RESEARCH PAPER

Alpha 1 Antitrypsin Deficiency in Children with Chronic Liver Disease in North India

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Objective: We attempted to determine the role of alpha-1-antitrypsin (AAT) deficient variants as an etiologic factor for chronic liver disease in North Indian children.

Design: This study investigated 1700 children (682 retrospectively and 1018 prospectively) (840 CLD, 410 neonatal cholestasis and 450 without liver disease) for AAT deficiency.

Setting: Tertiary referral center, All India Institute of Medical Sciences, New Delhi.

Patients: Of 1250 liver disease patients, 98 (7.8%) were suspected to be AAT deficient on the basis of screening tests (low serum AAT levels and/or absent/faint alpha-1-globulin band on serum agarose electrophoresis and/or diastase resistant PAS positive granules on liver biopsy).

Main outcome measures: AAT deficient Z or S allele in suspected patients.

Results: Z or S allele was not observed on phenotyping (1700 subjects), or with PCR-RFLP, SSCP and sequencing done in 50 of 98 suspected AAT deficient patients. A novel mutation G-to-A at position 333 in exon V was found in two siblings having positive immunohistochemistry for AAT on liver biopsy, both of whom had significant liver disease with portal hypertension.

Conclusion: In conclusion, AAT deficiency as an etiologic factor for chronic liver disease in childhood appeared to be uncommon in North India.

Key words: Etiology, Novel mutation, Phenotyping

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Since the first description of Alpha-1-Antitrypsin (AAT) deficiency by Laurell and Eriksson in 1963 (1), major advances have been made in the understanding of the genetic and clinical aspects of this disorder. Studies done among Caucasian children suggest that AAT deficiency is among the commonest etiological factor associated with chronic liver disease (CLD) in childhood. The allele frequency of Z or S deficient alleles in West varies from 0.004 to 0.1%(2-4). These individuals as well as their descendants in other parts of the world were described to be at the highest risk.

In India, AAT deficiency is being diagnosed on the basis of low serum AAT levels, Periodic Acid Schiff (PAS) positive diastase resistant granules on liver histology and absent or faint alpha 1 globulin band on serum electrophoresis. Based on these criteria, up to 8% of children were suspected to have AAT deficiency associated CLD(5-8). However, these techniques have issues of low specificity with high false positivity(9) and no confirmed case based on either Isoelectric focusing (IEF) or PCR based assay has ever been reported in India. In view of this background, we attempted to determine prevalence of AAT deficiency among pediatric chronic liver disease patients in North India using diagnostic tests

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(IEF and genotyping) that are considered gold standard.

METHODS

The study (retrospective: 1991-1999, prospective: 2000-2004) was conducted in pediatric liver disease patients (up to 15 years) mostly residents of North India, at the Pediatric Gastroenterology Clinic, Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, India The caretakers of the patients who were worked up for AAT genotype, gave consent to participate in the study.

CLD was diagnosed on the basis of clinical, biochemical, ultrasound picture, endoscopic evidence of varices and when feasible liver histology. Subsequent diagnostic workup was for specific etiologies. Neonatal cholestasis was defined as conjugated bilirubin 2 mg/dL or more than 20% of total bilirubin (whichever is less)(10). For neonatal cholestasis, workup was done as previously described(11). The investigation protocol is shown in ***Fig.*****1**

Alpha-1-antitrypsin workup

Screening: Reduction of serum levels(12) and/or a faint or absent alpha-1-globulin band on routine gel electrophoresis and/or histopathological evidence of PAS positive diastase resistant granules on liver biopsy were indicators of suspected AAT deficiency state.

Phenotyping: Isoelectric focusing (IEF) (Phast System®, Amersham Biosciences, Sweden) was standardized using polyacrylamide gel slabs at a narrow pH gradient of 4.2-4.9(13) followed by silver staining for detecting the protein bands. Reference sera for 21 allelic variants were provided by Prof. Magne K.Fagerhol, Oslo, Norway.

Genotypic characterization: The Pi Z (14) and S(15) genotypic characterization was done by polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) and single strand conformation polymorphism (SSCP)(16) on DNA extracted from whole blood. Sequencing was done commercially for confirmation.

Serum and genomic DNA from five control blood samples were obtained from adults volunteers aged 26-49 years with no history or evidence of CLD. Their bilirubin levels, liver enzymes, serum AAT levels and total proteins were within normal limits. All had a PiMM phenotype.

