SELF-TOF Mass Spectrometry Evaluation of Variant Transthyretins for Diagnosis and Pathogenesis of Familial Amyloidotic Polyneuropathy

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BACKGROUND: Mass spectrometric analyses are valuable for detection of transthyretin (TTR) variants, which cause familial amyloidotic polyneuropathy (FAP). However, those methods require an immunoprecipitation step with an anti-TTR antibody and are not suitable for quantitative detection. We investigated the usefulness of SELDI-TOF mass spectrometry (MS) without an immunoprecipitation step.

METHODS: We used ProteinChips with chromatographic capture formats to detect TTRs. We attempted to correlate the intensity of mixed samples of amyloidogenic TTR (ATTR) V30M to wild-type (WT) TTR. We analyzed the proportion of ATTR V30M in amyloid-laden cardiac tissues from FAP patients, and also evaluated samples from FAP patients with 16 other TTR mutations.

RESULTS: Detection of ATTR required only 3 h of SELDI-TOF MS analysis. We determined that SELDI-TOF MS was suitable for quantitative detection of ATTR V30M and demonstrated that the proportion of ATTR V30M to WT TTR was 46.6% in amyloid-laden cardiac tissue from a FAP patient who died 10 years after liver transplantation. With this method, we identified 12 of 17 TTR variants. Small mass shifts and low concentrations of variants prevented ATTR detection. By changing the analytical conditions, we achieved detection of low concentrations of ATTR Y114C in serum.

CONCLUSIONS: SELDI-TOF MS is a reliable tool for quantitative evaluation of TTR variants, in both tissue amyloid deposits and body fluids. This method is useful for the diagnosis and investigation of the pathogenesis of FAP.

Familial amyloidotic polyneuropathy (FAP)7 is an autosomal dominant form of fatal hereditary amyloidosis, the most common cause of which is mutated amyloidogenic transthyretin (ATTR) (1–3). The duration of the disease is one of the most important prognostic factors for patient survival after liver transplantation, which has become a well-established treatment method for halting progression of FAP-related clinical symptoms (4, 5). Mass spectrometric analyses using tools such as electron spray ionization-mass spectrometry (6, 7) and MALDI-TOF mass spectrometry (MS) (8) allow identification of variant TTR forms in serum, because most of the amino acid substitutions manifest changes in the molecular mass of TTR. However, these methods require substantial time to analyze the variant proteins and also sometimes fail to detect TTR because of technical difficulties related to the cumbersome purification of an immunoprecipitate using an anti-TTR antibody.

SELDI-TOF MS combines chromatography with MS and uses arrays with various surface chemistries (9, 10). To develop a reliable method for quantitative detection of variant TTR proteins, we evaluated usefulness of SELDI-TOF MS performed as a 1-step procedure, without the immunoprecipitation step.

For evaluation of variant TTR proteins, we used 93 serum specimens from 70 patients with FAP ATTR V30M, 15 sera from 15 asymptomatic carriers of ATTR V30M, 6 sera and 6 cerebrospinal fluid (CSF) specimens from 6 patients with FAP ATTR Y114C, 20 sera from healthy volunteers, and 1 serum specimen each from other FAP patients with TTR mutations. Informed consent was obtained from each participant. All studies using human samples were in accordance with the current revision of the Helsinki Declaration. Tissue-deposited ATTR was extracted as previously described (11). Recombinant TTRs were expressed and purified as described previously (12). We analyzed samples using 3 different ProteinChip surfaces: a

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7 Nonstandard abbreviations: FAP, familial amyloidotic polyneuropathy; ATTR, amyloidogenic transthyretin; TTR, transthyretin; MS, mass spectrometry; WT, wild-type; CSF, cerebrospinal fluid.
strong anion exchange array (Q10), a weak cation exchange array (CM10), and ProteinChip preactivated with epoxide groups (PS20). All ProteinChip types were prepared according to the manufacturer’s instructions. Samples (serum diluted 50-fold and CSF and aqueous humor diluted 10-fold with binding buffers) were loaded and incubated for 60 min at room temperature on a shaker. For reduction of the ProteinChips, samples were treated on-chip with reducing agent and 50 mmol/L dithiothreitol solution in 50 mmol/L NH₄HCO₃, and then air dried at 70 °C. For the PS20 ProteinChip, SELDI-TOF MS immunoassay was performed as in a previous study (13). All analyses were performed with JMP version 5.1 (SAS Institute Japan). P-values of <0.05 were regarded as statistically significant.

