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Original scientific paper

MATRIX METALLOPROTEINASE 9 GENOTYPE MODULATES ASTHMA CONTROL IN PEDIATRIC ASTHMA PATIENTS

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ABSTRACT. Matrix metalloproteinases, particularly MMP9, play a pivotal role in asthma pathology by influencing extracellular matrix remodeling and inflammation. This study examined 100 Serbian pediatric asthma patients to explore the correlation between MMP9 3' UTR polymorphisms and MMP9 protein levels, and their impact on therapy response and asthma control. The analysis revealed two key polymorphisms (rs13925 and rs20544) in the MMP9 gene's 3'UTR, with higher frequencies of the rs20544 T allele and TT genotype in patients with well controlled asthma. Positive correlations were found between MMP9 serum levels and blood leukocyte count, and CRP levels. Patients with not well controlled disease exhibited significantly higher MMP9 levels than those with well controlled asthma (p=0.027), indicating MMP9's potential role in asthma therapy response.

Keywords: Asthma, MMP9, 3'UTR polymorphisms, response to therapy, asthma control

INTRODUCTION

Asthma is a chronic lung disease affecting more than 260 million people of all ages, worldwide (GLOBAL BURDEN OF DISEASE STUDY 2019, 2020). It is present in approximately 1 in 10 children making it the most common chronic lung disease in children (DHARMAGE *et al.,* 2019). Many genetic, environmental, and socio-economic risk factors play a role in pathogenesis and therapeutic response in asthma (HERNANDEZ-PACHECO *et al.,* 2020). Asthma symptoms are caused by chronic inflammation and narrowing of small airways, along with

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airway hyperresponsiveness and remodeling. Airway remodeling in asthma presents structural changes in the large and small airways, and consists of cellular and extracellular matrix changes, as well as epithelial cell apoptosis, airway smooth muscle cell proliferation, and fibroblast activation (HOUGH *et al.,* 2020, VARRICCHI *et al.,* 2022).

Matrix metalloproteinases are zinc-dependent proteolytic enzymes considered as major players in airway remodeling, extracellular matrix deposition and degradation, and function and migration of inflammatory cells. Their production and secretion are tightly regulated at different levels: the gene transcriptional level, micro RNAs, the activation of the latent form of the enzyme, and the inactivation by specific endogenous inhibitors (RAEESZADEH-SARMAZDEH *et al.,* 2020, GRZELA *et al.*, 2016a).

MMP9 is considered the most important MMP in asthma pathology. The imbalance between MMP9 and endogenous tissue inhibitor of metalloproteinase-1 is considered a major theory to explain the progression of asthmatic airway remodeling (CHUNG *et al.*, 2019; OHBAYASHI *et al.*, 2005). An increased level of MMP9 is regarded as an indicator of the harm inflicted on the bronchial epithelium by inflammation. Elevated levels of MMP9 were found in bronchial biopsies, bronchoalveolar lavage fluid (BALF), and sputum of asthmatic patients when compared to healthy controls (CHUNG *et al.*, 2019; VENTURA *et al*., 2014). It is important to understand which patients may be most responsive to those therapies, and to that end, the usefulness of MMPs as biomarkers is just beginning to be investigated, particularly concerning MMP9 as a marker of neutrophilic airway inflammation in allergic asthma.

The aim of our study was to investigate the possible correlation between 3' UTR polymorphisms in the MMP9 gene with levels of MMP9 protein as well as their involvement in response to therapy and asthma control.

