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The transthyretin cDNA sequence is normal in transthyretin-derived senile systemic amyloidosis

L. Christmanson[1], C. Betsholtz[1], Å. Gustavsson[2], B. Johansson[3], K. Sletten[4] and P. Westermark[3]

[1]*Department of Pathology and* [2]*Department of Rehabilitation Medicine, University of Uppsala,* [3]*Department of Pathology, University of Linköping, Sweden* and [4]*Department of Biochemistry, University of Oslo, Oslo, Norway*

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A variety of mutations leading to amino acid substitutions have been described in the transthyretin gene in association with different familial amyloidoses and have been implicated to be involved in the pathogenesis of amyloid deposits. However, there has been disagreement whether or not a transthyretin mutation is present in the most common form of transthyretin-derived amyloid, namely senile systemic amyloidosis. Therefore, the cDNA sequence of liver transthyretin was determined in a 91-year-old patient with typical senile systemic amyloidosis. This sequence was completely normal and lacked any variation. We conclude that in senile systemic amyloidosis factors other than the presence of a sequentially variant transthyretin must determine the amyloid fibril formation.

Transthyretin; Prealbumin; cDNA; Polymerase chain reaction

1. INTRODUCTION

Amyloid is a pathological fibrillar substance, deposited between cells. The exact spatial structure of the amyloid fibril is not known although a β-pleated sheet conformation is believed to be of importance [1]. Several different small proteins can aggregate to amyloid fibrils. There are 2 groups of amyloid syndromes in which the main fibril protein is transthyretin (TTR, previously prealbumin) or fragments thereof. These comprise most forms of familial amyloidosis [2–5] and the age-related senile systemic amyloidosis (SSA) [6,7]. In the TTR-derived familial amyloidoses, point mutations in the TTR gene, leading to single amino acid substitutions have invariably been found. Several different substitutions spread over the TTR molecule have been connected to amyloid syndromes [3–5,8–10]. How the amino acid substitutions are connected to the fibril formation has so far not been explained.

The familial amyloidoses are comparably rare. SSA, on the other hand, is a very common disorder affecting about 25% of individuals over 90 years old. However, few of these have more than very mild disease [11,12]. In spite of this frequency, it has been claimed that SSA might also be a hereditary disorder linked to an amino acid substitution in the TTR molecule [13]. We have now determined the cDNA sequence of TTR in a patient with advanced SSA and show that the sequence is identical with previously published normal TTR genomic DNA and cDNA sequences.

Correspondence address: P. Westermark, Department of Pathology, University Hospital, S-581 85 Linköping, Sweden

Abbreviations: AA, fibril protein in secondary systemic amyloidosis; AL, fibril protein in primary (plasma cell dyscrasia-associated) amyloid; ATTR, amyloid fibril protein derived from TTR; FAP, familial amyloidotic polyneuropathy; SSA, senile systemic amyloidosis; TTR, transthyretin

2. MATERIALS AND METHODS

2.1. *Patient*

The studies were performed on a 91-year-old man (patient 4) who died from cardiac insufficiency. Amyloidosis was not suspected clinically but at autopsy, an enlarged heart (490 g) with the appearance of amyloid infiltration was found. Myocardial and liver tissue were frozen at −70°C. Pieces of various tissues were fixed in formalin and embedded in paraffin. Congo red stained sections from the myocardium revealed massive amyloid infiltration, and small amyloid deposits were found in several other organs. Immunohistochemical examination revealed a reaction of the amyloid with a previously characterized antiserum to ATTR [12]. This antiserum does not react with amyloid of AA or AL-type. The clinical appearance and the histopathological and immunohistochemical findings were typical of SSA [12].

2.2. *Protein studies*

Only about 2 g of myocardium was available for amyloid fibril extraction. This was performed as described [7]. The fibrils in SSA have previously been shown to contain fragments of TTR and full length TTR (TTR-like protein) [7,14,15]. Since the fragments do not react with antiserum to TTR [14], the TTR-like protein of patient 4 was partially purified by covalent chromatography and gel filtration as described [7,15]. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed as described [16]. For Western blot analysis [17] proteins were transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA). With the latter, rabbit antiserum to TTR (DAKO, Copenhagen) was used, diluted 1:200. The protein band, corresponding to TTR, was also cut-out and applied directly to a 477A automatic sequence analyzer (Applied Biosystems) with an on-line 120A PTH amino acid analyzer for N-terminal analysis.

