

Novel methods for detecting amyloidogenic proteins in transthyretin related amyloidosis

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1. ABSTRACT

Transthyretin (TTR)-related familial amyloidotic polyneuropathy (FAP) is an autosomal dominant form of fatal hereditary amyloidosis. Until 25 years ago, tools for diagnosis of FAP were restricted to clinical manifestations and pathologic methods, and a small number of patients in the restricted endemic areas could be diagnosed with this disease. However, owing to progress in biochemical and molecular genetic analyses, this disease is now believed to occur worldwide. As of today, reports of about 100 different points of single or double mutations, or a deletion in the TTR gene have been published, and several different phenotypes of FAP have been documented, even for the same mutation in the TTR gene. Since liver transplantation has been established to halt the progression of FAP, rapid and reliable diagnostic system for FAP is needed. We present here a new diagnostic procedure for the disease using current methods of molecular genetics and protein chemistry.

2. INTRODUCTION

Amyloidosis is a disorder of protein metabolism in which soluble proteins are deposited in tissues as abnormal insoluble fibrils (1-2). So far, 27 different precursor proteins have been identified in different kinds of amyloidosis (3). Familial amyloidotic polyneuropathy (FAP) is a hereditary form of amyloidosis which is most often caused by a mutation of transthyretin gene (4). After Andrade identified patients with FAP in Portugal in 1952 (5), many foci of FAP cases have been reported worldwide (6-10). Because patients with FAP show various serious systemic symptoms, such as cardiac and renal dysfunction, gastrointestinal disorders, glandular and autonomic dysfunction, and peripheral neuropathy, many trials have been attempted to treat these symptoms (11-19).

Recently, liver transplantation has become a well-established treatment for halting the progression of FAP-related clinical symptoms, because the main source of

serum variant TTR is shut out (20-27). However, there are several problems with this therapy: (a) it is expensive and requires lifelong administration of immunosuppressants, (b) the ATTR carriers with no clinical symptoms and the patients with advanced stage of FAP cannot undergo liver transplantation, (c) shortage of liver donors, and (d) further development of cardiac and ocular disorders are not prevented (4, 20, 28, 29). Therefore, we and other FAP research groups have been investigating the possibility of essential therapy for FAP on the basis of TTR amyloid formation mechanism (20). However, it will take time before the ongoing therapeutic projects become an actual essential therapy. At present, liver transplantation in FAP is performed worldwide, therefore the development and application of rapid and reliable diagnostic methods for FAP are imperative.

In this review, we describe current clinicopathological, biochemical, molecular genetic, and epidemiological aspects of TTR-related FAP, and present a novel diagnostic procedure for this disease.

3. TTR: STRUCTURE, FUNCTION AND GENE

TTR was first discovered in an electrophoresis of cerebrospinal fluid (CSF) (30) and was also later observed in serum (31). It was first called component X and then later named "prealbumin", a name that describes its movement in front of albumin in electrophoresis. In 1958 it was demonstrated that this protein is a carrier protein for thyroxine (32). Ten years later TTR was shown to bind to the retinol-binding protein (33), which is a carrier protein for retinol. In 1981, when it was very clear that TTR was not related to albumin, the name transthyretin was proposed for this protein to avoid confusion with the preforms of albumin, prealbumin and proalbumin (34). The name transthyretin describes the functions of the protein, transport of thyroxine and retinol.

TTR is synthesized mainly in the liver (35), but also in the epithelial cells in the choroid plexuses (36-37) in the brain, in α -cells of the pancreatic islets (38-40) and retinal pigment epithelial cells (41-42). The concentration of TTR in plasma is about 0.3 g/l and in CSF 0.17 g/l. The TTR plasma level is slightly higher in adulthood than in childhood and higher in men than in women (43). The half-life of plasma TTR is 2 days (44) and the main sites for TTR degradation are liver, muscle and skin (45). TTR is not degraded within the nervous system. The plasma TTR level is reduced in conditions with inflammation and protein malnutrition, while the TTR level in CSF is not changed (46-48).

The tetramer has a central channel which contains two binding sites for the thyroid hormone thyroxine. Due to negative cooperativity between the two sites, only one thyroxine molecule is bound per TTR tetramer (49). TTR carries about 44% of the total serum thyroxine (43) while the rest of the thyroxine in plasma is bound to thyroxine binding globulin and albumin. Seventy-nine percent of total thyroxine in CSF is bound to TTR (50). The TTR molecules that carry thyroxine represent only a few percent of the total TTR present in both plasma and CSF (50).

