Molecular Confirmation of Alpha 1-Antitrypsin Deficiency in Liver Transplant Setting: A Province-Wide Experience

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Abstract

Objective:

Patients suspected of Alpha 1-Antitrypsin (A1AT) abnormality based on low serum concentration are routinely confirmed via PCR testing of peripheral blood. Genotyping Formalin-fixed Paraffin-embedded (FFPE) tissue is a novel approach that could aid in detecting variant A1AT. We performed qPCR on FFPE liver explants with PASD and A1AT positive globules to confirm and estimate the frequency of A1AT deficiency in transplant cases.

Methods:

18 (12.68%) of 142 patients with end-stage liver disease showed PASD/A1AT positive globules. FFPE of the explants were tested via qPCR to detect S and Z alleles. A second age and sex matched control group consisting of 5 liver transplant patients with negative globules were included.

Results:

qPCR assay was successful with all the samples meeting QC parameters. All patients included in the study elucidated Z allele variants; 2 homozygous (11.1%) and 16 heterozygous (88.9%). The control group demonstrated normal wild-type MM allele.

Conclusion:

In conclusion, screening for A1AT deficiency using serum levels is not sufficiently sensitive to detect deficiency, especially in carriers. If A1AT testing was not performed preoperatively and
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the risk is high based on the PASD/A1AT positive globules in the explants, then molecular testing of FFPE tissue can be a viable method for confirming the diagnosis.

**Keywords:** Alpha-1 Antitrypsin; Liver Transplant; FFPE PCR; M and Z Allele; Gene Frequency; British Columbia

**Introduction**

Alpha-1 Antitrypsin (A1AT) deficiency is an inherited autosomal recessive multi-system disorder that primarily affect the liver and lungs(1). The disease is caused by a mutation in the SERPINA1 gene located at locus 14q32.13. The SERPINA1 gene encodes for the A1AT protein, a major plasma serine protease inhibitor that is mostly synthesized and secreted by the liver(2). It performs multiple physiological functions including protection of the lungs from proteolytic elastases released by white blood cells in response to environmental factors (e.g. smoking) and other inflammatory processes(3), and potentially even the severity of SARS CoV-2 infection(4). Multiple point mutations at the A1AT gene locus result in an enzyme that is structurally and functionally different from wild-type (M) allele. Each mutated allele harbors variable risk of lung or liver disease phenotype, ranging from silent to severe. Primarily in the Z allele these mutations produce a misfolded A1AT protein that accumulate in the endoplasmic reticulum of the hepatocytes making them susceptible to environmental stressors with subsequent liver injury and fibrosis(5). This accumulated A1AT protein is not secreted in the peripheral blood and therefor, the serum concentration can be used as screening tool for A1AT deficiency(6). The
accumulated A1AT in the hepatocytes from the Z allele can be highlighted under light microscopy in a periportal distribution using Periodic Acid Schiff after Diastase digestion (PASD) or via A1TT immunohistochemistry(7).

The Z allele (NM_000295.4: c.1096G>A, p.Glu366Lys) is the most common disease causing allele, with up to 98% of A1AT deficiency harboring the Z allele(3). The homozygous form (ZZ) accounts for most of the severe cases affecting both the liver and lungs and in certain cases, necessitates transplantation. Heterozygous carriers of Z allele (MZ) are healthy in most cases, however, they still retain a slight risk of chronic liver damage, and to a lesser degree, lung disease(8, 9). In the liver, this is demonstrated by the presence of PASD/A1AT positive globules(7). The second most common allele is the S allele (NM_000295.4: c.863A>T, p.Glu288Val)(10). Additionally, a few other rare alleles are also described, some of which show normal M phenotype on isoelectric focusing, such as the M Malton and M Duarte(11). Those alleles are more common in non-Northern European populations and can demonstrate a certain risk of disease expression(12). Non-Z variants of A1AT can cause disease if present in associated with the Z allele (such as SZ), albeit at a much lesser expression than homozygous Z(13).

