DETECTION OF ALPHA-1 ANTITRYPsin GENE MUTATIONS BY POLYMERASE CHAIN REACTION IN PATIENTS WITH CHRONIC OBSTRUCTIVE PULMONARY DISEASE

DETEKCIJA MUTACIJA GENA ZA ALFA-1-ANTITRIPsIN PRIMENOM LANČANE REAKCIJE POLIMERAZE KOD PACIJENATA SA HRONičNOM OPlStrUKTivNOM BOLESTI PLuČA

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Summary

Introduction. The alpha-1 antitrypsin deficiency is the best described genetic cause of chronic obstructive pulmonary disease. The study of the alpha-1 antitrypsin deficiency, as the most important genetic risk factor for chronic obstructive pulmonary disease, is an important step in developing a strategy for the prevention and treatment of this disease. The aim of the study was detection of homozygous and heterozygous deficient gene alleles (protease inhibitor Z and protease inhibitor S) for alpha-1 antitrypsin in the group of patients with chronic obstructive pulmonary disease with the predominance of lung emphysema, as well as determination of a positive correlation between the serum levels of alpha-1 antitrypsin and the corresponding alpha-1 antitrypsin genotype. Material and Methods. The study included 90 patients, mutually unrelated individuals, hospitalized due to lung emphysema. The control group included 10 subjects, with no clinical signs of lung emphysema, but with a family history of chronic obstructive pulmonary disease. We attempted to identify the most common deficient alleles (protease inhibitor Z and protease inhibitor S) and the concentration of alpha-1 antitrypsin in the serum of the examinees. The polymorphism between the two allelic forms, protease inhibitor Z and protease inhibitor S, was detected by real-time polymerase chain reaction. Results. Protease inhibitor MM genotype alpha-1 antitrypsin was present in all 90 patients with the diagnosis of pulmonary emphysema, and the serum levels of alpha-1 antitrypsin were within the range of reference values. In the control group, there were two cases with mutated protease inhibitor MZ genotype, and in these 2 subjects the serum level of alpha-1 antitrypsin was at the lower limit of reference values. Conclusion. In patients diagnosed with lung emphysema, protease inhibitor MM genotype alpha-1 antitrypsin and normal serum alpha-1 antitrypsin levels, the genetically-determined deficiency of alpha-1 antitrypsin is not responsible for the development of chronic obstructive pulmonary disease. Key words: Mutation; alpha-1-Antitrypsin; Polymerase Chain Reaction; Pulmonary Disease, Chronic Obstructive; Polymorphism, Genetic; Pulmonary Emphysema; Risk Factors

Sažetak

Uvod. Deficit alfa-1-antitripsina je najbolje opisan genetski uzročnik hronične opstruktivne bolesti pluća. Istraživanje deficitalnog alfa-1-antitripsina, kao najvažnijeg genetskog faktora rizika za hroničnu opstruktivnu bolest pluća, predstavlja važan korak u razvoju strategije za prevenciju i lečenje ove bolesti. cilj istraživanja bio je detekcija homozigotnih i heterozigotnih deficitarnih alela (protein inhibitor Z i protein inhibitor S) gena alfa-1-antitripsina u grupi pacijenata sa hroničnom opstruktivnom bolesti pluća sa predomljajom emfizema pluća, kao i utvrđivanje pozitivne korelacije između nivoa serumskog alfa-1-antitripsina i odgovarajućeg alfa-1-antitripsin genotipa. Materijal i metode. Istraživanjem je bilo obuhvaćeno 90 bolesnika, međusobno nesrodnih osoba, hospitalizovanih zbog emfizema pluća. Kontrolnu grupu je činilo 10 ispitanika, bez kliničkih znakova emfizema pluća, ali sa pozitivnom porodičnom anamnezom hronične opstruktivne bolesti pluća. Svim ispitanicima vršena je identifikacija najčešćih deficitarnih alela (alfa-1-antitripsina Z i alfa-1-antitripsina S) i određivana koncentracija alfa-1-antitripsina u serumu. Polimorfizam između dve aleln formi protein inhibitor Z i protein inhibitor S detektovano je korišćenjem real-time polymerase chain reaction. Rezultati. Kod svih 90 bolesnika sa dijagnozom emfizema pluća bio je prisutan deficit alfa-1-antitripsina, a nivo serumskog alfa-1-antitripsina bio je u opsegu referentnih vrednosti. U kontrolnoj grupi identifikovana su dva slučaja sa mutiranim protein inhibitor MZ genotipom, i kod ova dva ispitanika serumski nivo alfa-1-antitripsina bio je na donjoj granici referentnih vrednosti. Zaključak. Kod bolesnika sa dijagnozom emfizema pluća, protein inhibitor MM genotip alfa-1-antitripsina bio je na donjoj granici referentnih vrednosti, genetski determinisan nedostatak alfa-1-antitripsina nije odgovarao za nastanak hronične opstruktivne bolesti pluća. Ključne reči: mutacija; alfa-1-antitripsin; PCR; hronična opstruktivna bolest pluća; polimorfizam gena; plućni emfizem; faktori rizika
Protease inhibitor MM (PiMM) phenotype/genotype is characteristic for homozygous persons with a normal Pi M allele who have a normal plasma α1-AT concentration that provides adequate antiproteinase protection. The homozygous persons with PiZZ genotype have a deficient Pi Z allele responsible for 95% of severe α1-AT deficiency cases [1, 5, 6].