MATERIAL AND METHODS

Subjects

The study included 100 asthmatic children and adolescents (5-21 years old) diagnosed and treated at the Department of Pulmonology and Allergology of the University Children's Hospital in Belgrade from 2013 to 2015. All procedures performed in the presented study were in accordance with the guidelines of The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by the Ethical Committee of the University Children's Hospital. Written informed consent was obtained from all patients' parents. Asthma was diagnosed according to the applicable Guidelines of the Global Initiative for Asthma (GINA). The skin prick tests for allergens were performed according to the European Academy of Allergy and Clinical Immunology (EAACI) guidelines. The disease severity was evaluated based on asthma control questionnaire (ACQ) scores, the use of asthma control medications, and the results of spirometry (forced expiratory volume in one second - FEV1 and forced vital capacity - FVC). Patients with mild asthma had intermittent symptoms, were treated with a low dose of inhaled corticosteroids, and had normal lung function (FEV1 >80% predicted), while patients with severe asthma had frequent symptoms, were treated with medium to high doses of inhaled corticosteroids in combination with beta 2 agonists, and had impaired lung function (FEV1 <80% predicted). The criteria for well controlled asthma were: the ACQ below 0.75, normal lung function and absence of eosinophilia in induced sputum. No patients with very poorly controlled asthma were enrolled in this study.

Genotyping

All patients were genotyped for *MMP9* gene 3′ UTR variants by direct sequencing. Deoxyribonucleic acid (DNA) was extracted from whole blood using a GeneJET Genomic

DNA Purification Kit (Thermo Scientific, USA) following the manufacturer's protocol. The 3′ UTR of *MMP9* gene was amplified by polymerase chain reaction (PCR) using primers: 5′-GTA TAT GTG GGA GAA TTA GAA TCA-3′ (forward) and 5′-ACA TGC ATA CAT ACG TGC ATA C-3′ (reverse). The PCR was conducted in a 25 μL reaction mixture containing: 50 ng of DNA, 1× Reaction buffer with Mg (Kapa Biosystems, USA), 200 μM deoxynucleotide triphosphates, 0.5 pmol of each primer, and 1.25 U Taq DNA polymerase (Kapa Biosystems, USA). The amplification conditions were: 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 for10 min. The obtained PCR products (409bp) were purified using a GeneJET PCR Purification Kit (Thermo Scientific, USA) according to the manufacturer's protocol and sequenced using an ABI Prism BigDye Terminator Kit (Applied Biosystems, USA) and a 3130 Genetic Analyzer (Applied Biosystems, USA). Sequences were analyzed using the Sequencing Analysis software (Applied Biosystems, USA).

Measurement of MMP9 in serum samples

Concentrations of MMP9 were measured in serum samples (dilution applied was 1:100) using Human MMP9 SimpleStep ELISA commercially available kit (ab246539 Abcam). Standards and samples were added to the wells, followed by the antibody mix. After incubation, the wells were washed to remove unbound material. Tetramethybenzidine (TMB) Development Solution was added and further catalyzed by horseradish peroxidase (HRP) during incubation period, generating blue coloration. This reaction was stopped by the addition of Stop Solution completing any color change from blue to yellow. The signal was generated proportionally to the amount of bound analyte and the intensity was measured at 450 nm using a microplate reader. The results were calculated in comparison with the standard curve. Each sample was run in duplicate, and the average was calculated. Assay sensitivity was 22.17 pg/ml. MMP9 assay was specific for both native and recombinant human MMP9 protein in serum, plasma (heparin), saliva, cell culture supernatant, and cell and tissue extract samples only. Recombinant tissue inhibitors of metalloproteinases (TIMP1) were prepared at 9000 pg/mL and assayed for cross-reactivity. No cross-reactivity was observed. The standard curve range for MMP9 was from 105.47 pg/ml to 6750 pg/ml. Intra-assay statistics revealed % CV = 2.5, while inter-assay statistics was % CV=6.0.

Statistical analysis

Statistical analysis was performed by Statistical Package for Social Sciences 20.0 (SPSS Inc., Chicago, Illinois, USA). Data were expressed as percentages and means±standard deviation (SD). Differences in categorical data between groups were analyzed by Fisher's Exact and Chi-square tests. To analyze the normality of continuous data the Kolmogorov-Smirnov test was used. Dependent on data distribution, differences between independent groups of continuous data were analyzed by Independent-Samples T-test, One-way Analysis of Variance (ANOVA), Mann-Whitney U test, and Kruskal-Wallis test. The degree of association between continuous data was calculated using Spearman's rank correlation coefficient. P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

This study included 100 asthmatic children and adolescents with well or not-well controlled asthma. No patients with very poorly controlled asthma were enrolled in this study. The demographic and clinical characteristics of the study group are presented in Table 1.

