

Diagnostic Radioimmunoassay for Familial Amyloidotic Polyneuropathy Before Clinical Onset

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Abstract

The purpose of this study is to develop an early diagnostic method for familial amyloidotic polyneuropathy (FAP) before clinical manifestations appear around the age of 30 yr. Amyloid fibrils isolated from type I FAP (FAP^I) of Portuguese, Swedish, and Japanese origins consist of a variant transthyretin (TTR) that contains a methionine-for-valine substitution at position 30 or a mixture of normal TTR and this variant form. The variant TTR is present in the serum of FAP^I patients and can be measured by a radioimmunoassay (RIA) based on a nonapeptide (positions 22–30) derived from the variant TTR. Serum levels of the variant TTR in 45 Japanese FAP^I patients range from 4.71 to 17.61 mg/dl with a mean value of 9.18 mg/dl. The variant TTR is not present in the serum of 100 normal individuals, in four cases of primary and six cases of secondary amyloidosis, nor in 26 non-inheriting members of families with FAP^I. The variant TTR level is measured in 24 children of 15 FAP^I patients as well. The variant TTR is already present in nine symptom-free children with the mean serum level of 11.90 mg/dl, but it is not present in 15 other children. FAP^I patients can be differentiated from non-FAP by this noninvasive diagnostic method even within families. The RIA can be applied worldwide to this intractable disorder for early diagnosis during childhood and for appropriate genetic counseling.

Introduction

Family trees carrying FAP have been reported from various countries including Portugal, Israel, Finland, Germany, Sweden, Brazil, U. S. A., and Japan (1). Type I FAP (FAP^I)¹ of Japanese origin is characterized by dissociated sensory disturbance and autonomic dysfunction in the early course, followed by motor

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1. *Abbreviations used in this paper:* AF_J, amyloid fibril protein from Japanese patients with familial amyloidotic polyneuropathy; AF_{TTR}Met-30, amyloid fibril protein of familial amyloidotic polyneuropathy, which contains a methionine-for-valine substitution at position 30 of transthyretin; CNBr, cyanogen bromide; FAP, familial amyloidotic polyneuropathy; FAP^I, type I FAP; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; SAF_{TTR}Met-30, serum amyloid protein of familial amyloidotic polyneuropathy that contains a methionine-for-valine substitution at position 30 of transthyretin; TFA, trifluoroacetic acid; TTR, transthyretin.

neuropathy (2). The illness is always progressive to death either from cardiac or renal failure associated with the systemic deposition of amyloid fibrils.

The diagnosis of advanced cases depends on characteristic clinical manifestations together with demonstration of amyloid deposition at biopsy. However, there has been no definite diagnostic method for this progressive disorder before symptoms appear. The mean age of onset is 34 ± 5 (SD) yr and patients tend to have one or two children before they develop clinical manifestations of the disease. Since this disorder is transmitted by autosomal dominant inheritance and the clinical onset is postmarital, it is important to develop an early diagnostic method to predict possible transmission of the disease. A simple and definite diagnostic test for the disease before the childbearing years is needed for appropriate genetic advice to patients and their families.

We have reported that amyloid fibril protein from Japanese patients with FAP^I (AF_J) is a variant transthyretin² (TTR) that contains a single amino acid substitution of a methionine for a valine at position 30 (3). This variant TTR is present in the serum of FAP^I patients along with normal TTR (4). Furthermore, we have developed a radioimmunoassay (RIA) based on a nonapeptide (positions 22–30) of the variant TTR (5).

Here we report the results of the RIA for the variant TTR, and serum levels of total TTR and total protein in 45 FAP^I cases at different stages of illness. Their ages of onset and duration of illness are considered along with serum levels of the variant TTR. Noninheriting members of families with FAP^I, cases of primary and secondary amyloidosis, and normal individuals have been also investigated. To explore the applicability of this RIA for early detection of carriers of FAP^I, we have studied 24 symptom-free children of 15 FAP^I patients as well.