Disease prevalence is variable and substantially different in each population. Unfortunately, accurate data is scarce and outdated as many cases are left undiagnosed due to carrier states and the incomplete penetrance of the disease. There is however an increase in the prevalence of Z and S allele variants among individuals of Northern European descent(3). In British Columbia, approximately 230000 individuals are carriers of Pi*S allele (2.6%), while 115000 are carriers of the Pi*Z allele (1.3%)(14).
A1AT deficiency has a bimodal distribution with differing presentations between adults and infants. It presents in infancy as neonatal hepatitis/cholestasis and in adulthood as chronic liver disease and/or chronic lung disease(8). In the liver, it can also result in hepatocellular carcinoma and not uncommonly, fulminant liver failure(1). Liver and lung disease phenotypes occur independently and may present at different ages in the same individual(15). Various algorithms are available and can be utilized to test populations for A1AT deficiency. British Columbia adapts a modified version of a protocol by Snyder et al whereby serum A1AT level is used as an initial test followed by PCR analysis and/or Isoelectric focusing of peripheral blood if the serum level is lower than 1.15 g/L. Gene sequencing is to be performed if PCR or Isoelectric Focusing yield ambiguous results(16). No specific therapy is available for A1AT deficiency yet, and the mainstay of management remains supportive with emphasis on delaying or preventing the need for transplantation. Liver transplantation is the only definitive therapy and leads to phenotypic conversion(15). New emerging gene therapies are gaining momentum such as the use of siRNA and stem cell technology to assist in reversing genetic aberrations in the A1AT gene(17).

It is not uncommon to under detect A1AT positive globules in liver explants of patients with clinically presumed liver pathology aetiologies other than A1AT when either the serum A1AT is within normal range or when A1AT is not tested preoperatively. This may result in an unsatisfying diagnosis of ‘cryptogenic’ liver cirrhosis in cases with homozygous or heterozygous mutant alleles, resulting in the loss of opportunity to diagnose, and subsequently screen the family of affected individuals. With the advent of advanced molecular testing, genotyping of A1AT via molecular testing of Formalin-Fixed Paraffin-Embedded (FFPE) tissue is a novel and theoretically feasible approach if preoperative assessment was not conducted and the suspicion of A1AT deficiency is high based on histological assessment(18).
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In this study, we performed real-time PCR assay of FFPE liver explant tissue in specimens that showed PAS-positive, diastase-resistant and A1AT immunoreactive globules to estimate the frequency of A1AT deficiency in liver transplant patients in the province of British Columbia.

Methods

The study had research ethics approval by the institutional review boards (UBC Ethics #H19-02913). All the patients who received liver transplant surgery for end-stage liver disease in British Columbia, Canada from May 1, 2016 to May 1, 2019 were accrued from the electronic medical records of the only liver transplant center in British Columbia. A total of 142 patients had liver transplants performed during the stated period, including 2 cases preoperatively attributed to A1AT deficiency. The mean age at transplant is 54 (33-72). Of those, 91 cases had no postoperative PASD done and thus were performed for the purpose of this study. Of the 142, 18 patients (12.68%) demonstrated periportal globules positive for PASD (Figure.1a-b) and A1AT immunohistochemical stain (Figure.1c). Interestingly, one of the cases (case #07) that was negative for PASD+ globules on the pre-transplant biopsy demonstrated the globules on the hepatectomy sections. Another case (case #18) was identified on a failed liver allograft. Of the eighteen patients, 7 patients (38.9%) had normal pre-transplant A1AT serum levels, 3 patients (16.7%) had a low serum A1AT levels and 8 patients (44.4%) were not tested preoperatively for A1AT serum concentration. Normal serum A1AT level is defined as a concentration ranging from 0.9 to 2.0 g/dl. The aetiologies attributed to transplantation are details in Table 1.
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Figure 1. a, b, c. Hematoxylin and Eosin (H&E) stain (a), Periodic Acid Schiff after diastase digestion (b) and Alpha-1 Antitrypsin immunohistochemical stain (c) show Alpha-1 Antitrypsin globules from case 8 (40x magnification).
The 18 (n=18) patients were selected for targeted genotyping via real-time PCR amplification and melting curve analysis of FFPE hepatic explant tissue to detect S and Z allele variants. Z Allele is defined as (NM_000295.4;c.1096G>A, p.Glu366Lys), whereas the S allele is defined as (NM_000295.4: c.863A>T, p.Glu288Val). Sanger sequencing of rare alleles variants is to be performed in cases with a negative PCR result.