Protease inhibitor Z mutation, which is labeled with glutamic acid (Glu) 342 GAG → lysine (Lys) AAG, occurs in the exon V of the gene for α1-AT and represents the substitution of glutamic acid with lysine at position 342, i.e. the transition of guanine to adenine at the nucleotide position g.3297 G >A [5]. This type of mutation provides secretion of only 10 – 15% of the normal amount of α1-AT from hepatocyte to homozygote. Consequently, α1-AT polymer formation occurs, resulting in the fact that such large molecules cannot be exported from the liver and remain in it [6–8].

Protease inhibitor S mutation, which is labeled with valine (Val) GTA GAA, is formed in the exon III inhibitor serpin peptidase (Pi gene) and represents substitution of valine and glutamic acid at amino acid position 264, i.e. the transition of adenine into thymine at the nucleotide position g.2958 A > T [9]. Hepatocytes decompose a great part of the new α1-AT, which reduces the secretion of α1-AT, thus creating a state of deficiency [8].

Several studies have shown a correlation between the serum levels of α1-AT and the corresponding α1-AT genotype. Thus, the PiZZ genotype of α1-AT has 10 – 15%, PiSZ 51%, PiMZ 83%, PiSS 93%, PiMS 97% and PiMM 100% normal serum concentration of α1-AT [9].

The α1-AT deficiency has been identified in all populations around the world. In the population of our country the most common alleles are Pi M variants with an allelic frequency of 0.9805 (98.05% Pi M). The frequency of Pi S variant is 0.0067 (0.67% Pi S), and Pi Z variant 0.0128 (1.28% Pi Z) [9].

Considering the fact that in our country there are no studies that precisely define the frequency of an allele of the gene for α1-AT in the group of patients with COPD with a predominance of pulmonary emphysema, the goal of the study was detection of homozygous and heterozygous deficient

### Table 1

| Table 1. Single-nucleotide polymorphism (SNP) genotyping Assay AAT E342K orable AAT E264K-set of primers and probes specifically designed to a target sequence |
|---|---|
| **SNP Genotyping Assay AAT** | **Probe sequences (reporter sequence)**<br>**Sekvence genskih praba** | **Sekvence prajmera** |
| PiM-5′-VIC-ACCATCGACGAGAAG-3′ | Forward/Uzvodni-5′-GGCTGGGAT-CGCCTTACACAGT-3′<br>Reverse/Nizvodni-5′-GATGGATATG-3′<br>CCTCTAAAAACATGG-3′ | PiZ-5′-FAM-CATCGACAAGAAAG-3′ |
| **SNP Genotyping Assay PiM-5′-FAM-GATGATATCGTGAGTGTTTCATT-TACCCAGGTGCTAGATT CCCCTCATC-3′ | PiS-5′-VIC-GATGATATCGTGAGTGTTTCATT-CAGGCGCTGATGTT CCCCTCATC-3′ | Forward/Uzvodni-5′-GGCTGGGAT-CGCCTTACACAGT-3′<br>Reverse/Nizvodni-5′-GATGGATATG-3′<br>CCTCTAAAAACATGG-3′ |