We believe that our diagnostic RIA is useful for early diagnosis and appropriate premarital genetic advice to FAP^I patients and their families.

Methods

Subjects. The subjects studied here consist of 100 normal individuals aged from 2 to 70 yr with a mean age of 30.8 ± 10.8 (SD) yr; 45 Japanese FAP^I cases (22 males and 23 females) aged from 25 to 68 yr with a mean of 41.6 ± 11.8 ; 24 children of FAP^I patients (11 males and 13 females) aged from 7 to 22 yr with a mean of 15.3 ± 3.4 ; 26 noninheriting members of families with FAP^I aged from 31 to 69 yr with a mean of 46.9 ± 11.4 ; 4 cases of primary amyloidosis aged 43, 49, 55, and 56 yr; and 6 cases of secondary amyloidosis associated with rheumatoid arthritis or polymyositis aged 36, 46, 48, 53, 59, and 59 yr. All the children of FAP^I patients were in good health up until the time when the serum studies were performed.

2. The name transthyretin has been suggested by the Nomenclature Committee of the International Union of Biochemistry and the International Union of Pure and Applied Chemistry for the protein commonly called prealbumin.

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Radioimmunoassay for the variant TTR. The nonapeptide amide (Gly-Ser-Pro-Ala-Ile-Asn-Val-Ala-Met-NH₂) corresponding to positions 22–30 of the variant TTR was synthesized by solid-phase techniques, conducted on a *p*-methylbenzhydrylamine resin. COOH-terminal methionine amide was changed to a mixture of homoserine and homoserine lactone by using 300-fold M excess of cyanogen bromide (CNBr) (Nakarai Chemicals Ltd., Kyoto, Japan) (6). COOH-terminal homoserine lactone was converted into homoserine by incubation in 0.1 M Tris-HCl (pH 8.0) at 40°C for 24 h. The COOH-terminal homoserine form of the nonapeptide was designated as FAP nonapeptide. Purification of FAP nonapeptide was performed by reverse-phase high performance liquid chromatography (HPLC) on a 4.6 × 250-mm column of octadecylsilane (TSK gel ODS 120A) (Toyo Soda Corp., Tokyo, Japan). A linear gradient of acetonitrile (CH₃CN) from 0 to 60% in 0.1% trifluoroacetic acid (TFA) was employed for 40 min at a rate of 2.0 ml/min. Correct synthesis was confirmed by amino acid and sequencing analyses. Tyr⁰-FAP nonapeptide was synthesized in a similar manner and used for radioiodination.

FAP nonapeptide (9.7 mg) was conjugated with bovine thyroglobulin (9.6 mg) by the glutaraldehyde method. The resulting antigenic conjugate was emulsified with Freund's complete adjuvant and then used for immunizing New Zealand White rabbits. They were boosted every 2 wk and bled 1 wk after each injection. Tyr⁰-FAP nonapeptide was radioiodinated by the lactoperoxidase method (7). The ¹²⁵I-labeled FAP nonapeptide was purified by reverse-phase HPLC.

RIA buffer consisted of 0.05 M sodium phosphate (pH 7.4), 0.08 M NaCl, 0.25% BSA, 0.025 M EDTA · 2 Na, and 0.05% NaN₃. Samples or FAP nonapeptide standard solutions (100 μl) were mixed with solutions of anti-FAP nonapeptide serum (200 μl, final dilution of 1:50,000) and ¹²⁵I-labeled ligand (200 μl, 18,000 cpm). The mixture was incubated at 4°C for 40 h. Bound and free ligands were separated by using 1 ml of 23% polyethyleneglycol. Samples were routinely assayed in duplicate.