Table 1. Demographic and clinical characteristics of patients.

Number of patients, n	100
Age, years (mean±SD)	12.7±3.8
Males/Females, %	57/43
Body mass index, kg/m^2 (mean±SD)	19.9±4.2
Spirometry, % (mean±SD)	
FEV1	87.7±14.9
FVC	86.4±11.9
FEV1/FVC	1.02±0.1
Positive skin prick test, %	91
Sputum cytology, % (mean±SD)	
Eosinophils	19.4±17.6
Neutrophils	37.6±17.7
Lymphocytes	19.6±11.9
Monocytes	7.4±4.43
Macrophages	16.3±9.1
Blood leukocyte count, cell/L (mean±SD)	$7.5x10^9$±$2.1x10^9$
Blood leukocyte distribution, % (mean±SD)	
Granulocytes	57.1±15.7
Lymphocytes	33.9±10.4
Monocytes	7.1±7.5
CRP, mg/L (mean±SD)	3.4±8.2
Asthma type, %	
Mild	53
Severe	47
Asthma control, %	
Well controlled	65
Not well controlled	35

SD-Standard Deviation; FEV1-Forced Expiratory Volume in 1 second; FVC-Forced Vital Capacity; CRP-C-reactive protein.

We have identified three polymorphisms in the 3'UTR of *MMP9* gene: rs13925, rs20544 and rs9509. Since polymorphism rs9509 was present in only 2 patients this polymorphism was not further analyzed. The distribution of rs13925 and rs20544 is given in Table 2. Variants rs13925 and rs20544 in 3′ UTR of the *MMP9* gene and total MMP9 serum levels were analyzed in all patients (Tab. 2). The distribution of obtained alleles and genotypes were analyzed in respect to the following clinical characteristics: lung function, inflammatory parameters (distribution of sputum and blood immune cells and level of CRP) and disease severity. Neither of the obtained p values were statistically significant ($p>0.05$, data not shown). Obtained concentrations of MMP9 in serum samples were in the range of 58.1-562.1 ng/mL and did not differ between genotypes ($p=0.326$ for rs13925 and $p=0.887$ for variant rs20544). Carriers of A allele had a slightly higher level of MMP9 in serum compared to carriers of G allele (180.4±97.1 ng/mL vs 160.3±101.1 ng/mL). However, this difference was not statistically significant ($p=0.391$). A similar level of MMP9 in serum was observed between carriers of the C allele and the T allele ($p=0.635$). We also tested the association between MMP9 serum levels and clinical characteristics of patients by Spearman's correlation coefficient. Positive correlations of MMP9 serum levels with the count of blood leukocytes and the level of CRP ($r=0.440$, $p=0.001$ and $r=0.543$, $p<0.001$, respectively) were found. Among leukocytes, a positive correlation between granulocytes and MMP9 levels was noticed, however, statistical significance was not reached ($r=0.265$, $p=0.060$).

Table 2. The distribution of alleles and genotypes frequencies of *MMP9* gene 3′ UTR variants and their associations with MMP9 serum levels in asthmatic patients.

***MMP9* gene variant**		**Frequencies, %**	**MMP9, ng/mL (mean±SD)**	**P value**
rs13925	**Alleles**			
	G	85.5	160.3±101.1	0.391
	A	14.5	180.4±97.1	
	Genotypes			
	GG	73	160.6±102.2	0.326
	GA	25	158.0±102.9	
	AA	2	236.6±69.3	
rs20544	**Alleles**			
	C	38.5	179.4±170.7	0.635
	T	61.5	154.7±88.1	
	rs20544			
	CC	14	190.4±127.4	0.887
	CT	49	173.6±122.7	
	TT	37	145.3±64.8	