The TTR molecule contains four binding sites (one on each monomer) for the 21 kDa retinol-binding protein (RBP) (51). However, probably because of negative cooperativity, only two RBP molecules are bound to each TTR tetramer (52). The exact location of the RBP binding sites on the TTR molecule is not known (53). RBP is a carrier protein for retinol and will only bind to TTR when it has bound retinol (54). Binding of RBP to TTR is thought to prevent its glomerular filtration (55-57).

The human TTR gene was localized at 18p11.1-q12.3. The structure of the human TTR gene was first determined by Tsuzuki *et al.* (58). cDNA coding for human TTR was cloned from a cDNA library prepared from human liver; genetic analysis used the restriction enzyme *Nsi*I. In 100 abnormal TTR genes, 0, 36, 40, and 24 points of mutation were detected in exons 1, 2, 3, and 4, respectively; a deletion in the TTR gene also occurred. The main sites of production of TTR confirmed by *in situ* hybridization methods are the liver, retinal pigment epithelium, choroid plexus of the brain, and visceral yolk sac endoderm (59).

4. TTR-RELATED FAP

FAP is a fatal hereditary amyloidosis in which amyloidogenic mutated transthyretin (ATTR), apolipoprotein A-I (AApoA-I), and gelsolin (Agel) have been identified as FAP-related amyloidogenic proteins (11-19). Of these proteins, ATTR is the most common throughout the world (7,10). Andrade first reported a large group of patients with FAP ATTR Val30Met in Portugal in 1952 (5), and other large foci have been discovered in Japan and Sweden (6,8). Until 20 years ago, FAP was thought to be a disease restricted to endemic occurrence in those areas. However, owing to progress in biochemical and molecular genetic analyses, this disease is now believed to occur worldwide (10). The presence of patients with TTR-related FAP has been confirmed in more than 30 countries, with FAP ATTR Val30Met patients verified in more than 15 countries (10) (Figure 1). Only FAP ATTR Val30Met has large foci in the world, although the reason for this is not known. Holmgren *et al.* performed an epidemiological study in the northern part of Sweden and estimated that the number of ATTR Val30Met gene carriers in a total population of 500,000 in the area was approximately 7500, although the penetrance of the mutation was as low as about 2% (60). By 1994, 1233 FAP ATTR Val30Met patients from 489 pedigrees had been diagnosed at Centro de Estudos de Paramidoidose in Porto, Portugal (61). So far, more than 1500 FAP patients have been registered in Portugal (personal communication). In Japan, Araki *et al.* first reported a focus of patients with FAP ATTR Val30Met in Arai district, Kumamoto (8), and Kito reported another focus in Nagano in Honshu Island (12). More than 350 FAP patients were found in the two endemic foci (4,13). In addition, 44 FAP kindreds with Val30Met TTR were traced (62-64). They were genealogically independent and were geographically scattered throughout Japan (65).

Table 1. Initial Clinical Symptoms in Patients with FAP ATTR Val30Met

Symptoms	Numbers of Patients
Sensory disturbances in lower limbs	52
Gastrointestinal symptoms	38
Erectile dysfunction	5
Faintness or syncope	5
Muscle weakness in lower limbs	3
Abnormal laboratory data for urine	5
Decrease in visual acuity	4
Arrhythmias	5

Initial symptoms of 117 patients from among the 169 patients with FAP ATTR Val30Met registered from 1968 to 2003 in Arao district, Kumamoto, Japan.



Figure 1. Distribution of FAP in the world. Locations of foci of FAP ATTR Val30Met patients described in previous reports and obtained from personal communications are presented.

