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# Alpha-1-antitrypsin Phenotypes and Neutrophil Elastase Gene Promoter Polymorphisms in Lung Cancer

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**Abstract** Imbalance between neutrophil elastase and alpha-1-antitrypsin (AAT) leads to emphysema in smokers as well as in patients with inherited alpha-1-antitrypsin deficiency. AAT as a proven inhibitor of apoptosis may play role in lung cancer (LC) progression. The aim was to analyse AAT protein variants and polymorphism in promoter region of the neutrophil elastase gene (ELA2) in patients with primary lung cancer. AAT phenotypisation by isoelectric focusing method and ELA2 gene promoter characterization by DNA sequencing were performed in 66 patients with primary lung cancer. Results showed that the frequency of M1 allele and PiM1 homozygotes in LC patients was significantly higher when compared to the healthy subjects ($f$=0.6360 and 0.7424 respectively). The most frequent ELA2 promoter region genotypes in LC patients were −903TT and −741GG. There were significantly more patients with intermediate and high ELA2 genotype activity, compared to those with low activity (91% vs. 9%, respectively). In conclusion, we found that PiM1 homozygosity could be associated with the lung cancer, probably due to increased synthesis of this anti-apoptotic protein. Non-MM variants of AAT and ELA2 genotypes with predicted intermediate or high activity could also represent a risk factor for aggressive form of lung cancer associated with extrathoracic metastases.

**Keywords** Alpha-1-antitrypsin · Neutrophil elastase · Polymorphisms · Lung cancer

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## Introduction

Lung cancer (LC) is the most common cancer in terms of incidence and mortality with 1.35 million new cases per year and 1.18 million deaths. The highest rates of lung cancer were recorded in Europe and North America [1] and current efforts are directed towards detection of multiple genetic and epigenetic abnormalities that lead to this disease. Based on clinical and histological criteria, lung cancer is classified into two major subtypes: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).

The most important environmental risk factor for lung cancer development is tobacco smoking which causes 87% of lung cancer deaths [2], but the risk of lung cancer varies widely among smokers [3]. It has been shown that smoking is an important risk factor for chronic obstructive pulmonary disease (COPD) and LC by induction of inflammation and oxidative stress in the lung [4]. The oxidation of AAT by cigarette smoke could lead to a relative deficiency of elastase inhibitor and has been suggested as a mechanism contributing to the development of emphysema in non-deficient AAT individuals [5, 6]. In addition, the proin-

flammatory cytokines released in this milieu elevate epithelial apoptosis resistance [7].

Alpha-1-antitrypsin (AAT) or SERPINA1 is a highly polymorphic, acute-phase glycoprotein synthesised in hepatocytes and subsequently secreted into the plasma. It is also produced, in smaller quantities, by alveolar macrophages, circulating monocytes and lung-derived epithelial cells. AAT is the archetype of the serpins family (SER*ine* P*roteinase* In*hibitors*). It is encoded by the protease inhibitor (Pi) locus on chromosome 14q32.1. Hepatic AAT gene expression as acute phase protein is controlled by different cytokines, such as interleukin-1, tumour necrosis factor and most effectively by the interleukin-6 family. Its main target molecule is neutropil elastase and imbalance between these two counterparts could lead to lung tissue destruction. The variants of AAT are classified by the Pi (P*rotese* i*nhibitor*) system. According to the AAT serum level and function, all AAT variants are categorized as normal, deficient, null and dysfunctional variants. Normal variants of AAT have a normal serum level and functional activity to inhibit neutrophil elastase. The most common are M1 ($Ala^{213}$), M1 ($Val^{213}$), M2 and M3, and M4 is less frequent. Deficient variants are associated with alpha-1-antitrypsin deficiency (AATD) in plasma. Several deficient variants of AAT have been identified and the most common are Z and S. Individuals who are homozygous for AATD gene can develop liver or lung disease.

