Molecular Analyses of an Acidic Transthyretin Asn 90 Variant

Maria João Mascarenhas Saraiva,*'† Maria do Rosário Almeida,*'† Isabel Longo Alves,*'†
Paulo Moreira,* MaryAnn Gawinowicz,‡ Pedro Pinho Costa,* Silke Rauh,§ Angelika Banhzoff,§
and Klaus Altland§

*Centro de Estudos de Paramiloidose, Hospital de Santo António, and †Bioquímica, Instituto de Ciências Biomédicas, Universidade do Porto, Porto, Portugal; ‡Department of Medicine, College of Physicians and Surgeons, Columbia University, New York; and §Institute of Human Genetics, University of Giessen, Giessen, Germany

Summary

A mutation in transthyretin (TTR Asn 90) has been identified in the Portuguese and German populations. This variant has a lower pI and was found by screening analyses in 2/4,000 German subjects and in 4/1,200 Portuguese by using either double one-dimensional (D1-D) electrophoresis with isoelectric focusing (IEF) or hybrid isoelectric focusing in immobilized pH gradients (HIEF) as the final separation step. The Portuguese population sample was from the area where TTR Met 30-associated familial amyloidotic polyneuropathy (FAP) prevails, and it was divided into (a) a group of 500 individuals belonging to FAP kindreds and (b) a group of 700 collected at random. HIEF showed two particular situations: (1) one case, from an FAP kindred, was simultaneously carrier of the Met 30 substitution and the acidic variant, and (2) one individual, from the randomly selected Portuguese sample, had only the acidic monomer. Comparative peptide mapping, by HPLC, of the acidic variant carriers and of normal TTR showed the presence of an abnormal tryptic peptide, not present in the normal TTR digests, with an asparagine-for-histidine substitution at position 90 explained by a single base change of adenine for cytosine in the histidine codon. This was confirmed at the DNA level by RFLP analyses of PCR–amplified material after digestion with SphI and BsmI. In all carriers of the Asn 90 substitution, no indicators were found for an association with traits characteristic for FAP.

Introduction

Human plasma transthyretin (TTR, previously referred to as prealbumin) plays an important physiological role in the transport of vitamin A and thyroid hormones. Severe impairment of these specific functions, with pathogenic consequences, due to structural changes in TTR are not known at present. In contrast, several pathogenic mutant TTRs have been described in association with the familial amyloidoses of dominant inheritance. In these cases, the deleterious effect is the deposition of TTR as amyloid fibrils in connective

tissue, leading to neuropathy (usually referred to as familial amyloidotic polyneuropathy, or FAP) or to cardiomyopathy or simultaneously to both. The most widespread TTR mutation is a substitution—of methionine (Met) for valine (Val)—at position 30, found in FAP patients in both Portugal (the largest focus) and other countries.

The changes produced in the three-dimensional structure by the different mutations are not known, and future comparative physicochemical studies between amyloidogenic and nonamyloidogenic TTR mutations will address this issue. Using double one-dimensional PAGE (D1-D PAGE), Altland et al. (1981, 1987) have demonstrated different nonpathogenic TTR variants in the German population. Using these techniques, the present study was performed to extend the search for nonpathogenic TTR variants in the Portuguese population and to define the substitutions involved.

Subjects and Methods

About 1,200 blood samples from the Portuguese population were collected and screened for TTR variants by D1-D PAGE with hybrid isoelectric focusing (HIEF) as the final separation step (Altland and Banzhoff 1986). Five hundred individuals belonged to FAP kindreds with the Met 30–associated variant and included both patients and asymptomatic at-risk family members. Blood from the remaining 700 individuals was collected at random in the resident population of the area of Póvoa de Varzim, where FAP prevails. For comparative purposes, blood was available from two nonrelated German individuals, carriers of a nonpathogenic acidic TTR variant detected in a screening of 4,000 samples (Altland et al. 1987).

Comparative peptide mapping of both variant plasma TTR and normal TTR was performed by high-performance liquid chromatography (HPLC) using procedures described elsewhere (Saraiva et al. 1990). Exons 2 and 3 of the TTR gene were amplified, and digestion with restriction enzymes was performed according to methods described elsewhere (Almeida et al. 1990). Plasma TTR and RBP (retinol-binding protein) concentrations were measured by single radial immunodiffusion using Partigen immunodiffusion plates purchased from Behring Corp. (Germany).

Results

The screening for nonpathogenic mutations started with samples taken from FAP patients and from their asymptomatic at-risk relatives. In one sample, an acidic variant was detected together with the normal TTR monomer. The father of the proband had FAP. The variant was indistinguishable, by its pI, from two other acidic TTR variants previously found in two nonrelated German families (Altland et al. 1987). Family studies followed, to study the inheritance of the TTR variant. Figure 1 shows the results obtained.

