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Andrzej Witusik[1*], Łukasz Mokros[2*], Janusz Szemraj[3], Piotr Kuna[4], Tadeusz Pietras[2]

[1]Grażyna and Kiejstut Bacewicz Memorial Academy of Music in Łódź, Faculty of Composition, Theory of Music, Conducting, Eurhythmics and Music Education, Music Therapy Course, Łódź, Poland
[2]Department of Clinical Pharmacology, 1st Chair of Internal Medicine, Medical University of Łódź, Łódź, Poland
[3]Department of Medical Biochemistry, Medical University of Łódź, Łódź, Poland
[4]Department of Internal Diseases, Asthma and Allergy, Medical University of Łódź, Łódź, Poland
*The contributions of the first two authors are equivalent

The -463G/A and -129G/A myeloperoxidase-encoding gene polymorphism in chronic obstructive pulmonary disease

Abstract

Introduction: Neutrophils are involved in the pathogenesis of chronic obstructive pulmonary disease (COPD). Myeloperoxidase is an important bactericidal granulocytic enzyme. It is of interest to question whether or not the polymorphic variants of the myeloperoxidase-encoding gene are associated with the risk of developing COPD.
Material and methods: The study determined the risk of COPD development in 186 COPD patients and 220 healthy subjects in the context of two selected polymorphic sites of the promoter region of the myeloperoxidase-encoding gene.
Results: It has been demonstrated that the AA genotype of locus -463 in the myeloperoxidase-encoding gene increases the risk of developing COPD (OR: 2.87; CI: 1.651–4.997). This genotype also correlates with a higher gene expression in patients (0.56 ± 0.12 *vs* 0.31 ± 0.18 in patients with AG genotype and 0.29 ± 0.17, p < 0.01 in those with GG genotype). In healthy individuals, the AA genotype was also characterized by increased expression of the myeloperoxidase-encoding gene (0.41 ± 0.16 *vs* 0.29 ± 0.15 for AG genotype, p < 0.01 and 0.25 ± 0.16 for GG genotype p < 0.01). Patients with the AA genotype had a significantly higher gene expression than healthy subjects with this genotype.
Conclusions: The polymorphic site -129 of the myeloperoxidase-encoding gene was unrelated to the development of COPD. The gene expression did not differ for the individual genotypes. Our studies indicate that the polymorphism of the myeloperoxidase--encoding gene may be related to chronic obstructive pulmonary disease.

Key words: genotype, gene expression, polymorphic sites

Introduction

Chronic obstructive pulmonary disease (COPD) inflammatory disease of the respiratory system which leads to irreversible airway obstruction and the formation of *emphysematous bullae* [1]. The neutrophil is one of the important cells involved in airway inflammation in COPD [2]. Neutrophils are phagocytes which secrete numerous inflammatory mediators, including reactive oxygen species [3]. An enzyme important for the antimicrobial defense of neutrophils is myeloperoxidase (EC 1.11.1.7) [4, 5]. This enzyme belongs to the group of hemoproteins catalyzing the formation of hydrogen peroxide, chloride anions of toxic hypochlorites and, in lesser amounts, of tyrosine and nitrotyrosine radicals, singlet oxygen and ozone [4–6]. The above products kill bacteria and pathogenic fungi and cause damage to the genetic apparatus of some viruses such as HIV-1 [5]. The products of catalytic activity of myeloperoxidase also damage the host's tissue [5]. An unfavorable contribution of this enzyme's activity to the pathogenesis of many inflammatory diseases has been noted. These diseases include chronic obstructive pulmonary disease, ARDS, Alzheimer's disease, multiple sclerosis, myocardial infarction, stroke and many others [5].

