *MMP9* gene are associated with clinical phenotype and responsiveness to treatment in children with asthma.

Materials and Methods

Subjects

This study has encompassed 127 children with asthma who were diagnosed and treated at the Private Practice Cebelica in Maribor, Slovenia, over a period of 12 consecutive months. At the inclusion into the study, patients were steroid naïve (22%) or were put off controller medication for 1 month (78%).

All subjects underwent spirometry, bronchoprovocation test with methacholine, and measurement of fractional exhaled nitric oxide (FeNO). Asthma diagnosis and phenotypes (persistence and severity of the disease) were defined according to the Global Initiative for Asthma (GINA) guidelines [2]. Asthma control was evaluated based on asthma control test (ACT) and the disease was considered uncontrolled if ACT scores were under 20 at least once during 12 months of follow-up. The skin prick tests to common inhalant, environmental, and food allergens were done according to the European Academy of Allergy and Clinical Immunology (EAACI) guidelines [17]. The size of the weal of 3 mm in diameter or more was considered as a positive finding. The patients were then randomly put on treatment with either fluticasone propionate (200 or 400 mcg, according to age) or montelukast (5 or 10 mg, according to age).

Analysis of *MMP9* Gene 3′ UTR

Peripheral blood samples were taken from all patients. Genomic DNA was extracted from the whole blood using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, USA) following manufacturer's instructions.

Amplification of the 3′ UTR of the *MMP9* gene was performed by polymerase chain reaction (PCR) using forward primer 5′-GTA TAT GTG GGA GAA TTA GAA TCA-3′ and reverse primer 5′-ACA TGC ATA CAT ACG TGC ATA C-3′. The following program was used for amplification: 95 °C for 10 min; 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s; and 72 °C for 10 min. The reaction was performed in a 25 µL volume containing 50 ng of DNA, 1× Reaction buffer with Mg (Kapa Biosystems, Boston, USA), 200 µM deoxynucleotide triphosphates, 0.5 pmol of each primer, and 1.25 U Taq DNA polymerase (Kapa Biosystems, Boston, USA).

The patients were genotyped for variants in the *MMP9* gene 3′ UTR by direct DNA sequencing. Amplified DNA was purified using GeneJET PCR Purification Kit (Thermo Scientific, Waltham, USA) according to the

manufacturer's protocol. The obtained PCR products (409 bp) were sequenced using ABI Prism BigDye Terminator Kit (Applied Biosystems, Foster City, USA) on 3130 Genetic Analyzer (Applied Biosystems, Foster City, USA). Sequences were analyzed using the Sequencing Analysis software (Applied Biosystems, Waltham, USA).

Statistical Analysis

Statistical analysis was performed using Statistical Package for Social Sciences 20.0 (SPSS Inc., Chicago, Illinois, USA). Data were expressed as percentages and mean ± standard deviation (SD) for continuous variables and percentages for categorical variables. To test the normality of parameters one sample Kolmogorov–Smirnov test was used. Differences between groups for categorical data were tested by χ^2 analysis, while for continuous data-independent samples Kruskal Wallis test was used. p values < 0.05 were considered statistically significant.

Results

In this study, 3′ UTR of the *MMP9* gene was analyzed in 127 asthmatics aged 5–19 years. Demographic and clinical characteristics of the study population are presented in Table 1.

Two variants in the 3′ UTR of the *MMP9* gene were detected, rs13925 and rs20544. The obtained distributions of alleles and genotypes for both variants are presented in Table 2.

Table 1 Description of the study population

Age, years (mean ± SD)	12.1 ± 2.9
Males/females, *n* (%)	72 (57)/55 (43)
Positive skin prick test, *n* (%)	102 (80.3)
FEV1, % (mean ± SD)	95.5 ± 12.8
FEV1/FVC, % (mean ± SD)	89.3 ± 5.9
FeNO, ppb (mean ± SD)	42.1 ± 31.1
Asthma therapy, *n* (%)	
Fluticasone propionate	37 (29.4)
Montelukast	90 (70.6)
Asthma control, *n* (%)	
Good	88 (69.3)
Poor	39 (30.7)
Asthma type, *n* (%)	
Mild	40 (31.5)
Moderate	57 (44.9)
Severe	30 (23.6)

SD standard deviation, *FEV1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *FeNO* fractional exhaled nitric oxide, *ppb* parts per billion

