RESEARCH NOTE

Discordant results between biochemical and molecular transthyretin assays: lessons learned from a unique testing algorithm at the Mayo Clinic

HONEY V. REDDI[1]*, BRITTANY C. THOMAS[1], KURT S. WILLKOMM[1], MATTHEW J. FERBER[1], KANDELARIA M. RUMILLA[1], KIMIYO M. RAYMOND[2], JOHN F. O'BRIEN[2] and W. EDWARD HIGHSMITH[1]*

[1]*Molecular and* [2]*Biochemical Genetics Laboratories, Department of Laboratory Medicine and Pathology, Mayo Clinic School of Medicine, Rochester, MN 55905, USA*

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Introduction

Genetic testing involves the detection of genetic changes that allows for appropriate diagnosis, management and treatment of a variety of inherited conditions, involving either direct testing of the DNA itself (molecular) or evaluation of the gene products such as enzymes and other proteins (biochemical). In a two-component test, discordance between biochemical and molecular results can cause a significant delay in test turn-around time with wasted costs, as tests are repeated and the potential for sample mix-up is thoroughly investigated. Our study evaluating possible causes for discordance in transthyretin testing at Mayo Clinic identifies domino liver transplants to be the most prevalent cause for discordant results, emphasizing the need for proactive communication between the clinician and testing laboratories.

Familial amyloidosis (Hawkins 2003) can be caused due to mutations in a number of genes including transthyretin (*TTR*), apolipoprotein A-I (*APOA1*), apolipoprotein A-II (*APOA2*), gelsolin (*GSN*), fibrinogen (*FGA*) and lysozyme (*LYZ*), resulting in deposition of amyloid in a variety of tissues. Of these, mutations in the transthyretin gene account for ~90% of the familial amyloidosis (Saraiva 2002). TTR is a normal serum protein synthesized primarily in the liver and involved in the transport of thyroxine. Functional TTR is a homotetramer and mutations in the *TTR* gene results in altered TTR structure, allowing amyloid deposition most frequently in the heart and peripheral nervous system (Saraiva 2002). Amyloidosis due to mutations in transthyretin - *TTR* gene (ATTR) is not solely due to inherited changes in the *TTR* gene and majority of ATTR seen in octogenarians is due to deposition of TTR with the wild type sequence, so called senile systemic amyloidosis (SSA) (Westermark *et al.* 2003; Ruberg and Berk 2012) or 'age-related' amyloidosis.

Diagnosis of amyloidosis involves Congo red staining of biopsied tissues to confirm amyloid deposition, which demonstrates a characteristic apple-green birefringence under polarized light. Amyloid protein typing is carried out either using immunohistochemical staining of an affected tissue biopsy with antisera to TTR, κ and λ light chains, and amyloid A (Fernandez-Flores 2011; Satoskar *et al.* 2011) or liquid chromatography and tandem mass spectrometry (LC/MS/MS) of tryptic digests of microdissected amyloid plaques (Vrana *et al.* 2009; Linke 2012). Detection of one of the proteins involved in the hereditary forms of amyloidosis requires follow-up genetic testing to confirm the diagnosis (Shiller *et al.* 2011).

Noninvasive testing interrogates the molecular weight of circulating TTR in serum to screen for the presence of inherited amyloidogenic missense changes in the TTR protein, wherein amino acid changes are reflected by a shift in mass/charge of the intact TTR protein detected by mass spectrometry (MS) (Sethi *et al.* 2012). Variants with amino acid changes giving mass shifts of greater than 10 Da from the wild-type mass of 13,800 Da are easily detected, with a specific mass shift associated with each amino acid change (figure 1A) (Connors *et al.* 2003; Bergen *et al.* 2004; O'Brien and Bergen 2009). A negative test result decreases the likelihood of hereditary ATTR; however, as MS cannot specify the type of amino acid substitution, follow-up with full gene sequencing is required when clinical suspicion remains high. Sanger sequencing performed by the Mayo Clinic Molecular Genetics Laboratory (MGL) identifies an estimated 99% of the disease causing mutations as well as benign

*For correspondence. E-mail: Honey V. Reddi, honeyreddi@gmail.com; W. Edward Highsmith, highsmith.w@mayo.edu.