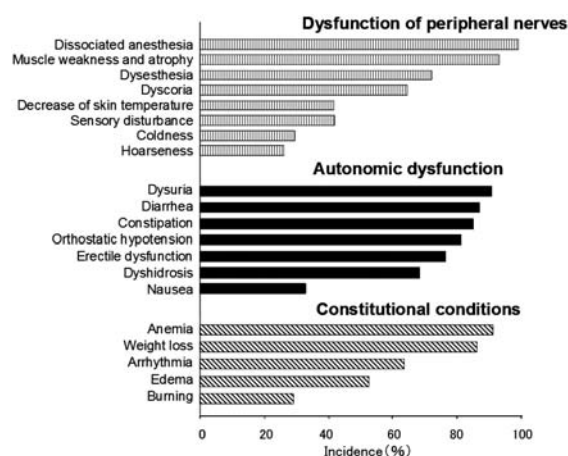


Figure 2. Clinical manifestations in patients with FAP ATTR Val30Met. One hundred sixty-nine patients with FAP ATTR Val30Met were registered from 1968 to 2003 in Arao district, Kumamoto, Japan.

Recently, several phenotypes of FAP, including the polyneuropathic, oculoleptomeningeal, and cardiac types, have been reported, and heterogeneity of clinical symptoms is recognized even for the same mutation of the TTR gene. Tashima *et al.* described the phenotypes of patients with FAP ATTR Val30Met, which could be classified into four types, with different dominant impairments, as follows: sensory, motor, autonomic, and visceral organ (11) (Table 1).

Among the confirmed 100 points of mutation or deletion in the TTR gene, 13 mutations are nonpathological forms (10). More than 20 points of mutation in the TTR gene have now been reported from the long islands of Japan, from the south to the north (13). Abnormal TTRs induce FAP, which can be classified into several phenotypes, such as neuropathic, oculoleptomeningeal, and cardiac types (Figure 2). Several types of ATTR mutants do not cause neuropathy, although they do induce other symptoms of the FAP disorder.

5. LIVER TRANSPLANTATION IN FAP

For treatment of FAP, liver transplantation has been reported to halt the progression of clinical manifestations (66). According to the data in the Familial Amyloidotic Polyneuropathy World Transplant Registry (FAPWTR), 71 centers in 17 countries have performed orthotopic liver transplantation (OLT) for FAP. During 2006, approximately 60 OLTs were performed worldwide. By the end of 2006, 1,316 patients have undergone 1,386 OLTs. Survival of these patients has been excellent (overall 5-year survival of 79%) and comparable to the survival of patients who underwent OLT for other chronic liver disorders. However, a longer follow-up is needed to compare the outcome after OLT with the natural course of the disease. The main cause of death was related to cardiac problems (39%) (67).

The use of sequential liver transplantation with resected livers from FAP patients started in Portugal; more than 552 patients have received FAP patients' livers (67). Recently, it has been reported that patients who underwent sequential liver transplantation using FAP patient's liver started to show amyloid deposits with or without clinical manifestations of FAP in less than 10 years after the surgery (68-70). However, we do not know whether all of the second recipients eventually will show the symptoms of FAP in the future.

6. SENILE SYSTEMIC AMYLOIDOSIS

In addition to mutated TTR related amyloidosis, senile systemic amyloidosis (SSA) induced by wild-type TTR amyloid deposits has been focused in the recent attention (71). Patients suffering from SSA show congestive cardiac failure caused by massive cardiac amyloid deposits as well as in other organs, the lung, kidney and gastrointestinal tracts (72-73). To make correct diagnosis of SSA, we should demonstrate the presence of ATTR deposits histopathologically and the absence of TTR mutation by genetic and proteomic analyses. Since the subcutaneous fat biopsies were amyloid-positive only in 20% of SSA patients (72), cardiac biopsy and physiological examinations should be needed when the diagnosis is uncertain. Although most of amyloid fibrils in hereditary type of TTR related amyloidosis are full length TTR, more than 90% of TTR is fragmented in SSA (74-75). From the tissues with amyloid deposits in SSA patients, a series of C-terminal TTR fragments with N-termini ranging from amino acids from 46 to 55 were identified.

