

Myeloperoxidase gene (MPO) polymorphisms in patients with age-related macular degeneration (AMD)

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SUMMARY

Myeloperoxidase gene (MPO) polymorphisms in patients with age-related macular degeneration (AMD) borreliosis

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Oxidative stress is one of the main mechanisms playing an important role in the etiology of age-related macular degeneration (AMD). Myeloperoxidase (MPO) is an enzyme involved in the formation of hypochlorous acid and other reactive oxygen species. We tried to assess the effect of two polymorphisms in the nucleotide sequence located in the promoter region of MPO gene on the expression of this gene at the mRNA level.

The aim of the study was to find a correlation between polymorphism occurring nucleotide sequence of the gene and gene expression of myeloperoxidase in patients with a form of "wet" AMD.

Materials and methods. We analyzed two polymorphisms in the nucleotide sequence in the promoter region of MPO gene, at position -463, -129. Gene expression at the mRNA level were studied by real time PCR. The study group consisted of 120 patients with the figure of 'wet' and not 300 people diagnosed with AMD as a control group.

Results. Statistical analysis showed no statistical differences in genotype distributions and allele frequencies between patients with AMD and the control group. It has been shown that the presence of GG genotype in the MPO gene -463 polymorphism is correlated with elevated expression of the gene test.

Conclusions. Sequence polymorphism-463 G / A -129 and MPO gene is not a risk factor for AMD.

Key words: AMD, myeloperoxidase; gene polymorphism, gene expression

STRESZCZENIE

Polimorfizm genu mieloperoksydazy u chorych ze zwyrodnieniem plamki ocznej związanej z wiekiem

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Stres oksydacyjny jest jednym z głównych mechanizmów odrywających istotną rolę w etiologii zwyrodnienia plamki ocznej związanej z wiekiem. Mieloperoksydaza (MPO) jest enzymem zaangażowanym w powstawanie kwasu podchlorynowego i innych reaktywnych form tlenu. W przedstawionych badaniach podjęliśmy próbę oceny wpływu dwóch polimorfizmów sekwencji nukleotydowej zlokalizowanych w rejonie promotorowym genu MPO na ekspresję tego genu na poziomie mRNA.

Celem przeprowadzonych badań było znalezienie korelacji pomiędzy występującym polimorfizmem sekwencji nukleotydowej genu a ekspresją genu MPO u pacjentów z postacią „mokrą” AMD.

Materiał i metody. Analizowano dwa polimorfizmy sekwencji nukleotydowej znajdujące się w rejonie promotorowym genu MPO, w pozycji -463, -129. Ekspresję genu na poziomie mRNA badano metodą real time PCR. Grupę badaną stanowiło 120 pacjentów z postacią „mokrą” oraz 300 osób których nie zdiagnozowano zwyrodnienia plamki ocznej związanej z wiekiem jako grupę kontrolną.

Wyniki. Przeprowadzona analiza statystyczna wykazała brak różnic statystycznych w rozkładzie genotypów i częstości alleli między pacjentami ze zwyrodnieniem plamki ocznej związanej z wiekiem a grupą kontrolną. Wykazano, że występowanie genotypu GG w polimorfizmie -463 genu MPO jest skorelowane z podwyższonym poziomem ekspresji badanego genu.

Wnioski. Polimorfizm sekwencji G-463/A and -129 genu MPO nie jest czynnikiem ryzyka wystąpienia zwyrodnienia plamki ocznej związanej z wiekiem.

Słowa kluczowe: zwyrodnienie plamki ocznej związanej z wiekiem, mieloperoksydaza, polimorfizm genowy, ekspresja genów

Age-related macular degeneration (AMD) is the leading cause of visual loss in individuals over 60 years old, in developed countries. About 11 million people are affected by AMD worldwide and its incidence increases with age [1, 2]. According to data from Germany, the disease develops in 30% of subjects above 75 years of age [3, 4]. Although the disease presents a serious social problem, its pathogenesis is still unclear.