Myeloperoxidase (MPO) is encoded by the gene localized in the 17q23.1. fragment of chromosome 17 in the cluster coding for numerous growth hormones [7]. Myeloperoxidase is one member of a gene family of mammalian peroxidases that also includes eosinophil peroxidase, lactoperoxidase, thyroid peroxidase, and prostaglandin H synthase [7]. The mature enzyme is a 140-kDa dimer of identical halves, each containing two polypeptide chains of 108 and 466 amino acids resulting from post-translational excision of 6 amino acids from a single polypeptide precursor. Each half-molecule contains a covalently bound heme that exhibits unusual spectral properties [7]. The significance of polymorphism of the myeloperoxidase-encoding gene in numerous human diseases has been demonstrated. Among others, the myeloperoxidase gene polymorphism plays a role in the risk of gastric cancer [8], preeclampsia [9], lung and prostate cancer [10], coronary heart disease [11], periodontitis [12], chronic lymphocytic leukemia and multiple myeloma [13], as well as in many other pathologies. In our own studies, we have not demonstrated the correlation of the myeloperoxidase gene polymorphism with macular degeneration [14].

Research and discussion of whether polymorphism of the gene coding for myeloperoxidase plays a role in the pathogenesis of COPD is warranted. Neutrophils and mediators produced by them are one of the main pathogenic pathways of this disease [15]. On the one hand, myeloperoxidase is an enzyme of the antimicrobial defense system but on the other, its harmful effect on the tissue has been demonstrated [16–18].

The purpose of this study was to evaluate the frequency of alleles and two polymorphic sites of the myeloperoxidase-encoding gene.

Material and methods

In our study, we examined 186 patients with COPD and 220 healthy controls. There were no statistical differences in sex and age between the groups of patients and the healthy controls. The study exclusion criteria for the patients were as follows: a history of malignant tumors, collagenosis and systemic vasculitis, type 2 diabetes mellitus, bronchial asthma, and asthma and COPD overlap. All of the subjects forming the study group and the control group were smokers or ex--smokers with a minimum number of 10 smoking pack-years. The age of the participants ranged from 45 to 70 years. The group of patients with

COPD included 50 at risk level A, 56 at level B, 34 at risk C, and 46 at risk level D. The allele frequency distribution was not analyzed with respect to the level of COPD risk because of an insufficient size of the groups.

In the group of 186 patients, the mean age was 62 years of age with a standard deviation of 7 years, and in the control group the mean age was 60 with a standard deviation of 9. The differences were not statistically significant. The youngest patient was 48 years old while the youngest healthy subject was 45 years old. Among 186 patients, 56 were women and 130 were men. The control group consisted of 66 women and 154 men. Gender distribution did not differ between the two groups.

Reagents

All reagents used were of analytical grade. The restriction enzymes as well as Taq DNA polymerase were procured from Promega (Madison, WI, USA). Oligonucleotides were synthesized at IDT (Coralville, IA, USA).

DNA extraction

Blood DNA was purified on a QIAamp spin column (Qiagen, Hilden, Germany) using the protocol for DNA isolation from body fluids provided by the manufacturer and modified as follows: 5 μg of RNA poly(A) (Pharmacia Biotech, Uppsala, Sweden) was added to 1 mL of serum to serve as a carrier to improve the recovery of small amounts of DNA. Lysis was ensured by adding 20 μL of Qiagen Proteinase K solution and 1 mL of AL buffer (QIAamp® DNA mini kit). After 10-min of incubation at 56°C, 1 mL of ethanol was added. The mixture was loaded on the QIAamp spin column and centrifuged at 20000 g for 1 min. The column was washed twice by adding 500 μL of AW buffer (QIAamp® DNA mini kit) and centrifuged at 20000 g for 1 min. Finally, DNA was incubated for 5 min at room temperature with 50 μl of AE buffer and eluted by centrifugation.

Polymorphism of MPO gene (-463, -129)

The isolated DNA was used for amplification of -463 and -129 SNP sequences of MPO human gene. The PCR reactions and conditions were identical for both polymorphisms by applying starters: 5'CGGTATAGGCACACAATGGTGA 3', 5'GCAATGGTTCAAGCGATTCTTC 3' specific for -463 polymorphism and 5'TGGGCAACAGAGCA-AGATAA3', 5'CTCTTT CTCCTCCCCCACTG3' specific for -129. Amplification was performed in a total volume of 25 μL containing 50ng of