SD-Standard Deviation; MMP9-Matrix metalloproteinase 9

Further, the patients were divided into two subgroups according to the asthma control and clinical characteristics, *MMP9* gene 3′ UTR variants and MMP9 serum levels were compared (Tab. 3). There were no significant differences between subgroups in terms of age, sex and body mass index. All patients had both FEV1 and FVC above 80% of the predicted value. Of patients with not well controlled disease, 91.4% had severe asthma ($p<0.001$). Among inflammatory markers, there were significant differences in distributions of blood granulocytes and lymphocytes ($p=0.014$ and $p=0.034$, respectively), while the level of CRP and the distribution of sputum cells were similar in both subgroups. Although the distribution of alleles and genotypes for *MMP9* gene 3′ UTR variants did not statistically differ between subgroups ($p>0.05$), higher frequencies of the T allele and TT genotype, rs 20544, were noticed in patients with well controlled asthma. Patients with not well controlled disease had statistically significantly higher levels of MMP9 compared to patients with well controlled disease ($p=0.027$).

MMP9 is the major matrix metalloproteinase in asthma involved in matrix remodeling and inflammation. We have previously shown that polymorphisms in the 3'UTR of the MMP9 gene can affect response to therapy in pediatric patients (DRAGICEVIC *et al.*, 2018). We aimed to confirm these results in another cohort of patients but also to correlate the presence of polymorphisms with MMP9 protein levels. In addition, we analyzed the correlation of the presence of 3'UTR polymorphisms and protein levels with clinical characteristics and patients' asthma control. We identified the same polymorphisms in the 3'UTR as in our previous study, and the frequency of the rs31925 polymorphism was similar between the two cohorts. The difference was observed in the prevalence of C and T alleles rs20544 between the patient populations we analyzed (C allele 50.4 vs 38% in the current study). This could be because of difference in allele prevalence between general populations since they came from different countries. The allelic frequencies obtained in this study were similar to those in the general population published previously and were in Hardy-Weinberg equilibrium. The presence of either polymorphism did not affect lung function, inflammatory parameters, or disease severity. MMP9 levels did not differ between genotypes, indicating that these polymorphisms do not affect MMP9 expression.

Table 3. Comparisons of clinical characteristics, allele and genotype frequencies of *MMP9* gene 3′ UTR variants and MMP9 serum levels in asthmatic patients with well and not well controlled disease.

	Well controlled (n=65)	Not well controlled (n=35)	P value
Age, years (mean±SD)	12.7±3.8	12.7±3.7	0.978
Males/Females, %	55.4/44.6	60/40	0.678
Body mass index, kg/m^2 (mean±SD)	19.2±3.6	21.0±5.0	0.092
Spirometry, % (mean±SD)			
FEV1	89.1±14.6	85.2±15.7	0.297
FVC	86.8±11.9	85.8±12.3	0.708
	Well controlled (n=65)	**Not well controlled (n=35)**	**P value**
FEV1/FVC	103.1±13.2	99.1±10.9	0.133
Sputum cytology, % (mean±SD)			
Eosinophils	17.2±16.2	23.4±19.6	0.089
Neutrophils	38.3±18.1	36.1±17.1	0.554
Lymphocytes	19.2±12.1	20.3±11.9	0.418
Monocytes	7.9±4.7	6.3±3.9	0.094
Macrophages	17.8±10.1	13.4±5.8	0.058
Blood leukocyte count, cell/L (mean±SD)	$7.4x10^9$±$2.2x10^9$	$7.7x10^9$±$2.2x10^9$	0.105
Blood leukocyte distribution, % (mean±SD)			
Granulocytes	54.1±17.5	62.8±9.5	**0.014***
Lymphocytes	35.6±10.2	30.9±10.4	**0.034***
Monocytes	7.5±8.2	6.5±6.3	0.527
CRP, mg/L (mean±SD)	3.6±10.1	2.8±2.2	0.108
Asthma type, %			
Mild	76.9	8.6	**<0.001***
Severe	23.1	91.4	
Alleles, %			
rs31925			
G	87.7	81.4	0.292
A	12.3	18.6	
rs20544			
C	35.4	44.3	0.227
T	64.6	55.7	
Genotypes, %			
rs31925			
GG	75.4	68.6	
GA	24.6	25.7	0.144
AA	0	5.7	
rs20544			
CC	10.8	20.0	
CT	49.2	48.6	0.399
TT	40.0	31.4	
MMP9, ng/mL (mean±SD)	138.3±68.8	209.6±133.5	**0.027***