Family screening: Father (*n*-43), mother (*n*-48) and siblings (*n*-32) of the 50 suspected AAT deficiency patients (undergoing genotypic characterization) were also investigated for their AAT status (Pi phenotyping and mutation screening).

Statistical Analysis

The clinical and laboratory records of all patients were entered in a database on a regular basis and appropriate consistency checks with reference ranges put in for quality assurance purposes.

Analysis was done using "STATA" version 8.0 (STATA Corporation, Texas, USA) statistical packages.

RESULTS

The serum samples from 1700 patients were available for investigating AAT deficiency. Out of these, 682 patients were recruited retrospectively (1991-1999) and 1018 prospectively (2000-2004). A total of 840 subjects had evidence of underlying CLD and 410 had NC. Remaining 450 subjects, who had either no CLD (*n*-404) or NC (*n*-24) or were Hepatitis B surface antigen (HBsAg) carrier (*n*-22) with no histological or biochemical evidence of ongoing hepatic inflammation, were also part of this study and labeled as "without liver disease", and served as controls.

Etiology of CLD and NC: Among 840 patients with definite CLD (3 months – 15 years), chronic viral hepatitis (HBV/ HCV/ mixed infection) (*n*-238; 28.3%) and metabolic liver diseases (MLD) (*n*-159; 18.9%) were the most common etiologies. In 237 patients (28.2%), no etiologic label could be assigned (***Table* I**).

A total of 410 patients fulfilled the diagnostic criteria of NC (***Table* II**). Obstructive causes contributed to over one-third (141; 34.4%) cases followed by infective etiology (66; 16%). Almost one-third (30.7%) children presenting with NC

