Alpha 1 Antitrypsin Deficiency in Infants with Neonatal Cholestasis

Maryam Monajemzadeh1,2, MD; Reza Shahsiah1, MD; Mohammad Vasei1, MD; Parin Tanzifi1, MD, Nima Rezaei3-4, MD, PhD; Mehr Najaﬁ5-6, MD, Narjes Soleimanifar1, Maryam Eghbali1

1. Department of Pathology, Tehran University of Medical Sciences, Tehran, Iran
2. Infectious Disease Research Center, Children’s Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
3. Research Center for Immunodeficiencies, Children’s Medical Center, Pediatrics Center of Excellence, Tehran University of Medical Sciences, Tehran, Iran
4. Department of Immunology, Molecular Immunology Research Center, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
5. Children’s Medical Center Hospital, Tehran University of Medical Sciences, Tehran, Iran
6. Department of Pediatrics, Tehran University of Medical Sciences, Tehran, Iran

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Abstract

Objective: Alpha1-antitrypsin deficiency (A1ATD) is the most important indication for liver transplantation in children. The gene frequencies vary in different ethnic groups. In the present study, we attempt to determine the frequencies of the most common defective alleles, Z and S, in Iranian children suffering from idiopathic neonatal cholestasis. Eighty-seven infants were typed for Z and S alleles.

Methods: In a single center study, 87 consecutive liver biopsies from infants with cholestasis were reviewed and patients with neonatal cholestasis enrolled in the study and cases with confirmed biliary tract atresia excluded. Formalin fixed paraffin embedded blocks were used for DNA extraction. AAT genotype was determined by polymerase chain reaction (PCR) assay and amplification of the two most common deficiency variants, S and Z alleles, and then sequencing of PCR products.

Findings: There were 48 (55.2%) males and 39 (44.8%) females, with a median age of 60 days. Out of 87 of the study subject, 2 (2.2%) were heterozygous for the S allele, and no ZZ, SS or MZ individual was found in the patients. No other polymorphism was found in the sequencing results.

Conclusion: In comparison to other populations, AAT deficiency seems not to be an important etiologic factor for neonatal cholestatic liver disease in Iran; however, further studies are recommended to estimate the true mutant gene frequencies.

Key Words: Alpha1-antitrypsin Deficiency; Liver; Biopsy; Cholestasis; PCR; DNA Sequencing; Iran

Introduction

Alpha1-antitrypsin deficiency (A1ATD) is among the most common Mendelian hereditary liver diseases in Caucasians that affects 1 in 1800 live births[1-3]. It is the most common and most important indication for liver transplantation in children[1,2,4]. A1AT is a glycoprotein synthesized mainly by the liver and is the major circulating protease inhibitor[2,5]. Preventing lung damages, the main physiological function of A1AT is acting against neutrophil elastase[4]. Its concentration is determined by the 2 alleles of the SERPINA1 gene (SERine Proteinase INhibitor A1) located on the
long arm of chromosome 14 which is inherited as autosomal codominant fashion. There are more than 100 genetic variants, of these genes of which about 30 alleles have clinical implication. The normal gene is designated Pi (Protease inhibitor) M which is the common allele in the population. The two most common and deleterious deficiency alleles are PiS and PiZ resulting from a single base-pair substitution in exon III (PiS allele) and in exon V (PiZ allele).

Deficient gene frequency varies in different ethnic groups, with the high frequencies in Scandinavia and the frequency in UK and US populations is about 1%-2%. Its frequency is low in Malaysia and India. The prevalence of MM, MS, and MZ genotypes are estimated to be 86, 9, and 3%, respectively among whites.

Accumulation of intracellular mutant A1AT molecule triggers a series of events which lead to hepatocyte death, inflammation, fibrosis and cirrhosis. Neonatal cholestasis is one of the clinical manifestations of A1ATD; therefore, in an infant with cholestasis, this disease should be in differential diagnoses list and work up should be performed and Pi typing is recommended irrespective of the serum A1AT concentration. Genotyping is the method of choice in diagnosis of A1ATD.

Although A1ATD is a frequent disorder, it is poorly recognized in practice, especially in patients with liver disease; moreover, a deficient phenotype will not always cause liver disease development.

Liver disease represents a major health problem among children, so in the present study we attempt to determine the prevalence of the most common defective alleles, Z and S, in children suffering from idiopathic neonatal cholestasis. We hypothesized that by testing individuals within a specific disease category, the chance of finding more cases with mutation would be higher in comparison to general healthy population.

