Isoelectric Focusing and PCR-RFLP Joined Techniques for Alpha1-antitrypsin Deficiency Detection

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Summary

53 persons suspected to alpha1-antitrypsin deficiency detection (AATD) were investigated for ZZ, MZ, ZS, SS, and MS alleles analysis by serum protein electrophoresis (SPE), measurement of trypsin inhibiting capacity (TIC), isoelectric focusing (IEF), polymerase chain reaction (PCR), and IEF/PCR-RFLP techniques. The result clearly shows by using SPE and TIC techniques only 35.85 % and 50.08% of AATD cases can detect respectively while, IEF/PCR-RFLP joined technique can detect 100% of AATD cases. However, since IEF/PCR-RFLP joined technique can determine the protein structure and alleles gene mutation together, they can detect up to 100% of AAT. Hence for detection of AATD the IEF/PCR-RFLP technique is recommended.

Key words: isoelectric focusing, α1-antitrypsin, α1-antitrypsin deficiency, alleles, PCR-RFLP

Introduction

Alpha1-antitrypsin (AAT), also known as α1 -protease inhibitor is a serine protease inhibitor. It serves as the major inhibitor of neutrophile elastase, a powerful

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proteolytic enzyme stored in neutrophile leukocytes. AAT is a glycoprotein and synthesized predominantly in liver, from where it is transferred to plasma (Cox 1995, WHO 1996). The structural gene locus encoding AAT is called Pi (proteinase inhibitor) and is located on chromosome 14q32.1. AAT is coded by a single gene. This gene was composed of five exons and four introns. In this regard demonstrated that all of the information coding for AAT protein were contained in exon II to V, whereas exon I and a small 5' portion of exon II coded for a non-translated region of 5'mRNA to the translation start codon ATG (Brantly 1988). α1-antitrypsin is a polymorphic protein and over 90 genetic variants have been identified in the Pi gene, the most common of which is the Z mutation resulting in the substitution of lysine for glutamate at position 342 (Glu342Lys). In approximately 15% of homozygous patients (Z/Z), the abnormal protein is not secreted from the liver, thus predisposing to cirrhosis, obstructive pulmonary disease, and emphysema. The S variant, resulting in substitution of valine for glutamate at position 264 (Glu264Val), is a less common mutation. Homozygosity and heterozygosity for the S mutation have no phenotypic effect, however, a compound heterozygote for the Z and S mutations may be symptomatic (Brantly 1988, WHO 1996). Neither severe (homozygous Pi ZZ) nor partial AATD (heterozygous Pi MZ) can be reliably detected by measuring serum AAT concentrations, since both phenotypes can be associated with normal serum AAT levels under stimulatory conditions such as inflammation, estrogens, major surgery, malignancy, and chronic liver disease (Cox 1995). Laboratory diagnosis based on biochemical and molecular methods is suggested (Brantly 1988, Carrell 1982, Pierce 1988).

In this study we were attempted to establish the joined technique of IEF and PCR-RFLP for detection of AATD and confirmed it by genotyping of patients suspected to the deficiency.

Materials and Methods

Sample preparation and tests. In the study, 53 patients suspected to AATD were selected. Blood samples were collected in two tubes. One tube with anticoagulent for DNA extraction and the other tube without anticoagulent for serum protein electrophoresis (SPE), measurement of trypsin inhibiting capacity (TIC) and isoelectric focusing (IEF) tests. SPE was performed according to Bishop *et al* method (1992). TIC test was performed according to Dietz *et al* (1974). The antitryptic proteins of serum inhibit the hydrolysis of α-N-benzoyl-DL-arginine-ρ-nitroanilide (BAPNA) by trypsin in Tris buffer. The reaction was stopped by adding acetic acid and the absorbance was then read at 405nm. For IEF the sample was reduced and alkylated with the following modifications. 20µl of serum was mixed with 40µl of 40mM Dithiotheitol and incubated at room temperature for 30min.The reduced serum was then alkylated by mixing with 60µl of 20mM Iodoacetamide. The sample was incubated at room temperature for a further 1h before focusing. Polyacrylamide gel was made according to the Qureshi method (1982). IEF was performed at 4˚C using a Pharmasia system and a 3000 power supply. 0.1M glumatic acid and 1M NaOH were considered as the anolyte and catholyte, respectively. Prefocusing, sample application and sample separation steps were accordance with table 1.