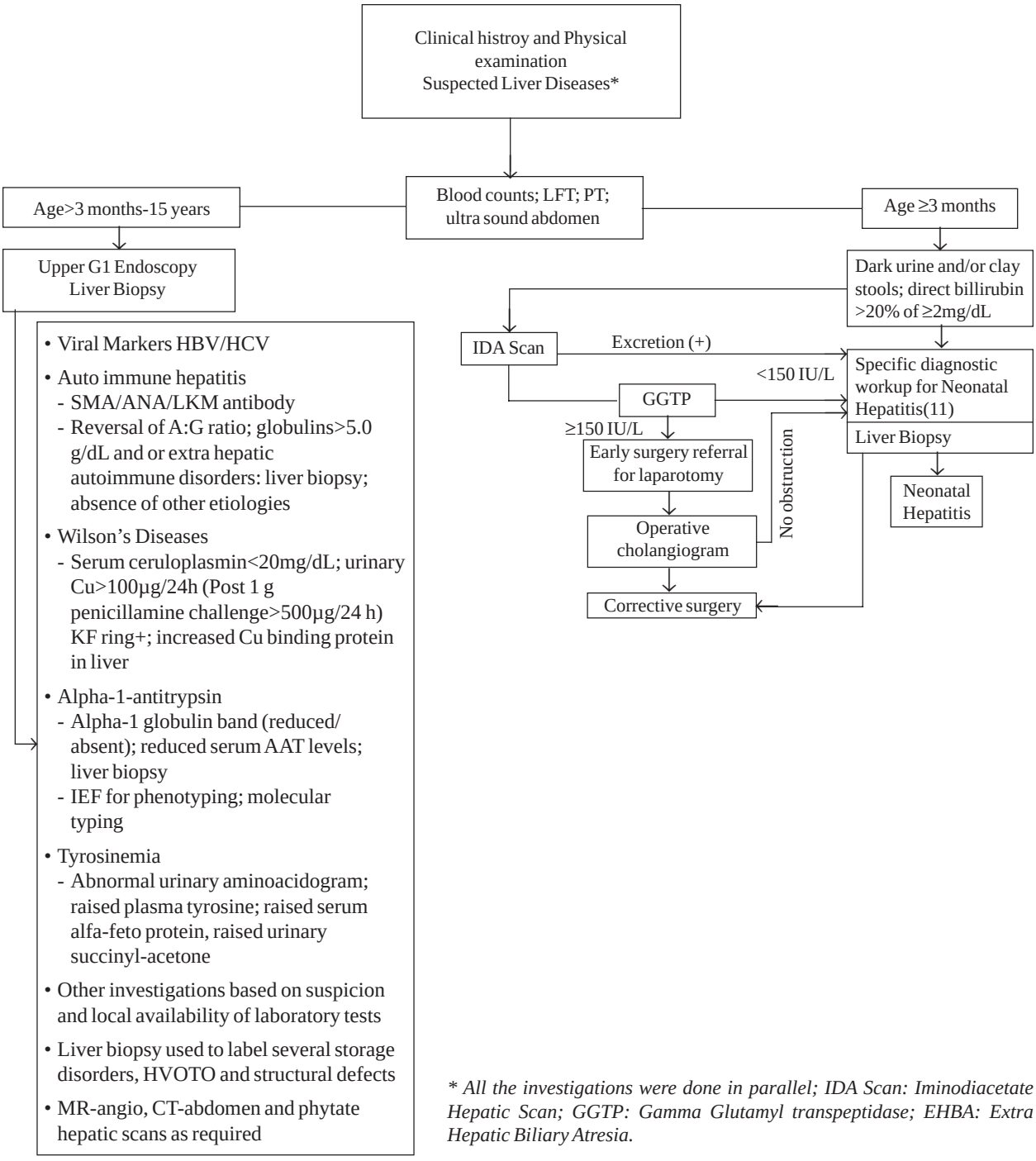

FIG.1. *Investigation protocol for chronic liver disease and neonatal cholestasis.*

could not be assigned any specific etiology.

Suspected Alpha 1 antitrypsin deficiency: Ninety eight (CLD-78; NC-20) of 1250 (7.8%) liver disease patients were suspected to be AAT deficient on the basis of screening tests. Forty-nine liver biopsies were possible in these 98 cases (CLD: 34; NC: 15). There were 9 biopsies with histopathological suggestion of AAT deficiency with presence of PAS positive diastase resistant granules in the liver biopsy. Four of these biopsies had evidence of lipofuscin material and in one PAS positive granules

were present in the cytoplasm. On follow up liver biopsies, PAS positive granules were not observed in three of five [CLD: 1; NC: 2] study patients. A faint/ absent alpha-1-globulin band was documented in 88 children (CLD: 72; NC: 16) and 20 children had low serum AAT levels. Five of these 20 children with low AAT serum levels had severe malnutrition at the time of presentation.

Underlying conditions in the group of children with suspected AAT deficiency: CLD *c*ases suspected to be AAT deficient (*n*-78) were also worked up in parallel for other etiologies. They had chronic viral hepatitis B/C (*n*-18); autoimmune hepatitis (*n*-5); Wilson's disease (*n*-3); other MLD's [Gaucher's disease (*n*-1), hemochromatosis (*n*-1), presumed bile acid metabolic defect (*n*-1). Byler's disease (presumptive; *n*-1) and hereditary fructose intolerance (*n*-1)]; biliary cirrhosis (histological diagnosis, *n*-2); hepatic venous outflow tract obstruction (HVOTO; *n*-1); miscellaneous hepatic disorders (*n*-5) and unknown etiology (*n*-39). Similarly, NC patients (*n*-20) were diagnosed as: extra hepatic biliary atresia (EHBA) (*n*-3); cytomegalovirus (CMV) with neonatal hepatitis (*n*-3); hepatitis B associated (*n*-1); suspected MLD's (*n*-1); sepsis (*n*-1), Down syndrome (*n*-1); and unknown etiology (*n*-10). This etiological spectrum was very similar to the overall etiological profile of CLD and NC patients. Clinical and biochemical profile of the patients suspected to be AAT deficient in the initial screen (78 CLD and 20 NC) and the rest of the liver disease patients (762 CLD and 390 NC) were similar and did not help to differentiate the two groups.

Phenotypic characterization for AAT deficiency: IEF was done in all 1700 children (CLD-840; NC-410; and children with no liver disease-450). Out of 1700, 1697 had PiMM phenotype and other variants of AAT were observed in 3 children. M1E phenotype was present in a single patient who had unknown CLD. His mother and 3 siblings also had M1E phenotype. Child 2 with MP phenotype had autoimmune hepatitis (ASMA and ANA positive). Third child with MC phenotype had a history of sepsis and had acute Hepatitis E virus (HEV) infection without CLD. The family screening could not be done for the 2nd and 3rd child.