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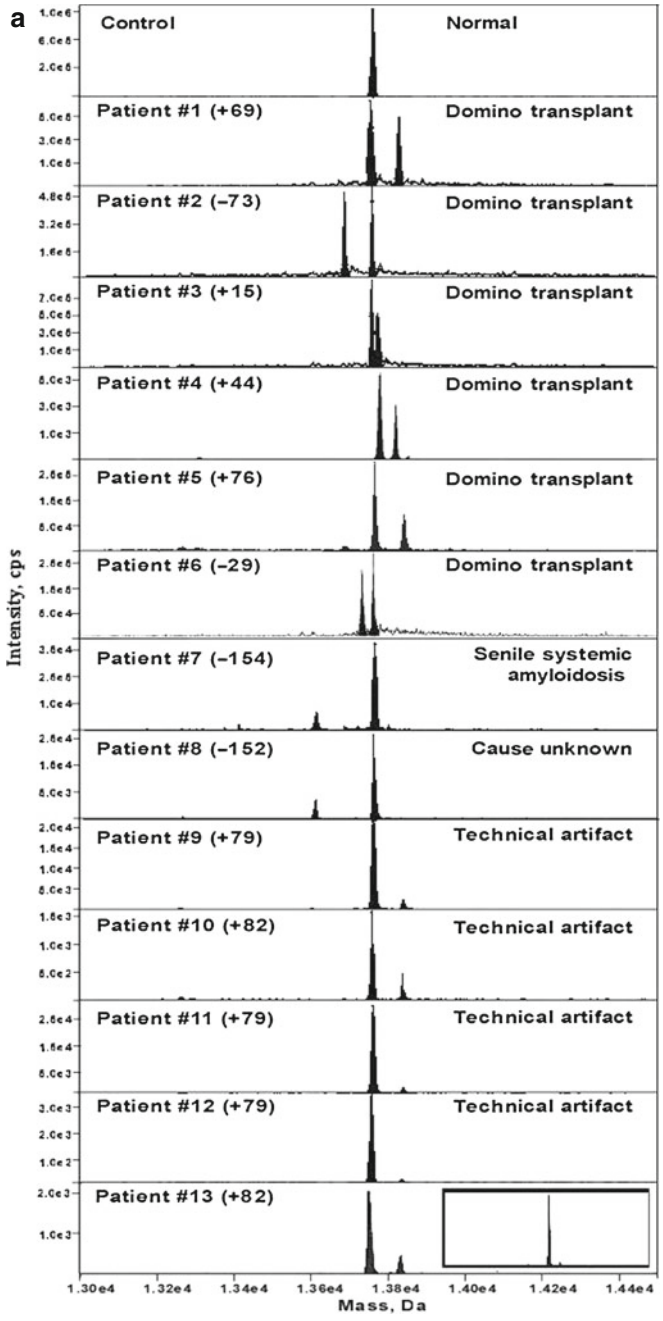

Figure 1A. Mass spectrometry results of 13 cases of transthyretin testing which exhibited presence of variant peaks with no detectable germline mutations. Of the 206 cases with MS results, 13 cases (patients 1–13) had structural changes evidenced by an extra peak (mass listed) in addition to the wild-type peak at about 13,800 Da (normal). Normally, variant peaks are observed due to mutations in the TTR gene that result in the production of mutant protein. Patients with domino transplants will also have variant peaks due to the presence of mutant protein from the donor liver (patients 1–6). In some cases, variant peaks could be due to the presence of fragmented TTR protein in addition to wildtype as seen in patients with SSA (patient 7) or due to other unknown reasons (patient 8). Variant peaks may also be observed if the posttranslational modifications of TTR such as the addition of sulphite in particular are not completely reduced (patients 9–13). In the case of patient 13, we show the initial MS peaks (patient 13) and after reduction (inset) to demonstrate that the variant peak in these cases is due to incomplete reduction of sulphite on the TTR.

polymorphisms which give rise to mass shifts in the MS assay (Benson *et al.* 2002). Though MS for TTR is offered as a screening test that is reflexed to sequencing for follow-up of abnormal TTR detected in serum, full gene sequencing can be independently ordered if the clinical suspicion of familial ATTR is high.