Table 1. *Isoelectric focusing program for α-antitrypsin*

Step	Potential (V)	Current (mA)	Power (W)	Temp. (˚C)	Duration (V.h)
Perfocusing	2000	4	8	15	750
Sample application	750	8	14	15	750
Sample separation	3000	16	14	4	2200

DNA preparation and PCR assay. Genomic DNA was isolated from blood samples according to method of Cox (1980) and was preserved in sterile distillated water at 4˚C until use. PCR was used to amplify the S and Z mutations fragments on the genomic DNA. Primers P7553 (5'-CGT TTA GGC ATG AAT AAC TTC CAG C-3') and P7720 (5'-GAT GAT ATC GTG GGT GAG AAC ATT T-3') were used to amplify a 149-bp fragments from S mutation. Primers P9966 (5'-ATA AGG CTG TGC TGA CCA TCG T-3') and P10063 (5'-GAA CTT GAC CTC GAG GGG GAT AGA C-3') were used to amplify a 133-bp fragments from Z mutation. All PCR amplifications were performed with 10X reaction buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$, pH8.3) in a 25µl total volume containing DNA template (1µg), oligonucleotide primers (30pmol), dNTPs (20nmol) and 4U of *Taq* DNA polymerase. The reaction mixture was heated for 5min at 95˚C for initial denaturation. 35 PCR cycles were performed with 1min denaturation at 95˚C, 1min annealing at 50˚C, and 2min extention at 74˚C. A blank amplification containing all reagents without sample DNA was included in the experiments to check the presence of contaminating DNA from cloned or previously amplified DNA.

Restriction enzyme digestion. Before the restriction enzyme digestion, the performance of the PCR procedure including the blank amplification was tested by subjecting sample of the PCR products to electrophoresis in a 3% agarose gel (Figure 5). 15µl of samples were digested overnight in 5µl of α1-casein (1g/L) and 10U of the respective restriction enzyme. After 15h, 10U more of enzyme was added and the digestion continued for 2-3h. For digestion of PCR product with *Xmn*I and *Taq*I, the buffers recommended by the supplier were used. Both digested and undigested samples were electrophoresed in 20% ethidiume bromide stained polyacrylamide gel.

Results

A SPE gel pattern is shown in figure 1. In pH8.6, five protein fractions are detected. Albumin is at the anodal (+) end and followed by α1-, α2-, β-, and γ-globulin fractions. AAT is a major component (about 90%) of the α1-globulin therefore, increase or decrease αı-globulin indicate the increase or decrease of AAT. Also, two selected densitometry patterns of SPE are shown in figure 2. The SPE densitometry study indicated that 19 (35.85%), 31 (58.49%) and 3 (5.66%) persons are diagnosed as AATD at <2%, 2-4.5% and >4.5%, respectively. Number and percent of persons, who are diagnosed by TIC as follows: 27 (50.06%), 25 (47.17%) and 1 (1.89%) at <1.8, 1.8-2.4 and >2.4, respectively.

Figure 1. *Serum protein electrophoresis*

Pi-typing of α1-antitrypsin was performed by IEF in a narrow pH gradient, 4.2-4.9. A typical isoelectric focusing pattern obtained for Pi phenotyping is shown in figure 3. The Pi bands were obtained in the pH range of 4.42 to 4.7 and are seen as series of sharp protein bands. It demonstrates a gel pattern with the typical microheterogeneity of some common αı-antitrypsin variants of clinical importance.

Figure 4 also shows a schematic drawing indicating the position and number of the bands. As it is shown in figures 3 and 4, different patterns due to the variation of the content of sialic acid and the length of the polypeptide chain are detected.

Figure 2. *Selected densitometry patterns of SPE. A) normal sample, B) deficient sample*

Figure 3. *Pi-typing AAT by IEF in a narrow pH gradient, 4.2-4.9*

Figure 4. *A schematic drawing from Pi-typing AAT in a narrow pH gradient, 4.2-4.9*

PCR produced fragments of exons II & V α1-antitrypsin gene pattern (figure not shown). This pattern indicating that PCR reaction has been performed correctly. The results of typing for the S and Z mutations are shown in figures 5 and 6, respectively. Homo- and heterozygous persons and persons who did not have the mutations showed different patterns.