**Abbreviations**

- α1-AT: α1 antitrypsin
- COPD: chronic obstructive pulmonary disease
- Real-Time PCR: real-time polymerase chain reaction
- PI (gene): Serine (or cysteine) protease inhibitor
- Glu: glutamic acid
- Lys: lysine
- Val: valine
- DNA: deoxyribonucleic acid
- 6-FAM: 6-carboxyfluorescein
- VIC: 50-fluorescein

**Introduction**

Chronic obstructive pulmonary disease (COPD) is the leading cause of morbidity and mortality in the world and represents a significant and growing social and economic problem worldwide. According to the World Health Organization, COPD was globally the third on the list of leading causes of death, with 3 million lives lost in 2016 [1].

In recent years, the risk factors for COPD have been in the focus of numerous studies dealing with these issues, given that the identification of risk factors is an important step in developing strategies for the prevention and treatment of any disease. In 80% of cases, COPD is caused by a combination of smoking and genetic predispositions. The best described genetic cause of COPD is the alpha-1 antitrypsin (α1-AT) deficiency [1].

The α1-AT deficiency is a potentially lethal genetic disorder that leads to the development of a disease primarily with pulmonary and hepatic clinical manifestations. Instructions on the production of α1-AT protein, which is a protease inhibitor (Pi) from serpin superfamily, are given by the SERPI NA1 gene. This gene is located in the long arm of chromosome 14 at position q31-32.3 [1, 2]. The normal gene for α1-AT (Pi M) possesses extremely high polymorphism, with more than 123 different alleles determined at the protein or genome level [2].

The two most common mutations in the "deficiency" are Pi Z and Pi S, and the rare ones: PiMMalton, PiMPittsburg Mduarte, Pi Null Null and others [2-4].
gene alleles (Pi Z and Pi S) for α₁-AT in patients with COPD, with predominance of pulmonary emphysema, determination of the incidence of polymorphisms Pi S and Pi Z in the gene for α₁-AT, and evaluation of its connection with the risk for COPD in the population of Vojvodina. In addition, in patients with COPD and prevailing emphysema the following aims have also been set: determination of serum concentrations of α₁-AT and establishing a positive correlation between the level of serum α₁-AT and the corresponding α₁-AT genotype.

**Material and Methods**

The research was conducted at the Institute of Pulmonary Diseases of Vojvodina in Sremska Kamena as well as in the Deoxyribonucleic acid laboratory of the Institute of Forensic Medicine, Clinical Center of Vojvodina in Novi Sad. The test protocol was approved by the Ethics Committee of the Institute of Pulmonary Diseases of Vojvodina and the Ethics Committee of the Faculty of Medicine in Novi Sad. Selected patients who meet the necessary criteria for inclusion, after being fully informed about the type of examination and detailed introduction to the planned procedure, have confirmed in writing that they voluntarily agreed to participate in the study.

The sample included two groups of subjects, the first group of patients with lung emphysema, and a control group of healthy subjects without clinical signs of emphysema, but with a positive family history of COPD (mother or father were suffering from bullous emphysema and had a α₁-AT deficiency).

**Table 2.** Distribution of α₁-antitrypsin genotypes in 100 analyzed subjects by using the real-time PCR method

| Number of respondents (n) | Alph1-antitrypsin genotype Genotip alfa1-antitripsina | M/M | M/Z | Z/Z |
|---------------------------|--------------------------------------------------------|-----|-----|-----|
| Group of patients with the diagnosis of lung emphysema/Grupa pacijenata sa dijagnozom emfizema pluća | 90 | 90 | 0 | 0 |
| Glu 342 Lys Polymorphism Glutaminska kiselina 342 Lizin polimorfizam | (100%) | (0%) | (0%) |
| Group of respondents with a positive family history, without lung emphysema/Grupa ispitanih sa pozitivnom porodičnom anamnezom, bez emfizema pluća | 10 | 8 | 2 | 0 |
| Glutaminska kiselina 342 Lizin polimorfizam | (80%) | (20%) | (0%) |

