Determination of alpha 1-antitrypsin phenotypes and genotypes in Iranian patients

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Abstract

Alpha 1-antitrypsin (AAT) or alpha 1-protease inhibitor (PI) is the principal inhibitor of proteolytic enzyme in serum. Its phenotypic variability has been reported to be associated with liver, lung diseases and rheumatoid arthritis in humans. There is much documentation about high risk phenotypes of PI in some regions of the world, however, there are no reliable reports on these phenotypes and genotypes and their related diseases in Iranian population. The aim of this study was to determine PI phenotypes and genotypes in Iranian patients suffering from PI deficiency. For this purpose, whole blood samples from 307 patients suspected of diseases related to PI deficiency, and 156 healthy persons were examined. PI phenotypes and genotypes were determined by isoelectric focusing (IEF) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), respectively. Allele frequencies from patients and normal subjects were compared. For reliability, a family study of the patients was also carried out. The PI phenotype frequencies of all six possible combinations of M, S and Z haplotypes in patients were: MM, 77.20%; MS, 6.18%; MZ, 7.17%; SS, 3.91%; ZZ, 4.56%; SZ, 0.98% and in normal subjects were: MM, 78.20%; MS, 5.76%; MZ, 15.38%; SS, 0.64%; 0% for ZZ and SZ. Analysis of data showed that there was a significant difference between patients (with liver, lung diseases and rheumatoid arthritis) and control subjects ($p< 0.05$). In Conclusion, the allelic frequencies of S and Z in the patient group were 7.49% and 8.63%, while in the normal subjects were 5.13% and 4.17%, respectively. This is the first report of the prevalence of high risk alleles (Z and S) in patients suspected of PI deficiency and related diseases in Iran.

Keywords: *Alpha 1-antitrypsin deficiency; Phenotypes; Genotypes; IEF; PCR-RFLP*

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INTRODUCTION

Alpha 1-antitrypsin (AAT), a glycoprotein with a molecular weight of 52-kDa, is a serine protease inhibitor (PI) which is found in the highest concentration in plasma and comprises 90% of the total alpha-1-globulin in plasma. The major function of AAT is to inhibit the activity of elastase generated by neutrophils in the lung (Carrell *et al*., 1982; Morse *et al.*, 1977). The main effect of PI deficiency is destruction of the pulmonary alveoli resulting in chronic pulmonary disease or emphysema (Brantly *et al*., 1988; Crystal, 1990; Lomas *et al*., 2004). The connection between deficiency and liver diseases in children was first described in 1969 (Sharp *et al*., 1969).

Clinical studies have shown that occurrence of liver diseases in individuals with PI deficiency is bimodal, affecting children during neonatal life or early infancy and less commonly adults during late middle life (Perlmutter, 1991; Berg *et al*., 1972; Sveger, 1976). The PI gene is highly polymorphic with more than 90 different alleles described in different populations (Crystal, 1989; DeMeo *et al*., 2004). The PI phenotype represents the codominant expression of the two parental haplotypes. The most common PI haplotypes in white populations is the M type (PiM) with an allele frequency of 0.95 and normal PI levels, S type (frequency, 0.02-0.03) with a mild reduction in PI level and Z types (frequency, 0.01-0.03) with a severe reduction in PI level (Blanco *et al*., 2001; Luisetti *et al*., 2004). Two mutant alleles, S and Z, are responsible for most of the diseases associated with PI deficiency. A large majority of individuals with severe PI deficiency have the ZZ genotype (PiZZ). PiZZ is associated with 10-15% of normal PI activity and confined

Table 1. Selected Primers for PCR.

Mutation	Position	Primer sequence (Genbank, accession No: K02212)
S (Forward)	P9504	5'-CGTTTAGGCATGAATAACTTCCAGC-3'
S (Reverse)	P9629	5'-GATGATATCGTGGGTGAGAACATTT-3'
Z (Forward)	P11916	5'-ATAAGGCTGTGCTGACCATCGTC-3'
Z (Reverse)	P12014	5'-GAACTTGACCTCGAGGGGGGATAGAC-3'

Mutated bases in the primers are underlined.

predominantly to Caucasians, but rarely found in African-Americans or East-Asians (Blanco and Bustillo, 2001). Homozygous PiSS reduces PI activity by 50%-60%. However, heterozygous PiSZ individuals display 30-35% of normal PI activity. The type of diseases associated with heterozygous PiSZ is similar to that associated with the ZZ homozygote state (Fischer *et al*., 2000). The classic approach for the detection of the PiS and PiZ genes is at the protein level through a combination involving the determination of isoelectric point (pI) of serum proteins using the isoelectric focusing (IEF) technique, serum PI levels and eventually family studies. In this study, all possible PI phenotypes were initially identified by IEF. Furthermore, ambiguous results were investigated by PCR-RFLP.

