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LIVER DISEASE AND HPLC QUANTIFICATION OF DISIALOTRANSFERRIN FOR HEAVY ALCOHOL USE: A CASE SERIES

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Abstract

Background—It had previously been suggested that individuals with cirrhosis may have a pattern of transferrin glycosylation that interferes with the interpretation of carbohydrate-deficient transferrin (CDT) testing for heavy alcohol use. The goal of this case series was to evaluate the prevalence of liver disease among individuals with poor resolution of transferrin glycoforms by high performance liquid chromatography.

Methods—We reviewed the electronic medical records of 35 consecutive patients with poor chromatographic resolution of disialotransferrin from trisialotransferrin, and recorded information on diagnosed liver disease, liver function testing, and other factors.

Results—Thirty of the 35 subjects with poor chromatographic resolution of the transferrin glycoforms had sufficient data in the medical record for some estimation of liver function. Of these 30 subjects, 25 had previously diagnosed liver pathology. Of the remaining 5 subjects, 2 had liver imaging results suggestive of benign tumor; the remaining 3 had mildly elevated bilirubin and aminotransferase activity, and low albumin.

Conclusions—Liver abnormalities, but not necessarily cirrhosis, are common in individuals with poor chromatographic separation of transferrin glycoforms, which might lead to false positive results on CDT testing. However, the chromatographic-based assay can detect this issue, minimizing the reporting of false positives, but not necessarily assisting in valid detection of heavy drinking.

Keywords

liver disease; carbohydrate-deficient transferrin

INTRODUCTION

Transferrin is an abundant serum glycoprotein and functions as the main transporter of iron from sites of absorption to sites of utilization (de Jong et al., 1990). It is synthesized in the liver, and has two asparagine residues that are typically modified by glycosylation. The major structure at each glycosylation site is a biantennary oligosaccharide, with each antenna being capped by a sialic acid residue (Leroy, 2006). Thus the predominant transferrin glycoform is tetrasialotransferrin. Other glycoforms that exist under normal conditions include hexasialo-, pentasialo-, trisialo-, and disialo-transferrin. Stibler

discovered that chronic heavy drinking can cause a decrease in transferrin sialylation (Stibler et al., 1978), which came to be known as carbohydrate-deficient transferrin, and was developed as a biomarker for chronic heavy drinking (Anton, 2001). Further research demonstrated that heavy drinking was associated with a complete absence of one oligosaccharide, resulting in an elevation of disialotransferrin (due to a normal biantennary structure at the other N-glycosylation site) (Flahaut, 2003). These insights into the effects of heavy alcohol use on transferrin eventually led to more specific assays for carbohydrate-deficient transferrin as an alcohol biomarker. One such assay involves a high performance liquid chromatography (HPLC) separation of transferrin glycoforms (Helander et al., 2003), with disialotransferrin being quantified as a percentage of total transferrin (referred to as %dCDT in the remainder of this report). This assay has been suggested as the reference standard for carbohydrate-deficient-transferrin testing worldwide (Jeppsson et al., 2007).

The HPLC-based %dCDT assay is approximately 95% specific for heavy drinking if levels are greater than or equal to 1.7%, but there are known factors that limit the utility of the test (Bergstrom and Helander, 2008). Most pertinent to this report, Arndt et al reported on a series of 21 subjects with poor chromatographic resolution of disialotransferrin from trisialotransferrin (i.e., di-tri bridging). In acquiring clinical information about these cases, they discovered that 19 had evidence of liver disease, and hypothesized that an alteration in transferrin glycosylation due to liver cirrhosis was the cause of the poor separation (Arndt et al., 2008). This was in contrast to an earlier hypothesis that the di-tri bridging was caused by a genetic variant (Helander et al., 2003). Our laboratory performs the same HPLC assay for %dCDT, and is affiliated with a university medical center, with access to detailed electronic medical records. We retrospectively evaluated the electronic medical records of patients who on clinical %dCDT testing had chromatograms indicative of di-tri bridging, with a particular focus on assessments of liver function.