Recent studies showed that AAT could have antiapoptotic role in alveolar cells which represents a novel protective mechanism of AAT against emphysema [8]. However, the question arises whether inhibition of apoptosis in lung tissue by AAT, under certain conditions, becomes a pathological mechanism that leads to LC development.

Human neutrophil elastase (NE) is single chained glycoprotein with 218 amino acid residues which belongs to chymotrypsin family of serine proteases. The gene coding for neutrophil elastase, ELA2, is located on chromosome 19p13.3 [9]. In humans, ELA2 gene is expressed in bone marrow, during promyelocytic differentiation [10]. Neutrophil elastase mRNA can not be detected in mature polymorphonuclear neutrophils isolated from the systemic circulation. Mutations in ELA2 gene have been associated with severe congenital neutropenia and cyclic neutropenia [11, 12]. The active NE is stored within cytoplasmatic azurophilic granules of the neutrophil until extruded into phagolysosomes. It acts either intracellulary to degrade ingested host pathogens or extracellulary in the breakdown of extracellular matrix components at inflammatory sites. It has been shown that the immunoreactive-neutrophil elastase (ir-NE) produced by lung cancer cell might facilitate the invasion of cancer cell either by directly dissolving the tumour matrix or indirectly by activating a protease cascade [13].

Several polymorphisms at the promoter region of ELA2 gene were identified so far: −903(T/G), −741(G/A), −832 (G/T), −789(C/T) and extra 52 bp STS relative to the transcription initiation site. Polymorphisms −903(T/G) and −741(G/A) were associated with lung cancer risk [14]. Luciferase activity assays showed that ELA2 promoter constructs with −903T/−741G genotype had higher activity compared with the −903G/−741A construct. Based on this results, predicted activity of ELA2 genotypes are classified as: low ELA2 activity genotype (−903TG), intermediate ELA2 activity genotypes (−903TT/−741AG and −903TT/−741AA) and high ELA2 activity genotypes (−903TT/−741GG) [15].

According to the physiological roles of AAT and NE in lung tissue, it is reasonable to investigate the role of both AAT and NE in the etiology of lung cancer. Our intention was to study AAT variants at protein level and polymorphism in promoter region of the neutrophil elastase in LC patients.

## Materials and Methods

### Study Subjects

Investigation was performed on patients with primary lung cancer, admitted to the Institute of Lung Disease and TB, University Clinical Centre of Serbia, Belgrade and Zvezdara University Medical Center, Belgrade, Serbia. The protocol has been approved by the local research ethic committees and informed consent was obtained from all participants of the study.

The study included 66 patients, diagnosed with primary lung cancer within one year prior to the inclusion in the study. Patients were histologically classified as patients with small cell lung cancer (SCLC, $n=18$) and patients with non small cell lung cancer (NSCLC, $n=48$). For each patient the following information were collected: smoking status and tobacco use, personal and familiar history of COPD, familiar history of LC, and long-term exposure to air pollution (smog, car exhaust gases, dust in coal mine, metal dust pollution). Tobacco use was categorized into pack/years (one pack/year equals one pack of cigarettes per day for one year. Control group for investigation of AAT phenotypes was healthy blood donors from our previous study [16].

### Methods

Blood samples were taken from all patients. Sera were separated by centrifugation and stored at −80°C until analyzed. Genomic DNA was isolated using QIAamp DNA Mini Kit (Quiagen).

**Table 1** Selected characteristics of investigated LC patients (data expressed in %, except age)

| | All ($n$=66) | Men ($n$=54 ) | Women ($n$=12) | P* |
|---|---|---|---|---|
| Age (mean±SD) | 58.67±7.84 | 57.96±7.81 | 61.83±7.48 | 0.174 |
| Histology type of LC: | | | | 0.100 |
| NSCC | 72 | 70 | 92 | |
| SCC | 28 | 30 | 8 | |
| Smokers | 97 | 100 | 83 | 0.031 |
| Personal history of COPD | 13 | 17 | 0 | 0.128 |
| Air pollution | 40 | 44 | 17 | 0.050 |
| Familiar history of LC | 12 | 11 | 18 | 0.296 |
| Familiar history of COPD | 3 | 4 | 0 | 0.667 |
| Extrathoracic metastases | 43 | 45 | 33 | 0.203 |

P*: statistical difference between men and women

Pi phenotype was determined using isoelectric focusing (pH range 4.2–4.9) method described by Kishimoto et al. [17].