Additionally, a second age and sex matched control group consisting of 5 patients who also received liver transplants during the same period but who did not demonstrate PASD positive globules were also included. All the 5 cases are of European descent and therefore have the highest risk of Z-allele. The mean age for the control population at the time of transplant is 50 (27-71). Given the absence of the PASD positive globules, the Z variant was not expected in any of the 5 cases; these were tested primarily to rule out technical issues that could potentially lead to the false positive detection of Z allele in the FFPE liver samples. The predicted allele frequency, from the BC population (1.3%), was used as the comparator prevalence rate for Z containing genotypes (2.6%), the pre-requisite for most PASD positive hepatocyte inclusions.

Descriptive and simple statistical tests, that do not require a commercial statistical software package, were employed.

To process the FFPE tissue, 0.5 um sections of formalin fixed paraffin embedded hepatic tissue were placed in an Eppendorf tube with 100ul of 0.1 mm glass beads (Biospec), 700ul of PBS, 0.36% SDS and 15U of Proteinase K (Roche), and incubated at 55C overnight with shaking. The sample was bead lysed in a Qiagen TissueLyser LT at 50 setting for 3 minutes. After 5s centrifugation, 400ul of supernatant was extracted on the MagNA Pure compact (Roche, Pleasanton CA), and eluted in 50ul. Targeted genotyping was performed using real-time
polymerase chain reaction (PCR) amplification and melting curve analysis to detect the S and Z alleles.

**Results**

Real-time PCR assay of the FFPE tissue was successful with the PCR products robustly amplifying and meeting quality control parameters in each case. All the patients included in the study elucidated Z allele variants; 2 of which are homozygous (11.1%) and 16 heterozygous (88.9%). None showed S allele. All the cases in the control group demonstrated normal wild-type MM allele. This is summarized as follows:

- PASD positive samples: n=18/142 or 12% (7.6% - 19%); all tested positive for the Z allele
- Expected maximum PASD positive rate based on predicted BC population prevalence (ethnicity dependent Z allele frequency, based on Canadian 2016 Census and gnomAD population frequency=2.6%)
- PAS negative samples (n=5; all tested negative for the S and Z alleles)

**Table 1:** Summary of the clinical and molecular findings

<table>
<thead>
<tr>
<th>Case#</th>
<th>Age</th>
<th>Sex</th>
<th>Pre-transplant Serum A1AT Levels (g/L)</th>
<th>Clinical Diagnosis</th>
<th>A1AT Allele</th>
<th>Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>44</td>
<td>F</td>
<td>0.16</td>
<td>Autoimmune Hepatitis</td>
<td>ZZ</td>
<td>ZZ</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>2</strong></td>
<td>54</td>
<td>F</td>
<td>0.22 and 0.18</td>
<td>A1AT Deficiency and Alcoholic liver disease</td>
<td>ZZ</td>
</tr>
<tr>
<td><strong>3</strong></td>
<td>33</td>
<td>F</td>
<td>0.69 and 0.71</td>
<td>Primary Biliary Cholangitis and A1AT deficiency</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>4</strong></td>
<td>65</td>
<td>M</td>
<td>1.01 and 0.93</td>
<td>Alcoholic liver disease</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>5#</strong></td>
<td>40</td>
<td>M</td>
<td>1.2</td>
<td>Alcoholic liver disease</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>6</strong></td>
<td>38</td>
<td>M</td>
<td>0.88, 0.75 and 0.51</td>
<td>Alcoholic liver disease</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>7</strong></td>
<td>48</td>
<td>F</td>
<td>1.08</td>
<td>Alcoholic liver disease</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>8</strong></td>
<td>39</td>
<td>M</td>
<td>Not performed</td>
<td>Alcoholic liver disease</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>9</strong></td>
<td>67</td>
<td>M</td>
<td>Not performed</td>
<td>Alcoholic liver disease</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>10#</strong></td>
<td>71</td>
<td>M</td>
<td>1.16</td>
<td>Non-alcoholic Steatohepatitis</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>11</strong></td>
<td>56</td>
<td>M</td>
<td>0.99</td>
<td>Non-alcoholic Steatohepatitis</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>12</strong></td>
<td>70</td>
<td>F</td>
<td>0.93</td>
<td>Non-alcoholic Steatohepatitis</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>13</strong></td>
<td>56</td>
<td>M</td>
<td>Not performed</td>
<td>Non-alcoholic Steatohepatitis</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>14</strong></td>
<td>58</td>
<td>M</td>
<td>Not performed</td>
<td>Non-alcoholic Steatohepatitis</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>15</strong></td>
<td>56</td>
<td>F</td>
<td>0.94</td>
<td>Chronic Hepatitis C</td>
<td>MZ</td>
</tr>
<tr>
<td><strong>16</strong></td>
<td>72</td>
<td>M</td>
<td>Not performed</td>
<td>Chronic Hepatitis C</td>
<td>MZ</td>
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<tr>
<td>17</td>
<td>48</td>
<td>M</td>
<td>Not performed</td>
<td>Chronic Hepatitis C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MZ</td>
</tr>
<tr>
<td>18</td>
<td>63</td>
<td>F</td>
<td>Not performed</td>
<td>Chronic transplant rejection and hepatitis C (19 years apart)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MZ</td>
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F Female; M Male; A1AT Alpha 1-Antitrypsin; g/L grams per deciliter