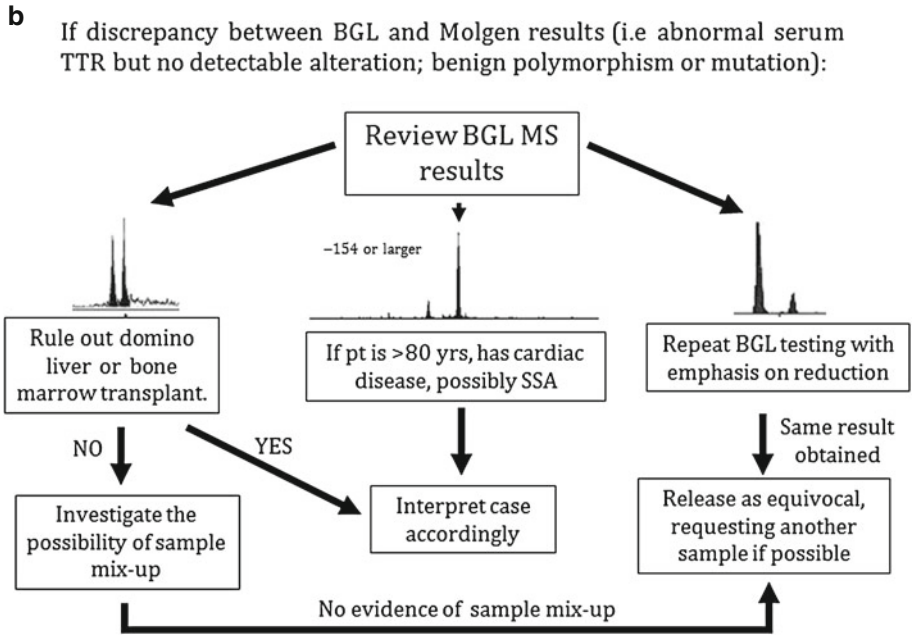

Figure 1B. Schematic for proposed course of action to be taken when a discordant result is observed within the molecular genetics laboratory.

Multiple factors including both technical and biological could potentially negatively impact the results of genetic testing and yield either false positive or false negative results. Technical errors, such as those in sample processing, analysis, or interpretation impact test outcome and when discordance between biochemical and molecular test results occurs, sample mix-up is typically one of the first possibilities to be investigated. Reextracting DNA, repeating testing and double-checking order paperwork can cause a significant delay in test turn-around time. In addition, substantial costs can be incurred by the laboratory, much of which will ultimately prove to be wasted when other explanations for discordant results are found.

Organ and bone marrow transplant or blood transfusions of the patient at the time of testing also contribute to discordant results (Hackbarth *et al.* 2010). Domino liver transplant is an organ conserving maneuver in which an individual with a metabolic disorder, such as ATTR, undergoes transplant from either a living or cadaver donor. The liver from the ATTR patient is healthy even though it produces abnormal TTR, and can be transplanted into a high-risk patient (either due to malignancy or advanced age) with end-stage liver disease who otherwise would not have been a candidate for a cadaveric liver (Dyer and Bobrow 1999; Nunes *et al.* 2004). Because the recipients of the ATTR liver have life expectancies shorter than the time needed to develop disease symptoms from the domino transplant, it is possible to perform two life-saving transplant procedures with a single donor organ. From a genetic testing perspective, however, if not known by the laboratory at the time of testing, domino liver transplant in a patient being tested for serum transthyretin leads to a conundrum, since the patient will have abnormal serum TTR by mass spectrometry but no germline mutations on follow-up sequencing using DNA isolated from the peripheral blood (Stangou *et al.* 2005). Similar discordant results could also be obtained in cases of patients who are recipients of bone marrow from ATTR patients (Chee *et al.* 2010).