TABLE I ETIOLOGIC FACTORS ASSOCIATED WITH CLD IN NORTH INDIAN CHILDREN [1991-2004]

Etiologic Factors	*n* (%)
Chronic viral hepatitis	238 (28.3)
Hepatitis (HBV)	206 (24.5)
Hepatitis C (HCV)	29 (3.4)
Mixed infection (HBV/HCV)	3 (0.3)
Autoimmune hepatitis	52 (6.2)
Metabolic liver diseases	159 (18.9)
Wilson's disease	76 (9)
Glycogen storage disease	23 (2.7)
Hereditary fructose intolerance	14 (1.6)
Lipid storage disorder	13 (1.5)
Gaucher's disease	6 (0.7)
Bile acid metabolic defect	6 (0.7)
Tyrosinemia	2 (0.2)
Hematochromasis	4 (0.4)
Organic academia	4 (0.4)
Galactosemia	2 (0.2)
Niemann Pick disease	2 (0.2)
Byler's disease	2 (0.2)
Indian childhood cirrhosis	2 (0.2)
Suspected MLD	1 (0.1)
AAT Deficiency	2 (0.2) (Novel mutation)
Hepatic venous outflow tract obstruction	55 (6.5)
Biliary Tract with CLD‡	36 (4.2)
Miscellaneous	63 (7.5)
Primary Hepatic malignancy	19 (2.2)
Congenital hepatic fibrosis	11 (1.3)
Drug induced*	8 (0.9)
Celiac disease/ cystic fibrosis	5 (0.5)
Non Alcoholic Steato-Hepatitis	4 (0.4)
Other Hepatic Disorders**	16 (1.9)
Unknown etiology	237 (28.2)
Total chronic liver disease	840 (100)
No evidence of CLD†	404
HBsAg Carriers#	22
Incomplete workup	136
Total Registered	1402

*Figures in parenthesis are percentage: ‡Gallstones (18), cholelithiasis (12), biliary cirrhosis (6) * Histologic evidence with duration of more than 6 months; anti TB therapy(4), Valproic acid(3), chemotherapy for acute leukemia (1); ** Histiocytosis (3); Non cirrhotic portal fibrosis (6); Rubella (3); Kala-azar associated liver disease (4); †Details provided in text; #No histological or biochemical evidence of liver disease.*

TABLE II ETIOLOGICAL FACTORS ASSOCIATED WITH NEONATAL CHOLESTASIS [1991-2004] [*N*=410]

Etiological Factor	*N* (%)
Obstructive Causes	141 (34.4)
Biliary atresia	114 (27.8)
Biliary atresia with CMV infection	8 (1.9)
Choledochal cyst	19 (4.6)
Non Obstructive Causes	264 (64.4)
Infections	66 (16.3)
CMV	53 (13.1)
Toxoplasma	1 (0.24)
Rubella	2 (0.5)
HSV	1 (0.24)
CMV/ Toxoplasma	1 (0.24)
CMV/ HSV	1 (0.24)
HBV	5 (12)
HCV	1 (0.24)
Metabolic*	11 (2.7)
Hypothyroidism (conjugated)	22 (5.4)
Bile acid metabolic defects/ Byler's disease	16 (3.9)
Miscellaneous	23 (5.6)
Sepsis	7 (1.7)
Down syndrome	3 (0.74)
Postintestinal surgery‡	2 (0.5)
Caroli's disease	2 (0.5)
Alagille's syndrome	2 (0.5)
Polycystic liver/ kidney diseases	2 (0.5)
Immunodeficiency	1 (0.24)
Hemangioendothelioma liver	1 (0.24)
Autoimmune hepatitis	1 (0.24)
Unknown Etiology	126 (30.7)
Undifferentiated	5 (1.2)

*Figures in parenthesis are percentage: *Galactosemia(5), Hereditary Fructose intolerance (1), Fatty Acid Oxidation Defect (1), Tyrosinemia (1), Cystic Fibrosis (1), Suspected MLD (2); ** Neonatal Cholestasis but could not be differentiated into obstructive/ non obstructive etiologies** Excluded from the analysis; ‡Intestinal obstruction and required corrective surgery, small bowel resection, †Total neonates registered:529; No cholestasis 5, Incomplete workup 95 and familial hyperbilirubinemia 19.*

Genotypic characterization for AAT Deficiency: Genetic workup was done for 50/98 children suspected to be AAT deficient on screening. Two of the 98 patients were siblings and hence both were included for complete genetic workup. Of the remaining 96 suspected AAT deficient patients, 48 unrelated children (CLD-32, NC-16) were selected through a computer generated random process. S or Z mutation was not found in any of the 50 patients by PCR. Two patients (siblings) showed a shift in band pattern in exon V on SSCP and sequencing confirmed a single base substitution (G to A) at position 333. Index '1' (younger; female) had homozygous mutation and her sibling '2' (elder; male) had heterozygous base substitution. The mutation converts valine to methionine at position 333 in exon V.