MS peaks derived from wild-type (WT) TTR and ATTR V30M were most clearly detected at pH 7.0 on the Q10 ProteinChip (see Figure 1 in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol55/issue6). It took only 3 h to obtain results without immunoprecipitation. TTR-related peaks obtained with the Q10 ProteinChip were identical to those ob-

Fig. 1. Mass spectra of TTR variants.

Serum specimens were analyzed with the PCS 4000 SELDI-TOF MS instrument (Bio-Rad Laboratories) by using the following protocol, unless otherwise specified: ion focus mass, 13 800 m/z; laser energy, 2000 nJ; matrix attenuation, 500 m/z; sample rate, 800; shots/pixel, 5; partition, 1 of 4; and acquired mass range from 0–100 000 m/z. Baseline smoothing: smoothing before fitting baseline, window 25 points. Baseline width: automatic. These baseline settings were default. External calibration of the instrument was performed by using the All-in-1 protein molecular mass standard (Bio-Rad Laboratories). Conditions: (A–S), Q10 ProteinChip in 50 mmol/L phosphate buffer, pH 7.0, and (T) CM10 ProteinChip in 50 mmol/L glycine-HCl buffer, pH 3.0, with on-chip reduction with dithiothreitol. All sample donors were heterozygous for FAP, except for (A), a healthy volunteer and (C) a patient homozygous for FAP ATTR V30M. Arrows indicate variant peaks. Asterisks indicate broad peaks. (U), Correlation between theoretical and measured mass changes.
tained with TTR-specific immunoaffinity analysis using the anti-TTR antibody-coated PS20 ProteinChip and were depleted by immunoprecipitation using the anti-TTR antibody–coated protein A beads (see online Supplemental Fig. 2). ATTR V30M was also identified in CSF and aqueous humor (see online Supplemental Fig. 2). ATTR V30M was also identified in patients with the ATTR V30M form of the transthyretin (TTR) gene. The change in mass of ATTR V30M from WT TTR in those samples was 31.19 (0.93) m/z.

We attempted to correlate the ratio of the intensity derived from ATTR V30M and mixed samples of ATTR V30M and WT TTR that were purified from serum specimens obtained from a healthy volunteer and a patient homozygous for FAP ATTR V30M, respectively, as described previously (14). Ratios obtained showed good correlation with ATTR V30M/WT TTR (see online Supplemental Fig. 4). However, this method did not enable us to discern values <20% and higher than 80% because larger peaks hid smaller peaks. Using the regression equation obtained, we calculated the relative values of ATTR V30M to those of total TTR in serum and tissue specimens of FAP patients. We did not find a significant difference in ATTR V30M/total TTR in serum specimens from FAP patients and asymptomatic heterozygous ATTR V30M carriers: 51.76 (3.24)% vs 52.82 (2.07)%., respectively. We also determined the proportion of ATTR V30M in cardiac autopsy tissues from a patient with FAP ATTR V30M who did not have a liver transplant and a patient who had received a liver transplant. Values for ATTR V30M in cardiac tissues of these patients were more than 80% and 46.6%, respectively (see online Supplemental Fig. 5). ATTR V30M detected in patients who underwent liver transplantation should definitely be the protein synthesized and deposited before the operation.

We also confirmed that mass shifts of variant TTRs measured by SELDI-TOF MS corresponded to theoretical mass changes (Fig. 1 and Table 1). However, we could not determine mass shifts for 5 of 17 variants by using the Q10 ProteinChip in 50 mmol/L phosphate buffer pH 7.0. There are 2 possible reasons for this result. The first is that these mass shifts were too small for differentiation of the variants from WT TTR. The second is that the variant concentrations were too low.