Figure 5. *PCR-amplified normal and S mutation-bearing alles before and after cleavge with XmnI followed by polyacrylamide gel electrophoresis. Lane 5 do not digested*

Figure 6. *PCR-amplified normal and Z mutation-bearing alles before and after cleavge with TaqI followed by polyacrylamide gel electrophoresis. Lane 1 do not digested*

Discussion

Diagnosis of AATD has traditionally been based on biochemical methods. Recently molecular techniques are applicable to detection of the deficiency. The use of each method to diagnose AATD has been problematic. Results obtained from SPE and TIC studies clearly demonstrate these assays don't suffice for diagnosis of AATD. Because A_1AT is a positive acute phase protein and it rises during normal pregnancy and a wide range of diseases. Therefore, falsely normal levels during acute illness may have seen. Serum A_1AT may even show a decreased in patients with severe protein loss or in improper storage of specimen (Cox 1995).

IEF is fast and simple method. The variants were easily recognized by the present technique. Therefore, this technique is suitable for the phenotyping of Pi variants. The protein separation can be further improved by hybride IEF in immobiline, with use of an ultra-narrow pH gradient (Jeppsson & Einarsson 1992) but interpretation of the banding pattern obtained by IEF is difficult and requires specially trained personnel. Alternative methods of diagnosis involve determination of the disease-causing mutations in DNA samples by PCR-based assays. Alternative methods of diagnosis involve determination of the disease-causing mutations in DNA samples by PCR-based assays. The most widely used techniques for this are PCR combined with ASO (Petersen *et al* 1988) and the ARMS assay (Newton *et al* 1989). PCR/ASO probe for the Z and S mutations is very reliable, but this technique is labor intensive, and requires the use of two radioactively labeled probes and is not suitable for routine use. The ARMS assay may seem like a good alternative to the PCR/ASO probe assay. It is non-radioactive and is more easily performed but this technique cannot detect heterozygous alleles of Z and S (MZ, MS, -S, -Z and ZS).

In our experience we used IEF technique, along with PCR-RFLP for the detection of AATD. The most critical step in the new assay is the restriction enzyme digestion step (Lindeman 1991). Another very important element is inclusion of control sites for restriction enzyme cleavage including a control site will indicate when digestion is incomplete, thereby avoiding problems with false-negative or false-positive results. The control sites in our assay are placed in such a way that the cleaved-off fragments are a different size (16bp in the S and 11bp in the Z assays) from the fragment cleaved off at the mutation site (22bp in the both assays). Given this difference in fragment sizes, we can distinguish unambiguously between cases of incomplete digestion and true heterozygosity. In contrast to the problems we encountered in optimizing the restriction enzyme digestion step. Principles of S and Z mutation assays are shown in figure 6. PCR-amplification produces a 149-bp fragment of exon III of the αý-antitrypsin gene in the S mutation assay and a 97-bp fragment of exon V of the αý-antitrypsin gene in the Z mutation assay. Changes in dinucleotide content of the primers pairs result in the creation of *Xmn*I site (GAANNNNTTC) and a *Taq*I site (TCGA) in S and Z mutations, when the normal sequence is copied by PCR. These sites serve as an internal control of restriction enzyme digestion. Therefore, after cleavage with *Xmn*I, amplified S mutation-bearing alleles are 133bp, amplified normal alleles are 111bp, after cleavage with TaqI, amplified Z mutation-bearing alleles are 86bp, and amplified normal alleles are 64bp. This clearly illustrates that individuals who are heterozygous for the respective mutations can be distinguished unambiguously from normal persons and from subjects who are homozygous for the mutation question. Note that alleles with the S mutation will appear normal in the Z mutation assay, and vice versa (Figures 7

and 8, lanes 4).

In conclusion, to make use of SPE and IC techniques can detect only 35.85 % and 50.08% of AATD patients. Then these techniques cannot be suitable for diagnosis of AATD. But if we used IEF technique, along with PCR-RFLP, we can detect AATD by 100%. Hence we recommended the joined technique of IEF/PCR-RFLP for detection of AATD in routine use.

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