Vol. 153, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1988 Pages 198-202

RESTRICTION FRAGMENT ANALYSIS CONFIRMS THE POSITION 33 MUTATION IN TRANSTHYRETIN FROM AN ISRAELI PATIENT (SKO) WITH FAMILIAL AMYLOIDOTIC POLYNEUROPATHY

Daniel R. Jacobson[a], Frances Santiago-Schwarz[b], and Joel N. Buxbaum[a,b]

Research Service, New York V.A. Medical Center and Departments of Medicine[a] and Pathology[b], New York University Medical Center, New York, NY[a]

Received March 31, 1988

Summary: Transthyretin isolated from amyloid fibrils from an Israeli patient with Familial Amyloidotic Polyneuropathy was sequenced by two research groups. One laboratory reported a position 49 Thr→Gly substitution, while the other noted a Phe for Ile interchange at amino acid 33. We used a transthyretin cDNA probe to study DNA from this patient by Southern blotting. The DNA displayed the unique Bcl I restriction site predicted by the mutation in codon 33. Because of the close size of the normal (6.40 kb) and variant (6.27 kb) fragments, the variant was more easily demonstrated after digestion with both Bcl I and Sph I, which generated two easily resolvable fragments of 2.39 and 2.27 kb.

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant disease characterized by multiple organ dysfunction resulting from widespread amyloid deposition. Affected patients often develop peripheral and autonomic neuropathy, as well as cardiac, renal, gastrointestinal, and ophthalmologic dysfunction. Differences in age of onset and rapidity of disease progression exist both within and between affected kindreds. Except for one kindred, in which the amyloid material consists of apolipoprotein AI (1), the amyloid fibrils in all cases studied thus far consist of transthyretin (also referred to as prealbumin or thyroxine-binding prealbumin).

Normal transthyretin is a tetrameric protein composed of four identical subunits (each 127 amino acids) which carries thyroxine and retinol-binding protein (2,3). The gene has been sequenced (4,5), and consists of four exons and three introns spanning 7.0 kb. In all affected kindreds in

Abbreviations
FAP: familial amyloidotic polyneuropathy
SSC: salt, sodium citrate; 1xSSC = 0.15 M NaCl, 0.015 M sodium citrate.

0006-291X/88 $1.50

which the transthyretin protein has been studied, a variant form has been found in the fibril (6-12). However, both normal and abnormal transthyretin are present in the serum, and may be found in the tissues as well. The most common mutation, present in the Portuguese-Japanese-Swedish type of FAP (also called FAP type I), is a position 30 substitution of valine for methionine (7-9). Other kindreds have mutations at positions 54/55 (reference 6 and unpublished observations), 36/37 (6), 84 (10), 60 (11), and 77 (12). These mutations may affect interactions between transthyretin subunits or disrupt the secondary structure of the polypeptide so that the molecule is no longer soluble under physiologic conditions and forms the Congo Red-binding amyloid fibrils which constitute the tissue deposits. Accurate descriptions of the molecular variants associated with the disease should provide a better understanding of the pathogenesis of amyloidosis, and may lead to simple tests for the mutant gene within affected families.

The Ashkenazic Israeli FAP patient "SKO" has been described previously (13). Extracted amyloid fibrils were subjected to amino acid sequence analysis in two laboratories. One group, which isolated amyloid from the thyroid and spleen, found a substitution of glycine for the threonine normally found at position 49 in the transthyretin molecule (14), while the other identified a position 33 substitution of phenylalanine for isoleucine (15). The latter, corresponding to a T to A transversion at the first position of the codon, predicts the creation of a new restriction site for the endonuclease Bcl I (figure 1). We studied DNA from this patient to clarify the abnormality present and to develop a simple test for this transthyretin variant.

Materials and Methods

A human liver library of cDNA inserted into the Pst I site of pkt218 was provided by Dr. Stuart Orkin and grown in E. coli MC 1061. The library was screened using two sets of synthetic oligonucleotides constructed according to the known amino acid sequence of transthyretin. Plasmid DNA from positive clones was isolated by cesium chloride density gradient centrifugation, and inserts were separated by Pst I digestion and agarose gel electrophoresis. Two inserts were subcloned into M13 mp 8 and 9 for sequencing by the dideoxy method (16). The sequence of one clone corresponded to the reported sequence of human transthyretin cDNA (17) from base pair 29 to base pair 405, which codes for part of the hydrophobic leader sequence plus all of the secreted protein except for the last 12 amino acids. The clone included part of the first and last exons and all of exons 2 and 3. The insert excised from this clone, or the intact M13 clone, was labelled by nick translation for use as a probe in Southern blotting.