Oxidative stress is a major factor in the pathogenesis of AMD. High oxygen concentrations, prolonged exposure to light and the presence of photosensitizers are factors favoring the generation of reactive oxygen species (ROS) in the region. Oxidative stress is also required for age-related accumulation of lipofuscin in pigmented epithelium cells located in the vicinity of the macula. This pigment accumulates in much larger quantities in AMD-affected eyes than in healthy ones. Increased exposure to free oxide radicals results in enhanced activity of enzymes causing degradation of free radicals. A variety of genes including catalase (CAT), manganese superoxide dismutase (MnSOD or SOD2), glutathione peroxidase 1 (GPX1) are involved in the first line of defence against oxidative stress which may be an important mechanism in the development of AMD.

Oxidative stress might also be modulated by enzymes like myeloperoxidase (MPO). The H_2O_2 generated by MnSOD can be further converted to highly damaging hydroxyl radicals via myeloperoxidase, a product of the MPO gene [5]. MPO is a metabolic/oxidative lysosomal enzyme found in neutrophils and monocytes [6]. It generates ROS endogenously by functioning as antimicrobial enzyme, catalyzing the reaction between H_2O_2 and chloride to generate hypochlorous acid, a potent oxidizing agent. Hypochlorous acid further reacts with other biological molecules to generate secondary radicals [7]. Thus, ultimate levels of potentially cytotoxic ROS may depend in part upon the balance between activities of MnSOD, catalase and MPO, determining further generation of ROS or detoxication of H_2O_2 . Activity levels of these enzymes are likely affected by functional polymorphisms in the genes encoding them. MPO gene spans over 11 kilobases, has 12 exons and is located on chromosome 17. Different allelic variants of MPO have shown to alter the expression levels of mRNA. Polymorphism at the -463 and -129 positions can potentially affect the expression and protein levels of MPO [8].

This study is an attempt to assess the functional polymorphisms in -463 and -129 positions of the myeloperoxidase gene in patients with wet form of AMD. The correlation between MPO gene expression and polymorphisms of -463 and -129 gene was analyzed.

MATERIALS AND METHODS

In the study 120 patients with wet form of AMD and 300 healthy controls were examined. There were no statistical differences in sex and age between analyzed groups of patients and healthy controls.

Patients study exclusion criteria were: diagnosed acute inflammation of eye, chronic inflammation, diabetes mellitus or/and rheumatoid polyarthritis and patients with a body temperature higher than 38°C for at least two weeks. Pregnant and lactant women were also excluded from the study based on ethical and legal requirements.

Reagents

All used reagents 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 buffer AL (QIAamp® DNA mini kit). After 10-min 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 buffer AW (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 buffer AE and eluted by centrifugation.

Polymorphism of MPO gene (-463, -129)

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'TGGGCAACAGAGCAAGATAA3', 5'CTCTTTCTCCTCCCCACTG3' 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 *AclI* restriction enzyme at 37°C and separated on 3% agarose gel. The digestion of homozygote GG yields three fragments 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 MPO human gene was analyzed by direct sequencing PCR product. Amplification was performed under the same conditions using 0.1 µg genomic DNA, 200 µM each dNTP, 5x GoTaq buffer solution, 1U GoTaq polymerase (Promega, Madison WI USA), 0.5 µM primers. Amplification product 129 bp was sequenced using specific primer labelled with biotin molecule 5'ATTTTCAGG '3' provided 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 patients' whole blood cells using the Trizol reagent (Invitrogen, Groningen, Netherlands) method, a single-step purification protocol [9]. 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 of the following PCR primers were designed using software PrimerExpress (Applied Biosystem) forward 5'CCACCAAACCGATCACCAT 3', reverse 5'CACTCCTCGCCTG CATCAT 3'forward, 5'AGCCACATCGCTCAGACAC 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 s, 56°C for 1 min. and 72°C for 1 min. for 40 cycles. SYBR Green I fluorescence emission data were captured and mRNA levels were quantified using the critical threshold (Ct) value. Analysis was 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 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 [10]. Amplification specific transcripts were further confirmed by obtaining melting curve profiles.

