Danish type gelsolin related amyloidosis: 654G-T mutation is associated with a disease pathogenetically and clinically similar to that caused by the 654G-A mutation (familial amyloidosis of the Finnish type)

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Abstract
Background—Familial amyloidosis of the Finnish type (FAF, Finnish hereditary amyloidosis) is caused by a 654G-A mutation in the gelsolin gene on chromosome 9 resulting in the expression of mutant Asn-187 gelsolin which is abnormally proteolytically processed generating amyloidogenic fragments that polymerise into amyloid fibrils. We have recently shown that in a Danish and a Czech family with a clinical syndrome similar to FAF, including corneal lattice dystrophy, cranial neuropathy and skin changes, the disease is caused by another mutation at the same position, namely 654G-T predicting a Tyr for Asp substitution at 187 in secreted gelsolin.

Aim—To undertake a closer examination of the Danish subtype of FAF and report immunohistochemical and biochemical findings.

Results—Immunostaining of plasma gelsolin isolated from heterozygous FAF of the Danish subtype revealed a pattern similar to that found in FAF-Asn 187. The > 60 kDa gelsolin species contain an epitope characteristic of the amyloid forming region as revealed by an amyloid specific antibody, whereas the ∼ 50 kDa fragments are devoid of it. Compared with the wild-type gelsolin peptide (Asp-187), the corresponding mutant peptide (Tyr-187) showed dramatically increased fibrillogenesis as revealed by quantitative thioflavine-T based fluorimetry; ultrastructurally, amyloid-like fibrils were formed by the mutant peptide. Immunohistochemistry showed that antibodies directed against residues 231–242 of secreted gelsolin, representing the carboxy terminus of the sequence forming the amyloid protein (residues 173–243) laid down in the tissues in a fibrillar form in FAF, specifically labelled the amyloid deposited in rectum and skin in the Danish (654G-T) subtype.

Conclusions—The 654G-T mutation in the gelsolin gene gives rise to an amyloid disease clinically and pathogenetically similar to that caused by the 654G-A mutation.

Keywords: amyloidosis; Finnish familial amyloidosis; gelsolin mutation 654G-T; fibrillogenesis

Familial amyloidosis of the Finnish type (FAF, Finnish hereditary amyloidosis, amyloid polynecuropathy type IV) is a dominantly inherited systemic amyloid disease characterised by cranial neuropathy, corneal lattice dystrophy, skin changes, and renal and cardiac manifestations.1–3 The disease is caused by a point mutation, 654G-A, in the gelsolin gene4–6 resulting in the expression of amyloidogenic Asn-187 gelsolin and the accumulation of a 71 residue fragment of mutant gelsolin, Ala173-Met243, as amyloid fibrils in the tissues.7 8 The Asn-187 gelsolin mutation has been found in all FAF patients so far described from Finland, as well as in occasional American, Dutch, and Japanese families.9–14 In contrast, in a Danish family and a Czech family with clinical syndromes similar to FAF, we found another mutation, namely 654G-T, in the gelsolin gene10 predicting a Tyr for Asp mutation at position 187 in secreted gelsolin.10 We have now undertaken a closer examination of the Danish subtype of FAF and report the immunohistochemical and biochemical findings.

Methods
Tissue and plasma samples
We studied biopsy specimens from skin and rectum from three siblings of a Danish family with familial amyloidosis with slowly progressive cranial neuropathy and corneal lattice dystrophy.15 The 654G-T mutation in gelsolin in this family was identified by allele specific
oligonucleotide slot-dot hybridisation and by sequencing. Plasma gelsolin was isolated from a 57 year old male patient of the same Danish family. He had suffered from cranial amyloidotic polyneuropathy for about 10 years at the time of sampling. As controls we used plasma gelsolin isolated from Finnish subjects with FAF (two heterozygous (male) and one homozygous (female) patients with an identified 654G-A gelsolin mutation), plasma from healthy controls, and tissue samples from patients with FAF (Asn-187; three cases), AA amyloidosis (two cases), and AL amyloidosis (one κ and one λ case).