**Subjects and Methods**

**Patients**
The present study was conducted in the pathology department of the Children’s Medical Center Hospital, the main tertiary university hospital of children, affiliated to Tehran University of Medical Sciences. From April 2007 to March 2012, all patients with history of neonatal cholestasis due to non obstructive etiologies were collected from pathology archives; their charts were reviewed, and one hundred patients between 1 to 3.5 months were enrolled in the study. Cases with diagnosis of biliary tract obstruction confirmed during intraoperative cholangiography, and those with insufficient tissues were excluded from study.

Age, gender, clinical manifestations, clinical impression, pathology diagnosis, serum aminotransferase and A1AT levels were retrieved from medical records.

Hematoxilin and eosin, tricrome, PAS (with and without diastasis treatment) and reticulin-stained slides were reviewed and cases with sufficient amount of tissue were selected. Biopsies were re-examined and degree of fibrosis, inflammation, bile ductular proliferation, steatosis and extramedulary hematopoiesis were recorded.

Neonatal cholestasis was defined as jaundice within the first 4 months of life with conjugated bilirubin more than 20% of the total serum bilirubin. The biliary atresia was diagnosed according to Fischler et al. If the serum A1AT concentration was lower than 1.13 g/L, the case was considered as suspicious for A1ATD.

Neonatal hepatitis was diagnosed after thorough physical examination, laboratory evaluations and complete history that reveals no clear underlying cause of cholestasis. Final diagnoses were based on clinical and biochemical data, imaging tests and liver biopsy.

The study population was all Iranian and cases from other nearby countries were not enrolled in the research; however, Iran is composed of different ethnicities such as Persian, Turks and Kurds and as our study was retrospective the detailed ethnicity of cases was not clear.

**Nucleic Acid Purification**

Two 5 µm sections of each paraffin block were made, using disposable blades, and then the sections were transferred to DNase/RNase free 1.5-ml Eppendorf tubes by means of a disposable plastic applicator. Nucleic acids were extracted, using Roche High Pure polymerase chain reaction (PCR) Template Preparation Kit (Roche...
Table 1: Primers and polymerase chain reaction product characteristics

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence 5’--&gt; 3’</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATS Forward</td>
<td>ATACCTGGGCAATGCCAC</td>
<td>150</td>
</tr>
<tr>
<td>ATS Reverse</td>
<td>AGCTTCTTGGTGACACCTGAG</td>
<td></td>
</tr>
<tr>
<td>ATZ Forward</td>
<td>GGGATCAGCCTTACAACGTG</td>
<td>154</td>
</tr>
<tr>
<td>ATZ Reverse</td>
<td>GGGTTTGTTGAACCTGACCTC</td>
<td></td>
</tr>
</tbody>
</table>

ATS: Alpha one antitrypsin S allele; ATZ: Alpha one antitrypsin Z allele

Diagnostics, Indianapolis, IN), as instructed by the manufacturer. Tissue lysis buffer and proteinase K were added to tubes and checked until no tissue particle became discernible. The specimens then were brought to columns after addition of binding buffer and alcohol. Washing steps then were performed to remove inhibitors. Extracted DNA was eluted into a final volume of 200 µL and stored in −20°C until PCR carried out.

PCR

Amplification of two targets were performed which contain PiS, PiZ alleles. Table 1 shows primers characteristic. The PCR conditions for both reactions were as follow: initial denaturation at 95°C for 1 min and then 40 cycles of 95°C for 10s and 60°C for 30s; real-time acquisition was performed during annealing/extension phase on green channel. The final steps of both products involved an extension phase at 72°C for 5 min and melting curve generation by raising temperature from 60°C to 95°C at a rate of 18°C/s. Meanwhile, real-time acquisition was performed on green channel. Positive and negative controls were included in each run. The PCR reactions were performed in a 20 ml reaction containing 0.2 mM of each forward and reverse primers, 10 ml Premix Ex Taq (Takara Bio, Ostu, Shiga, Japan) and 9 ml of sample. All PCR products were sent for purification and sequencing to Macrogen (Geum chun-gu, Seoul, Korea), where the sequencing was performed by ABI 3730 XL machine employing forward primer. Finally, sequence nucleotides were aligned by means of alignment explorer of MEGA software, version 4.0.2[18].