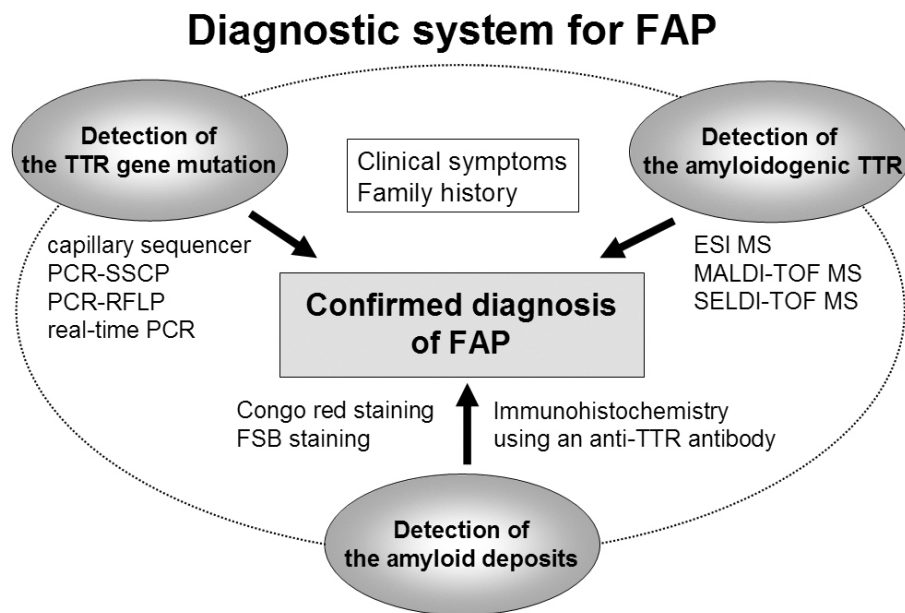


Figure 3. Novel rapid and reliable diagnostic system for FAP. Three kinds of independent analyses to detect (1) amyloid deposits in tissues, (2) genetic mutations of the TTR gene, and (3) variant TTR in serum or cerebrospinal fluid (CSF) are performed for rapid and reliable diagnosis for FAP.

7. NOVEL DIAGNOSTIC SYSTEM FOR FAP

FAP diagnosis in the early stage is absolutely imperative for the treatment of FAP, because duration of the disease is one of the most important prognostic factors for patients' survival after liver transplantation (23). However, the diagnosis is sometimes late, especially in late-onset patients who tended to have unclear family history, and in FAP patients with atypical clinical manifestations (76). To make a diagnosis of FAP in such patients sooner, we established a novel rapid diagnostic system for FAP (Figure 3). It included histopathological, genetic and proteomic techniques used to detect amyloid deposits in tissues, genetic mutations of the TTR gene, and variant TTR in serum or cerebrospinal fluid (CSF).

In addition, in Japan, liver transplantation from a living donor (LTLD) has been mainly performed due to shortage of cadaver donor livers (77). Since a donor for an FAP patient is often the patient's family member who may have a mutated TTR gene, establishment of the reliable diagnostic method is necessary to confirm that the potential donors do not have a mutated TTR gene.

7.1. Histopathological and immunochemical/chemical analyses

First of all, patients suspected with FAP should be examined for the presence of amyloid deposits in the tissues by means of histopathological methods. Biopsy materials, such as subcutaneous fat, submucosa of the gastrointestinal tracts, skin, and peripheral nerves, are subjected to histological staining with Congo red or thioflavin S. These stains intercalate between beta-strands of amyloid-forming proteins allowing detection of amyloid deposits. In the patients with small amyloid deposits,

repeated biopsies should be required to confirm the diagnosis. In addition to the commonly used Congo red staining, (*E,E*)-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy) styrylbenzene (BSB) and (*E,E*)-1-fluoro-2,5-bis-(3-hydroxycarbonyl-4-hydroxy) styrylbenzene (FSB) can be employed to detect a small amount of amyloid deposits in biopsy specimens, since these two chemical agents bind to amyloid fibrils more strongly than Congo red (78). ¹³C-labeled BSB and FSB also have been demonstrated to have a potential utility for detection of amyloid beta plaques by MRI and scintigraphy in Alzheimer patients (79): these agents are amyloidophilic and without benzidine structures, which are known to have carcinogenic effects *in vivo*.

Most commonly, immunohistochemical determination of the chemical type of amyloid is the first step in classifying amyloid, where a panel of antibodies recognizing different amyloid types is used to reveal the origin of deposited protein (80, 81). However, it was reported that in some cases misdiagnoses may occur, especially when immunohistochemical staining is performed in the absence of standardized antibodies and appropriate positive controls (81).

Extraction of amyloid fibril proteins from frozen and formalin-fixed tissues or from amyloid-containing histopathological tissue sections followed by immunostaining or amino acid sequence analyses is another useful way to characterize amyloid deposits (82-86), especially in cases when the immunohistochemical data are negative or inconclusive.