Statistical analysis

Statistical analysis of the collected material utilized descriptive methods as statistical conclusion. In order to describe the studied groups of patients and controls, structural indexes were calculated in qualitative analysis of characteristics. Differences in allele and genotype frequencies between AMD patients and controls were analysed by applying the χ^2 test. To reject the null hypothesis, a probability of 0.05 was used. Differences in normally distributed continuous variables were compared using Student's t-test and for non-normally distributed data by the Mann-Whitney test. Two-sided P-values less than 0.05 were considered significant.

RESULTS

Polymorphism of MPO gene (-463, -129)

This study enrolled 120 patients with wet form of AMD and 300 healthy controls. The frequency of genotypes in the studied polymorphism -463, -129 in patients with AMD and the control group has been presented in tables 1 and 2. There is no statistically significant difference in the distribution of genotypes in the studied -463 gene polymorphism between patients with AMD and controls group ($\chi^2 = 1.76$, $P = 0.419$). No skewing was observed in gene frequency between controls and AMD patients ($\chi^2 = 0.032$, $P = 0.862$) (table 1). The single-point analysis showed no association with AMD. The -129 MPO SNP was genotyped in the same group of patients with AMD and healthy controls (table 2). There is no statistically significant difference in the distribution of genotypes ($\chi^2 = 3.54$, $P = 0.715$) and gene frequency between patients with AMD and controls ($\chi^2 = 0.871$, $P = 0.352$). Single-point analysis did not reveal any association with studied polymorphism with AMD (table 2).

Table 1. Genotype distributions and alleles frequencies of the -465 MPO gene polymorphism in healthy subjects and patients with AMD

Allele frequency	Allele frequency		Genotype distribution		
	A (%)	G (%)	AA n (%)	AG n(%)	GG n(%)
AMD patients	16.7	83.3	11 (9.2%)	27 (22.5 %)	82 (68.3 %)
Control group	19.0	81.0	30 (10%)	54 (18 %)	216 (72 %)

Table 2. Genotype distributions and alleles frequencies of the -129 MPO gene polymorphism in healthy subjects and patients with AMD

Allele frequency	Allele frequency		Genotype distribution		
	A (%)	G (%)	AA n (%)	AG n(%)	GG n(%)
AMD patients	19.6	80.4	4 (3.3%)	39 (32.5%)	77 (64.2%)
Control group	18.0	82.0	6 (2.0%)	96 (32.0%)	198 (66.0%)

Expression of MPO gene on mRNA level

In this study, we used real-time RT-PCR method to compare MPO gene expression in wet form of AMD patients and healthy controls. The mean values for mRNA levels of the studied gene were standardized by the mRNA level of GADPH on the same sample. Relative gene expression was calculated using $2^{-\Delta\Delta ct}$ method. The method was able to detect MPO mRNA expression in the cells isolated from whole blood. The MPO gene expression on mRNA level was compared with genotype distribution (A/A; A/G; G/G) in polymorphism at -463 position (fig. 1) and (A/A; A/G; G/G) in polymorphism at -129 position in each of the studied groups (fig. 2). Homozygotes GG in polymorphism at -463 position in AMD patients and healthy controls presented higher expression of MPO gene as compared to homozygote AA (2.3 and 1.45 fold higher respectively). Expression of MPO gene in AMD patients with GG genotype in polymorphism

-463 was 1.56 fold higher than in controls with the same genotype. No significant difference in MPO expression level in polymorphism at -129 position between AMD patients and healthy controls was observed. Pearson's correlation coefficient was used to evaluate the relationship between gene expression of MPO and genotype distribution. There is no correlation between MPO mRNA expression and genotype distribution in AMD patients.