SD-Standard Deviation; FEV1-Forced Expiratory Volume in 1 second; FVC-Forced Vital Capacity; CRP- C-reactive protein; MMP9-Matrix metalloproteinase 9; *Statistically significant P value

In our previous study, we found an association of MMP9 genotype rs20544 TT with better asthma control. In this study, a higher frequency of the T allele and TT genotype was found in patients with well controlled asthma. Although this trend was not statistically significant, it confirms our previous findings and suggests that this polymorphism is a novel predictive biomarker for asthma control and indirectly, response to therapy. There were some differences in patient asthma control assessment between the two studies, so this could also influence the statistical significance of our findings. In this study, we did not find a correlation between rs13925 AA and AG with better asthma control. This may be due to the small cohort size, and investigation on a larger multicenter scale would be valuable.

MMP9 serum levels were observed to positively correlate with both the number of blood leukocytes and the level of CRP (r=0.440, p=0.001 and r=0.543, p=0.001, respectively). Neutrophils and macrophages are the main sources of MMP9 in inflammatory reactions and our results support this with this since we discovered a strong link between MMP9 levels and leukocyte counts (NAIK *et al.*, 2017).

Patients with asthma have elevated levels of MMP9 and exhaled breath condensate MMP9 levels correlated with pulmonary functions and other inflammatory markers in exhaled breath such as IL-6, IL-4 and IL-10 (KARAKOC *et al.*, 2012; TIOTIU *et al.*, 2018). In our current study, a statistically significant difference in serum MMP9 levels (p=0.027) between patients with not well controlled and well controlled disease were observed. The patients with higher levels of MMP9 had not well controlled disease. MMP9 levels are considered an indirect disease progression marker for the assessment of airway remodeling, and in our case they also correlate with disease control, which could indirectly give information about response to treatment. Patients were on corticosteroid therapy corticosteroid therapy does not affect remodeling and MMP9 and should be considered alternative therapy in patients with high MMP9 levels, alternative or additional (INGRAM *et al.*, 2015). The levels of MMP9 in induced sputum are known to be elevated in asthmatics and are not affected by inhaled corticosteroids (VENTURA *et al.*, 2014; GRZELA *et al*., 2016b; CHUNG *et al.*, 2019). Moreover, the MMP9/TIMP1 ratio imbalance in the induced sputum may be the cause of the degradation of extracellular matrix proteins, which could indicate airway inflammation and injury, and contribute to disease progression and severity (CHUNG *et al.*, 2019). It has been shown that higher levels of MMP9 can be observed in severe persistent asthmatic patients compared to moderate persistent asthmatics, and that higher levels of MMP9 associate with lower lung function (MATTOS *et al.*, 2002). Patients with not well controlled, unstable asthma usually have persistent inflammation which could contribute to poorer response to therapy, higher counts of MMP9 producing cells (such as eosinophils, mast cells, and alveolar macrophages), leading to increased MMP9 levels (CHUNG *et al.*, 2019; MING *et al.*, 2023). Moreover, it has been reported that MMP9 may also participate in the chronic airway inflammation not only in classical asthma, but also in specific types of asthma – cough variant and allergic asthma (MA *et al.*, 2014; KHALEEL MOHAMMED *et al.*, 2024). There are several limitations to our study. Since the balance between levels of MMP9 and TIMP1 is also important for tissue remodeling in asthma, it would be beneficial to assess the levels of TIMP1. As mentioned above, a larger patient cohort could further strengthen our findings. There are several other polymorphisms reported in the MMP9 gene that could also contribute to the effects of MMP9 in asthma (PINTO *et al.*, 2010). It would be interesting to analyze the whole MMP9 gene in order to identify potential biomarkers that could be used to better predict response to therapy.

MMP9 deserves further attention to elucidate its role in asthma control and response to therapy, as well as a potential target for asthma treatment.

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