| Number of respondents (n) | Alph1-antitrypsin genotype Genotip alfa1-antitripsina | M/M | M/S | S/S |
|---------------------------|--------------------------------------------------------|-----|-----|-----|
| Group of patients with the diagnosis of lung emphysema/Grupa pacijenata sa dijagnozom emfizema pluća | 90 | 0 | 0 | 0 |
| Glu 264Val Polymorphism Glutaminska kiselina 264 Valin polimorfizam | (0%) | (0%) | (0%) |
| Group of respondents with a positive family history, without lung emphysema/Grupa ispitanih sa pozitivnom porodičnom anamnezom, bez emfizema pluća | 10 | 0 | 0 | 0 |
| Glutaminska kiselina 264 Valin polimorfizam | (0%) | (0%) | (0%) |
The study included 90 patients (71 men and 19 women), aged 32 to 83 years, unrelated individuals with their last residence in the territory of Vojvodina, who were hospitalized at the Institute of Pulmonary Diseases in Sremska Kamenica due to lung emphysema. Clinical diagnosis of pulmonary emphysema in all patients was made by functional examination of the respiratory system and radiological or computerized tomography (CT) by verification of pulmonary parenchyma reduction and bullous changes.

The control group included 10 respondents (5 males and 5 females), aged 34 – 51, without clinical signs of emphysema, but with a positive family history of COPD (mother or father suffered from bullous emphysema and had a deficiency of $\alpha_1$-AT), who presented with difficulty breathing, persistent cough with or without sputum production, repeated respiratory infections, rapid onset of respiratory function problems, or wanted a routine pulmonological examination. All subjects were tested for serum $\alpha_1$-AT concentration and identification of the most common deficiency alleles (Pi Z and Pi S) of $\alpha_1$-AT gene was attempted.

The concentration of the serum $\alpha_1$-AT of patients was determined by the radial immunodiffusion using HUMAN Alpha 1-Antitrypsin NL BINDARIDTM Radial Immune Diffusion (RID) kit (Birmingham, UK), to which the surface of the agar added monospecific antibody for the $\alpha_1$-AT and control serum of human $\alpha_1$-AT[10].

The polymorphism resulting from the difference in a single nucleotide between two allelic forms Pi S and Pi Z was detected by using a highly specific and precise method of real-time polymerase chain reaction (PCR) that combines conventional PCR amplification and fluorimetry. The blood samples were taken into the anticoagulant tubes of ethylene diamine tetraacetic acid (EDTA), followed by permanent blood stains on the Flinders Technology Associates (FTA) cards, which were used for further analysis.

After surface disinfection with ultraviolet (UV) radiation and of fittings using 70% ethanol, isolation of nuclear deoxyribonucleic acid (DNA) from individual blood stain samples was performed using the Chelex-100 reagent, supplemented with Proteinase K, according to the proposed protocol[11, 12].

One μl of the diluted sample of isolated nuclear DNA was amplified by real-time PCR method by adding 12.5 μl TaqMan Universal PCR Master Mix (all 4 deoxyribonucleoside triphosphates, AmpliTaq Gold DNA polymerase, Mo$^{2+}$ ions buffer) (Applied Biosystems, Foster City, CA, USA), 1 μl SNP Genotyping Assay set of primer and probe specifically designed for the target sequence (SNP Genotyping Assay AAT E342K or AAT E264K) (Table 1) and deionized water, made according to the protocol recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA). During the detection of Pi Z-allele, the TaqMan assay for Pi Z-allele was flagged with the FAM signal molecule (6-carboxyfluorescein), and for Pi M-allele VIC-signal molecule (50-fluorescein), while during the detection of Pi S-allele, TaqMan test for Pi S-allele labeled with VIC, and for Pi M-allele FAM. The difference in signal intensity for each tested allele before and after amplification was used to determine the presence of normal and/or mutated alleles. The final PCR mixture volume was 25 μl and contained about 250 ng of genomic DNA.