DNA, 50 pM PCR primers, dNTP, each at a concentration of 80 μM and 1U of GoTaq polymerase (Promega). PCR conditions were as follows: 94°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec (35 cycles). The final elongation step was 10 min at 72°C. The amplification products of 350 bp length (-463 polymorphism) were digested with AciI restriction enzyme at 37°C and separated on 3% agarose gel. The digestion of homozygote GG yields three fragments of 169 bp, 120 bp and 61 bp, heterozygote GA 289 bp, 169 bp, 120 bp and 61 bp fragments and homozygote AA 289 bp, 61 bp fragment. The genotyping of the -129 SNP sequences of the MPO human gene was analyzed by directly sequencing the PCR product. Amplification was performed under the same conditions using 0.1 μg genomic DNA, 200 μM each dNTP, 5 × GoTaq buffer solution, 1U GoTaq polymerase (Promega, Madison WI USA), 0.5 μM primers. Amplification of the product of 129 bp was sequenced using a specific primer labelled with biotin molecule 5'ATTTCAGG '3' by DNA sequencing service IBB PAN (Warsaw Poland).

RNA purification and real time RT-PCR

The human myeloperoxidase and GADPH gene expression was quantified by real-time PCR using ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA USA) according to the manufacturer's protocol. Total cellular RNAs were extracted from the patients' whole blood cells using the Trizol reagent (Invitrogen, Groningen, Nederlands) method, a single-step purification protocol [19]. Polyadenylated RNA was isolated using an Oligotex kit (Qiagen, Chatsworth, CA USA). 50 ng of poly(A) RNA was then used for cDNA synthesis using the TaqMan Reverse Transcription Reagents kit (Applied Biosystem) according to the manufacturer's protocol. Briefly 2.5, 2.0; 1.5, 1.0; 0.5 and 0.25 μL of synthesized cDNA were amplified in triplicate for both GADPH and each of the target genes to create a standard curve. Likewise, 2 μl of cDNA was amplified in triplicate in all isolated samples

for each primer/probe combination and GADPH. Each sample was supplemented with both respective 0.3 μM forward and reverse primers, fluorescent probe, and made up to 50 μL using qPCR™Mastermix for SYBIR Green I (Eurogentec Seraing Belgium). All following PCR primers were designed using software PrimerExpress (Applied Biosystem) forward 5'CCACCAAAAC-CGATCACCAT 3', reverse 5'CACTCCTCGCCTG CATCAT 3'forward, 5'AGCCACATCGCTCAGA-CAC 3', reverse 5' GCCCAATACGAC CAAATCC 3' specific for mRNA of human myeloperoxidase and GADPH respectively. GADPH was used as an active and endogenous reference to correct for differences in the amount of total RNA added to the reaction and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR. Each target probe was amplified in separate 96-well plate. All samples were incubated at 50°C for 2 min. and at 95°C for 10 min. and then cycled at 95°C for 30 sec, 56°C for 1 min. and 72°C for 1 min. for 40 cycles. SYBR Green I fluorescence emission data were captured and m-RNA levels were quantified using the critical threshold (C_t) value. Analysis were performed with ABI Prism 7000 (SDS Software). Controls without RT and with no template cDNA were performed with each assay. To compensate for variations in input RNA amounts, and to maximize efficiency of reverse transcription, GADPH mRNA was quantified and results were normalized to these values. Relative gene expression levels were obtained using the $\Delta\Delta C_t$ method [20]. Amplification-specific transcripts were further confirmed by obtaining melting curve profiles.

Results

The frequencies of the investigated genotypes of locus -463 in the myeloperoxidase-encoding gene in the study population are summarized in Table 1 and Table 2. The AA genotype at locus -463 of the myeloperoxidase-encoding gene has a 2.87-fold increased risk of developing COPD

Table 1. Frequency of genotypes and alleles at -463 polymorphic site of the MPO-encoding gene in COPD and in subjects without COPD. The distribution of genotypes in the group of patients with COPD differs from the distribution in healthy subjects (Chi^2 = 114.19, p < 0.001)

	Allele frequency		Genotype distribution		
	A [%]	G [%]	AA [n%]	AG [n%]	GG [n%]
COPD patients	46	54	45 (25)	77 (41)	64 (34)
Healthy subjects	24	76	22 (10)	40 (18)	158 (72)

COPD — chronic obstructive pulmonary disease

Table 2. Frequency of genotypes and alleles at -129G/A genomic site of the MPO-encoding gene in COPD patients and in the healthy group. The distribution of genotypes in the group of patients with COPD does not differ from the distribution in healthy subjects (Chi^2 = 5.48, p = 0.064)