Immunoreactive FAP nonapeptide in the serum. 15 ml of the serum from a biopsy proven FAP patient (64-yr-old, female) was dialyzed against 500 ml of 30 mM sodium phosphate buffer (pH 7.0). The dialysate was applied to an Affi-Gel Blue column (2.3 × 48 cm; Bio-Rad Laboratories, Richmond, CA) preequilibrated with the above phosphate buffer. The column was washed with 480 ml of phosphate buffer at a rate of 10 ml/h. The bound proteins were eluted with a linear gradient of sodium chloride from 0 to 1.0 M (total volume, 500 ml) and then 180 ml of 0.5 M sodium thiocyanate. Each 8-ml fraction was collected. 5 μl of aliquots were lyophilized, and assayed for FAP nonapeptide by RIA. 20 μl of aliquots were also monitored by double immunodiffusion in two dimensions with 20 μl of anti-TTR serum (Behringwerke AG, Marburg, Federal Republic of Germany) (8). The fractions exhibiting FAP nonapeptide immunoreactivity (tubes 19–23 in Fig. 2) were further submitted to fast protein liquid chromatography (FPLC) on a Mono Q column (0.5 × 5 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) preequilibrated with 20 mM Tris-HCl buffer (pH 7.6). The bound proteins were eluted with a linear gradient of sodium chloride (0–0.5 M) for 40 min. The flow rate was 1.0 ml/min and the fraction size was 0.5 ml/tube. All the fractions were monitored by the RIA for FAP nonapeptide and double immunodiffusion for TTR. The fractions exhibiting FAP nonapeptide immunoreactivity (tubes 56–61 in Fig. 3) were purified by rechromatography on the Mono Q column.

The fractions exhibiting FAP nonapeptide immunoreactivity were concentrated and desalted by the use of a Sep-Pak ODS cartridge (Waters Assoc., Milford, MA) with an elution solution of 60% CH₃CN in 0.1% TFA. The eluate was cleaved by CNBr, incubated in 50 mM Tris-HCl (pH 8.0), and digested with trypsin (treated with 1-L-tosylamido-2-phenylethyl chloromethyl ketone, TPCK) (Worthington Biochemical Corp., Freehold, NJ) at 37°C for 2 h with an enzyme/substrate ratio (wt/wt) of 1:50. The reaction was stopped by the addition of 1 M acetic acid. The tryptic digests were analyzed by reverse-phase HPLC on the column of TSK gel ODS 120A. A linear gradient of CH₃CN (0–60%) in 0.1% TFA was employed for 40 min. The flow rate was 2.0 ml/min and each 1-ml fraction was collected. 5-μl aliquots of fractions were subjected to the RIA for FAP nonapeptide. Authentic FAP nonapeptide was chromatographed on the same condition of HPLC and its elution position was

compared with that of the fraction exhibiting FAP nonapeptide immunoreactivity.

Serum level of the variant TTR. 5 μl of the serum of subjects were lyophilized. The serum was treated with 3 mg of CNBr in 70% formic acid at room temperature for 36 h and then lyophilized. The resulting fragments were incubated in 100 μl of 0.1 M Tris-HCl to convert COOH-terminal homoserine lactone into homoserine. The fragments were then digested with 8 μg of TPCK-treated trypsin at 37°C for 2 h. Thereafter, soy trypsin inhibitor was added to the buffer to stop the reaction. The serum samples were diluted with RIA buffer and were assayed for FAP nonapeptide by RIA. The amount of the variant TTR was calculated by multiplying the results of the RIA by the ratio of the molecular weight of the variant TTR (*M_r* = 13,745) to that of FAP nonapeptide (*M_r* = 830).

Recovery of the RIA. Serum TTR was purified from the FAP¹ patient by a two-step procedure using affinity chromatography on Affi-Gel Blue and anion exchange FPLC on Mono Q, and then it was submitted to amino acid analysis. The variant TTR has two methionine residues in comparison to one methionine residue in normal TTR. Therefore, the ratio of the variant TTR to normal TTR was determined by the molar quantity of a methionine residue. Since 5 μl of FAP¹ patient's serum usually contained 500 ng of the variant TTR, studies on the recovery of the RIA were performed by using the same amount of the variant TTR. Purified serum TTR (1,200 ng) containing 500 ng of the variant TTR was added to 5 μl of three separate serum samples obtained from three normal individuals. Three separate normal sera prepared as above were cleaved by CNBr and then trypsinized. The sera were assayed for FAP nonapeptide by RIA and the amount of the variant TTR was calculated. The recovery rate was computed from the results of the RIA divided by 500 ng.