DISCUSSION

Association studies have examined individual SNPs within MnSOD, CAT, GPX1 genes as risk factors for the development of AMD usually by assessing SNPs for differences in allele frequencies [11, 12]. MnSOD and microsomal epoxide hydrolase gene polymorphism has only been shown to be associated with wet AMD in a Japanese study [11]. Results obtained

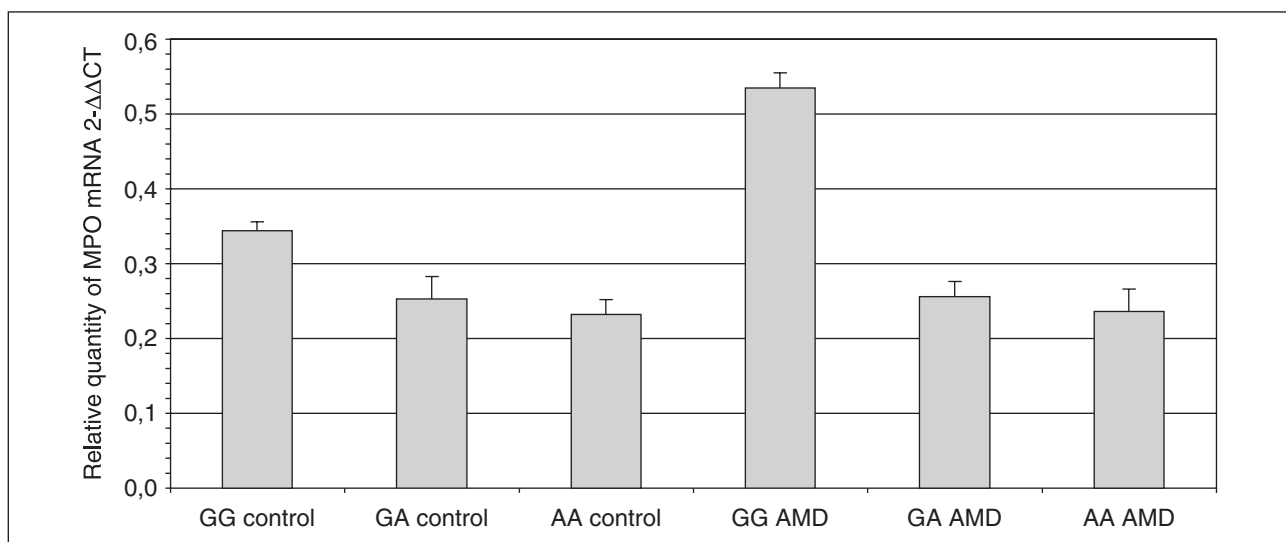


Fig. 1. Quantitative real time PCR analysis of human MPO mRNA ($2^{-\Delta\Delta ct}$) in healthy controls and AMD patients with wet form. Correlation expression level of MPO gene with polymorphism -463