Since transthyretin testing includes both biochemical and molecular test components, we use this as an example to understand the possible causes for discordance and attempt to establish measures that will minimize or prevent wasted costs and decrease turn-around time for this gene assay.

Materials and methods

We reviewed all cases of TTR full gene sequencing at the MGL during the period, April 2004 through January 2012. Data was evaluated to identify samples that were sequenced as a follow-up to abnormal MS results from the Biochemical Genetics laboratory (BGL), focussing on samples with abnormal serum TTR as identified by MS and no detectable germline mutations by full gene sequencing. For both full gene sequencing and MS testing peripheral blood was used. For Mayo Clinic patients, information from the electronic medical record (EMR) was extracted to identify potential causes for discordant results, tabulated without patient identifiers and maintained in a confidential manner per guidelines of the Mayo Clinic Institutional Review Board.

Table 1. Evaluation of all transthyretin tests, sequencing and serum MS at the Clinical Molecular and Biochemical Laboratories, Mayo Clinic.

TTR testing: 2843 sequencing and 1293 MS tests at Mayo Clinic (April 2004 through January 2012)

206 cases		Structural change in TTR identified by MS suggestive of a germline mutation
35%	72 of 206	Pathogenic alteration in TTR gene accounting for observed structural change
59%	121 of 206	Benign polymorphism in TTR gene accounting for observed structural change
6%	13 of 206	No detectable mutation or polymorphism to account for observed structural change

Cases with discordant results (13 of 206)

Patient	Gender	Age (yrs)	Clinical symptoms	Mass difference observed	Cause of discordant results
1	M	67	Hepatocellular carcinoma	+69	Domino liver transplant
2	F	52	Hepatocellular carcinoma	−73	Domino liver transplant
3	M	72	Cryptogenic cirrhosis	+15	Domino liver transplant
4	F	60	Liver and renal failure	+44	Domino liver transplant
5	F	70	Cirrhosis of the liver	+76	Domino liver transplant
6	M	63	Hepatocellular carcinoma	−29	Domino liver transplant
7	M	87	TTR amyloid deposits, cardiomyopathy, congestive heart failure	−154	Senile systemic amyloidosis
8	M	58	Autonomic neuropathy, nerve biopsy amyloid negative, death from cardiac failure	−152	Not known
9	F	56	IgA multiple myeloma, BM biopsy +ve for TTR amyloid	+79	Technical artifact
10	F	62	Medical history not available	+79	Technical artifact
11	F	27	Medical history not available	+82	Technical artifact
12	F	5	Medical history not available	+79	Technical artifact
13	F	84	Medical history not available	+82	Technical artifact

Results

A total of 2483 samples were sequenced for the presence of mutations in all four exons of the TTR gene in MGL and a total of 1293 samples were evaluated for serum TTR by MS testing in BGL during the period of April 2004 through January 2012. BGL samples, 206 were reflexed to sequencing due to the detection of abnormal serum TTR protein suggestive of a germline variant and form the cohort of samples evaluated are outlined in table 1. Full gene sequencing detected a pathogenic alteration in 35% (72 of 206) and a benign polymorphism in 59% (121 of 206) of cases. However, we did find that 6% (13 of 206) of cases had no detectable germline mutations accounting for the observed mass shift (figure 1A).

Review of clinical history revealed that six of 13 patients (46%) of discrepant cases were recipients of domino liver transplants (figure 1A, patients 1–6) and one of 13 had SSA (figure 1A, patient 7). One patient had a variant peak (−154 kDa) similar to that observed with the patient with SSA (patient 8, figure 1A) and five of 12 (38%) of cases had variant peaks whose peak height reduced on repeat analysis (patient 13, inset demonstrates repeat analysis with emphasis on reduction) but did not completely disappear suggesting a possible reduction artifact.