7.2. Genetic Testings

To detect the Val30Met mutation in TTR gene, which is the most frequent pathogenic mutation in FAP, we

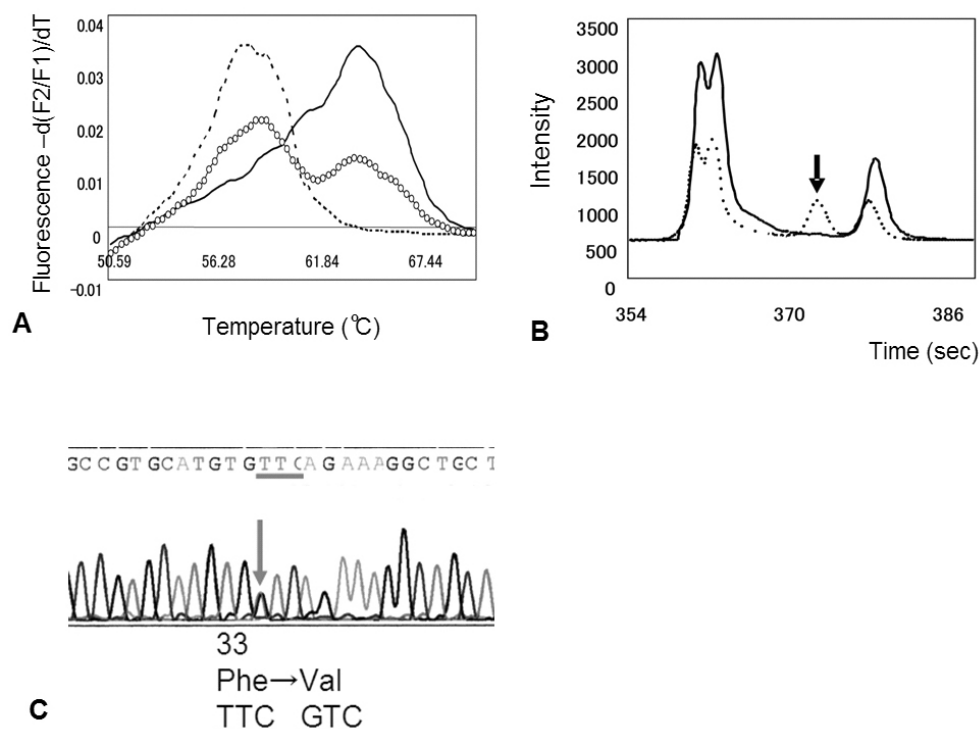


Figure 4. Genetic testings for TTR-related FAP. A. Detection of ATTR Val30Met by a real-time PCR genotyping assay based on the melting curve analysis. Solid line: normal subject; dotted line: FAP ATTR Val30Met homozygote; and circles: FAP ATTR Val30Met heterozygote. B. Nonradioisotopic SSCP analysis of an FAP ATTR Tyr114Cys heterozygote. Solid line: normal subject; dotted line: FAP ATTR Tyr114Cys heterozygote. The arrow points to an additional peak. C. DNA sequence analysis of exon 2 of the TTR gene. The substitution of a T to a G at the first position of codon 33 of TTR gene indicated the Phe33Val mutation.

employ a real-time PCR genotyping assay based on the melting curve analysis. The Real-time PCR genotyping assay was found to be highly reliable, rapid, cost-effective, and suitable for high-throughput analysis (81-82 87-88). This assay requires only a single preparation step before the actual analysis by using, for example, an anchor probe (5'-TGTGGCCGTCATGTGT-3'-FITC, in which the underlined nucleotide is the normal nucleotide) and a sensor probe (LC Red 640-5'-CAGAAAGGCTGCTGATGACACCTGGGAGCCATTTGCCTCTGGG-3'-OH). Because a single mismatch can significantly reduce binding affinity of the probe to the TTR gene, the melting temperature is reduced when the amplified gene encodes ATTR Val30Met. By using this method we can determine homo- or heterozygosity of the Val30Met mutation in FAP ATTR within one hour (Figure 4A). Specific probes detecting other point mutations of the TTR gene can be designed to analyze those mutations rapidly.