MATERIALS AND METHODS

Patients and blood sampling: In this study, 307 patients with diseases related to PI deficiency (138 men and 169 women), admitted to three hospitals in Tehran, were randomly collected. The patients were divided as follows: 105 cases of individuals with PI deficiency were suffering from lung diseases, 87 cases from liver diseases, 70 cases from rheumatoid arthritis, 38 cases from glomeronephritis and 7 cases from cutaneous diseases. As a control group, 156 healthy adults (89 men and 67 women) who lived in the hostels of the universities were also included. Mean age of patients was 22.15 ± 17.19 and it was 30.8 ± 8.6 years for healthy persons. About 42.7% of patients and 12.6% of the control group were cigarette smokers. 10 ml of whole blood was collected from each individual divided to tubes. Samples containing anticoagulant were used for DNA extraction and sample without anticoagulant was used for IEF. An informed consent was taken from each individual before blood collection.

Isoelectric focusing (IEF): IEF was performed at 4°C on 10 µl of each serum sample using flat bed polyacrylamide gels in a pH gradient of 4.2-4.9, with a power supply of 3000 voltage (Phast gel dry IEF; Pharmacia, Uppsala, Sweden). The major variants of PI were placed between pH 4.5 and 4.7. After staining, bands were compared with the control samples corresponding to all six possible phenotypes (Jeppsson *et al*., 1982).

PCR amplification and restriction enzyme digestion: Genomic DNA was isolated from whole blood according to the standard method (Maniatis *et al*., 1989) and was preserved in sterile distilled water at 4°C until the PCR assay.

For PI genotyping, PCR-mediated mutagenesis was performed as described previously (Andresen *et al*., 1992). Briefly, Target DNA (0.2 µg) from the PI genome was amplified by PCR using two sets of mutated primers, Genbank K-02212 (MWG Biotech, Germany), (PCR kit was from Fermentas UAB, Lithuania), in a DNA thermal cycler (Corbett Research Company, Australia). The sequence of primers used for PiS and PiZ typing were as shown in Table 1. This table shows that mutated bases in the primer sequences as underlined. The fragment was amplified in a reaction volume of 25 µl containing 10 pm of primers, 200 µm dNTPs of 1U of *Taq* polymerase. Each reaction mixture was subjected to 35 cycles of 1 min at 95°C, 1 min at 50°C and 2 min at 72°C, followed by initial denaturation for 5 min at 95°C, and final extension for 9 min at 72°C. PCR products (148 bp for the S-type and 97 bp for the Z-type) were checked by electrophoresis on a 3% agarose gel. In order to test for the S and Z allele mutations, PCR products were digested by *Xmn*I and *Taq*I as previously described (Andresen *et al*., 1992). Upon digestion, the cleaved products were submitted to electrophoresis on 20% polyacrylamide gel and visualized after being stained by ethidium bromide. On the basis of the unique bands of the

Table 2. PCR products of S and Z alleles cleaved by restriction enzymes, *Xmn* I and *Taq* I respectively.

	Z-typing		S-typing	
PI Genotyping	Restriction fragments produced by *Taq* I digestion (bp)	Fragment before digestion (bp)	Restriction fragments produced *by Xmn* I digestion (bp)	Fragment before digestion (bp)
PiMM	64	97	111 (M)	149
PiMZ	64 (M)& 86 (Z)	97	111 (M)	149
PiZZ	86	97	-	97
PiMS	64 (M)	97	111 (M) & 133 (S)	149
PiSS	-	149	133	149
PiSZ	86 (Z)	97	133 (S)	149

Each PI genotype can be distinguished by unique combinations of restriction fragments obtained by S-typing and Z-typing.
(-): The band was not digested.

restriction fragments, six possible different genotypes; PiMM, MS, MZ, SS, ZZ, and SZ, were distinguished (Table 2).

Statistical analysis: The statistical analyses of the results were evaluated by Chi-Square with Fisher's Exact test for estimating ODD ratio, $P < 0.05$ was considered to be significant.

RESULTS

After carrying out IEF and PCR-RFLP, six different phenotypes were identified (Fig. 1 and 2). The first phenotype was homozygous for the M alleles (MM). The second and third were homozygous for PI deficient alleles (ZZ or SS). The fourth and fifth phenotypes were heterozygous for the M allele and a PI deficient allele (MZ or MS), and finally the sixth one was heterozygous for the two PI deficient alleles (ZS).