Discussion

Genetic diagnosis has a significant impact on patient care and management, particularly for inherited disorders. It is therefore of prime importance that testing and reporting of results is done with the utmost care and quality. Analysis of the 206 samples which had a structural change in TTR by MS identified that 35% had a pathogenic alteration and 59% had a benign polymorphism that could account for the structural change observed (table 1). In those cases with benign polymorphisms, it may be concluded that the clinical phenotype observed was not due to mutant TTR. For 6% of cases with structural changes in the TTR protein wherein no detectable germline mutation was present, domino liver transplant was identified to be the cause of discordance in 46% of these cases (six of 13, patients 1–6, figure 1A). An organ transplant, such as liver, particularly when testing for TTR, is a critical piece of information that needs to be communicated to the testing laboratory. Retrospectively, we now know that this information could have avoided a significant delay in test turn-around time and wasted costs, given that tests were repeated, and the potential for sample mix-up was thoroughly investigated and ruled out. Domino liver transplants are becoming more common, as the range of disorders with livers suitable for transplantation, such as methylmalonic acidemia (http://ucsdnews.ucsd.edu/newsrel/health/2011_

09hemming-gish.asp) and maple syrup urine disease (http://whsc.emory.edu/home/multimedia/videos/domino-liver-transplant.html) expand, emphasizing the need for transplant information upfront.

We observed two cases (patients 7 and 8), in which a minor peak was seen by MS indicating a loss of 154 Da. The first two amino acids at the amino terminus of the circulating form of TTR, Gly–Pro, have a combined mass of 154 Da, suggesting that the MS is detecting an uncommon cleavage product. One of the patients (7) with the 154-Da peak had a diagnosis of SSA, however, patient 8 with a 152-Da peak, was not of the age typically associated with senile amyloidosis. Cardiac amyloid deposits in SSA have been reported to be composed of wild-type TTR including both full length and C-terminal fragments (Westermark *et al.* 2003). It is the presence of the C-terminal fragments that account for the shift in MS observed in these cases; however, their clinical or diagnostic significance is unknown.

About a third of our cohort (38%) had variant peaks with mass shifts of +79–82 which had low peak heights (<25% of the height of the wild-type peak) (patients 9–13), suggesting it was not due to a heterozygous amino acid substitution. In the event of a heterozygous change, we expect to see two peaks of more or less equal height as observed in patients 1–6 (figure 1A). TTR undergoes various posttranslational modifications resulting in different masses when measured by MS including the addition of sulphite (+80), cysteine (+119), cysteineglycine (+176) and glutathione (+305). TTR in the plasma is reduced with dithiothreitol (DTT) to simplify the mass spectra by removing adducted species prior to loading on to a TTR immuno-affinity column. Complete reduction of TTR results in a single peak on the spectra, indicative of wild-type full-length TTR. Incomplete reduction results in an additional peak with molecular weight equal to the wild type plus the adduct, typically sulphite. As the incomplete reduction peak is typically small, it is distinguishable from abnormal TTR which demonstrates almost equal height peaks in case of a heterozygous mutation (figure 1, patients 1–6 compared to patients 7–13). In the case of patients 9–13, we therefore hypothesized that the extra peak observed was due to partial or incomplete reduction of sulphite. This was validated by the significant decrease in peak heights observed on repeat MS (figure 1A, patients 9–12), after retreatment with DTT. In the case of patient 13, this effect is clearly seen, wherein the variant peak almost disappeared on repeat analysis (figure 1A, patient 13, inset).

Based on our findings, it is important to be cognizant of the following points while interpreting TTR test results: (i) the knowledge of a liver transplant or age-related systemic disease in the individual being tested and; (ii) the possibility of technical or procedural artifacts within the method. Within the MGL at Mayo Clinic, we have now initiated steps that ensure that clinical information with regard to organ transplants, liver in particular, is sought prior to repeating tests when discordant biochemical and molecular results are obtained. In addition, if a discordant result is obtained and the mass shift is approximately +80 Da, we repeat the MS testing with emphasis on the reduction before repeating the more expensive molecular tests (figure 1B).

Our experience identifies possible causes for discordance in TTR testing that can be extrapolated to other multi-component testing algorithms, highlighting the critical role of communication within the testing laboratories as well as between the testing laboratories and the ordering clinician.

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