The ELA2 promoter region polymorphisms were analyzed by PCR-direct DNA sequencing, using the ABI Prism BigDye Terminator Kit (Applied Biosystems) with following primers: 5′ CGCAGTGAGTGCCCGACAC 3′ and 5′ CTGCCAAACCTAGACCTGA 3′.

### Statistical Analysis

The $\chi^2$ was used to assess whether control group and patients group were in Hardy-Weinberg equilibrium for AAT gene. Investigation of differences in the frequencies of AAT phenotypes and alleles between patients and controls was performed using the $\chi^2$ test (2×2 contingency table). Fisher exact test was used when $n$<5. $P$ values of <0.05 were considered as significant. For statistical analysis, STATISTICA 6.0® software was used.

## Results

A total of 66 patients with primary lung cancer were included in this study. There were 54 men and 12 women, with mean age 58.67±7.84. Small percentage of LC patients had personal or family history of COPD (3%), and family history of lung cancer (12%). Approximately half of all patients (43%) with primary LC had extrathoracic metastases (liver, spleen, pancreas, bone, brain). Selected characteristics of study subjects are given in Table 1.

The relative frequencies of AAT phenotypes and alleles in LC patients are presented in Table 2. These data were compared with control group from our previous study. No deviation from Hardy-Weinberg equilibrium were detected in either study groups ($\chi^2$=4.71; d.f.11; $p$=0.97 for control, and $\chi^2$=6.00, d.f.14, $p$=0.97 for LC patients). When differences in the distribution of the allele frequencies and Pi phenotypes between patients and controls were com-

**Table 2** Alpha-1-antitrypsin phenotypes and alleles in 66 LC patients

| Phenotype | Obtained relative frequency | Allele | Obtained relative frequency |
|---|---|---|---|
| M1 | 0.5606 | M1 | 0.7424 |
| M2 | 0.0303 | M2 | 0.1288 |
| M3 | 0.0000 | M3 | 0.0985 |
| M1M2 | 0.1667 | Z | 0.0152 |
| M1M3 | 0.1515 | S | 0.0076 |
| M2M3 | 0.0303 | P | 0.0076 |
| M1Z | 0.0152 | | |
| M2Z | 0.0000 | | |
| M3Z | 0.0152 | | |
| M1S | 0.0152 | | |
| M2S | 0.0000 | | |
| M3S | 0.0000 | | |
| M1P | 0.0152 | | |
| M2P | 0.0000 | | |
| M3P | 0.0000 | | |

Hardy-Weinberg equilibrium: $\chi^2$ =6.00, d.f.14, $p$=0.97

**Table 3** Frequencies of ELA2 gene alleles, genotypes and predicted ELA2 genotype activity in LC patients

| | Observed | |
|---|---|---|
| | Number | Frequency |
| ElA2 alleles | | |
| −903 | | |
| G | 6 | 0.0454 |
| T | 126 | 0.9545 |
| −741 | | |
| A | 35 | 0.2651 |
| G | 97 | 0.7348 |
| ELA2 genotypes | | |
| −903 | | |
| G/G | 0 | 0.0000 |
| T/G | 6 | 0.0900 |
| T/T | 60 | 0.9100 |
| −741 | | |
| G/G | 34 | 0.5151 |
| G/A | 29 | 0.4394 |
| A/A | 3 | 0.0454 |
| Predicted ELA2 genotype activity | | |
| Low | 6 | 0.0909 |
| Intermediate | 29 | 0.4394 |
| High | 31 | 0.4697 |

pared, M1 allele was found to be more frequent in patients than in control group ($f$=0.6360 and 0.7424 respectively, $p$=0.021). In addition, homozygotes for PiM1 were found to be significantly more frequent in LC patients than in the controls ($f$=0.5606 and $f$=0.4118 respectively, $p$=0.029). Allele M2 was less frequent in patients than in control group ($f$=0.1288 and 0.2059 respectively, $p$=0.043).