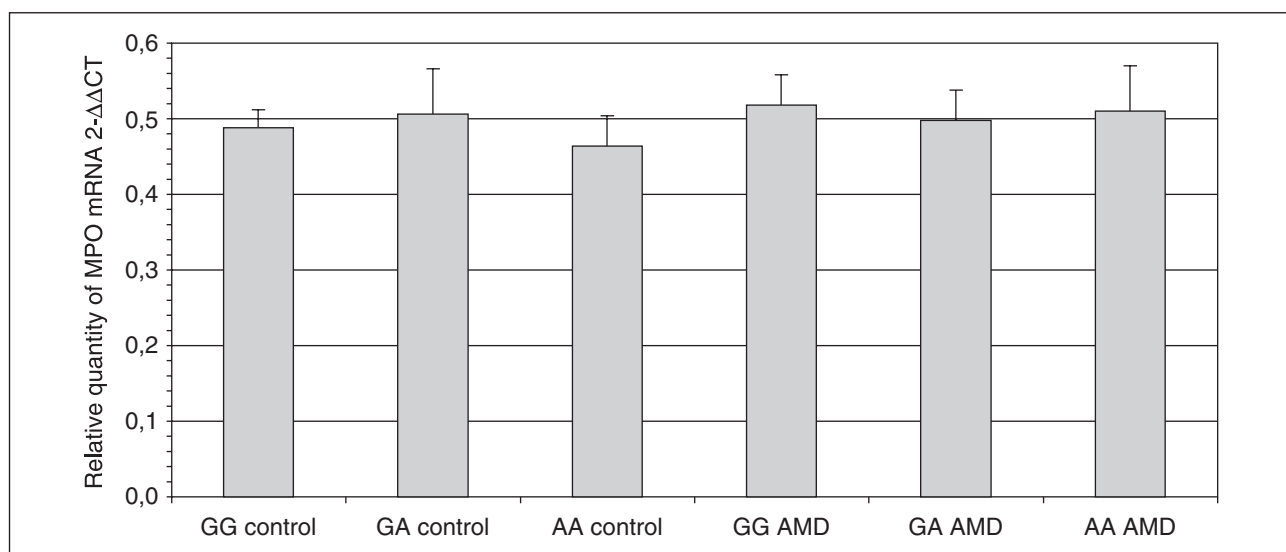


Fig. 2. Quantitative real time PCR analysis of human MPO mRNA ($2^{-\Delta\Delta Ct}$) in healthy controls and AMD patients with wet form. Correlation expression level of MPO gene and polymorphism -129

by *Esfandiary et al.* [12] suggest that manganese superoxide dismutase gene polymorphism (V16A) is associated with exudative age-related macular degeneration. *Esfandiary et al.* [12] assessed the same SNP and two other intronic SNPs but were unable to demonstrate significant differences between cases and controls.

MPO, a catalyst for the reaction yielding a potent oxidant such a hypochlorous acid (HOCL) causes tissue damage through its strong oxidative activity. In this study, we evaluated the potential role of functional myeloperoxidase (MPO) promoter polymorphisms in the occurrence of AMD. Several single nucleotide polymorphisms have been identified in MPO locus, one of which is located in the promoter region (-463 G/A) and leads to the loss of a Sp1 transcription binding site in an Alu hormone-responsive element [8]. The presence of -463G allele, occurring with high frequency, causes increased expression of the enzyme, whereas -463A allele decreases enzyme expression by destruction of the transcriptional factor binding site. The functional polymorphism of MPO is associated with inflammatory processes [13] and neurodegenerative diseases [14]. The G allele of -463 polymorphism of MPO gene is associated with acute myelocytic leukemia [15], susceptibility to lung cancer [16] and to Alzheimer's disease in females [17], but the A allele presumed associated with lower levels of ROS, has been associated with decreased risk of breast cancer among women [18]. Another MPO gene polymorphism -125G/A has influence on myocardial infarction in a Swedish population [19]. The A allele of the MPO -129G/A promoter polymorphism is associated with a reduced risk of MI in women. A number of studies have addressed its possible asso-

ciation with multiple sclerosis [20, 21]. Polymorphisms at the -463 and -129 positions in MPO promoter can potentially affect the expression of MPO on mRNA and protein level [8]. Our study has been the first one to assess the correlation between MPO gene expression and two functional SNP's G-463A and G-129A of the gene encoding MPO in AMD patients/control material. Statistic analysis has shown that there is no statistically significant difference in the distribution of genotypes and genotype frequency between patients with AMD and controls in both analyzed polymorphisms. Real-time RT-PCR analysis of MPO gene expression on mRNA level demonstrated that homozygotes GG in polymorphism at -463 position in AMD patients and healthy controls presented higher expression of MPO gene as compared to homozygote AA (2.3 and 1.45 fold higher respectively). Expression of MPO gene in AMD patients with GG genotype in polymorphism -463 was 1.56 fold higher than in controls with the same genotype. No significant difference in MPO expression level in polymorphism at -129 position between AMD patients and healthy controls was observed.

CONCLUSIONS

Although the promoter polymorphism (-463 and -129) is not associated with wet form of AMD, other polymorphisms in MPO gene could be involved in this defect, and a comprehensive search for genetic variants in the gene may show such correlation.

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