According to our results, the PI phenotype frequencies in patients were: MM, 77.20%; MS, 6.18%; MZ, 7.17%; SS, 3.91%; ZZ, 4.56% and SZ, 0.98% while in normal individuals the frequencies were: MM, 78.20%; MS, 5.76%; MZ, 15.38%; SS, 0.64% and 0% for ZZ and SZ (Table 3).

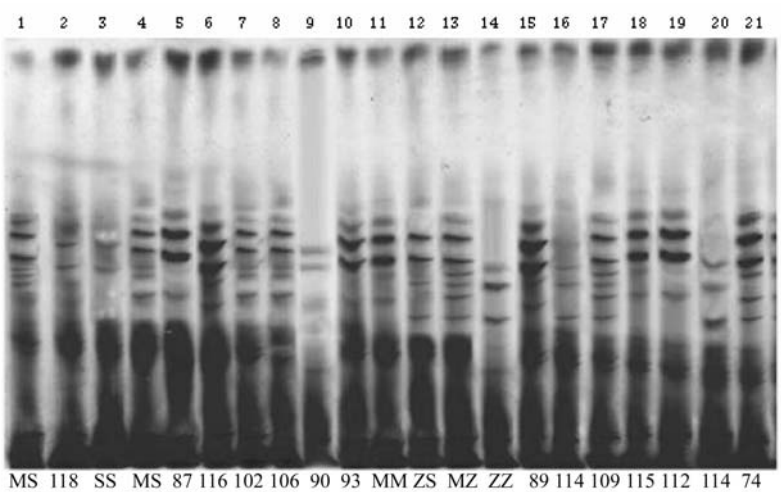

Figure 1. PI typing of the samples by IEF in a narrow pH gradient 4.2-4.9. Electrophoretic pattern of the serum controls of PiMM(Lane 11), MS(Lane 1 and 4), MZ(Lane 13), SS(Lane 3), ZZ(Lane 14) and ZS(Lane 12) prepared from the reference laboratory of Prof. Cox, from Canada. Other numbers represent different patients with PI deficiencies as follows: Lane 2: Woman from Eastern Azarbuyjan of Iran, 33 years old, with rheumatoid arthritis (RA). Lane 5: Woman from booshehr, 31 years old, with (RA). Lane 6: Man from Kohkiloyeh & Boyerahmad, 37 years old, with renal disease. Lane 7: Boy from Tehran, 7 years old, with liver disease. Lane 8: Girl from Haraat of Afghanistan, 5 years old, with liver disease. Lane 9: Boy from Zanjan, 5 years old, with liver disease. Lane 10: Woman from Kerman, 40 years old, with renal disease. Lane 15: Boy from Kurdestan, 8 years old, with liver disease. Lane 16 and 20: Woman from Kurdestan, 24 years old, with liver disease. Lane 17: Girl from Eastern Azarbuyjan, 6 years old, with liver disease. Lane 18: Man from Golestan, 21 years old, as the normal subject. Lane 19: Woman from western Azarbayjan of Iran, 13 years old, with lung disease. Lane 21: man from Guilan, 50 years old, with lung disease.

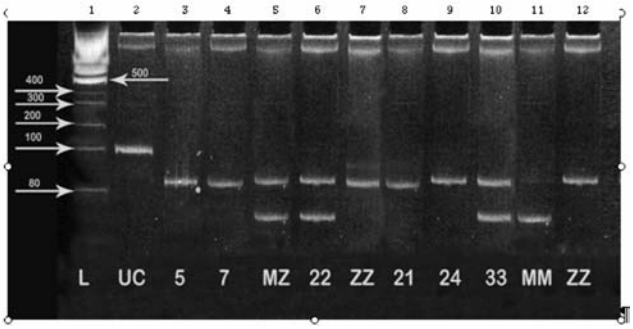

Figure 2. Z-typing of the samples. Ethidium bromide stained acrylamide gel showing the results of PI typing by RFLP-PCR using *Taq* I digested PCR fragments. L(Lane 1): Ladder DNA marker, UC(Lane 2): uncut PCR fragment, MZ(Lane 5) and ZZ(Lanes 7 and 12) and MM(Lane 11) are the standard controls of PI genotypes. Other numbers represent different patients with PI deficiency.

The allelic frequencies in the patients were: M, 83.88%; S, 7.49% and Z and 8.63%, and in the healthy controls these were: M, 90.70%; S, 5.13% and Z, 4.17% (Table 4). The results were confirmed by family studies showing that some cases of AAT related diseases were seen in their relatives.

