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ORIGINAL ARTICLE

Polymorphic Variants 279R and 668Q Augment Activity of Matrix Metalloproteinase-9 in Breath Condensates of Children with Asthma

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Abstract Matrix metalloproteinase (MMP)-9 is involved in pathophysiology of asthma, mainly asthma-associated airway remodeling. Exhaled breath condensates (EBC) of asthmatics contain increased amounts of MMP-9 with activity higher, than in healthy controls. The increased activity of MMP-9 may originate from its excessive production and activation, but may also result from variations in MMP-9 structure, which are determined by single nucleotide polymorphisms (SNPs). In this pilot study we aimed to assess the possible influence of two functional MMP-9 polymorphisms, Q279R and R668Q, on enzymatic activity of MMP-9, measured in EBC of asthmatic children. The concentration and activity of MMP-9 were analyzed in EBC of 20 children with allergic asthma using specific standard ELISA and novel immunoenzymatic activity assay. The SNPs of MMP-9 were assessed using real-time PCR-based genotyping test. We have found that MMP-9 concentration in breath condensates of children with stable asthma was slightly higher in ELISA, than in the activity assay. Moreover, these results and activity-to-amount ratio have revealed some relationship with a presence of specific 279R and/or 668Q MMP-9 gene variants. Our observation suggests that at least in some patients MMP-9 hyperactivity may result from genetic predisposition, determined by polymorphic variants of MMP-9 gene. Moreover, it supports previous reports postulating significance of MMP-9 in pathogenesis of asthma. However, this issue still requires further studies.

Keywords ASTHMA · Enzyme activity · Exhaled breath condensate · Matrix metalloproteinase · Airway remodeling · Single nucleotide polymorphism

Introduction

Matrix metalloproteinase (MMP)-9 is a member of zinc-dependent proteases family, which is responsible for physiological turnover of extracellular matrix and tissue repair. Also, it is engaged in pathomechanism of various diseases (Hadler-Olsen et al. 2011), including cancer progression and metastasis (Deryugina and Quigley 2006), development of aortic aneurysm (Grzela et al. 2011), or delayed wound healing (Grzela et al. 2014). Furthermore, it has been postulated that MMP-9 may be involved in pathophysiology of asthma, especially asthma-associated airways remodeling (Cataldo et al. 2002; Grzela et al. 2016a).

Numerous studies have shown increased MMP-9 levels in blood, sputum and bronchoalveolar lavage of asthmatic patients (Cataldo et al. 2002; Gagliardo et al. 2009). Recently, increased concentration and activity of MMP-9 have also been found in exhaled breath condensates (EBC) of adults and children with allergic asthma (Barbaro et al. 2014; Grzela et al. 2015; Karakoc et al. 2012).

MMP-9 amount depends on balance between its production and degradation. However, when analyzed as a

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single parameter, it does not provide any information regarding actual activity of this enzyme. The assessment of MMP-9 enzymatic activity in biological samples is more complex issue. MMP-9 is secreted by various cells, including neutrophils, eosinophils, monocytes and mast cells, as an inactive pro-enzyme, which is then converted to its active form. Noteworthy, activated enzyme may still be suppressed by its natural inhibitors, including α2-macroglobulin, tissue inhibitors of MMPs (TIMPs), or serpins. Therefore, its in vivo activity obviously results from the amount of secreted pro-enzyme, but is also controlled by specific and unspecific MMPs modulators (Litwiniuk et al. 2012; Nagase et al. 2006).

On the other hand, the activity of MMP-9 may also be due to some variations in molecular structure of this enzyme. These variations correspond to some single nucleotide polymorphisms (SNPs) of gene encoding for MMP-9 (Hu et al. 2005; Pinto et al. 2010). Although presumable role of selected MMP-9 polymorphisms in asthma was investigated previously, the practical relevance of these data still remain unclear (Grzela et al. 2016a). Therefore, the aim of our pilot study was to assess relationship of two functional SNPs of MMP-9 gene, Q279R and R668Q, and actual concentration and enzymatic activity of MMP-9 in EBC collected in small group of children with mild asthma.