Tissue from patient SKO was provided by Dr. Mordechai Pras (13). DNA was isolated (18) from kidney obtained at autopsy. Other genomic DNA samples were obtained from three unrelated kindreds with FAP and from several controls without amyloidosis. Southern blots were performed using stan-

dard conditions (19). 10-20 micrograms of genomic DNA was digested with a 5-fold excess of restriction enzyme. For double digests with Sph I and Bcl I (New England Biolabs, Beverly, MA) samples were digested first with Sph I at 37° C for four hours. Bcl I was then added and the sample incubated at 50° C for an additional four hours. The digested DNA samples were electrophoresed on 0.7% agarose gels in tris-acetate buffer, transferred to nitrocellulose filters, baked (80° C for two hours under a vacuum), prehybridized, and hybridized overnight. Filters were washed in 2xSSC[1], 0.1% SDS for 10 minutes at 20° C, then for two hours in 0.15xSSC, 0.1% SDS at 59° C. Hybridized filters were exposed to Kodak XAR-5 film at -80° C for three to ten days.

Results

After digestion with Sph I and Bcl I, DNA from SKO kidney revealed a restriction fragment not present in DNA isolated from controls or other kindreds with FAP (figure 2). The digested SKO specimen contained both the normal (2394 base pairs) and a mutant (2266 base pairs) allele (figure 3). Digests of DNA with Bcl I alone also revealed a unique pattern in SKO (data not shown), but the mutant fragment (6.27 kb) was difficult to distinguish from the normal (6.40 kb) since they differed by only 128 base pairs. Because of the Sph I site near the 3' end of exon 3, digestion with Sph I and Bcl I yielded smaller fragments which still differed by 128 base pairs, hybridized strongly with a cDNA probe, and were easily resolved electrophoretically. Digestion with Sph I alone yielded no polymorphic fragments in SKO (data not shown). SKO DNA did not contain the restriction

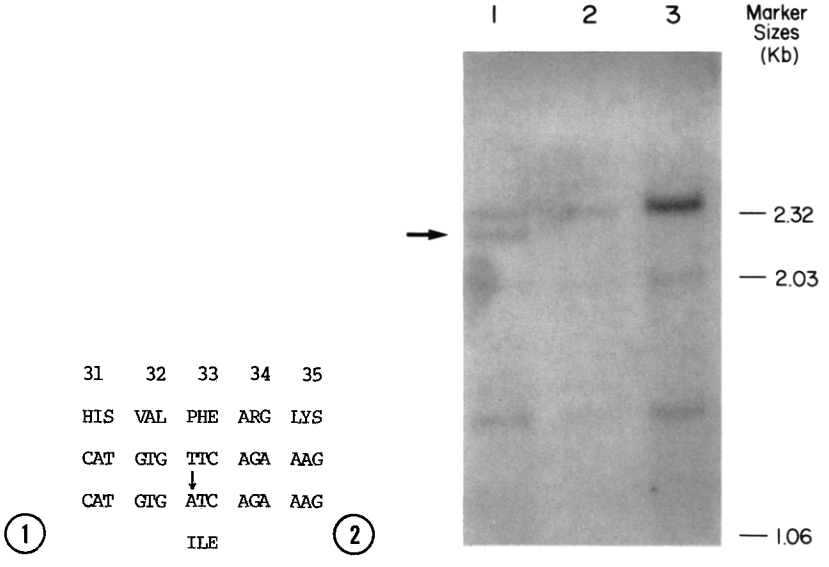

Figure 1. Position 33 mutation in SKO. Bcl I cuts at T^GATCA. Mbo I and Sau3A I cut at ^GATC.

Figure 2. Southern blot of Bcl I/SphI digests of SKO (lane 1) and control (lanes 2 and 3) DNA samples. Eight other unrelated control specimens showed a pattern identical to the pattern seen for the controls in lanes 2 and 3.

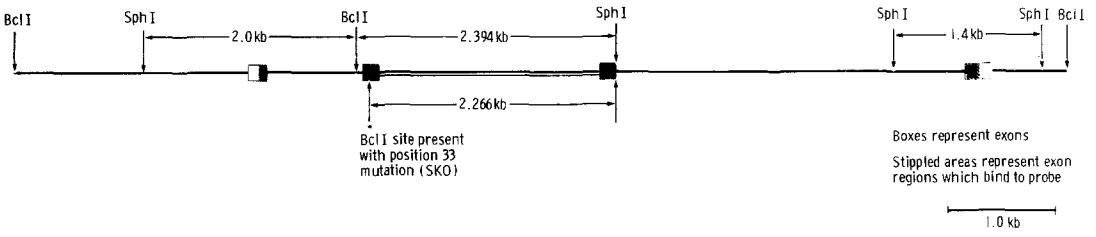

Figure 3. Bcl I/Sph I restriction map of the human transthyretin gene. Exons are indicated by boxes, and introns by lines. Coding regions of exons are stippled; non-coding regions are blank. The site present only in SKO DNA is indicated by an arrow with an asterisk. Sizes indicated are in kb.

digest differences found with any other previously described FAP-associated mutation, as shown by normal patterns after digestion with Nsi I, Alu I, Fnu4H I, and Pvu II (data not shown). None of 10 other samples tested demonstrated a Bcl I/Sph I restriction fragment length polymorphism.