It was observed that the 28-year-old woman (labeled NA in fig. 1) inherited the acidic monomer from her mother and transmitted it to two of her four offspring (not shown); one of her siblings, a 31-year-old man (labeled FA in fig. 1) was asymptomatic for FAP and had inherited the two TTR variants, the acidic variant from his mother and the Met-30 variant from his father, thus being a carrier of two mutant TTR monomers. There was no normal monomer in his serum. The pIs of the normal and acidic variant TTR monomer were calculated from the pH gradient profile
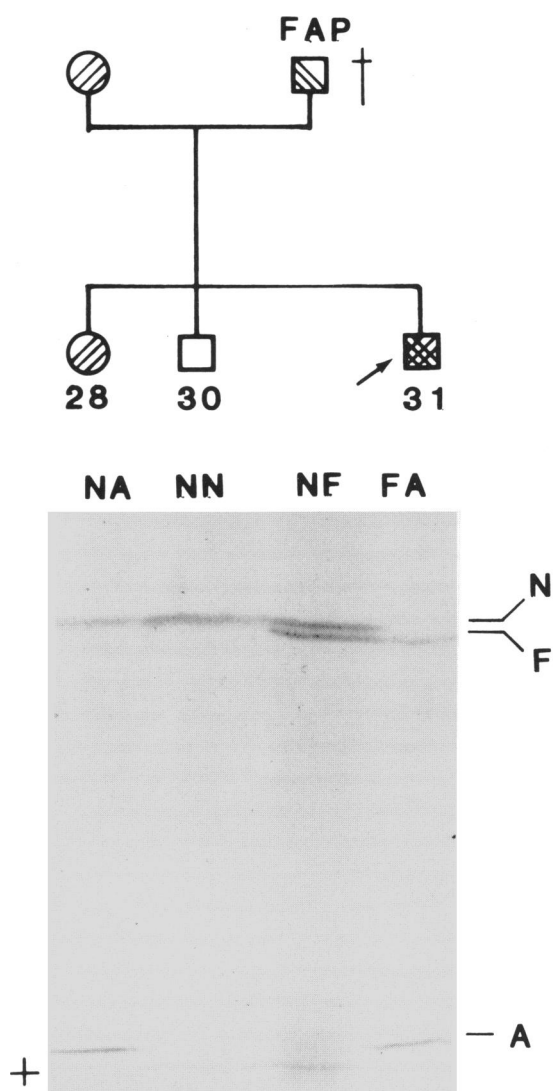

Figure 1 Demonstration by HIEF of acidic TTR monomer (A) and TTR Met 30 (F) in same kindred. NA = heterozygote for acidic monomer; NN = normal sibling; NF = heterozygote Met 30 FAP patient; FA = carrier of two variant monomers, without the normal monomer.

to be approximately 5.7 and approximately 5.43, respectively.

The screening for TTR variants continued within 700 individuals from the normal Portuguese population, and the acidic variant was found in three other samples. In one of these sera, the pattern only contained a major zone at the location of the acidic monomer, indicating homozygosity for the corresponding allele or compound heterozygosity for the latter and a silent gene. This sample was labeled AA.

All the heterozygotes for both the acidic variant and the normal TTR from both the Portuguese and the German population, as well as from the proband with no other than the acidic variant, were age 6–58 years, were healthy, and had no signs of FAP, suggesting that this variant is not associated with a pathogenic condition. Plasma TTR and RBP levels were measured in the variant carriers (NA, FA, and AA) and were compared with age-matched controls. TTR values were similar for the NA heterozygote individuals and the controls, being 284–295 mg/liter (normal 250–350 mg/liter); the AA and FA individuals, however, had low TTR concentrations—172 and 154 mg/liter, respectively. Also, their RBP levels were found to be low—20 and 23 mg/liter, respectively (normal 40–60 mg/liter).

Structural studies on isolated plasma TTR were next undertaken, to elucidate the nature of the change in the pI of the protein. Comparative peptide mapping was performed on isolated TTR from two of the carriers of the mutation (NA and FA, presented in fig. 1) and on TTR from normal plasma. Figure 2 shows the maps obtained by HPLC. A distinct abnormal peptide peak not observed in normal TTR (and indicated in the figure as 10*) was observed in the chromatograms of the two carriers of the acidic monomer TTR. Sequence analyses showed it corresponds to residues 81–103 of TTR, except for asparagine (Asn) at position 90 replacing histidine (His). In peptide 10*, a shoulder which corresponded with the position where peptide 4 with Met at position 30 is eluted (indicated as 4* in fig. 2) was also noted in the peptide map from the FA individual with two variant TTR monomers. Sequence analyses confirmed the presence of Met at position 30. We concluded that both carriers of the acidic monomer had an abnormal TTR in plasma, with an Asn-for-His substitution at position 90, along with normal TTR. The FA sample had both the Asn-for-His substitution at position 90 and a Met-for-Val substitution at position 30.