<table>
<thead>
<tr>
<th>TTR variant</th>
<th>Detection of variant TTR peaks</th>
<th>Best analytical conditions</th>
<th>Theoretical mass changes (m/z)</th>
<th>Measured mass changes (m/z)</th>
<th>Theoretical pI°</th>
<th>Measured pI</th>
<th>Ratio of intensity (variant/WT)</th>
<th>Predominant symptoms</th>
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<td>WT</td>
<td>+</td>
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<td>45.32</td>
<td>42.24</td>
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<td>PN, AN, heart</td>
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<td>+31.26</td>
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<td>4.61</td>
<td>1.00</td>
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<td>5.57</td>
<td>NE</td>
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<td>A25S</td>
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<td>NE</td>
<td>0.96</td>
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<td>+12.43</td>
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<td>ND</td>
<td>5.17</td>
<td>NE</td>
<td>-</td>
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<td>-</td>
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<td>CM10/pH 3</td>
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<td>5.35</td>
<td>5.69</td>
<td>0.49</td>
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</table>

Table 1. Characteristics of TTR variants.

*° pI, isoelectric point; Q10, ProteinChip with a strong anion exchange array; pH 7, 50 mmol/L phosphate pH 7.0; PN, peripheral neuropathy; AN, autonomic neuropathy; NE, not examined; ND, not detected; CTS, carpal tunnel syndrome; LM, leptomeningeal amyloidosis; CM10, ProteinChip with a weak cation exchange array; pH3, 50 mmol/L glycine-HCl pH 3.0.
compared with WT TTR concentrations. MS analysis did not enable us to ascertain mass differences in samples with small mass shifts. Although our results suggested the use of SELDI-TOF MS was associated with difficulty in distinguishing TTR variants whose mass differences from WT TTR were <15 Da, several TTRs within the 15-Da mass shift showed a broad single peak. When this broad TTR peak appears in SELDI-TOF MS analyses, genetic testing is warranted to evaluate whether patients have atypical TTR variants with small mass shifts. With regard to the second possibility, that concentrations were too low in our study, we could not identify 2 TTR variants, D18G and Y114C, by using the Q10 ProteinChip, although the variants’ theoretical mass changes were large enough. Both mutants reportedly had an atypical phenotype, leptomeningeal amyloidosis (15, 16). In a similar case the intensity of the peak of ATTR A25T, which corresponds to a leptomeningeal type of FAP (17), was also much lower than that of the WT TTR peak. Using recombinant TTR molecules in vitro, Sekijima et al. (18) demonstrated that the secretion rates of ATTR D18G, A25T, and Y114C were markedly reduced because of endoplasmic reticulum–associated degradation caused by significant low kinetic stability, which was defined by the rate of TTR tetramer dissociation. In addition, Mitsuhashi et al. (8) demonstrated that ATTR Y114C might be prone to dissociation to monomers and to degradation. These factors may contribute to the low TTR variant concentrations in serum.

Using the CM10 ProteinChip coated with cation exchanger under acidic conditions, we also confirmed the presence of ATTR Y114C in serum specimens (Fig. 1 T). We also demonstrated that recombinant ATTR Y114C had a higher isoelectric point than WT TTR by use of a Model 111 mini–isoelectric focusing cell with isoelectric focusing standards (Bio-Rad Laboratories) (see online Supplemental Fig. 6). These results suggest that ATTR Y114C, with a higher positive charge than WT TTR in acidic buffer, has strong binding affinity to the cationic chip. Mass spectra indicated that recombinant ATTR Y114C tended to exist as a double sulfocysteine–conjugated form without reduction, whereas other recombinant TTRs, such as WT TTR, and ATTR V30M, had no such sulfocysteine modification (see online Supplemental Fig. 7). However, we could not confirm these ATTR Y114C modifications in samples from FAP ATTR Y114C patients. Further investigations are needed to determine the specific chemical characteristics of ATTR Y114C. We also determined that ratios of intensities of ATTR Y114C to WT TTR in CSF specimens from FAP ATTR Y114C patients were significantly higher than those in serum samples (see online Supplemental Fig. 8). High ATTR Y114C concentrations in CSF may contribute to pathogenesis of leptomeningeal amyloidosis in loco.

Our results show that SELDI-TOF MS is a reliable tool for quantitative evaluation of TTR variants in body fluids and tissue amyloid deposits. We believe that SELDI-TOF MS is a more convenient means of identifying TTR variants for clinical diagnosis than previous methods using immunoprecipitates of TTR. For reliable clinical diagnosis of FAP, histopathological and genetic tests are also required because mass spectrometric analyses are not suitable for determination of the onset of FAP and may be unable to detect atypical FAP cases. However, the mass spectrometric analyses can clearly detect TTR variants in the most frequent cases of FAP ATTR V30M and can be used in genetic testing for screening of FAP and double-checking TTR variants.

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**References**