Materials and Methods

Our pilot study involved 20 children (13 male and 7 female, mean age: 13.4 ± 2.6) with stable, well controlled pharmacologically, mild allergic asthma, diagnosed according to recommendations of GINA—the Global Initiative for Asthma, Strategy for Asthma Diagnosis and Prevention (updated 2009, available on http://www.ginasthma.org). Patients with other systemic diseases or infections were not involved to the study. None of patients has used inhaled corticosteroids at least 3 weeks before EBC sample collection. The allowed medication included second generation H1 antihistamines and inhaled β2-mimetics (upon request). All children and their parents provided the informed consent to participate in the study, which was approved by the local ethics committee.

Based on inclusion and exclusion criteria, all patients underwent clinical examination and routine laboratory tests, including peripheral blood morphology, total IgE serum level and skin tests (or specific IgE) to verify the allergic background of asthma (Grzela et al. 2015). The effectiveness of asthma control was estimated by the presence of clinical symptoms, spirometric assessment and level of nitric oxide (NO) in exhaled air (Smith et al. 2005; Zeiger et al. 2006). The spirometry was performed using Lung Test 1000 device (MES, Krakow, Poland). The forced expiratory volume in the first second (FEV1) and forced vital capacity (FVC) were used for calculation of the FEV1/FVC ratio (Tiffeneau–Pinelli index) and, after adjustment to the patients age, presented as the standard deviation score. Data from large multicenter population studies were used as the reference values (Hankinson et al. 1999; Quanjer et al. 2010; Zapletal and Chalupova 2003). The NO concentration in exhaled air was analyzed using Sievers NO 280 device (GE Analytical Instruments, Boulder, CO, USA), and expressed in ppb (particles per billion).

Exhaled breath condensates were collected using ECoScreen condenser (Jäger, Höchberg, Germany), as described elsewhere (Zagorska et al. 2013, 2014). Briefly, after short adaptation to ambient condition, patients were subjected to the 15-min-long collection of breath condensate (approx. 700–1000 μl each). Then, collected samples were immediately deep frozen and stored at −70 °C, until being used for further assessment.

The MMP-9 activity in tested EBC samples was analyzed using QuickZyme Human MMP-9 Activity Assay (QuickZyme BioSciences, Leiden, Netherlands), according to procedure described previously (Grzela et al. 2015). In brief, to enable measurement of total MMP-9 concentration (including both, the active and latent form of MMP-9) samples were pretreated with p-aminophenyl mercuric acetate (APMA). The absorbance of tested samples was measured with the Microplate Reader 550 (BIO-RAD) immediately after addition of detection reagent (T_0) and then after 2 h of incubation at 37 °C (T_2). The MMP-9 concentrations in EBC were calculated based on the standard calibration curve of APMA-activated human recombinant MMP-9 and expressed in ng/ml, with the assay sensitivity of 0.01 ng/ml.

Simultaneously, the concentration of total MMP-9 protein in EBC was assessed using Human MMP-9 Quantikine ELISA Kit, according to detailed protocol provided by the manufacturer (R&D Systems, Minneapolis, MN, USA). The results of both, QuickZyme and ELISA assessments were used for calculation of MMP-9 activity-to-amount ratio (AAR).

To exclude possible contamination of collected EBC with the saliva, ten randomly selected samples were additionally screened for amylase content (Gaber et al. 2006; Zagorska et al. 2014).

DNA samples from patient's peripheral blood were isolated using GenElute Blood Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA), and subjected to genotyping for two functional polymorphisms of MMP-9: Q279R (rs17576) and R668Q (rs17577), using TaqMan SNP Genotyping Assay (Life Technologies, Carlsbad, CA, USA) with the ABI PRISM 7500 Real-Time PCR device

(Applied Biosystems, Foster City, CA, USA). Then, results of genotyping were compared to those obtained from analysis of breath condensates. The genotype frequencies of both selected polymorphisms were tested for Hardy–Weinberg equilibrium using the Pearson's Chi-square test.