Serum level of total TTR. The serum (5 μl) was applied to a single radial immunodiffusion plate for TTR (M-Partigen; Behringwerke AG, Marburg, Federal Republic of Germany). The area of a halo was measured after 48 h (9).

Amino acid analyses. Amino acid analysis was performed on a model 835 automated amino acid analyzer (Hitachi, Ltd., Tokyo, Japan). The peptides and proteins were hydrolyzed in 6 N HCl containing 0.1% phenol and 0.02% 2-mercaptoethanol at 110°C for 24 h.

Statistics. Statistical analysis was performed by an independent *t* test (Table I). *P* value of <0.05 was considered significant. The results were expressed as a mean ± SD.

Results

RIA for FAP nonapeptide. The antiserum raised against FAP nonapeptide could bind 35–40% of ¹²⁵I-labeled FAP nonapeptide at a final dilution of 50,000. A standard curve of the RIA is illustrated in Fig. 1. The sensitivity (effective dose, 10%) of the RIA for FAP nonapeptide was 21 pg/tube, and 238 pg of the peptide were required to reduce binding by 50% (effective dose). Intraassay and interassay coefficients of variation were 7.5 and 6.8%, respectively (*n* = 10). The serum of an FAP¹ patient exhibited a dose-response curve parallel with that of FAP nonapeptide. Normal human serum, on the other hand, did not show any crossreactivity. 1 μg of normal TTR, which was cleaved by

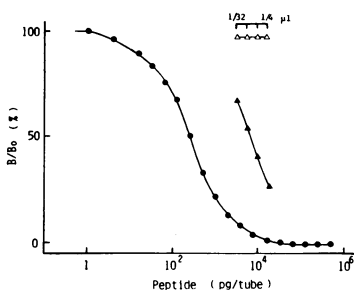


Figure 1. Dose-response curve of FAP nonapeptide (●), dilution curves of the serum from an FAP¹ patient (▲), and from a normal individual (Δ). The serum samples were diluted to the amount of 1/4, 1/8, 1/16, and 1/32 μl of the original serum samples.

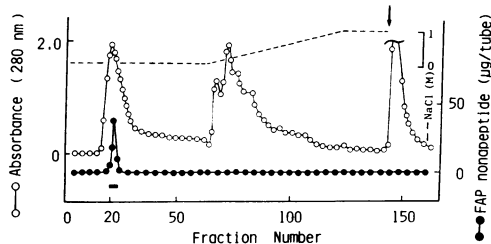


Figure 2. Separation of the serum protein from an FAP¹ patient by Affi-Gel Blue chromatography. The flow rate was 10 ml/h and the fraction size was 8.0 ml/tube. Equilibration buffer was 30 mM sodium phosphate (pH 7.0). At tube 60, a linear NaCl gradient (0–1.0 M, total volume 500 ml) was started. At tube 145, indicated by an arrow, buffer was changed to 0.5 M sodium thiocyanate. The black bar (tubes 19–23) represents fractions exhibiting TTR immunoreactivity, which was determined by double immunodiffusion in two dimensions.

CNBr and trypsinized, did not appreciably crossreact with the antiserum.