Discussion

The unique Bcl I/Sph I restriction fragment in DNA from patient SKO confirms the presence of the position 33 substitution previously found on amino acid sequencing. The unique band seen in the SKO sample (figure 2, arrow) and the normal bands seen in both the SKO and control specimens represent DNA fragments depicted schematically in the transthyretin restriction map (figure 3).

As an alternative to double digestion with Bcl I and Sph I, the variant fragment can be demonstrated by digestion with Mbo I (or Sau 3A I), which recognizes the central four bases recognized by Bcl I, "GATC" (figure 1). Digestion with Mbo I yielded even smaller fragments than those obtained after Bcl I/Sph I digestion, with a unique fragment of 571 bp seen in the SKO digest but not in controls (datanot shown). A drawback to this method, using a cDNA probe, is that the diagnostic fragment binds to exon 2 alone after Mbo I digestion, rather than to both exons 2 and 3, as after Bcl I/Sph I digestion; thus, the signal is weaker. In our hands, incubation with Bcl I plus Sph I yielded the clearest results, and we recommend this as the method of choice in testing for the position 33 mutation seen in SKO.

The reported position 49 glycine for threonine mutation cannot be addressed by simple restriction fragment analysis, as no known enzyme will detect the DNA change which would cause that substitution. The mutation cannot be explained by a single base change; one must hypothesize changes

in both the first and second positions of the codon. A DNA event more complex than a simple point mutation may have occurred, although no insertion or deletion large enough to be detected by Southern blotting was present.

The restriction pattern we identified in DNA from patient SKO confirms the position 33 mutation previously found on amino acid sequencing and provides a simple test for its presence. The reported method can be used in other newly identified FAP kindreds, and for testing of at-risk patients within known affected kindreds.

Acknowledgements

This work was supported by an Associate Investigator Award from the Veterans Administration (Dr. Jacobson), N.I.H. Training Grant CA09161 (Dr. Santiago-Schwarz), and N.I.H. Grant AM 01431.

References

1. Nichols, W.C., Dwulet, F.E., and Benson, M.D. (1987) Clin. Res. 35, 595A.
2. Kanda, Y., Goodman, D.S., Canfield, R.E., and Morgan, F.J. (1974) J. Biol. Chem. 249, 6976-6805.
3. Blake, C.C.F., Geisow, M.J., and Oatley, S.J. (1978) J. Mo. Biol. 121, 339-356.
4. Tsuzuki, T., Mita, S., Maeda, S., Araki, S., and Shimada, K. (1985) J. Biol. Chem. 260, 12224-12227.
5. Sasaki, H., Yoshioka, N., Takagi, Y., and Sakaki, Y. (1985) Gene 37, 191-197.
6. Jacobson, D.R., Santiago-Schwarz, F., Rosenthal, C.J., and Buxbaum, J.(1987) Clin. Res. 35, 594A.
7. Dwulet, F.E. and Benson, M.D. (1983) Biochem. Biophys. Res. Comm. 114, 657-662.
8. Tawara, S., Nakazato, M., Kangawa, K., Matsuo, H., and Araki, S. (1983) Biochem. Biophys. Res. Comm. 116, 880-888.
9. Saraiva, M.J.M, Birken, S., Costa, P.P, and Goodman, D.S. (1984) J. Clin. Invest. 74, 104-119.
10. Wallace, M.R., Dwulet, F.E., Conneally, P.M., and Benson, M.D. (1986) J. Clin. Invest. 78, 6-12.
11. Wallace, M.R., Dwulet, F.E., Conneally, P.M., and Benson, M.D. (1987) Clin. Res. 35, 419A.
12. Wallace, M.R., Dwulet, F.E., Williams, E.C., Conneally, P.M., and Benson, M.D. (1988) J. Clin. Invest. 81, 189-193.
13. Gafni, J., Fischel, B., Reif, R., Yaron, M., and Pras, M. (1985) Quart. J. Med. 55, 33-43.
14. Pras, M., Prelli, F., Franklin, E.C., and Frangione, B. (1983) Proc. Natl. Acad. Sci. USA 80, 539-542.
15. Nakazato, M., Kangawa, K., Minamino, N., Tawara, S., Matsuo, H., and Araki, S. (1984) Biochem. Biophys. Res. Comm. 123, 921-928.
16. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74:5463-5467.
17. Mita, S., Maeda, S., Shimada, K., and Araki, S. (1984) Biochem. Biophys. Res. Comm. 124, 558-564.
18. Blin, N. and Stafford, D.W. (1976) Nucl. Acids Res. 3, 2303-2308.
19. Alexander, R.J., Buxbaum, J.N., and Raicht, R.F. (1986) Gastroenterology 91, 1503-1510.