Table 2 The distribution of allele and genotype frequencies for variants rs13925 and rs20544

		n (%)
Alleles		
rs13925	A	39 (15.4)
	G	215 (84.6)
rs20544	C	128 (50.4)
	T	126 (49.6)
Genotypes		
	AA	2 (1.6)
rs13925	GA	35 (27.6)
	GG	90 (70.8)
	CC	33 (26.0)
rs20544	CT	62 (48.8)
	TT	32 (25.2)

The distribution of rs13925 and rs20544 genotypes was correlated with results of spirometry tests (FEV1 and FVC), methacholine challenge test, and ACT (Table 3). We found that the T allele and T genotype of variant rs20544 were associated with lower airway hyperresponsiveness ($p = 0.030$ for allele and 0.011 for genotype).

In order to assess the impact of detected variants on asthma progression, control, and response to treatment, the distribution of rs13925 and rs20544 genotypes was also correlated with results of spirometry tests (FEV1 and FVC) and ACT at two time points, 6 and 12 months after the beginning of therapy. When changes in FEV1 after 6 and 12 months of treatment were correlated with genotypes it was found that carriers of rs13925 AA genotype had significantly better lung function (Table 4). Since there were only two patients with this genotype, the results could not be considered statistically significant. However, when allele frequencies were correlated with changes in FEV1, carriers of A allele had statistically significant increase of FEV1 compared to carriers of G allele after the first 6 months of treatment (3.8 ± 9.2 vs 1.0 ± 14.1%; $p = 0.046$). The difference in change of FEV1 between carriers of A allele or G allele after 12 months of treatment was not observed (3.5 ± 9.6 vs 3.3 ± 13.8%; $p = 0.251$). The presence of variant rs20544 did not affect lung function of asthmatic patients. However, patients with TT rs20544 genotype had higher increase in ACT scores after 12 months of treatment compared to CC and CT carriers ($p = 0.049$). Also, T allele was associated with decrease in asthma symptoms after 12 months of treatment. The increase in ACT scores was significantly higher in patients carrying T allele compared to patients carrying C allele (1.3 ± 2.9 vs 0.5 ± 2.9%; $p = 0.030$). ACT scores obtained at 6th month of treatment were similar between carriers of A allele or G allele (1.6 ± 13.6 vs 1.3 ± 13.3%; $p = 0.131$).

The patients were stratified into groups according to the type of asthma therapy. Distributions of rs13925 and

Table 3 Correlation between rs13925 and rs20544 genotypes and clinical parameters in asthmatic patients

Variant	rs13925			
Genotype	AA	GA	GG	p^a
FEV1, % (mean ± SD)				
At baseline	85.5 ± 9.2	96.5 ± 10.3	95.3 ± 13.6	0.295
After 6 months of treatment	103.0 ± 8.5	98.7 ± 9.3	96.1 ± 10.7	0.538
After 12 months of treatment	102.0 ± 2.8	99.1 ± 10.5	98.8 ± 11.1	0.825
FEV1/FVC, % (mean ± SD)				
At baseline	84.0 ± 5.7	89.1 ± 4.8	89.4 ± 6.3	0.442
After 6 months of treatment	88.5 ± 3.5	90.1 ± 4.3	89.8 ± 5.6	0.846
After 12 months of treatment	86.0 ± 2.8	89.3 ± 6.1	89.6 ± 6.4	0.479
ACT, % (mean ± SD)				
At baseline	23.0 ± 2.8	22.9 ± 2.5	21.9 ± 3.4	0.407
After 6 months of treatment	22.0 ± 1.4	22.4 ± 3.1	22.5 ± 2.9	0.798
After 12 months of treatment	24.0 ± 1.4	22.7 ± 3.9	23.2 ± 2.5	0.944
PC20 mg/mL	0.2 ± 0.2	0.5 ± 0.5	0.6 ± 0.7	0.376
Asthma control, *n* (%)				
Good	2 (2.2)	27 (30.3)	60 (67.5)	0.335
Poor	0 (0)	8 (21.1)	30 (78.9)	
Variant	rs20544			
Genotype	CC	CT	TT	p^a
FEV1, % (mean ± SD)				
At baseline	97.4 ± 10.7	93.7 ± 14.3	97.1 ± 11.3	0.492
After 6 months of treatment	97.5 ± 8.4	96.8 ± 10.1	96.7 ± 12.3	0.867
After 12 months of treatment	100.4 ± 9.9	98.6 ± 10.5	97.9 ± 12.4	0.361
FEV1/FVC, % (mean ± SD)				
At baseline	89.4 ± 6.4	89.4 ± 5.5	88.9 ± 6.3	0.951
After 6 months of treatment	91.1 ± 5.3	89.7 ± 4.6	89.1 ± 6.3	0.187
After 12 months of treatment	90.5 ± 5.8	89.1 ± 5.9	89.0 ± 7.4	0.489
ACT, % (mean ± SD)				
At baseline	23.1 ± 2.7	21.9 ± 3.2	21.8 ± 3.2	0.136
After 6 months of treatment	22.8 ± 3.1	22.4 ± 2.7	22.3 ± 3.2	0.468
After 12 months of treatment	23.4 ± 3.1	22.7 ± 3.1	23.7 ± 2.1	0.156
PC20 mg/mL	0.4 ± 0.6	0.4 ± 0.8	0.8 ± 0.8	**0.011**
Asthma control, *n* (%)				
Good	26 (29.2)	42 (47.2)	21 (23.6)	0.437
Poor	7 (18.4)	20 (52.6)	11 (29)	