Results

The summary of patients' clinical characteristic was shown in Table 1.

The mean concentration of MMP-9 in EBC, measured by standard ELISA method, was 17.5 ± 9.2 ng/ml (median 15.0 ng/ml, with min/max = 5.0–40.0 ng/ml). In the same EBC samples the mean MMP-9 concentration, measured by QuickZyme activity assay, corresponded to 13.2 ± 8.4 ng/ml (median 10.8 ng/ml, with min/max = 2.3–36.2 ng/ml) of pre-activated recombinant MMP-9. In all tested EBC samples the MMP-9 concentration, calculated from its enzymatic activity, was lower than that from ELISA measurement. Nevertheless, both variables revealed significant positive correlation ($r = 0.96$). The mean AAR, calculated for all tested samples, was 0.72. However, it varied among patients, ranging 0.46–0.97 (Fig. 1). The dots corresponding to low MMP-9 concentrations and lowest AAR values (with mean 0.54) were located in light gray zone on Fig. 1. The high MMP-9 concentrations and highest AAR values (mean 0.9) were located in a dark gray zone of Fig. 1.

Table 1 The clinical characteristic of patients

Parameter	Mean value ± SD/median (min–max)
Age (years)	13.4 ± 2.6/14.5 (8–16)
Sex distribution (female/male)	7/13
Blood eosinophils ($\times 10^3$/μl)	0.30 ± 0.1/0.31 (0.1–0.5)
Total IgE serum level (kU/l)	948 ± 496/723 (325–1800)
Exhaled NO (ppb)	25.2 ± 12.3/21.6 (11.6–48.5)
$FEV1_{SDS}$	0.4 ± 0.2/0.3
$FEV1/FVC_{SDS}$	–0.2 ± 0.1/–0.2
Patients sensitized to:	–
Grass	16
Mites	12
Animals	11
Alternaria	5
Cladosporium	3
Monosensitized/polysensitized patients	8/12

Mean values ± SD

FEV1 forced expiratory volume in the first second, *FVC* forced vital capacity, *FEV1/FVC* Tiffeneau-Pinelli index, *SDS* standard deviation score (*Z* score)—value corrected in relation to the age

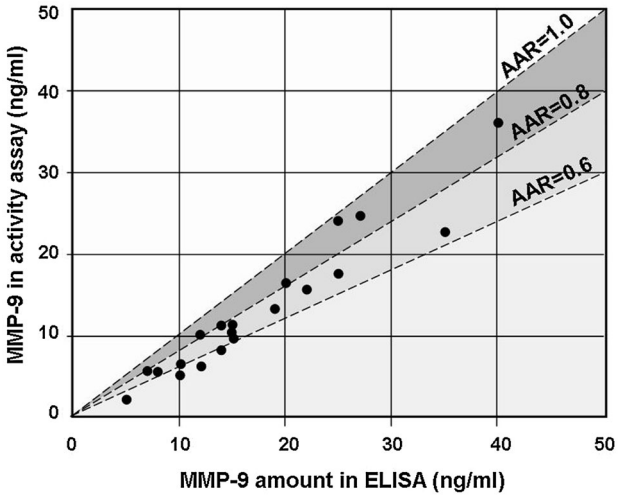

Fig. 1 The comparison of MMP-9 concentration in EBC samples, calculated from the results of QuickZyme activity assay (*vertical axis*) versus standard ELISA (*horizontal axis*). Each *dot* represents data from one EBC sample. *AAR* activity-to-amount ratio; *upper dashed line* (AAR = 1) corresponds to a condition where the MMP-9 concentration calculated from the activity assay is equal to that from ELISA. The arbitrary thresholds for AAR values (1.0, 0.8 and 0.6) were set as borders for *three gray zones*. The *dots* from the *dark gray area* correspond to the high AAR values, whereas those from *light gray*—to the *lower*

The observed allele frequencies of tested SNPs obtained in TaqMan Genotyping Assay did not reveal significant deviation from frequencies estimated by Hardy–Weinberg equilibrium.