The Asn-for-His substitution agrees with the observed shift of pI to a more acidic level, since, in the conditions employed, there is on the imidazole group of His a positive charge which is replaced by an electrically neutral residue in Asn. The amino acid substitution can be explained by assuming a single base substitution (i.e., A for C) at the first position of the codon for His 90 (CAT). The change creates a new restriction site for the restriction enzyme BsmI; at the same time, it abolishes the restriction site for the enzyme SphI. Figure 3 shows the various DNA patterns, obtained
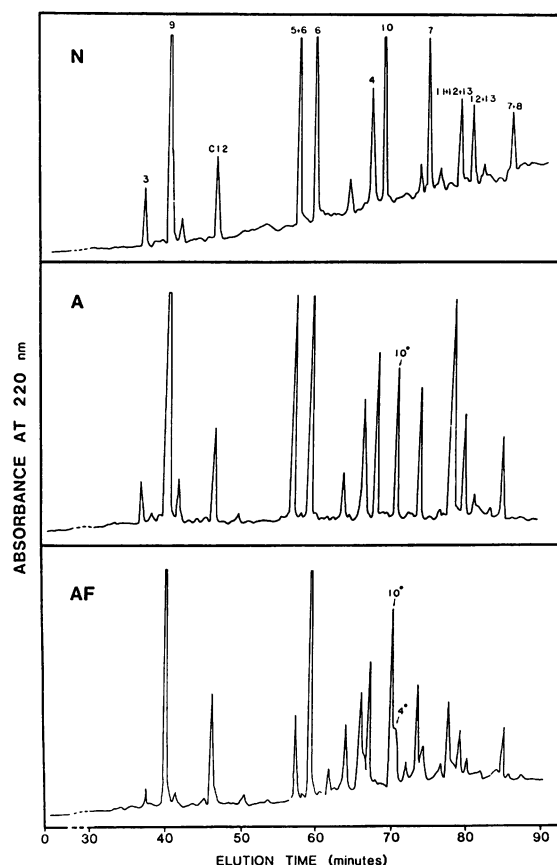

Figure 2 Comparative tryptic peptide maps of plasma TTR from (A) individual heterozygous for the acidic variant, (AF) individual with MET 30 and the acidic variant, and (N) normal plasma TTR.

after digestion with either SphI or BsmI, of the most representative samples of the present study; SphI digestion of DNA from a normal control and from an FAP patient resulted in DNA fragments of 170 and 77 bp, respectively, deriving from the amplified sequence of 247 bp; the carriers of the TTR Asn 90 monomer presented this uncleaved DNA piece because of a loss of the SphI restriction site. A sample from the AA individual with only TTR Asn 90 had only the uncleaved DNA fragment, whereas the heterozygous individuals presented additional bands resulting from digestion by SphI. The parallel analyses of the same samples by BsmI confirmed our interpretations of the results. Control and FAP DNA were not cut with the enzyme, whereas the DNA from the AA sample was completely cleaved, and the heterozygous samples had a mixed pattern.

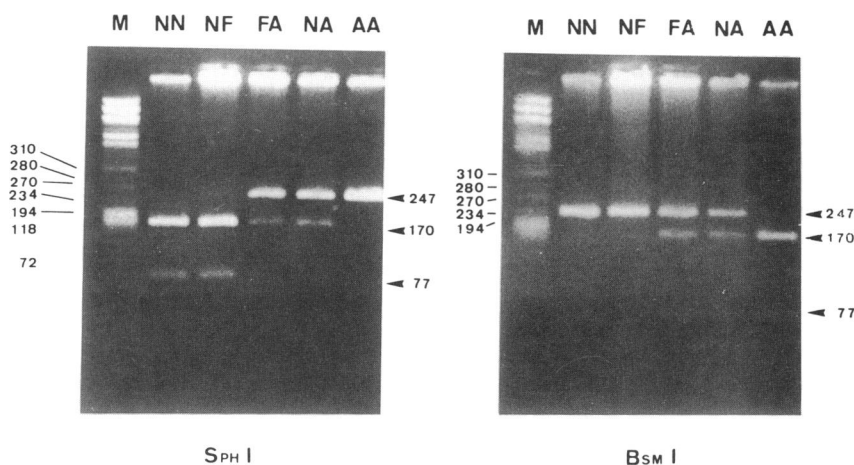RFLP analyses with SphI and BsmI were also per-

Figure 3 Demonstration by RFLP analyses of PCR-amplified material of A-for-C base change in exon 3 in subjects AA, NA, and FA, all of whom are carriers for the Asn 90 substitution. DNAs from an FAP patient (NF) and from a normal individual (NN) were included for control purposes. M = DNA size markers.

formed with the other available samples presenting the acidic TTR monomer after D1-D PAGE. In all tested samples, the DNA pattern supported the presence of the Asn 90 mutation, indicating that this variant is distributed in both the Portuguese and the German populations.