METHODS

Subjects

Subjects consisted of 35 consecutive patients at our affiliated university medical center, who were identified by the finding of di-tri bridging on their transferrin chromatograms (illustrated in Figure 1B) during routine clinical testing. Permission was obtained from the appropriate Institutional Review Board to review the electronic medical records of these subjects.

Determination of di-tri bridging

Serum %dCDT was measured by ion-exchange chromatography at the Clinical Neurobiology Laboratory at our institution (directed by one of the authors, RFA). A gradient salt and pH based chromatographic procedure separates transferrin into various isoforms depending on charge and molecular weight as illustrated in Figure 1A (Helander et al., 2003). Bridging (lack of adequate separation of disialo and trisialo-transferrin) was initially determined by laboratory personnel with experience in the interpretation of transferrin chromatograms. The authors collectively verified that bridging was present in each case.

Medical record review

Information from the electronic medical record was abstracted by one of the authors (SHS), who is an experienced Internal Medicine clinician. Data included basic demographics, previously diagnosed liver disorders and other medical conditions, alcohol, tobacco, and drug use, liver imaging studies and biopsy results if available, and laboratory values reflective of liver function. Of note, gamma-glutamyltranspeptidase (GGT) is no longer included in routine laboratory testing, and was not measured in the subjects included in this

report. Abstracted laboratory values included all parameters utilized in the calculation of the Model for End Stage Liver Disease (MELD) score (Kamath et al., 2001), and the Child-Turcotte-Pugh (CTP) score (Child and Turcotte, 1964; Pugh et al., 1973). MELD scores were calculated with the modification used by the United Network for Organ Sharing, with higher scores indicating more severe disease. The CTP score was categorized as class A, B, or C, reflecting increasing severity of disease. In addition to laboratory parameters, complete CTP scoring also includes degree of ascites and encephalopathy; however, since this information was not consistently available in the electronic records, these factors were not considered. As a result, the CTP scoring system we used may underestimate disease severity.

RESULTS

A normal transferrin chromatogram is illustrated in Figure 1A, in which the percentage of total transferrin existing in the disialo form (i.e., %dCDT) is estimated by determining the total area under the curve and the area attributable to disialotransferrin. Conversely, as illustrated in Figure 1B, di-tri bridging does not allow accurate quantification of disialotransferrin due to suboptimal separation from trisialotransferrin. We identified 35 patients from our medical center with di-tri bridging over a 9-month period, 30 of whom (23 males) had sufficient data for an estimation of liver impairment. Ten currently smoked and 20 records included mention of current or past heavy drinking (of note, the %dCDT would have been ordered by the health care provider to screen for heavy alcohol use in each case, even when this is not mentioned in the medical record). Four subjects were recorded to be current users of other drugs (2 marijuana, 1 cocaine, and 1 “polysubstance” without further description).

Table 1 includes a summary of the 30 evaluable cases. Twenty seven had at least mild elevation in aspartate aminotransferase (AST) or alanine transaminase (ALT). Twenty-five subjects had a previously diagnosed alcoholic (n=5) or non-alcoholic (n=20) liver disorder. Hepatitis C with or without a history of heavy drinking was the most common diagnosis, and was present in 15 subjects. Two of the 5 subjects without a previously diagnosed liver condition had imaging findings interpreted as benign liver tumors (including focal nodular hyperplasia on MRI with otherwise normal liver tests, and a 1×2 cm “hypodensity” on CT scan with a slight increase in AST). The remaining 3 subjects had some abnormalities in liver function testing, including elevations in total bilirubin, international normalized ratio (INR), or aminotransferase activity, or low albumin. There were 8 subjects with liver biopsy data in the medical record, 7 of whom had a diagnosis of Hepatitis C. Four were interpreted as cirrhosis. The remaining 4 had undergone liver biopsy at least one year prior to the finding of di-tri bridging and inclusion in this case series, and had variable degrees of fibrosis (2 with Stage 1 fibrosis, and 2 with Stage 2 fibrosis) (Batts and Ludwig, 1995).