After the preparation of the reaction mixture for the PCR, the sample plate was placed in the ABI Prism 7000 instrument Sequence Detection System (Applied Biosystems, Foster City, CA). The instrument was programmed using the software so that the initial heating of the mixture at 50°C lasted 2 minutes (activation of AmpliTaq Gold DNA polymerase), complete initial denaturation at 95°C before the first cycle lasted 10 minutes, followed by 45 cycles of PCR, each lasting for 1 minute and 15 seconds, denaturing at 95°C for 15 seconds, and hybridization/elongation at 60°C, for 60 seconds. The instrument read the fluorescence generated during the amplification. By measuring and comparing fluorescence signals, it is possible to determine the presence or absence of certain alleles in each test sample.

The collected data were entered into the computer database, and statistical data processing was performed using the statistical software JMP 7, SAS Institute, Cary, NC. A descriptive and comparative method was used to describe the general characteristics of the subjects as well as of the test results. The correlation between parameters and its presentation and interpretation of significance was done by using the linear correlation coefficient. Continuous variables are shown as mean values ± standard deviation. Student’s t-test was used to compare continuous variables with normal distribution. The probability value of \( p \leq 0.05 \) was considered significant.

**Results**

Out of the total number of (100) participants, 76 were males and 24 females. Graph 1 shows the gender distribution of patients with the diagnosis of pulmonary emphysema. Of the total number of patients with pulmonary emphysema, there were more men - 71 (79%), and almost four times fewer women - 19 (21%). The patients were aged from 32 to 83 years.

In regard to smoking, of the total number of patients with pulmonary emphysema, there were 15 women (17%) and 61 men (68%) who were smokers, of the average age of 65.6 (SD = 9.38), while 10 men (11%) and half of the women - 4 (4%) of the average age of 66 (SD = 13.75) years were non-smokers.

The control group included 10 respondents (5 males and 5 females), aged 34 – 51, without clinical signs of emphysema, but with a positive family history of COPD (mother or father suffered from bullous emphysema and had a $\alpha_1$-antitrypsin deficiency). In this group there were 2 smokers and 8 non-smokers.

The results of our study in the group of patients with pulmonary emphysema indicate that the average measured serum concentration of $\alpha_1$-AT in...
smokers was 1.66 g/l (0.49) and in non-smokers 1.80 g/l (0.43). There was no statistically significant difference in the average values of $\alpha_1$-AT between smokers and non-smokers ($p > 0.05$) (Graph 2).

Of the 10 examinees from the control group, in three subjects the serum level of $\alpha_1$-AT was 0.80, 0.81 and 0.93 g/l, and was at the lower limit of reference values. Five subjects had a serum $\alpha_1$-AT concentration below 2.0 g/l (the average serum concentration of $\alpha_1$-AT was 1.68 g/l). Only two subjects had the serum level of $\alpha_1$-AT above 2.0 g/l.

The results of the frequency of Pi $Z$ and Pi $S$ polymorphisms in 90 patients diagnosed with pulmonary emphysema, as well as in 10 subjects of the control group (Table 2).

Protease inhibitor MM genotype was present in all 90 (100%) patients with the diagnosis of pulmonary emphysema, while in the control group, 2 cases with mutated heterozygous PiMZ genotype were identified, as well as 8 cases with normal genotype PiMM. It is necessary to point out that, in addition to the heterozygous PiMZ genotype, both subjects had a serum deficiency of $\alpha_1$-AT. The serum level of $\alpha_1$-AT was 0.80 and 0.81 g/l, individually and was at the lower limit of the reference values. One of the heterozygous was also a perennial smoker.

**Discussion**

The only risk factor that comes from the host, i.e. a human, that is well known to be causally related with the development of COPD, is a genetically determined deficiency of $\alpha_1$-AT [13].

In all patients diagnosed with lung emphysema, the PiMM genotype $\alpha_1$-AT was present and the serum level of $\alpha_1$-AT was within the limits of the reference values, whereas the genetically-determined deficiency of $\alpha_1$-AT was not responsible for the development of COPD. PiMM genotype represents individuals who are homozygous with a normal Pi M allele and have a normal concentration of $\alpha_1$-AT in the plasma to provide adequate protection - antiprotease.