Bold value indicates statistical significance at $p < 0.05$

FEV1 forced expiratory volume in 1 s, *FVC* forced vital capacity, *ACT* asthma control test, *SD* standard deviation, *PC20* concentration of methacholine causing a 20% fall in FEV1

[a]Difference between three groups

rs20544 genotypes were correlated with response to therapy within each of the groups. We did not find association between analyzed variants and response to therapy either in group treated with fluticasone propionate or with montelukast ($p > 0.05$). Also, no association was observed when patients treated with montelukast were stratified according to therapeutical regimen (regular and episodic) ($p > 0.05$).

Discussion

As a leading matrix metalloproteinase involved in airway remodeling in asthma, MMP9 is of potential importance for disease phenotype and response to therapy. To the best of our knowledge, this is the first study examining the involvement of MMP9 3′ end variants as modulators of asthma phenotype and response to treatment. It has been previously

Table 4 Correlation between rs13925 and rs20544 genotypes and treatment outcome

Variant	rs13925			
Genotype	AA	GA	GG	p^a
dFEV1, % (mean ± SD)				
After 6 months of treatment	17.6 ± 0.7	2.2 ± 8.4	0.8 ± 14.9	**0.038**
After 12 months of treatment	16.5 ± 6.4	2.5 ± 8.9	3.5 ± 14.6	0.109
Between 6 and 12 months of treatment	13.5 ± 0.7	8.9 ± 10.9	8.9 ± 10.8	0.779
dFEV1/FVC, % (mean ± SD)				
After 6 months of treatment	4.5 ± 9.2	1.0 ± 4.4	0.42 ± 5.5	0.841
After 12 months of treatment	2.0 ± 8.5	0.1 ± 5.9	0.15 ± 5.6	0.938
Between 6 and 12 months of treatment	− 2.5 ± 0.7	− 0.9 ± 5.8	− 0.3 ± 4.6	0.438
dACT, % (mean ± SD)				
After 6 months of treatment	− 1.0 ± 4.2	− 0.5 ± 1.9	0.6 ± 3.3	0.180
After 12 months of treatment	1.0 ± 1.4	− 0.2 ± 3.0	1.3 ± 2.9	0.108
Between 6 and 12 months of treatment	2.0 ± 2.8	0.3 ± 3.8	0.7 ± 2.5	0.768
Variant	rs20544			
Genotype	CC	CT	TT	p^a
dFEV1, % (mean ± SD)				
After 6 months of treatment	0.2 ± 9.9	3.1 ± 16.6	− 0.4 ± 9.1	0.674
After 12 months of treatment	3.1 ± 9.3	4.9 ± 16.5	0.8 ± 8.6	0.508
Between 6 and 12 months of treatment	9.3 ± 11.9	8.9 ± 10.3	8.8 ± 10.60.2	0.913
dFEV1/FVC, % (mean ± SD)				
After 6 months of treatment	1.7 ± 6.2	0.3 ± 5.0	0.2 ± 4.5	0.568
After 12 months of treatment	1.1 ± 5.2	− 0.3 ± 6.3	0.1 ± 5.0	0.685
Between 6 and 12 months of treatment	− 0.6 ± 5.3	− 0.6 ± 5.5	− 0.9 ± 2.9	0.797
dACT, % (mean ± SD)				
After 6 months of treatment	− 0.3 ± 2.1	0.5 ± 3.6	0.5 ± 2.5	0.328
After 12 months of treatment	0.3 ± 2.6	0.7 ± 3.2	1.9 ± 2.6	**0.049**
Between 6 and 12 months of treatment	0.6 ± 3.2	0.2 ± 3.0	1.4 ± 1.9	0.076