# Genotyped and confirmed preoperatively

**Discussion**

It’s not uncommon to encounter cases of hepatic transplant whereby the liver shows exuberant periportal PASD+ globules highly suspicious for A1AT accumulation, with subsequent confirmation via A1AT immunohistochemistry(7, 19). A majority of these cases had either a negative preoperative screening result (i.e. a serum concentration within normal range) or no pre-transplant screening for A1AT deficiency. In our province, a negative A1AT screening result usually translates to no further clinical testing (in the absence of additional information eg PASD positive inclusions) and likely contributes to an underdiagnoses of heterozygous A1AT deficiency and a missed opportunity to screen family members of those carriers. Therefore, A1AT serum testing is not sufficiently sensitive to detect all heterozygous cases, or cases with rare allele variants which are more difficult to detect (e.g. M Malton allele). Recent studies suggest that heterozygous states (e.g. MZ) can still show subtle liver phenotype which may, solely or additively, lead to the development of chronic liver disease and subsequent cirrhosis requiring transplantation, further cementing the need to detect carriers of abnormal alleles and perform subsequent familial testing(8, 9).
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Our aim is to assess if such cases can be molecularly tested postoperatively on the liver explant specimen and therefore capture abnormal A1AT alleles that were not identified prior to transplantation.

The results of our study show that all of the cases that are positive for A1AT globules expectedly showed Z allele, both heterozygous and homozygous. This suggest that FFPE PCR testing of explant tissue is a valid method for the detection of Z allele and that PASD+ globules are highly reliable predictor for the Z alleles. It also demonstrates that preoperative A1AT serum testing alone is not sufficiently sensitive for the detection of this risk predisposing condition, given that most of our patients had normal A1AT serum level prior to transplant hepatectomy. It can also help explain a portion of cases labeled ‘cryptogenic cirrhosis’.

The use of PASD and A1AT positive periportal globules as a surrogate marker for the Z allele had been previously utilized in as early as 1980 by Clausen et al whereby liver biopsies were assessed for A1AT globules(19). 34 of 850 biopsies (4%) demonstrated PASD and A1AT positive globules, and therefore assumed to harbor a Z allele variant. Half of their positive biopsies showed either fibrotic or cirrhotic livers, suggesting that the Pi*Z allele in both homozygous and heterozygous forms correlate with the development of liver cirrhosis. The genotypes of these cases however were not confirmed with subsequent testing.