Clinical details of two patients with novel mutation at position at 333 in exon V: The index cases (1 and 2) had low serum AAT levels (Index 1:126mg/dL and Index 2: 108mg/dL) and absence of alpha-1 globulin band on serum agarose electrophoresis. Both of them had portal hypertension along with histological evidence of fibrosis and inflammation without PAS positive diastase resistant granules. However, immunohistochemistry revealed numerous rounded deposits of AAT in index 1 with homozygous mutation (***Fig.* 2**). In index 2 (heterozygous mutation), there were bands of fibrosis extending from central and portal regions and immunohistochemistry was weakly positive for AAT. Neither of them had other known etiological factor associated with liver disease.

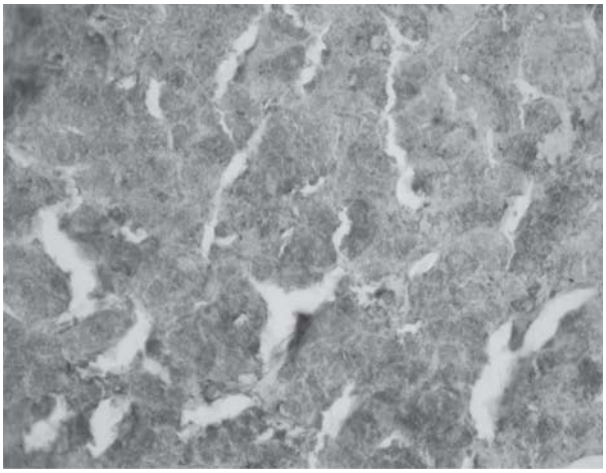

FIG.2 *Index 1(female, 120 months, homozygous mutation at position 333 in exon V); Immunohistochemistry (X20) showing numerous rounded alpha 1 antitrypsin (AAT) bodies.*

The siblings were born out of non-consanguineous marriage. The mother of these patients had normal band pattern on SSCP for both exon III and V. The father had expired at the age of 40 years in 1999 at our hospital. He was admitted with portal hypertension, grade II hepatic encephalopathy and hepatorenal syndrome. The liver biopsy could not be carried out but in the background of history of regular intake of alcohol for 16 years, he was labeled as having alcoholic liver disease. Genetic studies were not done for the father.

Quality assurance: At the inception of this study, no expertise was available to interpret IEF gels. Thus, 10 IEF gels including the gels containing M1E, MC and MP phenotypes and gel strip of patient with homozygous mutation at position 333 were sent to Alpha-1-Foundation Research Professor, University of Florida, School of Medicine, Florida. Also, five randomly chosen DNA samples (of the patients suspected to be alpha-1-antitrypsin deficient and chosen for detailed genetic analysis) were sent to Genetics and IVF Institute, Virginia, USA.

DISCUSSION

This study is an attempt at describing AAT deficiency associated liver disease in children from India based on IEF and genotyping. If we had based our diagnosis on low serum AAT levels and/or absence of alpha 1 globulin band on electrophoresis and/or liver biopsy features, 7.8% of liver disease patients would have been labeled as AAT deficient. However, when IEF and PCR-RFLP were done, phenotypes commonly associated with liver disease (Z and S) were not observed in any patient. The normal variants M1E, MC and MP, detected in 3 patients have not been described in association with pathogenesis of liver disease (17,18). On SSCP, 2 CLD patients who were also siblings were detected to have a novel G to A mutation at position 333. These observations indicated the rarity of AAT associated liver disease in North Indian children.

The screening techniques have limitations particularly in regions of low gene frequency and when the condition is rare or extremely uncommon (19). Furthermore, factors influencing the validity of individual screening tests are also operating. The AAT levels may drastically reduce in malnutrition, respiratory distress syndrome of neonates, cystic fibrosis, nephrotic syndrome and severe liver disease (20). Five of 20 suspected alpha-1-antitrypsin deficient subjects with low serum levels had severe malnutrition at the time of presentation. The electrophoretic alpha 1 lipoprotein's migratory behavior also varies with the duration of storage of serum and with variations of intermediary lipid metabolism(1). There are reports showing the presence of non-glycogenic PAS –positive material in the normal as well as the abnormal liver. Fisher, *et al*(21) reported a case of a patient with PiMM phenotype whose liver biopsy sections revealed both PAS positive globules and positive immunofluorescence. Lipofuscin granules frequently give PAS reaction, larger granules appear coated by a PAS-positive layer. In hemochromatosis, both in the primary idiopathic and in the secondary form associated with anemia, the PAS reaction is strong in both Kupffer and liver cells(22). In our study, four of nine patients with appearance like PAS positive granules on liver biopsy had evidence of lipofuscin material. In one of the biopsy samples, PAS positive granules were present in the cytoplasm. On 5 follow up liver biopsies, PAS positive granules were not observed in three [CLD: 1; NC: 2] study patients. Possibly, biliary concrements or plugs within dilated bile canaliculi or extracellular bile deposits on the first biopsy gave a PAS reaction, which disappeared as acute condition settled down. These three patients did not have any residual liver disease on follow-up. The PAS positive granules were also not observed in two siblings with novel G to A mutation at position 333. However, AAT inclusions were observed in both on immunohistochemistry. Thus, specificity of all the screening tests is particularly low in regious with rare possibility of the conditions, resulting in high possibility of false positive tests, as was observed in our study.