Using descriptive statistics, patients’ characteristics were analyzed and data were shown as mean and SD for quantitative variables. Statistical analyses were performed, using SPSS version 13 (SPSS Inc, Chicago, IL, USA). P-values less than 0.05 were considered as significant. The Ethics Committee of Tehran University of Medical Sciences approved the study, while the study was in accordance with the Helsinki Declaration of 1975.

Findings

One hundred liver biopsies were collected from a group of infants with idiopathic neonatal cholestasis which presented with cholestasis during infancy. The most (100%) common clinical manifestations was icterus and acholic stool was observed in 32 (27.8%) cases. Demographic data are shown in Table 2. Among them, 5 cases had insufficient tissue, and 87 satisfactory PCR and sequencing results were obtained. There were 48 (55.2%) males and 39 (44.8%) females, with a

Table 2: Demographic data of cases with idiopathic neonatal cholestasis before biopsy

<table>
<thead>
<tr>
<th>Clinical impression</th>
<th>Number of cases</th>
<th>Mean (SD) age at biopsy (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biliary tract atresia</td>
<td>42</td>
<td>64 (16)</td>
</tr>
<tr>
<td>Idiopathic neonatal hepatitis</td>
<td>21</td>
<td>67 (17)</td>
</tr>
<tr>
<td>Metabolic liver disease</td>
<td>18</td>
<td>54 (16)</td>
</tr>
<tr>
<td>Paucity of intrahepatic bile ducts</td>
<td>6</td>
<td>60 (15)</td>
</tr>
</tbody>
</table>

SD: Standard Deviation
Table 3: Demographic data of cases after biopsy and final diagnosis

<table>
<thead>
<tr>
<th>Final diagnosis (based on pathology findings and clinical data)</th>
<th>Mean (SD) age at biopsy (days)</th>
<th>Gender</th>
</tr>
</thead>
<tbody>
<tr>
<td>Idiopathic neonatal hepatitis</td>
<td>62 (18)</td>
<td>Female: 32 Male: 41</td>
</tr>
<tr>
<td>Neonatal hepatitis in favor of Metabolic liver disease</td>
<td>57 (15)</td>
<td>Female: 5 Male: 4</td>
</tr>
<tr>
<td>Paucity of intrahepatic bile ducts</td>
<td>68 (12)</td>
<td>Female: 1 Male: 2</td>
</tr>
<tr>
<td>Granulomatous hepatitis and CMV infection</td>
<td>48 and 67</td>
<td>Female: 1 Male: 1</td>
</tr>
</tbody>
</table>

SD: Standard Deviation

median age of 60 days (mean age of 62±31 days, ranging from 7 to 120 days). The diagnoses of these patients were based on clinicopathologic correlations and on variable laboratory methods which are shown in Table 3. The most (84%) common diagnosis was idiopathic neonatal hepatitis.

Thirty-eight cases had A1AT level analysis in their records; among them, one showed reduced level, while the remaining individuals had normal levels. The mean level of ALT, AST and alkaline phosphatase were 256±172, 295±202 and 1958±912 mg/dl, respectively. The mean level of alpha one antitrypsin was 160 mg/dl (109-260). No significant difference was found in serum levels of alpha one antitrypsin between patients with different pathologies. No patient had a final clinical or pathological diagnosis of A1ATD.

Using the PCR amplification and subsequent sequencing method, the frequency of S and Z alleles were determined. The result of the real-time fluorescent reaction and melt curves are shown in Fig. 1. An increase in the fluorescence signals corresponded to gene amplification and subsequent product accumulation (Fig. 1-left). Fig 1 (right) shows the melt curves of amplified DNA products.

Among 87 enrolled subjects, 2 (2.2%) were heterozygous for the S allele, while no ZZ, SS or MZ individual was found in the patients. No other polymorphism was found in the sequencing results.

**Discussion**

The spectra of liver manifestations of A1ATD are prolonged infantile cholestasis leading to juvenile cirrhosis and slowly progressive adult liver disease[2]. Histopathological alterations may be subtle in infants before 12 weeks after birth, while the pathological findings may be same as neonatal hepatitis; therefore, using the biopsy alone could underestimate A1ATD diagnosis[13].

Genotype frequencies in our series showed 2% MS, which is less than those detected in general
white populations (9% MS, 3% MZ)\cite{19}. Moreover, we did not find any MZ, ZZ or SZ in our study group. Previous studies have detected that 4.5-11% of patients with neonatal cholestasis have the ZZ genotype\cite{20,21}. This single center study showed that the distribution of mutant A1AT alleles differ significantly in our cases, compared to previous published studies in other populations\cite{14,22-24}.