**ANTIBODIES**

We used two different antigelsolin antisera (fig 1): murine monoclonal antigelsolin antibody (MoAb-2C4) raised using a chymotryptic 47 kDa C-terminal actin binding fragment of gelsolin (Sigma), and polyclonal rabbit antiamyloid antibodies (PoAb231–242) raised using synthetic VHVSEEGTEPEA amide corresponding to the carboxy terminus (residues 231–242) of the region forming the amyloid protein in FAF. The polyclonal antibodies were purified by immunoaffinity chromatography on Sepharose coupled to a gelsolin peptide corresponding to residues 231–241 in secreted gelsolin.

**IMMUNOBLOTTING**

For western blots, proteins were separated on 10% SDS-PAGE, electroblotted on nitrocellulose membranes, and detected with murine monoclonal or rabbit polyclonal antibodies using as second antibodies peroxidase or alkaline phosphatase conjugated rabbit or swine immunoglobulins to murine or rabbit immunoglobulins (Daka), respectively. Development was performed with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide or with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in dimethylformamide (AP Conjugate Substrate kit, Bio-Rad), respectively. As molecular standards, we used pre-stained protein marker, broad range (New England Biolabs) and pre-stained SDS page standards, low range (Bio-Rad).

**AFFINITY CHROMATOGRAPHY**

Isolation of gelsolin was carried out by immunoaffinity chromatography on a MoAb-2C4-Sepharose column using Prot On Kit 2 (Multiple Peptide Systems) in the presence of 1 mM EGTA. Affinity chromatography on immobilised cibacron blue F3GA was performed in the presence of 1 mM EGTA as described previously. For routine testing, 100 µl of plasma was chromatographed on 1.0 × 1.5 cm cibacron blue column.

**HISTOCHEMISTRY**

Tissue specimens were studied for the presence of amyloid by routine congo red staining and polarisation microscopy. Immunoperoxidase staining was carried out as previously described. For immunogold staining, tissue sections 1 µm thick were deparaffinised and after three washings incubated with 5% normal goat serum diluted in phosphate buffered saline (PBS), pH 7.2, containing 0.1% bovine serum albumin and 0.1% sodium azide. The sections were then incubated with the purified rabbit PoAb 231–242 antibodies for 30 minutes, followed by three washings with PBS. Gold labelled goat antirabbit IgG (Auro Probe LM GAR, Amersham) at 1:40 dilution was added and incubated for one hour, followed by three washings with water. The sections were developed by a silver enhancement kit (Inten SEM, Amersham).

**IN VITRO FIBRILLOGENESIS**

For fibril formation, synthetic peptides homologous to wild-type (SFNNGDCFILD-amide; residues 182–192) or mutant (SFNNGYCFILD-amide and SFNNGNCFILD-amide representing residues 182–192) gelsolin were used (Multiple Peptide Systems). The purity of the peptides was > 95% as judged by high pressure liquid chromatography on a Vydac C18 column (fig 2).
The peptides were dissolved in 10% acetic acid, pH 2.3, at a concentration of 5 mg/litre and analysed for Congo red positivity and green birefringence in polarised light after 15 minutes and overnight incubation at room temperature.

MONITORING OF FIBRIL FORMATION
Fibril formation was monitored by quantitative thioflavine-T fluorometry as described. In this study we used the excitation and emission maxima of 450 nm and 482 nm, respectively, and a Perkin-Elmer Luminescence LS 30 spectrometer.

ELECTRON MICROSCOPY
Negative staining electron microscopy was carried out on peptide samples that were dissolved in water or 10% acetic acid at a concentration of 5 mg/litre, incubated at room temperature for 15 minutes and then kept frozen at −20°C. After thawing, the samples were diluted 1:1 in water, applied to a copper grid coated with pioloform and carbon, and stained with phosphotungstic acid adjusted to pH 6.5 with potassium hydroxide. The grids were examined with a JEOL-JEM-100CX transmission electron microscope at an operating voltage of 60 kV.