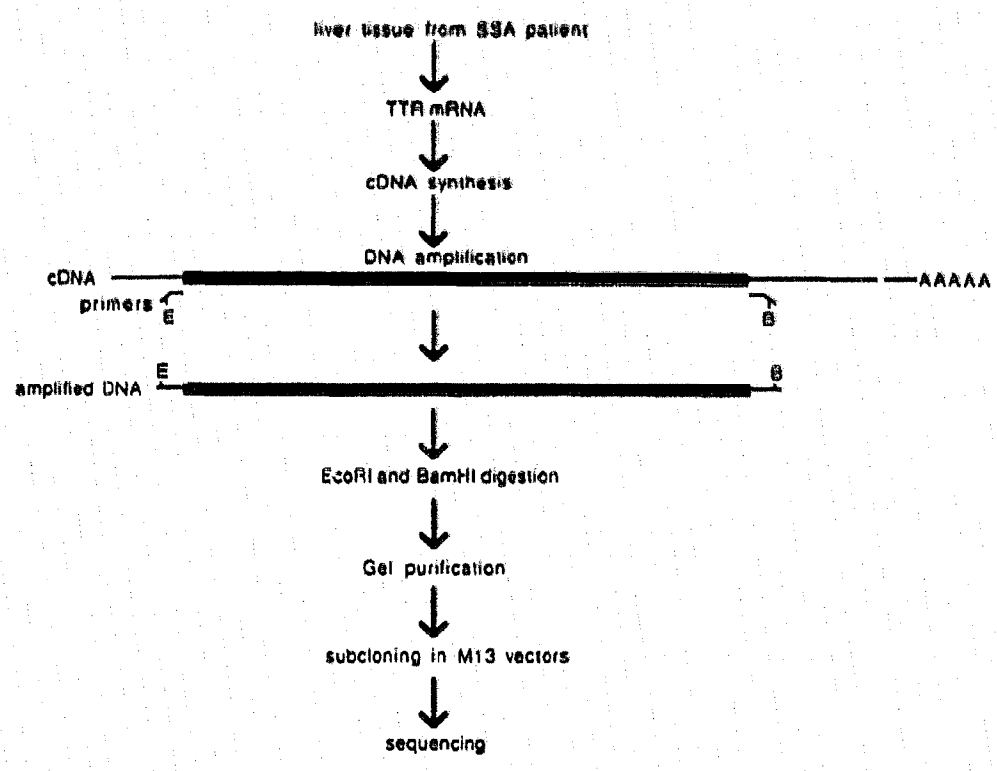

Fig. 1. Principle for the TTR cDNA synthesis, amplification and sequencing. E, *Eco*RI site; B, *Bam*HI site.

2.3. *Transthyretin cDNA*

Total cellular RNA was extracted from approximately 2 g of frozen liver tissue using the LiCl/urea method [18]. Poly(A)$^+$ RNA was enriched through chromatography on oligo(dT) cellulose (Pharmacia, Uppsala, Sweden) and used for oligo(dT)-primed first strand cDNA synthesis using an Amersham cDNA synthesis kit (Amersham, Buckinghamshire, UK). 10–50 ng of cDNA was used for polymerase chain reactions using a sense primer corresponding to the 5'-untranslated sequence of the human TTR cDNA sequence [19] immediately upstream of the translation initiation site (5'-AATCGAGAATTCAGTCCACTCATTCTTGGCAGG) and an antisense primer complementary to the 3'-untranslated sequence immediately downstream of the translation stop codon (5'-ATTCGAGGATCCCACTGGAGGAGAAGTCCCTCA). The primers included an *Eco*RI site and a *Bam*HI site respectively in their 5'-ends to facilitate subcloning of the amplification product. Thermostable DNA polymerase was purchased from Perkin Elmer Cetus (Norwalk, CT) and used as recommended by the manufacturer. The following PCR cycle temperature profiles were used: 94°C, 1 min; 54°C, 10 min; 72°C, 30 min, for 2 cycles, followed by 94°C, 1 min; 54°C, 2 min; 72°C, 4 min, for 28 cycles. Analysis of 10% of the amplification reaction product on a 1.5% agarose/TBE gel showed only one DNA band of the expected size (507 bp). The reaction products from each of 2 independent cDNA syntheses and subsequent amplification materials were purified by isotachophoresis [20], cleaved by *Eco*RI and *Bam*HI and subcloned in M13mp18/19 vectors. The complete sequences of 10 clones from the first experiment and additional 5 clones from the 2nd experiment were determined by dideoxynucleotide sequencing methodology [21] using Sequenase reagents (USB Biochemicals, Cleveland, OH). An outline of the strategy to reveal the TTR cDNA sequence is shown in Fig. 1.