Bold values indicate statistical significance at $p < 0.05$

dFEV1 change in forced expiratory volume in 1 s, *dFVC* change in forced vital capacity, *dACT* change in asthma control test, *SD* standard deviation

[a]Difference between three groups

shown that MMP9 expression can be regulated by interactions with miRNAs and that variants in either the gene or miRNAs can influence these interactions, but their role in asthma remains unknown. We have analyzed variants in the 3′ UTR of the *MMP9* gene and investigated their association with lung function, airway hyperresponsiveness, response to therapy, and asthma control.

Among variants in the 3′ end of *MMP9* gene which were detected in previous studies, we have identified variants rs13925 and rs20544. The obtained allelic frequencies were in concordance with the frequencies published for general European populations according to the 1000 Genomes and Single Nucleotide Polymorphism database (dbSNP). Variant rs1056628 was not found in analyzed group of Slovenian asthmatics and all patients were carriers of the A allele. According to literature data, variant rs1056628 was investigated only in Chinese population and observed frequency of the C allele was 16% [8]. The same study reported lower frequency of the T allele of the variant rs20544 (32.0%) compared to our finding (49.6%). The observed differences in allele frequency might be due to sample size, population stratification, and/or different genetic backgrounds.

Variant rs20544 affects a presumed site for interaction with hsa-miR-4673 [18]. No further analysis on interactions has been performed, but it should be investigated if the interaction really occurs and how it affects MMP9 function. We have shown that patients with TT genotype have significantly lower airway sensitivity to methacholine compared to the patients with CT and CC genotype. This variant was linked to general disk space height in patients with chronic low back pain, but it was not functionally characterized [19].

Additionally, the carriers of TT genotype had significant increase in ACT scores, indicating better response to treatment and asthma control after 12 months of treatment. Since this increase was not significant after 6 months of treatment, we can speculate that longer time is needed for the effects of therapy to show.

Variant rs13925 affects the sequence of the last exon in the *MMP9* gene, and does not lead to amino acid change. Since the change is near the end of the gene, it could influence RNA stability, editing, or function regulation. We have found only two patients with AA genotype for this variant. When the presence of this variant was correlated with asthma progression, it was found that the carriers of AA and AG genotypes had significant increase in FEV1 during the first 6 months of treatment. Since there were only two AA genotype carriers, these results should be taken with reserve, and they need to be confirmed on a larger cohort of asthmatic patients and for different populations. It should also be considered that for allele frequencies the same trend was observed as for the genotypes. After 12 months of treatment, this increase in FEV1 was no longer significantly different between genotypes which can be explained by the overall good response to treatment and well-controlled asthma in our group of patients.

The involvement of MMP9 in modulation of the response to asthma treatment was hypothesized, but its molecular basis has not been previously investigated. It has been suggested that MMP9 could serve as an indirect disease progression marker for assessment of airway remodeling, as it was found that its active form was significantly elevated in exhaled breath condensate (EBC) of children with stable asthma treated with corticosteroids [20]. Some data indicate that ICS treatment does not affect MMP9 activity in asthmatic patients. It is hypothesized that this high activity of MMP9 in asthmatics is in association with high IgE levels [20, 21].

The main limitation of our study is lack of data on MMP9 levels and/or activity, since it would be beneficial to correlate these parameters not only with the presence of different variants, but also with response to treatment. A larger cohort of patients would additionally improve our study by increasing its statistical power. It should also be noted that there may be other variants in the *MMP9* gene that could contribute to its function in asthma patients.

Conclusion

The main finding of our study is association of specific genotypes with better asthma control, and indirectly better response to treatment. The patients carrying rs20544 TT genotype and rs13925 AA and AG genotypes showed improvement in disease symptoms indicating better response to therapy in carriers of these variants. Based on these results, MMP9 deserves further research as a potential predictive biomarker for asthma.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical Approval and Informed Consent The study was in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained for all patients and the investigation was approved by the hospital ethical committee.

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