Based on HapMap data, both analyzed SNPs revealed low level of linkage disequilibrium ($r^2 < 0.4$).

MMP-9 activity, amount and AAR values, when analyzed in regards to each of tested SNPs, have shown marked association with the occurrence of respective alleles (Fig. 2). Interestingly, remarkable trend was also observed, when AAR values were assessed in respect to the combination of both analyzed MMP-9 SNPs. Thus, the lowest AAR values were found in EBC samples from patients with wild-type (reference) homozygous patterns for each of tested SNPs, whereas both polymorphic (effect) MMP-9 variants, 279R and 668Q, were associated with increasing values of AAR. Hence, the highest AAR values (mean 0.9) were observed in patients bearing combination of both, 279R and 668Q, effect alleles (Fig. 3). No differences in tested clinical parameters have been found between patients with various allelic patterns.

Discussion

Our pilot study has shown for the first time the direct correlation between two functional polymorphisms of gene encoding for MMP-9 (Q279R and R668Q) and actual

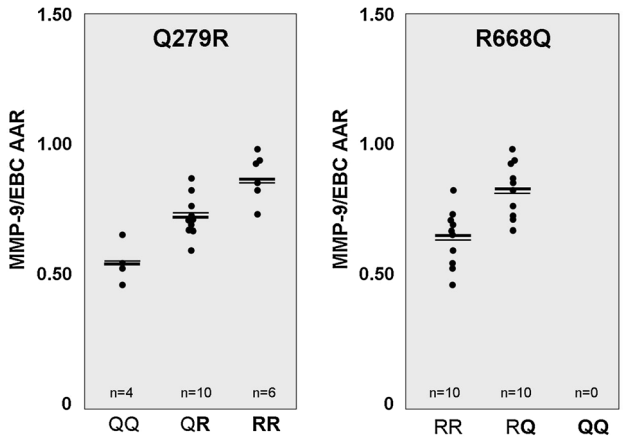

Fig. 2 The distribution of activity-to-amount ratio (AAR) for MMP-9 in EBC, in relation to respective MMP-9 polymorphisms—Q279R (*left graph*) and R668Q (*right graph*). Each *dot* represents AAR value of single patient. *Horizontal lines* show mean and median AAR values for patients group with respective polymorphic pattern. Effect alleles were shown in *bold letters*

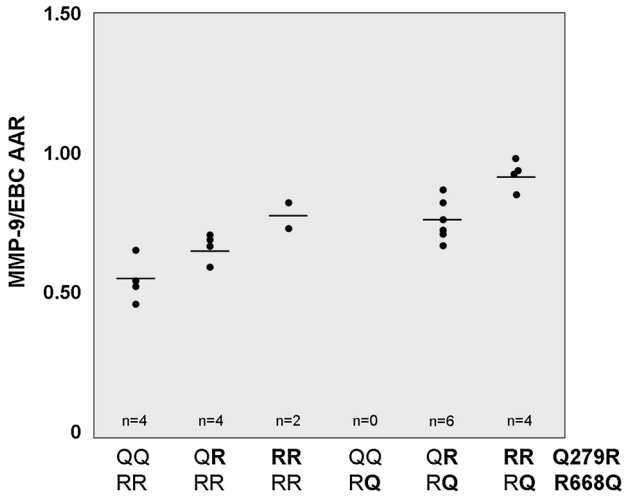

Fig. 3 The distribution of activity-to-amount ratio (AAR) for MMP-9 in EBC, in relation to combined MMP-9 polymorphisms (Q279R and R668Q). Each *dot* represents AAR value of single patient. *Horizontal lines* show mean AAR values for patients group with respective polymorphic pattern. Effect alleles were shown in *bold letters*

activity of this enzyme in EBC of children with mild, stable asthma. We have found that among some patients the total MMP-9 activity differed significantly, despite its similar amount in their EBC samples. Previous studies were mainly focused on single parameter—MMP-9 amount or its activity. Occasionally, even if that analysis concerned both parameters simultaneously, observed discrepancies in MMP-9 concentration were rather explained as result of some technical limitations of methods used for a study (Lowrey et al. 2008).