Identification of immunoreactivity for FAP nonapeptide. Immunoreactive FAP nonapeptide was identified by the assay of FAP nonapeptide through a two-step procedure of chromatography to isolate serum TTR. Affinity chromatography on an Affi-Gel Blue column was used as an initial step for purifying serum TTR from an FAP¹ patient. TTR was not adsorbed on the dye (Cibacron Blue F3GA), but most of the serum proteins, especially albumin, bound to the dye (10). Immunoreactivity for FAP nonapeptide was observed at the same position (tubes 19–23) as that of TTR, identified by double immunodiffusion in two dimensions using anti-TTR serum, as shown in Fig. 2. Tubes 19–23 were collected and applied to anion exchange FPLC on a Mono Q column. TTR was eluted later on the chromatogram and was well separated from other unretained materials on the Affi-Gel Blue resin column, since it was a strongly acidic protein (pI 4.7). A single immunoreactive peak for FAP nonapeptide was eluted at the position (tubes 56–61) corresponding to TTR as shown in Fig. 3. Therefore, it could be concluded that immunoreactive FAP nonapeptide originated from TTR. TTR purified as above was cleaved by CNBr and digested with trypsin. The resulting digests were separated by reverse-phase HPLC. A sharp immunoreactive peak was noted only at the position identical to authentic FAP nonapeptide as shown in Fig. 4. From the studies described above, this RIA was proved to be specific for SAF_{TTR}Met-30.³ Any cross-reactive substances were not detected by the RIA.

Validation of the quantitative precision of the RIA. The RIA is standardized quantitatively for FAP nonapeptide. Before calculation of the amount of the variant TTR, the quantitative precision of the RIA was examined. Quantitative basis of the RIA was validated with a mixture of normal and the variant TTR, since normal TTR did not appreciably crossreact with the antiserum. The amounts of normal and the variant TTR were determined by amino acid analysis, based on the fact that the former had one methionine residue and the latter had two. Total serum TTR (1,200 ng) containing 500 ng of the variant TTR was mixed with separate sera of three normal individuals. The mixture was assayed for FAP nonapeptide by RIA, and the results

3. After the suggested nomenclature of amyloid proteins, the amyloid fibril protein of familial amyloidotic polyneuropathy of Japanese origin is designated by AF_{TTR}Met-30 and its serum form by SAF_{TTR}Met-30.

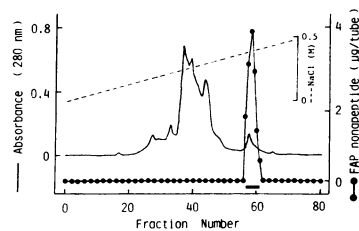


Figure 3. Anion exchange FPLC of FAP nonapeptide immunoreactive fractions obtained by Affi-Gel Blue chromatography. Tubes 19–23 in Fig. 2. were collected and applied to FPLC on a Mono Q column. The flow rate was 1.0 ml/min and the fraction size was 0.5 ml/tube. A linear gradient of NaCl (0–0.5 M) in 20 mM Tris-HCl (pH 7.6) was employed over 40 min. The black bar (tubes 56–61) indicates fractions exhibiting TTR immunoreactivity.

were 17.6, 18.1, and 18.8 ng. These results corresponded to 296, 305, and 318 ng of the variant TTR, respectively; therefore, the recovery rate was 61% as a mean. The RIA recovery was checked in every assay to validate the quantitative precision. The recovery rate was almost constant in every assay, ranging from 58 to 65%.

Serum levels of the variant and total TTR. Table I summarizes serum levels of the variant and total TTR in subjects. The variant TTR was not detected in normal individuals, nor in primary and secondary amyloidosis cases. The variant TTR was not present in all the 26 noninheriting members of families with FAP¹ who were above the age of usual onset of FAP¹. Serum levels of total TTR in noninheriting FAP¹ ranged from 24.8 to 41.5 mg/dl with a mean of 30.0 ± 7.7 (SD) mg/dl, which were within normal limits.