The frequencies of the SZ and ZZ as "at risk" genotypes of PI (Nukiwa *et al*., 1986) in liver, lung and rheumatoid patients were also considered (Table 5). For evaluation of the relationship between frequency of genotype and the type of disease, and to find the occurrence of causal genotypes in disease, ODD ratio (element of statistical regression analysis) was evaluated. This evaluation revealed that ZZ genotype could be a risk factor in liver disease (ODD RATIO: 4.962 with 95% confidence internal =1.613-15.260 and P =0.002).

DISCUSSION

There are two important reports regarding PI frequency in Iran, one focused on the normal population and second on inflammatory eye diseases. The first one (Walter *et al*., 1992) indicated that some rare alleles (S and Z) were found in 4 Iranian populations. The second one (Ghavami *et al*., 2005) indicated that some subtypes of PI (MS or MZ) were higher in patients than in normal groups. Both of these studies probably analyzed their samples just by IEF (not mentioned in the first report) without genotyping. IEF is an important technique used by researchers to determine of PI phenotypes, but in some cases it is difficult to classify the IEF patterns, so genotyping can help to confirm the correct phenotypes as has been the case in our study, where both methods were used. Among all six possible phenotypes (MM, MS, MZ, SS, ZZ, and SZ), two of them (ZZ, and SZ) are "at risk" and others are "not at risk" phenotypes. The other major goal of this study

Table 3. Frequency of AAT phenotypes in normal and patients.

Group / PI	(Frequency%)	
	Normal	Patients
	Number (Frequency%)	Number (Frequency%)
MM	132 (84.62)	237(77.2)
MZ	11(7.05)	22(7.17)
ZZ	1(0.64)	14(4.56)
SZ	0(0.0)	3(0.98)
SS	4(2.56)	12(3.91)
MS	8(5.13)	19(6.18)
Total	156(100)	307(100)

Table 4. Frequency of AAT alleles in normal and patients.

Group / Allele	Normal (%)	Patients (%)
M	90.70	83.88
Z	4.17	8.63
S	5.13	7.49

Table 5. Frequency of "at risk" phenotypes in the patients.

Phenotypes \ Patients	Liver Number/Total	Lung	R. Arthritis	Total
SZ	66.6% (2.87)	33.3% (1.106)	0% (0.70)	3
ZZ	69.2% (9. 87)	15.4% (2.106)	15.4% (2.70)	13

was to identify the above mentioned phenotypes. For this purpose, we collected 326 samples from the patients and after performing IEF, the ambiguous samples (19 cases) which didn't fit to the electrophoretic profiles of M, S and Z phenotypes, were excluded from this study.

There are also several reports on the of PI in other countries. For instance the frequency of Z allele in the white American population and some north European countries like Denmark and Netherlands are 1.4, 2.3 and 1.3%, respectively. However some authors state that the frequency of S allele in Spain and Portugal is 15%. Some reports show that S and Z alleles in the black races as well as in the East Asian races are rare or absent. They also show that the Z-allele frequency is zero in Japan, while it is 0.6% in India. Z and S alleles frequencies in Siberia are zero and 0.2%, while in the central Asia, they are 0.8% and 0.4%, respectively. The frequency of PiZ is highest in the Caucasian and is approximately 10 % (Luisetti *et al*., 2004; Blanco *et al*., 2001; World Health Organization, 1996 and 1997). It seems that Caucasians can be considered as the center of Z allele occurrence in Asia. In this study, the frequency of S and Z-allele for normal subjects and patients was found to be 5.13%, 7.5% and 4.17%, 8.63%, respectively. High frequencies of S and Z-alleles in the Iranian population could be due to some factors like immigration of Caucasians to Iran and mixing with other racial groups. In fact, according to the history of Iran, 120 years ago Caucasian region was a part of Iran. A previous study published as a WHO report (1996), showed that PiZ frequency in the Eastern Mediterranean region ranged from 0% in Jordan to 2.2% in Saudi Arabia and Iran while, PiS frequency ranged from zero to 5.15% in the aforementioned regions. According to our results, it seems that the WHO report correlates more with Saudi Arabia than Iran. In spite of equal values of PiS, the frequency of PiZ in the normal Iranian population is significantly higher than the above mentioned report. The relationship between the so-called at risk phenotypes of PI and the kind of disease also indicate that only PiZ phenotype could be considered as a risk factor for liver disease. In conclusion, this study is the first report to show the frequencies of high risk alleles (Z and S) in Iranian patients suspected of PI deficiency, using powerful techniques such as IEF and PCR-RFLP.

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