Only four out of 66 patients carried non-MM AAT variants (PiM1Z, PiM3Z, PiM1S and PiM1P). Comparison of characteristics between LC patients with MM variants and non-MM variants reviled that non-MM phenotypes were associated with extrathoracic metastases, and showed tendency for the presence of familiar history of LC. Non-MM individuals did not have personal or familiar history of COPD, as well as exposure to air pollution.

DNA sequencing of ELA2 promoter region revealed the presence of two polymorphisms: −903T/G and −741A/G. The most frequent genotypes in the patient group were −903TT and −741GG ($f$=0.9100 and $f$=0.5151, respectively). Genotypes associated with predicted intermediate and high levels of expression were present in 91% of lung cancer patients (Table 3).

All patients with non-MM phenotypes had ELA2 genotype with predicted high activity (Fig. 1a). On the other hand, in MM patients, genotypes with all three levels of predicted activity were present. The distribution of genotype activities in PiM subtypes showed that low activity genotype was associated only with the PiM1 subtype (Fig. 1b).

## Discussion

Multiple environmental and genetic factors may influence individual susceptibility to lung cancer. Lung tissue damage which could create a favorable host environment for carcinogenesis is generally thought to be caused by imbalance between AAT and neutrophil elastase. Beside the fact that decreased level of AAT and increased levels of NE can be genetically predetermined, balance between protease inhibitor and protease can be further disturbed by tobacco smoke which acts by inactivating AAT by oxidation and stimulating neutrophils to secrete more neutrophil elastase. Given all this as well as the newly discovered antiapoptotic role of AAT, it is reasonable to assume that this protein alone or in interplay with NE could play a part in lung cancer development.

Analysis of AAT phenotypes in patients included in our study showed that only 6% of them were carriers of deficient AAT alleles. Similar results were obtained in our previous case-control study [18], where no association was found between AATD and lung cancer, with exception of moderate AATD deficiency (PiMZ and PiMS) and squamous cell lung cancer. In a large USA case-control study,

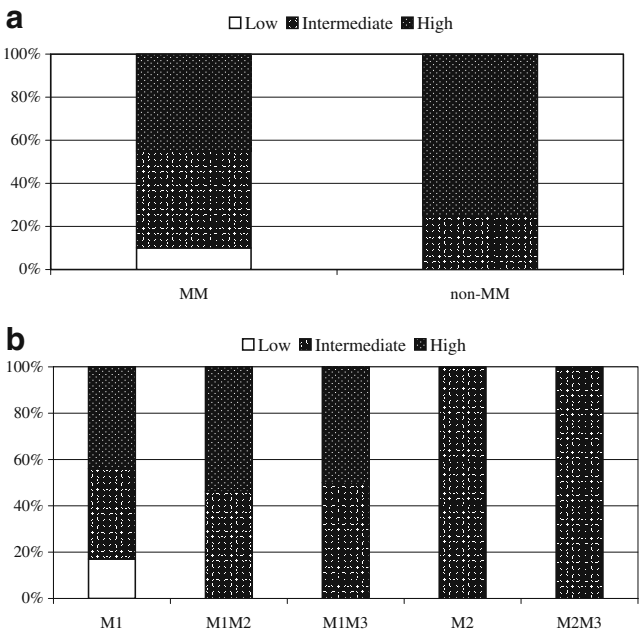


**Fig. 1** Percentage of ELA2 genotypes with predicted low, intermediate and high activity in LC patients with different phenotypes of AAT: **a**. MM and non-MM phenotypes; **b**. M-subtypes