On the other hand, all the FAP¹ patients had high serum levels of the variant TTR, ranging from 4.71 to 17.61 mg/dl with a mean of 9.18 ± 2.69 mg/dl. Their ages of onset were 23–67 yr with a mean of 36.3 ± 11.4 yr. Duration of illness ranged from 1 to 17 yr with a mean of 5.5 ± 3.6 yr. There was no significant difference in serum levels of the variant TTR between males and females, whose mean values were 10.12 and 9.82 mg/dl, respectively. Serum levels of total TTR in FAP¹ cases ranged from 10.6 to 40.0 mg/dl, which were significantly reduced as compared with normal individuals ($P < 0.001$). There were no correlations of the variant TTR level or total TTR level, with age of onset and duration of illness. The serum level of total protein in FAP¹ patients (6.4 ± 0.7 g/dl) was significantly lower than the control value of 7.3 ± 0.7 g/dl ($P < 0.002$).

The results of the RIA for the variant TTR in 24 children of 15 FAP¹ patients are shown in Table I. Nine children had the variant TTR and their serum levels ranged from 8.32 to 14.71 mg/dl. On the other hand, the variant TTR was not present in the other 15 children. There was no significant difference in serum levels of total TTR between the two groups of children.

Pedigrees of two FAP families are presented in Fig. 5, and

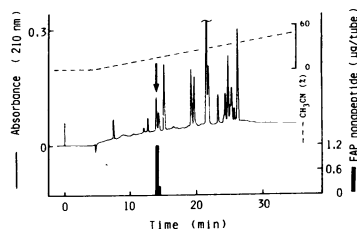


Figure 4. Tryptic peptide map for FAP¹ serum TTR cleaved by CNBr. Serum TTR (2.5 nmol) was cleaved by CNBr, trypsinized, and then applied to reverse-phase HPLC on a TSK gel ODS 120A column. The flow rate was 2.0 ml/min and the fraction size was 1.0 ml/tube. A linear CH₃CN gradient (0–60%) in 0.1% TFA was employed over 40 min. The arrow indicates the elution position of authentic FAP nonapeptide.

Table 1. Serum Levels of the Variant and Total TTR

Subject	Number	Age yr	TTR	
			Variant mg/dl	Total mg/dl
Normal individuals	100	2-70	0	30.5±4.5
Primary amyloidosis	4	43-56	0	35.0±6.5
Secondary amyloidosis	6	36-59	0	25.6±9.8
Noninheriting FAP ¹	26	31-69	0	30.0±7.7
FAP ¹ patients	45	25-68	9.18±2.69	22.4±6.6
Children of FAP ¹ patients	15	9-19	0	28.6±5.6
	9	7-22	11.90±2.33	27.0±4.7

Values are represented as a mean±SD.

showed the ages of the individuals tested and serum levels of the variant TTR. Some of the siblings in the second generation were omitted to preserve anonymity. The variant TTR was detected in patients, but not in unaffected siblings. In the third generation, four of nine children had the variant TTR, whereas five children did not have it. This incidence of the variant TTR was compatible to the autosomal dominant mode of inheritance of this disorder. Thus, differentiation even within kindreds of FAP¹ was possible by studying the serum level of the variant TTR, since the latter was present only in family members inheriting this disorder.

Discussion

Recent studies have been clarifying that FAP¹ results from the deposition of structurally abnormal TTR. Costa et al. first verified in 1978 that amyloid protein in FAP¹ of Portuguese origin was immunologically related to TTR (11). In 1983, Pras et al. reported that a variant TTR with a glycine-for-threonine substitution at position 49 was found in amyloid fibrils isolated from a Jewish FAP case (12). Also in 1983, another variant TTR with a methionine-for-valine substitution at position 30 was found in FAP¹ of Japanese, Portuguese, and Swedish origins (3, 13, 14). The characteristics of our studies were comparing peptide maps of amyloid protein obtained by CNBr and tryptic digestion with those of normal human TTR. This method was useful to determine the primary structure of amyloid protein related to TTR. After having studied AF₇, we applied the above peptide mapping procedure to the Jewish case. Amyloid fibril protein of Jewish FAP turned out to consist of a variant TTR with an isoleucine-for-phenylalanine substitution at position 33 and a small amount of normal TTR. In this way the original study by Pras et al. has now been revised (15). The variant TTR with a methionine-for-valine substitution is present in the serum of