3. RESULTS

3.1. *Transthyretin nature of the amyloid fibrils*

In order to prove the TTR-origin of the amyloid in patient 4, analysis of the constituent fibril protein was done. SDS-PAGE of the partially purified amyloid fibril protein revealed 3 closely situated bands of which the predominant had a mobility similar to monomeric TTR ($M_r \approx 14\,000$). Western blot analysis of the material in this band revealed a strong reaction with antiserum to TTR (Fig. 2). Furthermore, N-terminal amino acid analysis of the material gave the sequence NH_2-( )-Pro-Thr-Gly-Thr-Gly-Glu-Ser-, identical with positions 2–8 of TTR [19].

3.2. *cDNA sequence of transthyretin*

PCR using primers specific for the untranslated sequences flanking the TTR mRNA resulted in the amplification of a single DNA fragment (Fig. 3) of expected size. This fragment was purified from 2 independent experiments, subcloned and sequenced. Fifteen randomly picked TTR-positive M13 clones, 10 from the 1st and 5 from the 2nd experiment had a DNA sequence identical to that of normal TTR genomic [22] and complementary [19] DNA (Fig. 4).

4. DISCUSSION

An important question concerning the pathogenesis of SSA and other TTR-derived amyloidoses is whether or not TTR with normal primary structure can give rise to amyloid fibrils in vivo. In the most studied group of TTR-amyloidosis, the familial amyloidoses, mutant TTR has always been found, but the fibrils usually have

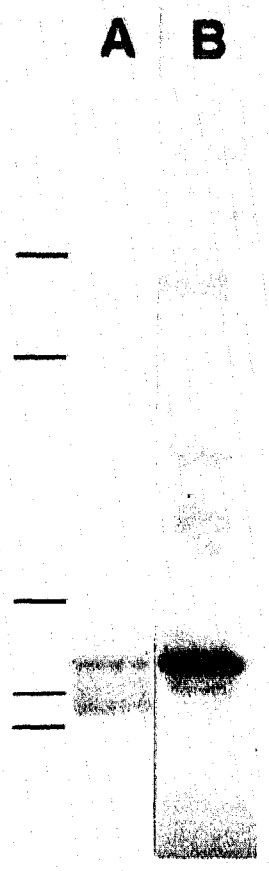

Fig. 2. (A) SDS-PAGE of partially purified amyloid fibril protein. The strong band with slightly slower mobility than lysozyme reacted strongly with anti-TTR antibodies in Western blot analysis (B). Molecular weight standards (bars) are (from above): bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; soybean trypsin inhibitor, 20.4 kDa; lysozyme, 14.4 kDa; and aprotinin, 6 kDa.

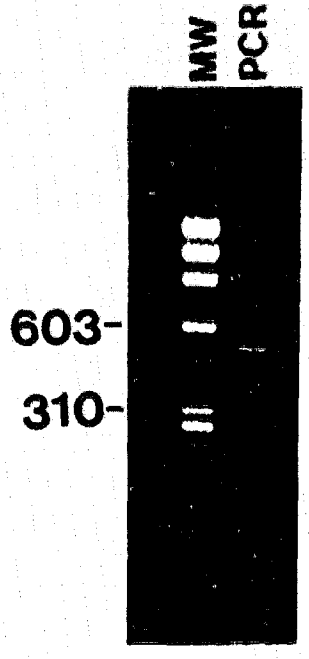

Fig. 3. Analysis of the PCR reaction using agarose gel electrophoresis followed by ethidium bromide staining shows essentially a single DNA amplification product of the expected size (507 bp). *Hae*III/ΦX174 restriction fragments are shown as molecular weight markers (MW). The sizes of 2 of the fragments are given in base pairs.