Yang at al [19] observed higher AATD allele carriers in squamous cell or bronchoalveolar carcinoma LC patients than in healthy USA Caucasians. Most of the patients included in their study had personal history of COPD, as well as a family history of LC, while in our study, only 3% of patients had personal history of COPD, and 12% had family history of LC. We suppose that these differences among the LC patients in these two studies are responsible for the lack of association between AATD and lung cancer in our study.

In comparison to the group of healthy blood donors significantly higher frequency of PiM1 homozygotes and M1 allele was observed in the group of LC patients. PiM1 variant is considered as a normal AAT variant, and not related to any disease so far. Recent studies described new biological roles of AAT, independent of their antielastase role, in the lower respiratory tract [20–23]. Discovery of AAT inhibitory role in apoptosis in alveolar endothelial cells (due to the inhibition of caspase-3, the main component of execution pathway of apoptosis) is particularly important [24], and it can represent a novel mechanism by which this protein protects the lungs from emphysema. However, the question arises whether inhibition of apoptosis in lung tissue by AAT, under certain conditions, becomes a pathological mechanism that facilitates LC progression. Our assumption is that PiM1, which is normal variant, could be associated with LC progression due to its antiapoptotic role. Moreover, elevated serum level of AAT as acute phase protein in malignancy may inhibit apoptosis under certain conditions, and facilitate the spread of malignant cells. In lung cancer pathogenesis, antiapoptotic effect of AAT could be enhanced by nicotine from tobacco smoke that plays a role in the inhibition of apoptosis [25].

The most frequent ELA2 genotypes in our LC patients were −903TT and −741GG ($f$=0.9100 and $f$=0.5151, respectively). This data are in concordance with data from the studies by Taniguchi et al and Park et al [14, 15]. When our LC patients were categorized based on the predicted activity of NE, there were significantly more patients with intermediate and high NE activity, compared to those with low activity (91% vs. 9%, respectively). The distribution of patients with predicted intermediate and high activity was almost equal (43% vs. 47%, respectively). This data suggest that intermediate and high NE activity could play a role in LC, which is in concordance with results of Yamashita and co-workers who showed that elevated levels of NE facilitate the invasion of cancer cell [26].

In or group of patients all carriers of non-MM alleles (PiMZ, PiMS and PiMP) dominantly had NE genotypes with predicted high activity. Given that all non-MM patients had extrathoracic metastases we can speculate that the inadequate inhibition of NE in the case of moderate alpha-1-antitrypsin deficiency facilitated the spread of malignant cells. Our data correlate with study by Yang et al [27] that revealed that the AATD genotypes and/or an excess of neutrophil elastase were significantly associated with lung cancer risk.

Largest percentage of patients who were PiM1 homozygotes had intermediate and high NE activity (39% and 44%, respectively). Also, ELA2 genotypes with predicted low activity were detected only in the PiM1 homozygotes (17%). We presume that under certain circumstances, independently of NE activity, M1 phenotype represents a risk factor for the occurrence of lung cancer. It is possible that the M1 phenotype, unlike the other AAT phenotypes manifested significant antiapoptotic effect that under certain conditions could trigger carcinogenic process.

In conclusion, our study for the first time showed that PiM1 homozygosity, probably independently of NE activity could represent a risk factor for lung cancer. Furthermore, cumulative effect of non-MM variants of AAT, and intermediate or high activity of NE could also represent a risk factor for aggressive form of primary lung cancer associated with extrathoracic metastases. It is obvious that relationship between alpha-1-antytrypsin and neutrophil elastase in lung cancer predisposition is not simple disturbance of protease-antiprotease balance. Further studies on larger number of patients are required in order to clarify the mechanism and role of AAT and /or NE in lung cancer.

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