Results
HISTOCHEMISTRY
Prominent amyloid deposits were found along the collagen fibres in rectal mucosa and the thickened basement membranes of the dermal eccrine glands (fig 3). The amyloid deposits were specifically stained by the antigelsolin antibody PoAb231–242 directed against the carboxy terminus of the region forming the amyloid subunit protein (figs 1 and 4). PoAb231-242 did not stain tissue amyloid in AA or AL amyloidosis.

PLASMA GELSOLIN
Plasma gelsolin from heterozygous Danish subtype FAF was isolated by affinity chromatography and subjected to immunoblotting with MoAb-2C4 antibody. In addition to normal sized gelsolin, lower molecular weight fragments were detected; the pattern in Danish heterozygous FAF was similar to that found in

![Figure 3 Dermal eccrine glands in Danish subtype (Tyr-187) familial amyloidosis showing prominent amyloid deposits in thickened basement membranes. Congo red staining, polarised light, magnification ×140.](image)

![Figure 4 Immunogold-silver staining with amyloid specific antibody PoAb 231–242 of a skin biopsy in Danish subtype (Tyr-187) amyloidosis. Amyloid located in basement membranes is specifically stained. Magnification ×134.](image)

![Figure 5 Comparison of heterozygous Danish subtype FAF (Tyr-187) and heterozygous Asn-187 FAF by immunoblotting by the monoclonal MoAb-2C4 antibody. Lane 1, FAF Asn-187; lane 2, Danish FAF (Tyr-187); STD, prestained molecular weight markers (kDa).](image)
heterozygous Finnish patients with the Asn-187 mutation (fig 5). The antibody PoAb 231–242, directed against the amyloid forming region of gelsolin (fig 1) recognised the 60 kDa gelsolin species, but not the 50 kDa fragments (fig 6), which are devoid of the amyloid forming sequence.

IN VITRO AMYLOIDOGENESIS

Synthetic mutant Tyr-187 (Danish type) and Asn-187 (Finnish type) peptide, corresponding to residues 182–192 of gelsolin, formed amyloid-like fibrils in vitro (fig 7, Danish-type).

Discussion

The Danish type of familial amyloidosis with cranial neuropathy and corneal lattice dystrophy was described in 1979 by Boysen et al in five siblings of a family who had lived in Denmark for several generations. No genealogical connections to Finland could be shown for the family. The clinical features of the disease are very similar to those described by Meretoja in the Finnish families with hereditary amyloidosis, FAF. In contrast to FAF, where the gelsolin mutation has been identified as 654G-A, a 654G-T mutation causes the Danish subtype of FAF. This mutation predicts a tyrosine for aspartic acid substitution at position 187 in secreted gelsolin.

The results of this study show that the biological consequences of the 654G-T mutation in gelsolin are very similar to those of the 654G-A mutation. Immunoblotting of plasma gelsolin in heterozygous Danish subtype FAF reveals a fragmentation pattern similar to that found in FAF (Asn-187), in addition to normal sized gelsolin, a series of lower Mr C-terminal fragments is found. Thus the substitution of aspartic acid at position 187 in gelsolin with either asparagine or tyrosine is associated with abnormal degradation. The most likely explanation for the abnormal fragmentation, and the generation of the disease specific 65 kDa fragment, is that residue 187 represents a critical site where a substitution of an amino acid with a charged side chain (Asp) or hydrophobic (Tyr) side chain induces a novel proteolysis site at 172–173 by the mechanism of local unfolding. This
Danish-type gelsolin related amyloidosis

99

Tyr-187 peptide were amyloid-like. Thus the as revealed by quantitative thioflavine-T based mutant Tyr-187 peptide was very fibrillogenic the corresponding wild-type gelsolin peptide, into insoluble amyloid fibrils. Compared with are highly fibrillogenic and rapidly polymerise gelsolin fragments containing the mutation site starting with Ala 173.


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doi: 10.1136/jcp.53.2.95