been shown to contain normal TTR as well [23,24]. In a recent study of the amyloid from one patient with typical SSA, we resolved the primary structure of the whole ATTR molecule and we were unable to find any sequentially abnormal TTR-component [15]. However, most of the amyloid was composed of TTR-fragments of varying length and a TTR-variant with an amino acid substitution in the lacking part of the molecule could theoretically have been present. To exclude this possibility, we found it important to determine the structure of TTR cDNA from another patient with proven SSA. For this study we chose material from a 91-year-old man (patient 4) with typical SSA. The TTR nature of the amyloid was shown by immunohistochemistry, Western blot analysis of the partially purified amyloid fibril protein, and N-terminal amino acid sequence analysis. All the cDNA sequences derived from this second patient were normal. Due to the absence of a polymorphic sequence within the TTR coding sequence that distinguishes the 2 TTR alleles in this patient, we cannot completely exclude the possibility that what we have amplified represents the transcription product of only one of the TTR alleles. However, if a mutant TTR allele was present, this must have been expressed at low level since in 2 independent experiments, all analyzed clones had the normal nucleotide sequence. In addition, the cDNA could be diluted 100-fold without loss of the TTR-amplification product. In combination with the previous reports on normal amino acid sequences of TTR purified from SSA patients [6,7,15] the present result strongly suggests that TTR with normal primary structure is the sole constituent of amyloid fibrils in SSA, which is the most common form of TTR-derived amyloidosis. This is an important finding since it shows that mechanisms other than variations in the primary structure of TTR are important for the fibril formation in SSA and probably also in other TTR-derived amyloid forms.

Recently, several investigations have reported a isoleucine-for-valine substitution in patients with amyloid disorder, resembling SSA in that it mainly affects the heart [13,25–27]. However, several facts indicate that these patients, rather than having SSA, represent a new form of familial amyloidosis with cardiomyopathy: all the patients studied were black, and the amyloid disease seems to have occurred at a much younger age than in SSA, which is a disease of the very old [12]. Therefore, in typical SSA, no variant TTR seems to be involved in the pathogenesis of the disease.

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                                                                    AGTCCACTCATTCTTGGCAGG
 1                                   10                                  20
Met Ala Ser His Arg Leu Leu Leu Leu Cys Leu Ala Gly Leu Val Phe Val Ser Glu Ala Gly
ATG GCT TCT CAT CGT CTG CTC CTC CTC TGC CTT GCT GGA CTG GTA TTT GTG TCT GAG GCT GGC
                                    30                                  40
Pro Thr Gly Thr Gly Glu Ser Lys Cys Pro Leu Met Val Lys Val Leu Asp Ala Val Arg Gly
CCT ACG GGC ACC GGT GAA TCC AAG TGT CCT CTG ATG GTC AAA GTT CTA GAT GCT GTC CGA GGC
                                    50                                  60
Ser Pro Ala Ile Asn Val Ala Val His Val Phe Arg Lys Ala Ala Asp Asp Thr Trp Glu Pro
AGT CCT GCC ATC AAT GTG GCC GTG CAT GTG TTC AGA AAG GCT GCT GAT GAC ACC TGG GAG CCA
                                    70                                  80
Phe Ala Ser Gly Lys Thr Ser Glu Ser Gly Glu Leu His Gly Leu Thr Thr Glu Glu Glu Phe
TTT GCC TCT GGG AAA ACC AGT GAG TCT GGA GAG CTG CAT GGG CTC ACA ACT GAG GAG GAA TTT
                                    90                                  100
Val Glu Gly Ile Tyr Lys Val Glu Ile Asp Thr Lys Ser Tyr Trp Lys Ala Leu Gly Ile Ser
GTA GAA GGG ATA TAC AAA GTG GAA ATA GAC ACC AAA TCT TAC TGG AAG GCA CTT GGC ATC TCC
                        110                                 120
Pro Phe His Glu His Ala Glu Val Val Phe Thr Ala Asn Asp Ser Gly Pro Arg Arg Tyr Thr
CCA TTC CAT GAG CAT GCA GAG GTG GTA TTC ACA GCC AAC GAC TCC GGC CCC CGC CGC TAC ACC
                130                                 140                 147
Ile Ala Ala Leu Leu Ser Pro Tyr Ser Tyr Ser Thr Thr Ala Val Val Thr Asn Pro Lys Glu
ATT GCC GCC CTG CTG AGC CCC TAC TCC TAT TCC ACC ACG GCT GTC GTC ACC AAT CCC AAG GAA

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TGAGGGACTTCTCCTCCAGTG
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Fig. 4. TTR cDNA sequence and the deduced protein sequence. The sequences of 10 individual clones were determined and found to be identical with each other and with that previously reported for normal human TTR.

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