Twenty-seven subjects had sufficient data for calculation of Child-Turcotte-Pugh and MELD scores. Four were classified with relatively milder disease (CTP Class A), including the patient with presumed focal nodular hyperplasia based on radiographic appearance, a patient with mild biochemical changes but known cirrhosis based on distant biopsy and recent imaging studies, and two patients with chronic Hepatitis C (one of whom had a liver biopsy one year prior with moderate inflammation and non-cirrhotic fibrosis). Thirteen subjects (10 of whom had known liver disease) were Class B, and 10 subjects (all of whom had known liver disease) were Class C by CTP criteria. There were three subjects who did not have sufficient data for calculation of CTP and MELD scores (INR was not measured in these cases). Two had a history of Hepatitis C and elevated aminotransferases during their hospitalization, but normal bilirubin and mildly reduced to low normal albumin. The third

was the patient with the “hypodensity” on CT scan 18 months prior to %dCDT testing, who had a minimal elevation in AST activity.

DISCUSSION

This case series supports the hypothesis of Arndt et al. (2008) that poor resolution of disialotransferrin from trisialotransferrin when screening for heavy drinking is associated with liver disease; however, this chromatographic pattern is probably not specific for alcoholic liver disease or cirrhosis (a very advanced form of liver disease). While we did not do genetic analysis of the subjects with the di-tri bridging pattern, the fact that it occurred in strong association with liver disease suggests our results are in line with that of Arndt and colleagues (2008), and that the most likely cause of this pattern is liver pathology rather than genetic variation per se. Of note, our lab rarely sees this pattern in populations where liver disease has been vigorously excluded (e.g., clinical trial subjects), but frequently in patients whose samples come from liver clinics and acute care hospitals. In testing for heavy drinking, di-tri bridging could lead to false positive results for alcohol use if not interpreted correctly, and this phenomenon may underlie prior findings on low specificity of alternative CDT immunoassays for heavy drinking among liver disease patients (DiMartini et al., 2001; Heinemann et al., 1998). However, as the HPLC assay enables detection of this effect, proper interpretation will minimize false positive results associated with liver disorders.

There are two compelling clinical indications for conducting additional research on the interactions of heavy drinking and liver disease on transferrin glycosylation. For one, such studies may enhance the accuracy of carbohydrate-deficient transferrin screens for chronic heavy drinking (e.g., the absence of one entire N-glycan may remain relatively specific for heavy drinking (Flahaut, 2003), even in the presence of bridging). In addition, while liver disease in general is known to cause abnormal protein glycosylation (Mehta and Block, 2008; Morelle et al., 2006), specific effects on glycosylation may depend on the etiology of liver disease (Blomme et al., 2009). Therefore, as suggested by Arndt et al, specific glycoforms of transferrin (as opposed to bridging, which could be caused by several transferrin variants) may indicate the presence of otherwise unsuspected alcoholic liver disease.

There are two important limitations to our conclusions. This case series included subjects with a variety of liver disease diagnoses, and results suggest that the chromatographic bridging pattern may be caused by both non-alcoholic and alcoholic liver disease. However, we were not able to accurately confirm if patients were actively drinking, and had little information on life time alcohol use. Also, although there was evidence that some subjects did not have clinically advanced liver problems, non-invasive estimates are imperfect measures of liver disease severity (D’Amico et al., 2006), and these scores are best used to estimate the severity of illness in patients with cirrhosis rather than differentiate cirrhotic from non-cirrhotic liver disease (e.g., even cirrhotic patients can be categorized as CTP Class A). Therefore, it remains possible that di-tri bridging is only present in subjects with advanced liver disease. Few of our subjects had undergone recent liver biopsy, which would be required to confirm di-tri bridging in patients with non-cirrhotic liver disease.

In summary, %dCDT is in general a highly specific test for chronic heavy drinking, but liver disease and perhaps other factors can result in suboptimal chromatographic resolution of disialotransferrin from trisialotransferrin. In such cases %dCDT can not be quantified in a reliable manner, and our practice is to report di-tri bridging as “suggestive of liver disease”. Additional research may establish the molecular basis for this effect, more definitively evaluate the role of acute and chronic alcohol intake on this phenomenon, and provide even more valid transferrin assays for heavy drinking with or without liver disease.