Discussion

We report here a mutation in TTR—i.e., TTR Asn 90—found in screening studies of the Portuguese and the German populations. The four kindreds found in Portugal in a small-size population, as well as the presumable homozygous case, suggest that the mutation might be rather frequent, at least in Portugal. The Portuguese population studied was restricted to the area where FAP prevails, in the north of the country; the frequency of the TTR Met 30 gene in that area is estimated to be at least 1/150 (according to our estimates), when patients and asymptomatic carriers are taken into account. Whether the Asn 90 mutation has the same frequency in areas where FAP is absent remains to be explored; if it is focused in the FAP areas, it is possible to find the two Met 30 and Asn 90 mutations present in the same individual, as in the case characterized in our study. Further screening studies covering broad areas of other countries and employing the methodology described here should address this question.

The presented data cannot distinguish between (a) homozygosity for the TTR Asn 90 allele and (b) heterozygosity for the latter and a silent allele in the individ-

ual having only the TTR Asn 90 monomer. When the DNA data showing no amplified fragments other than those produced with the A-for-C substitution in the codon for His 90 are taken into account, a silent gene would presumably be lacking the whole sequence of exon 3. With no material being available from the parents of the AA individual, in situ hybridization of chromosome 18 by exon-specific probes could in the future help to clarify the situation.

At this point it is difficult to predict the effects produced in the three-dimensional structure of TTR by the Asn 90 mutation. It might affect both thyroxine binding and RBP binding. Preliminary studies with the Met 30–Asn 90 subject revealed a strong reduction in T$_4$ binding (M. J. M. Saraiva, unpublished results). As far as the interaction of TTR with RBP is concerned, the low RBP levels found might be due to an altered TTR-RBP interaction, a fact that should be further investigated.

Taken together, the evidence so far suggests that the Asn 90 substitution does not produce pathogenic effects; it might be useful to follow the asymptomatic Asn 90–Met 30 individual, to see whether the second mutation delays the clinical expression and, after onset, whether it alters the amyloidogenicity of the Met 30 variant. In this context, it is interesting that recent studies by Skare ét al. (1989) have identified an *Sph*I polymorphism in an American patient with FAP, a polymorphism localized in the same gene region as in our cases. The patient's TTR was analyzed by conventional IEF, using the conditions employed in the present study, and the variant and normal monomers did

not separate well; in contrast, in the cases reported here there is a difference of almost 0.3 pH units between the pI of TTR Asn 90 and that of the normal TTR monomer, as would be expected from the corresponding amino acid substitution. The individual described by Skare et al. (1989) has FAP, whereas all our carriers of TTR Asn 90 have not. Further studies are needed to clarify this significant discrepancy.

Acknowledgments

References

Almeida MR, Alves IL, Sakaki Y, Costa PP, Saraiva MJM (1990) Prenatal diagnosis of familial amyloidotic polyneuropathy — evidence for an early expression of the associated transthyretin methionine 30. Hum Genet 85:623–626

Altland K, Banzhoff A (1986) Separation by hybrid isoelectric focusing of normal human plasma transthyretin (prealbumin) and a variant with a methionine for valine substitution associated with familial amyloidotic polyneuropathy. Electrophoresis 7:529–533

Altland K, Becher P, Banzhoff A (1987) Paraffin oil protected high resolution hybrid isoelectric focusing for the demonstration of substitutions of neutral amino acids in denatured proteins: the case of four human transthyretin (prealbumin) variants associated with familial amyloidotic polyneuropathy. Electrophoresis 8:293–297

Altland K, Rauh S, Hackler R (1981) Demonstration of human prealbumin by double one-dimensional slab gel electrophoresis. Electrophoresis 2:148–155

Saraiva MJM, Sherman W, Marboe C, Figueira A, Costa PP, Freitas AF, Gawinowicz MA (1990) Cardiac amyloidosis: report of a patient heterozygous for the transthyretin isoleucine 122 variant. Scand J Immunol 32:341–346

Skare JC, Saraiva, MJM, Alves IL, Skare IL, Milunsky A, Cohen AS, Skinner MA (1989) A new mutation causing familial amyloidotic polyneuropathy. Biochem Biophys Res Commun 164:1240–1246