Single-strand conformational polymorphism (SSCP) was applied to determine the exon in which a mutation was present (89). However, a conventional type of SSCP requires a radioisotope to perform the procedure. To avoid the need for a radioisotope, we recently developed SSCP analysis with capillary electrophoresis (SSCP-CE) (90) in which a forward primer labeled with Cy5 and a microchip (i-Chip 12; Hitachi Chemical Co., Ltd, Tokyo,

Japan) filled with a separation gel for SSCP (i-S gel 3, Hitachi Chemical Co., Ltd) are used. An example of SSCP-CE analysis of an FAP ATTR Tyr114Cys heterozygote is presented herein. As shown in Figure 4B, an additional peak is observed when the amplified gene encodes for ATTR Tyr114Cys. Using the SSCP-CE analysis, we can rapidly determine the exon in which a mutation in the TTR gene is present. To determine the mutation in the exon where the SSCP-CE shows the additional peak, the DNA sequencing analysis should be performed (Figure 4C).

7.3. Proteomic analyses

To screen or confirm the presence of a mutation in the TTR gene, we also perform mass spectrometric analysis using several tools, such as electron spray ionization-mass spectrometry (ESI-MS) (91-93), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (94-96), and surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS). These methods allow detection of variant TTR in serum, because a substitution of amino acid results in a change in molecular size of TTR. The changes of molecular size of known TTR variants are summarized in Table 2. When using ESI-MS and MALDI-TOF MS, we employed immunoprecipitation for isolation of TTR from serum. Several forms of normal and variant

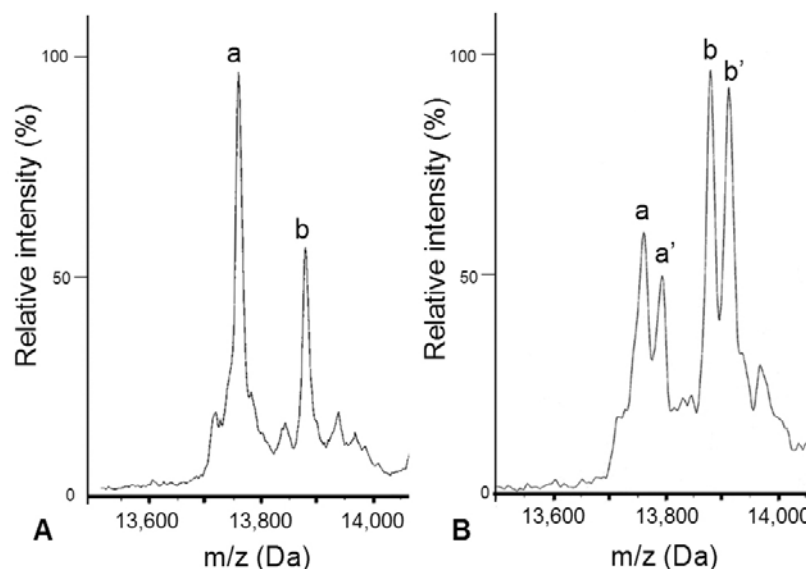


Figure 5. MALDI TOF-MS analysis to detect wild-type and variant TTR forms in serum samples. (A) Wild-type TTR forms in the serum sample of a healthy volunteer, analyzed by use of MALDI/TOF-MS with an anti-human TTR antibody. a: free form of wild-type TTR (13,762 Da), b: Cys-conjugated form of wild-type TTR (13,881 Da). (B) Wild-type and variant TTR forms in the serum sample of an FAP ATTR Val30Met patient, analyzed by use of MALDI/TOF-MS with an anti-human TTR antibody. a: free form of wild-type TTR (13,762 Da), b: Cys-conjugated form of wild-type TTR (13,881 Da), a': free form of variant TTR (13,793 Da), b': Cys-conjugated form of variant TTR (13,912 Da).