In contrast to them, in this report we have intentionally used two different methods for determination of MMP-9 concentration in EBC samples and then we have calculated the AAR. This approach appeared to be an easy method for assessment of specific catalytic efficacy of unknown amount of enzyme in biological material. Thus, we could compare catalytic efficacy of already determined amount of MMP-9 between various individuals. Furthermore, we were able to analyze the possible link between observed properties of enzyme and its molecular variations determined by two functional nucleotide polymorphisms.

The first of them, Q279R SNP (rs17576), is located in exon 6 of MMP-9 gene, encoding for sequence of fibronectin II domain, located close to the catalytic region of this enzyme. The replacement of single nucleotide (A to G) in this locus results in codon change for 279th aminoacid—from Q (Gln) to R (Arg). This change is supposed to modify the affinity of catalytic region of MMP-9 in regards to its binding efficacy for the specific substrate (Hu et al. 2005; Pinto et al. 2010). Obviously, it may also result in an increase of enzymatic activity. In fact, the presence of 279R allele was found to be associated with an increased enzyme stability (Bchir et al. 2015), which could explain association of 279R with higher risk of metastatic cancer (Hu et al. 2005), chronic obstructive pulmonary disease (Tesfaigzi et al. 2006) or cardiovascular diseases (Grzela et al. 2011).

The next of studied polymorphic variants of MMP-9, R668Q SNP (rs17577), is located in exon 13 and concerns the codon change for 668th aminoacid, from R (Arg) to Q (Gln). It is expected to modify the hemopexin-like domain of MMP-9 molecule, which is docking site for TIMPs (Nagase et al. 2006). Hence, this particular SNP may result in increased MMP-9 resistance to its natural inhibitors and higher in vivo activity of this enzyme (Sharma et al. 2012).

Based on these data we could expect that MMP-9 molecules of patients with both polymorphic variants should reveal activity and calculated AAR higher, than those from wild-type/reference homozygotes. Indeed, in our analysis, even despite very small group, we were able to observe such trend. On the other hand, due to the low number of individuals, which is the main limitation of our study, we could not fully verify our hypothesis. Although we expected highest AAR values in double homozygotes (279RR/668QQ), regrettably, none of children involved in the study revealed 668QQ homozygous pattern. Presumably, it was due to small patient group, but also low frequency of 668Q allele in general population (Pinto et al. 2010).

It has been suggested that increased level and/or activity of MMP-9 in respiratory tract may correlate with asthma severity. However, in our study we did not observe any significant differences between MMP-9 levels in regards to asthma severity, especially since our patients were recruited on a basis of stable clinical status, with mild symptoms.

Nevertheless, also some other reports did not confirm direct correlation between asthma severity and MMP-9/EBC levels (Grzela et al. 2016a, b; Turkeli et al. 2015). On the other hand, since mentioned studies concerned rather small groups, this issue still requires verification in large population of patients with various grades of asthma and/or asthma exacerbations. Noteworthy, our results may suggest that both, concentration and activity, should be assessed simultaneously, since even lower MMP-9 level with high AAR may have the same clinical relevance as higher MMP-9 concentration with low AAR.

In conclusion, since importance of MMP-9 SNPs was documented in various pathologies, we suppose that our observation may be of more universal relevance. Therefore, it could easily be verified in large population of patients with several other diseases, where MMP-9 was postulated as key effector molecule.

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Compliance with ethical standards

Conflict of interest All authors declare no conflict of interest.

Ethical approval All procedures performed in the study were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments.

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