The results of our research confirmed the literature allegations regarding the correlation between the level of $\alpha_1$-AT in the serum and the corresponding $\alpha_1$-AT genotype. The level of serum $\alpha_1$-AT in the subjects with PiMM genotype of $\alpha_1$-AT was 1.66 g/l (for smokers) and 1.80 g/l (for non-smokers) and was within the limits of the reference values. Floyd et al. reported that in subjects with PiMM genotype $\alpha_1$-AT with a diagnosed COPD, the average measured values of $\alpha_1$-AT were 1.39 g/l and 1.27 g/l in PiZZ subjects [14]. The serum $\alpha_1$-AT in the subjects with PiMZ genotype was 0.80 and 0.81, individually and was at the lower limit of the reference values. According to the literature, individuals with PiMZ genotype are characterized by a serum concentration of $\alpha_1$-AT of 0.87 g/l (0.5 – 1.2 g/l) [15].

However, there are still controversial opinions about whether people with heterozygous $\alpha_1$-AT genotype (especially people with PiMZ genotype for $\alpha_1$-AT) have predispositions for the development of emphysema. Floyd et al. consider that heterozygous individuals with PiMZ, PiSZ, and PiMS genotype for $\alpha_1$-AT exhibit a tendency to develop and can develop lung emphysema, especially if they are smokers, but do not have a greater risk than the rest of the population. If heterozygotes develop a pulmonary disease, this is because there was an additional impact of another host or environmental factors [14].

According to the recommendations of the most accepted guidelines for the management of COPD, Global Initiative for Chronic Obstructive Pulmonary Disease, it is recommended that in individuals who suffer from COPD before the age of 40 (emphysema in young people) or have a positive family history, $\alpha_1$-AT should be tested. The values less than 20% of predicted indicate that it is a congenital absence of this enzyme [13, 16].

The study of genetic risk factors for the development of COPD with prevalence of lung emphysema is an important step in developing a strategy for the prevention and treatment of this disease. Genetic discrimination is necessary for the detection of homozygous and heterozygous and the lack of a certain (Pi S and Pi Z) alleles of $\alpha_1$-AT gene. Based on this concept, a strategy for the prevention and control of COPD will be developed, targeted at the healthy and individuals with an increased risk for COPD predominantly with lung emphysema (positive family history, the level of serum $\alpha_1$-AT in the lower range of reference values but without clinically developed COPD with prevalence of lung emphysema), as well as those already suffering from COPD who need effective treatment and adequate care. In individuals with an inborn $\alpha_1$-AT deficiency, non-specific preventive measures as well as therapeutic procedures, with specific compensation of this enzyme inhibitor, should be applied. The compensation is administered to purified human $\alpha_1$-AT, which is administered as an intravenous infusion of 60 mg/kg body weight, once weekly or once a month [16, 17].

The main objectives of the strategy are to significantly reduce morbidity and mortality of people with COPD and improve their quality of life. Prevention has the greatest potential to reduce the incidence of COPD. Prevention in the general population is a sustainable strategy in the long run. However, at the same time, it is necessary to implement prevention at the individual and population levels.

By using screening, it is possible to prevent or modify risk factors, prevent the onset or progress of the disease, prevent incompetence, reduce mortality and improve quality of life, provided that effective, affordable and acceptable therapy is available to all who need it. The outcome of the disease can be improved by its early detection, appropriate therapy and effective rehabilitation. Screening with follow-up therapy for people with an increased risk for COPD associated with several risk factors is more cost-effective than focusing on individual risk factors. Appropriate application of knowledge at all levels of health care has multiple benefits for all [13].
Conclusion

Protease inhibitor MM genotype alpha-1 antitrypsin was present in all patients diagnosed with lung emphysema; the serum level of alpha-1 antitrypsin was within the limits of the reference values; the genetically-determined alpha-1 antitrypsin deficiency was not responsible for the development of chronic obstructive pulmonary disease.

In the group of patients with the diagnosis of pulmonary emphysema, there was no statistically significant difference in the average values of alpha-1 antitrypsin between smokers and non-smokers.

The results of our research confirmed the literature results regarding the correlation between the serum level of alpha-1 antitrypsin and the corresponding alpha-1 antitrypsin genotype. Investigations of genetic defects, the alpha-1 antitrypsin deficiency in individuals with a family history of chronic obstructive pulmonary disease will allow the identification of patients with a genetic predisposition to the development of chronic obstructive pulmonary disease, and its prevention and treatment before the onset of symptoms.

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