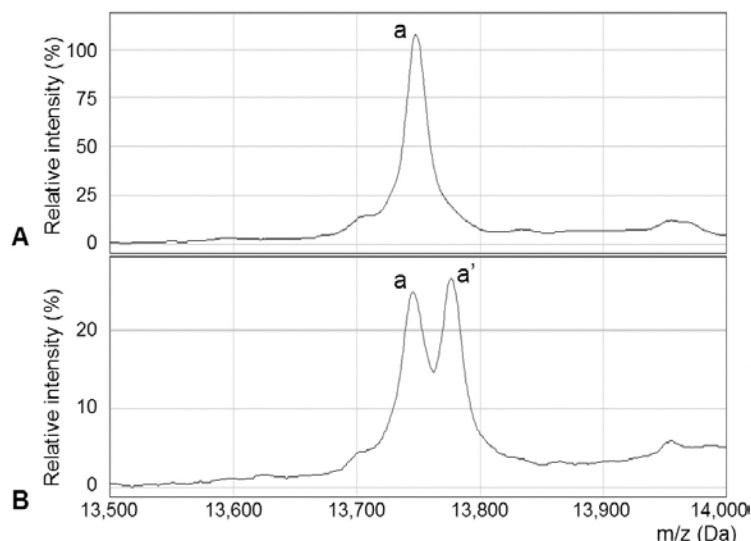


Figure 6. SELDI TOF-MS analysis for rapid detection of variant TTR. Mass spectras of serum TTR after on-chip reduction with DTT. (A) Wild-type TTR of a healthy volunteer. a: wild-type TTR (13,762 Da). (B) Wild-type and variant TTR of an FAP ATTR Val30Met patient. a: wild-type TTR (13,762 Da), a': variant TTR (13,793 Da).

TTR were detected in serum samples by these techniques, including unconjugated TTR and cysteine conjugated TTR (Figure 5A, 5B). In an FAP ATTR Val30Met patient, variant forms of TTR can be detected with a 32 Da higher molecular weight than those of wild type TTR (Figure 5B). Recently, we applied SELDI-TOF MS for rapid detection of variant TTR in patient's serum. By this technique, we can analyze the variant form of TTR

in a one-step procedure by omitting immunoprecipitation, with the almost same accuracy as ESI-MS and MALDI-TOF MS (Figure 6).

However, due to inherent limitations in resolving accuracy of the instrumentation, the applied mass spectrometric techniques do not allow precise identification of TTR variants that show mass differences below 10kDa.

Table 2. Mass changes of TTR variants

No	Variants	Mass Change	No	Variants	Mass Change	No	Variants	Mass Change	non-amyloidogenic	Variants / Polymorphisms	Mass Change
1	Cys10Arg	53	32	Gly47Ala	14	63	Lys70Asn	-14	1	Gly6Ser	30
2	Leu12Pro	-16	33	Gly47Val	42	64	Val71Ala	-28	2	Met13Ile	-18
3	Asp18Glu	14	34	Gly47Glu	72	65	Ile73Val	-14	3	Asp74His	22
4	Asp18Gly	-58	35	Thr49Ala	-30	66	Ser77Phe	60	4	His90Asn	-23
5	Asp18Asn	-1	36	Thr49Ile	12	67	Ser77Tyr	76	5	Gly101Ser	30
6	Val20Ile	14	37	Thr49Pro	-4	68	Tyr78Phe	-16	6	Pro102Arg	59
7	Ser23Asn	27	38	Ser50Arg	69	69	Ala81Phe	30	7	Arg104Cys	-53
8	Pro24Ser	-10	39	Ser50Ile	26	70	Ile84Ser	-26	8	Arg104His	-19
9	Ala25Ser	16	40	Glu51Gly	-72	71	Ile84Asn	1	9	Ala108Ala	0
10	Ala25Thr	30	41	Ser52Pro	10	72	Ile84Thr	-12	10	Ala109Thr	30
11	Val28Met	32	42	Gly53Glu	72	73	Glu89Gln	-1	11	Ala109Val	28
12	Val30Met	32	43	Glu54Gln	-1	74	Glu89Lys	-1	12	Thr118Met	30
13	Val30Ala	-28	44	Glu54Gly	-72	75	Ala91Ser	16	13	Thr119Met	30
14	Val30Leu	14	45	Glu54Lys	-1	76	Gln92Lys	0	14	Pro125Ser	-10
15	Val30Gly	-42	46	Leu55Arg	43	77	Val94Ala	-28			
16	Phe33Ile	-34	47	Leu55Pro	-16	78	Ala97Gly	-14			
17	Phe33Leu	-34	48	Leu55Gln	15	79	Ala97Ser	16			
18	Phe33Val	-48	49	His56Arg	19	80	Arg103Ser	-69			
19	Phe33Cys	-44	50	Leu58His	24	81	Ile107Val	-14			
20	Arg34Thr	-55	51	Leu58Arg	43	82	Ile107Met	18			
21	Lys35Asn	-14	52	Thr59Lys	27	83	Ala109Ser	16			
22	Ala36Pro	26	53	Thr60Ala	-30	84	Leu111Met	18			
23	Asp38Ala	-44	54	Glu61Lys	-1	85	Ser112Ile	26			
24	Trp41Leu	-73	55	Glu61Gly	-72	86	Tyr114Cys	-60			
25	Glu42Gly	-72	56	Phe64Leu	-34	87	Tyr114His	-26			
26	Glu42Asp	-14	57	Phe64Ser	-60	88	Tyr116Ser	-76			
27	Phe44Ser	-60	58	Phe64Ile	-34	89	Ala120Ser	16			
28	Ala45Asp	44	59	Gly67Glu	72	90	Val122Ile	14			
29	Ala45Ser	16	60	Ile68Leu	0	91	Deletion of Val122	-99			
30	Ala45Thr	30	61	Tyr69His	-26	92	Val122Ala	-28			
31	Gly47Arg	99	62	Tyr69Ile	-50	93	Asn124Ser	-27			