	Allele frequency		Genotype distribution		
	A [%]	G [%]	AA [n%]	AG [n%]	GG [n%]
COPD patients	25	75	4 (2)	60 (32)	122 (66)
Healthy subjects	26	74	6 (3)	69 (31)	145 (66)

COPD — *chronic obstructive pulmonary disease*

(95% CI: 1.651–4.997) as compared to AG and GG genotypes. Carriage of the AA and AG genotypes *vs* the GG genotype did not significantly affect the risk of developing COPD (OR: 0.813, 95% CI: 0.521–1.270). Carriage of the AA genotype relative to the GG genotype increases the risk of COPD in smokers (OR: 5050, 95% CI: 2.808–9.080). The AG genotype as compared to GG was also found to be a risk factor for COPD (OR: 4.752, 95% CI: 2.941–7.679). Carriage of the AA and AG genotype vs. the GG genotype is also a risk factor for the development of COPD (OR: 4.857, 95% CI: 3.187–7.406). In contrast, the AA genotype did not increase the risk of developing COPD relative to carriage of the AG genotype (OR: 1.0634, 95% CI: 0.562–2.001).

Genotypic distribution of the polymorphic locus -129 of the gene encoding MPO was not different in healthy subjects and patients and was not associated with the risk of developing COPD.

The myeloperoxidase-encoding gene expression for the -463AA genotype was significantly higher in both healthy subjects and COPD patients (Table 3). Patients with the AA genotype demonstrated a higher gene expression than healthy individuals with the AA genotype. The gene expression did not differ for the remaining genotypes.

No differences in gene expression were reported for the polymorphic variants of locus -129 of the myeloperoxidase-encoding gene (Table 4).

Discussion

The obtained results show the polymorphic locus -129 of the gene encoding human myeloperoxidase is not associated with the risk of developing COPD. The AA genotype at the polymorphic site -463 of the myeloperoxidase-encoding gene has a clear association with the occurrence of chronic obstructive pulmonary disease. It is also associated with an increased expression of mRNA of the enzyme-encoding gene and probably with the enzyme activity that was not studied in this

Table 3. Expression of the myeloperoxidase-encoding gene at site -463 according to the genotype in patients with COPD and in healthy subjects. The gene expression in the serum expressed as 2$^{-\Delta\Delta Ct}$

Genotype	Gene expression
COPD patients	
AA	0.56 ± 0.12[*, #]
AG	0.31 ± 0.18
GG	0.29 ± 0.17
Healthy subjects	
AA	0.41 ± 0.16[**]
AG	0.29 ± 0.15
GG	0.25 ± 0.16

*Significantly higher than gene expression for phenotypes AG and GG in patients, p < 0.01; **significantly higher than gene expression for AG and GG phenotypes in healthy subjects, p < 0.01; #Significantly higher in patients with AA phenotype than in healthy subjects with AA phenotype.
COPD — *chronic obstructive pulmonary disease*

Table 4. Expression of the myeloperoxidase-encoding gene at site -129 according to the genotype in patients with COPD and in healthy subjects. The gene expression in the serum expressed as 2$^{-\Delta\Delta Ct}$

Genotype	Gene expression
COPD patients	
AA	0.42 ± 0.12
AG	0.31 ± 0.18
GG	0.30 ± 0.17
Healthy subjects	
AA	0.41 ± 0.16
AG	0.29 ± 0.15
GG	0.31 ± 0.16

No inter-group differences in expression were observed. COPD — *chronic obstructive pulmonary disease*

research. As reported in the literature, neutrophil granulocytes are the most important cells responsible for the development of COPD [21, 22]. The mediators released by neutrophils damage the