FAP¹ of Japanese, Portuguese, and Swedish origins (4, 16, 17). This disorder results from the systemic deposition of amyloid fibrils that are composed of the serum variant TTR or a combination of normal TTR and its variant form. Therefore, detection of the serum variant TTR can be a new diagnostic method for this disorder. Identification of the variant TTR requires purification of serum TTR by affinity and anion exchange chromatography, and then separation of CNBr peptides by HPLC. This procedure is time consuming and requires a large amount of the serum sample because of a little yield of TTR. Moreover, the amount of the variant TTR cannot be determined by this method. Our RIA method is simple and quantitative for detecting the variant TTR, which is based on the finding that the variant TTR has an extra methionine at position 30.

Serum TTR from Japanese FAP¹ patients contains normal and the variant TTR. The structural difference between two TTR is only one amino acid replacement. Thus, it is difficult to differentiate the two immunologically with the antiserum against the whole variant. Since the variant TTR has a methionine-for-valine substitution, the protein is cleaved by CNBr at position 30. CNBr cleavage followed by tryptic digestion produces a nonapeptide (positions 22-30) from the variant as indicated in Fig. 6. On the other hand, normal TTR does not give the nonapeptide by this treatment. Once the RIA specific for the nonapeptide has been developed, the variant TTR can be differentiated from normal TTR. Homoserine lactone generated by CNBr cleavage has been converted to homoserine in order to establish a highly sensitive RIA. The nonapeptide with homoserine at the COOH-terminus is designated as FAP nonapeptide.

The variant TTR is present in all the 45 FAP¹ patients studied in our series, but is not present in normal individuals, other types of amyloidosis, nor in noninheriting members of families with FAP¹. Further studies on offsprings of patients revealed that the variant TTR is already present in half of their children, but is not present in the other half, as shown in Fig. 5. The variant TTR exists long before the clinical onset of the disease, which can be utilized to make early diagnosis of this illness even before the clinical onset of FAP¹.

The serum level of TTR is closely related to the nutritional status of the individual, since TTR has a rapid turnover rate, with a biological half-life of 2 d (18). Reduced serum levels of total TTR and total protein in FAP¹ patients are probably due to poor nutritional state caused by alternating diarrhea and constipation, severe epigastralgia, and vomiting. The wide variety of serum levels of the variant TTR (4.71-17.61 mg/dl) and total TTR (10.6-40.0 mg/dl) in patients at different stages of illness are partly related to their nutritional status.

Serum TTR in FAP¹ patients is a mixture of normal and the variant TTR in an approximately equal ratio. This ratio suggests that two TTR are the products of a co-dominant expression of two allelic genes in the heterogeneous state. It is

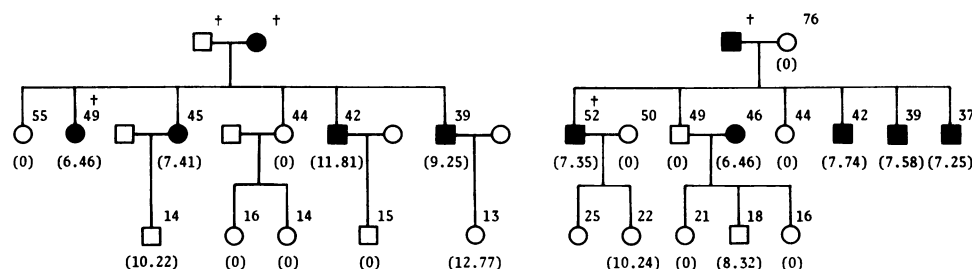


Figure 5. Samples of sibship and analysis by the RIA. Some of the siblings in the second generation are omitted to protect anonymity. The age is presented at the right upper side of the symbol. The serum level of the variant TTR is shown in parentheses. ■, affected; □, unaffected; and †, deceased.