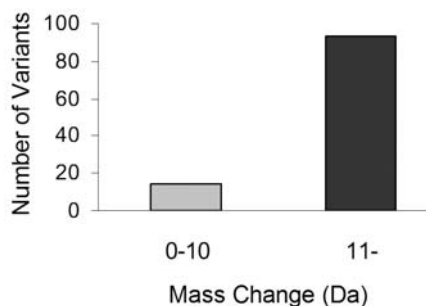


Figure 7. Number of TTR variants with mass changes below and above 10 Da. The mass spectrometric analyses for TTR can not precisely identify variants that show molecular size differences below 10 Da because of limitations in the resolving accuracy of the instrumentation.

Although the most common mutation, Val30Met and the most frequent polymorphism Gly6Ser cause +32 and -30 Da mass changes, respectively, and are clearly determined by means of mass spectrometry, TTR variants with mass changes not exceeding 10 Da (which are caused by 12 types of TTR mutations and 2 polymorphisms of TTR) can not be detected by these techniques (Table 2, Figure 7). On the other hand, the DNA sequencing analysis could not be almighty method because the analytical errors and performance imprecision still happen. In order to achieve accurate results on TTR mutation in FAP patients or variant TTR gene carriers, the application of both the genetic and proteomic methods is preferable to compensate the disadvantages and possible pitfalls in each of the techniques used.

Mass spectrometric analyses are valuable not only to screen and confirm a mutation in the TTR gene, but also to determine compound heterozygotes (95). Several compound heterozygotes of TTR mutation or polymorphism were reported using this method (97). In contrast, DNA sequencing of the TTR gene can not determine whether two different types of mutations are located on one allele (double mutations) or on different alleles (a compound heterozygote) (95).

Mass spectrometry is also useful to study ATTR metabolism in serum, CSF, and aqueous humor before and after liver transplantation (91, 93, 94, 96). Patients who underwent liver transplantation come to have predominantly wild type TTR in serum, as it is synthesized by the transplanted normal liver. However, it is well known that TTR is also synthesized by the choroids plexus and the retinal pigmental cells. In CSF and aqueous humor, variant TTR is still present (92, 94). Analyses in these fluids by proteomic methods are very helpful in revealing changes in the metabolism of both variant and wild type TTR (92, 94, 96, 98).

7.4. Other Diagnostic Methods

Ando *et al.* presented a histochemical method of diagnosis of ATTR Val30Met by using FAP patients' hair and monoclonal antibody supplied by Costa *et al.* (99,100). Also, Ando *et al.* demonstrated abundant abnormal ocular vessels in patients with FAP ATTR Val30Met, a finding which is of diagnostic value (101).

8. CONCLUSIONS

TTR-related FAP is not an insignificant disease,

and many more affected patients would likely be found if more careful and precise investigations were performed. Rapid and reliable methods are required to diagnose FAP in time for patients who need liver transplantation to halt this devastating disease. In this paper we presented a novel methodology combining histopathological, genetic and proteomic techniques for rapid diagnosis of ATTR-related FAP.

9. ACKNOWLEDGEMENT

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