respiratory epithelium, cause free radical and proteolytic tissue damage, and initiate respiratory tract remodeling typical of COPD [21, 22]. The increased expression of the AA genotype of locus -463 in the myeloperoxidase-encoding gene is associated in our studies both with an increased risk of developing COPD and with an increased expression of the enzyme mRNA. It is well known that neutrophils are the cells responsible for the development of COPD, however, the significance of the mediators released by myeloperoxidase is controversial. On the one hand, myeloperoxidase kills bacteria that cause exacerbations of infection in the course of COPD but, on the other hand, it damages the tissues of the respiratory tract [1, 2, 21, 22]. The lack of "tuning" the magnitude of the reaction involving the secretion of mediators by neutrophils against the infection may be responsible for the remodeling of the respiratory tract and the formation of emphysematous bullae [23]. Such a situation resembles an excessive immune response in autoimmune diseases, but this is only our hypothesis. Similarly, peroxidase deficiency is associated with a disease similar to chronic granulomatous disease and has a predisposition to severe Candida albicans infections [24]. It has recently been demonstrated that myeloperoxidase itself is a mediator of post-ischemic arrhythmogenic cardiomyopathy [25]. Conceivably, it is either through its own actions, or through the peptides resulting from its proteolytic degradation, a mediator of bronchial remodeling. The effects of myeloperoxidase on cardiac vessels and on the heart itself suggest that it contributes to the development of pulmonary hypertension and pulmonary heart disease in COPD, which requires further investigation. The role of HClO, the main product of myeloperoxidase enzyme activity in inducing bronchial remodeling and emphysema, cannot be ruled out, although there has been no research work on this subject to date. Undoubtedly, reactive oxygen species, including hypochloride produced by myeloperoxidase, have a well-established role as COPD development factors [26]. It is interesting what transcription factors affect the expression of the myeloperoxidase gene and what their association is with the polymorphic sites of the promoter region of the gene in question. The polymorphism of locus -463 in the myeloperoxidase-encoding gene is associated with a risk of developing Alzheimer's disease [27]. On the other hand, studies of Asian populations have shown that the AA genotype is associated with a reduced risk of lung cancer [28]. Also, the GG genotype reduces the risk of developing type 2 diabetes [29]. Subjects predisposed to developing lung cancer in the course of COPD may be those who carry the GG genotype. The AA/GA genotype at locus -463 promote the development of hypertension in people with a low BMI and without diabetes [30]. It would be interesting to check the blood pressure distribution in our study group, but this was not the subject of the research. The AA/GA variant reduced the risk of colorectal cancer in the Chinese population [31].

The disadvantage of our research was the fact that we did not determine myeloperoxidase activity and expression in sputum. This would give a more realistic and detailed picture of the correlations between the polymorphic variants of the myeloperoxidase gene and the enzyme activity, as well as its expression in the airways. On the other hand, COPD is a systemic disease and changes in its expression in the blood may have some significance in its pathogenesis. The studies need to be repeated in larger groups and in other populations coming from different regions. In addition, it needs to include the gene expression in induced sputum, or in bronchoalveolar lavage. In our work, we did not analyze the relationships between the polymorphic variants and the severity/risk of COPD. An analysis with division into subgroups would have resulted in there being too few patients to obtain reliable results. It would be interesting to see whether the genotype distributions in the non-smoking group would be similar to the distributions in smokers without COPD. Such studies, however, have not been conducted. That being said, in a study by Kowalski *et al.* concerning the polymorphic variants of the myeloperoxidase-encoding gene (the same as in our study), distributions of genotypes obtained in healthy subjects were similar to those obtained in our work for smokers (the control group in our study) [14]. The aforementioned study was also conducted in the Polish population. It indicates that the studied gene variants are unlikely to be associated with the risk of nicotine dependence syndrome. The distribution, which is similar to ours, supports the probable repeatability of the results we obtained. The contribution of genetic factors to the pathogenesis of nicotine addiction is likely to be low and involves only the genes coding for cytochrome C450 variants and receptor proteins in the central nervous system [32].

Conclusions

1. The polymorphism of locus -463 in the myeloperoxidase-encoding gene is associated

with the risk of developing COPD. Carriage of the AA genotype is associated with a 2.87-fold higher risk of COPD. Also, the myeloperoxidase gene expression in the blood is higher in subjects with the AA genotype, both healthy and sick; however, it is significantly higher in the patient group than in healthy subjects

2. The polymorphism of locus -129 in the myeloperoxidase-encoding gene has no association with the risk of COPD.

Funding statement

Conflicts of interest

The authors have no conflict of interest to report.

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