Moreover, not all patients who are heterozygous for the Z allele demonstrate PASD/A1AT positive globules, as is demonstrated by their follow-up study in 1984, showing that only 47% of patients with a confirmed (via Isoelectric Focusing) Pi*Z allele demonstrate PASD/A1AT positive globules (>3um) on their liver tissue histology(20). This suggest that the globules, albeit specific, are not sufficiently sensitive or reliable as a screening tool for heterozygous Z allele.
In 1997, Iezzoni et al performed PASD stain on liver explant specimens from cases with cirrhosis and found that 10% (17 of 171) demonstrated PASD and A1AT positive globules, which is similar to the 12% (18 of 142) we demonstrated in our study. The etiology for the majority of their cases were attributed preoperatively to causes other than A1AT deficiency. Furthermore, a subsequent Isoelectric Focusing elucidated homozygous or heterozygous Z phenotype in most of their cases (11 of 17; 65%), while one demonstrated SS phenotype (1 of 17; ~5%) and the remainder were of the MM phenotype (4 of 17; 25%). They concluded that an unsuspected PASD/A1AT positive globules, regardless of the presence or absence of other concomitant causes of chronic liver disease, should prompt the pathologist to alert the clinician of the possibility of an A1AT abnormality.

It is important to pay attention to the distribution of the globules; Qizilbash et al in 1983 had performed PASD on liver tissue from 500 autopsy cases and subsequently classified the PASD+ globules into type 1, whereby the globules are periportal in distribution and positive for A1AT immunoperoxidase stain, and type 2 which are centrilobular and negative for the aforementioned stain. The majority (10 out of 14; 71%) of the cases with type I globules showed cirrhosis and a few demonstrated dysplastic foci.

Lincoln et al. successfully validated real-time PCR assay to detect the S and Z alleles in FFPE liver tissue in 2018 and demonstrated that PCR testing of FFPE tissue is potentially a good alternative to peripheral blood tests.

Molecular DNA testing of FFPE tissue offer multiple advantages including the potential to test anatomic pathology specimens, eliminating the need to bring-in patients for additional peripheral blood sampling. Moreover, in certain instances (such as case #18), the liver specimen under investigation is not native to the patient, but instead is an allograft. Therefore, whole blood-based
genotyping may not accurately reflect the presence of A1AT deficiency in the patient due to potential pathology in the allograft liver. More recently, a case has been reported in the literature involving a patient who underwent allogeneic hematopoietic stem cell transplantation following liver transplantation(22). It is also possible that advances in immunology and transplant medicine could enable patients who have undergone allogeneic stem cell transplant to become eligible for liver transplantation in the future. In such cases, direct testing of liver tissue may provide a more reliable means of detecting A1AT deficiency cases that might otherwise be missed through whole blood-based genotyping.

PCR testing is relatively rapid, inexpensive and easier to interpret. One pitfall is detecting a false negative wild-type allele on real-time PCR when the actual liver histology shows convincing evidence of A1AT deficiency (i.e. classic PASD/A1AT positive globules). In such cases, additional testing such as Sanger sequencing can be performed on blood samples to detect rare alleles. Testing FFPE tissue is not exempt from limitations. It is not uncommon to encounter assay failures due to technical or specimen-related issues, such as small biopsies with low cellularity, improper fixation time, DNA fragmentation, and degradation. The latter is a significant factor in archived FFPE tissue, particularly after prolonged storage(23). In such cases, peripheral blood testing might be necessary. In addition, PCR testing does not detect rare allele variants such as M Malton, and therefore, a more definitive assay for rare allele detection might be required.

In conclusion, screening for Alpha 1-Antitrypsin deficiency using serum A1AT levels is not sufficiently sensitive to detect all A1AT deficiency carriers, leading to under diagnosis of the carrier status. If A1AT genotyping was not performed preoperatively and the risk is high based on the histological presence of PASD and A1AT positive globules in the explanted liver, then
molecular testing of FFPE tissue can be a viable method for confirming the diagnosis of A1AT deficiency. From our study, we also find that PASD and A1AT positive globules in the periportal distribution are highly reliable predictors for the presence of the Z allele. In addition, we advocate for the routine use and careful examination of the PASD special stain in explanted livers to detect A1AT globules with subsequent confirmation by A1AT immunohistochemistry, even in cases with negative PASD in the preoperative biopsies.

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