Large scale epidemiologic studies for carrier detection have shown at least 116 million carriers (Pi MS and Pi MZ) and 3.4 million deficiency allele combinations as Pi SS, Pi SZ and Pi ZZ in a total population of 4.4 billion worldwide\cite{22,23} with variable frequencies among different ethnic groups. Z allele frequencies are 1-2% in the US whites, 0.48% in the Africans, 0.4% in the central Asians, 1.5% in the Australia and New Zealand residents. S allele is found in 2-4% of the US whites, 3.1% in the Africans, 0.43% in the central Asians, 3.95% in the Australia and New Zealand residents\cite{22}.

Heterozygous PiZ state has a prevalence rate from 2% to 4% in different populations and is highest in the Northern and Western European descent. PiMS constitutes the most common heterozygous phenotype (70% of the heterozygotes) and the PiMZ accounts for about 28% and the SZ about 1% of phenotypes\cite{14}. In a study in Serbia, PiZ and PiS phenotypes were found more in infants with liver disease in comparison to healthy individuals\cite{24}.

The result of our study was in parallel with the results reported by Chongsrisawat et al and Lai et al, who investigated infants with neonatal cholestasis in Thailand and Taiwan, respectively, and did not find important association between A1ATD and infantile cholestasis\cite{9}. In addition, Arora et al in a study on 98 cases suspicious of A1ATD, based on serum level of A1AT, found no mutation and concluded that A1ATD is uncommon in North India\cite{10}. Similarly, in a multi-ethnic Southeast Asian population, Lee et al found that this deficiency was not account for a common cause of neonatal cholestasis in the Malaysian children\cite{9}. In contrast, in reviewing 58 individual countries, Serrer showed that A1ATD is found in different populations in the Middle East, including African blacks, Arabs and Jews, in Australia and New Zealand, in Europe, North America and in Central, Southeast and Far East Asia and concluded that A1ATD may affects any racial group worldwide\cite{23}.

Therefore, according to previous studies, the prevalence of mutations is highly affected by ethnic background, which could be a reason of the difference in our results in comparison to previous studies. However, any conclusion from this study is limited by low number of studied subjects. We also should remember that Iranians belong to different ethnicities such as Persians, Turks and Kurds and as our study was retrospective and our hospital, as a tertiary referral hospital admits patients from all regions of Iran, the detailed ethnicity of cases was not clear in many of cases but all cases were Iranians and no cases were from other countries.

In this study, we found two cases with MS genotype; however, the question remains to clear whether the liver disease in these two infants is due to heterozygous A1ATD or this finding is incidental. Although the role of homozygous A1ATD in liver disease is established, the association between heterozygous A1ATD and liver disease is doubtful. However, it is known that various environmental, genetic, coexisting liver disease and individual factors could influence A1ATD related liver diseases. On the other hand, it is believed that carriers of metabolic diseases are at higher risk of adverse health effects, while cases with MS phenotypes are classified as at risk population\cite{23}. These two infants may be prone to more severe phenotypes, but precise follow-up is needed. Moreover, a few reports suggested an association between PiMS phenotype and liver dysfunction in children due to loop-sheet polymerization and subsequent retention of A1AT in ERs that is milder than piZZ phenotype though\cite{23,25-28}. All carriers and deficient individuals could be at risk for adverse health effects\cite{23}; therefore, we cannot ignore these two cases, and dismiss the possibility of their role in cholestasis development. The impact of this heterozygosity on liver disease needs across-the-board screening of the whole disease population\cite{4}.

Previous studies on A1AT phenotypes in Iran, showed that patients with hepatitis B infection had higher frequencies of MS, MZ, M(1)Z, and M(1)S phenotypes\cite{29}. Higher frequencies of M1S, M2S, M1Z, and MV phenotypes were also documented in patients with uveitis\cite{30}. Both studies were performed through phenotyping, not genotyping, and performed in certain groups of
adult patients, which cannot be compared to our result.

Our study had some other limitations as follow: First, the results cannot be applied to the general population. Moreover, as we cannot detect common rare alleles by this method, we might have missed at-risk cases with other genetic variants leading to reduced or normal levels or presence of dysfunctional protein.

Conclusion

The present study provides new information on the frequency of S and Z alleles in a sample of Iranian infants with neonatal cholestasis, which reveals that A1ATD may be not as frequent as observed in the Western countries.

Acknowledgment

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Conflict of Interest: None

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