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Figure 1.

A) A normally resolved transferrin chromatogram. “di” indicates disialotransferrin, “tri” indicates trisialotransferrin, and so forth.

B) Poor chromatographic resolution of disialotransferrin from trisialotransferrin (i.e., di-tri bridging).

Table 1

Subject Characteristics

CTP Class ^a	Sex	Age	Clinical Liver Diagnosis	AST (12–38 IU/L)	ALT (10–45 IU/L)	Total bilirubin (0.2–1.3 mg/dL)	INR ^b (0.8–1.2)	Albumin (3.5–4.8 g/dL)	MELD ^c
A	F	49	Benign tumor [*]	23	23	0.8	1.0	3.7	7
	F	56	Hepatitis C	207	103	0.7	1.2	3.9	8
	F	57	Hepatitis C	102	67	0.8	1.2	3.1	8
B	M	57	Hepatitis C	167	78	0.5	1.1	3.7	8
	F	57	NASH	93	62	2.0	1.5	2.2	19
	F	57	Cryptogenic	51	25	4.6	1.5	2.5	17
	M	41	Cirrhosis, unspecified	140	114	1.3	1.6	2.4	12
	M	43	---	150	85	1.4	1.4	2.1	11
	M	45	---	53	46	1.2	1.2	2.7	12
	M	47	Hepatocellular cancer	123	39	5.6	1.5	2.1	13
	M	52	Hepatitis C	90	45	1.1	1.3	2.0	19
	M	53	Hepatitis C	270	141	4.2	1.6	2.8	17
	M	54	---	118	54	1.6	1.2	1.9	10
	M	57	Hepatitis C, alcohol	70	28	2.5	1.6	2.3	19
	M	64	Cirrhosis, unspecified	37	20	2.5	1.4	3.1	16
C	M	65	Cholangiocarcinoma	79	61	7.4	1.4	1.7	18
	M	69	Alcoholic liver disease	38	14	2.4	1.6	2.9	15
	F	46	Alcoholic cirrhosis	70	24	11.1	2.8	2.7	27
	F	53	Hepatitis C	92	46	2.5	1.8	2.4	17
	M	47	Hepatitis C, NASH	76	44	14.4	2.4	2.5	27
	M	49	Hepatitis C, alcohol	88	20	3.0	1.9	1.9	20
	M	50	Hepatocellular cancer	278	49	6.7	1.0	2.6	14
	M	53	Alcoholic liver disease	115	88	3.1	1.7	1.9	18
	M	54	Hepatitis C	84	55	4.7	2.4	2.9	23
	M	54	Hepatitis C	192	65	3.9	1.8	1.3	25
	M	57	Hepatitis C	195	104	3.4	1.8	2.0	21

CTP Class ^a	Sex	Age	Clinical Liver Diagnosis	AST (12–38 IU/L)	ALT (10–45 IU/L)	Total bilirubin (0.2–1.3 mg/dL)	INR ^b (0.8–1.2)	Albumin (3.5–4.8 g/dL)	MELD ^c
Unknown	M	61	Hepatitis C	248	121	24.2	2.4	3.5	30
	M	44	Hepatitis C	91	53	1.0	---	3.3	---
	M	55	Hepatitis C	91	53	1.0	---	3.7	---
	M	64	Benign tumor ^{**}	53	45	0.7	---	4.2	---

^aCTP class = Child Turcotte Pugh score, with A, B, and C suggesting mild, moderate, and severe liver disease respectively.

^bINR = International normalized ratio, a dimensionless measure, with higher levels suggesting decreased production of liver-synthesized blood clotting factors and greater risk for bleeding

^cMELD = Model for End Stage Liver Disease score with UNOS modification, with higher scores suggesting greater severity of liver disease. The MELD score is only clinically meaningful in liver disease patients, but in a perfectly healthy individual would typically be 6 or 7 (occasionally as high as 9 in females and 12 in males).

* Probable focal nodular hyperplasia (a benign proliferation of liver cells) on MRI

** Probable benign liver tumor (1×2 cm hypodensity on CT scan 18 months prior to %dCDT testing).