In comparison to studies done among South Asian children, the pediatric liver disease data from Caucasian population shows several AAT alleles (homozygous and heterozygous states) which are associated with liver diseases. In California, ZZ phenotype was found in 4.5% children with neonatal cholestasis(2) In Serbia(3), Pi*Z and Pi*S phenotypes were found to be 15 and 3 times higher

WHAT IS ALREADY KNOWN?

- Alpha 1 antitrypsin deficiency is prevalent in up to 8% of children with liver disease in India based on AAT serum levels, electrophoresis and/or liver biopsy.

WHAT THIS STUDY ADDS?

- Alpha 1 antitrypsin deficiency in children is uncommon in India. 'Z' or 'S' alleles could be altogether absent in our population.

respectively in newborns with liver disease as compared to their healthy counterparts in the population. Absence of AAT deficient alleles in highly selected North Indian child population with liver disease in the present study further confirmed the findings of epidemiologic surveys done in this region(4) . The results of the present study are also consistent to a previous study done in India(23).

Novel mutation: Most at–risk mutations in the AAT gene are single-base substitutions causing single amino acid modifications in the mature protein. The number of such single base substitution mutations is reported to be more often associated with emphysema than that associated with liver disease. Apart from Z, a few mutations like M_{Malton}, Siiyama and $Z_{Bristol}$were reported to be associated with liver disease (24-26).

In the current study, IEF in both the index siblings with mutation in exon V was similar to PiMM. Despite the mutation, the isoelectric point of AAT molecule might not have changed and hence the band movement was indistinguishable from PiMM allele. In an earlier report, variant $PiMM_{herleen}$ (CCC to TCC in Exon V) showed IEF pattern similar to PiMM despite the mutant AAT allele(27). It is also important to note that both homozygous (Index 1) and heterozygous (Index 2) states were associated with almost similar degree of liver disease. Immunohistochemistry indicated deposits of AAT in the hepatocytes to be denser in homozygous mutation (Index 1) than in heterozygous mutation (Index 2). It is difficult to explain the mechanism(s) involved in causing liver disease in Index 1 and her sibling but role of 333 mutation in the etiology of liver disease in these two siblings could not be ruled out completely.

For our patient population, we cannot be certain about occasional presence of mutations in exons and introns other than exon III and V that were not screened as part of current investigation. Such mutations associated with liver disease are however not reported so far.

In conclusion, the study indicated that the AAT deficiency alleles are uncommon in our population. In regions with very low prevalence of abnormal AAT alleles, diagnosis of AAT deficiency based on screening tests is not helpful to identify occasional patient with AAT deficiency alleles. In strongly suspected patients, IEF and molecular techniques should be used to diagnose the condition. We detected a single base substitution mutation at position Val333 Met in exon V in two siblings. The role of this novel mutation in etiology of liver disease could not be completely ruled out. In view of our findings, we do not recommend routine screening for AAT associated liver disease in our region. Further study in adult emphysema patients may clarify the role of AAT deficiency in Indians, although the possibility of it being a significant etiologic factor appears unlikely.

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Contributors: NKA: Study design, protocol preparation, results interpretation and manuscript editing; SA: Protocol preparation, manuscript writing, IEF standardization, analysis of data and other laboratory work; AA: Protocol preparation, PCR-SSCP, analysis of data and other laboratory work; PM: Study design and patients recruitment; MM**:** Laboratory support; MKD and VB:

Patient screening and recruitment; MK: Laboratory resource, genetic analysis and technical guidance; RK and MA: Serum agarose electrophoresis standardization; AK: Laboratory Support; SDG: Pathology; and SV: Special investigations for CLD

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