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SEEKING CONFIDENCE IN THE DIAGNOSIS OF SYSTEMIC AL (Ig LIGHT-CHAIN) AMYLOIDOSIS: PATIENTS CAN HAVE BOTH MONOCLONAL GAMMOPATHIES AND HEREDITARY AMYLOID PROTEINS

SHORT TITLE: types of amyloidosis

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ABSTRACT

Investigators in the United Kingdom have shown that hereditary amyloidosis can be misdiagnosed as Ig light-chain (AL) amyloidosis because family history is an ineffective screen and tissue-staining used to type amyloid is unreliable. Misdiagnosis of AL can lead to inappropriate use of chemotherapy and failure to diagnose a hereditary disease. Over a 3-year period we sought to determine how often both possible sources of amyloidosis occurred in the same patient. We employed an algorithm based on established data and patterns of amyloidosis in order to focus the screening effort. Of 178 consecutive patients referred for amyloidosis, 54 were screened by polymerase chain reaction techniques with primers designed to detect transthyretin, apolipoprotein AI, apolipoprotein AII, fibrinogen Aα and lysozyme variants. Three patients (6% of those screened and 2% of symptomatic patients) had both a monoclonal gammopathy and a hereditary variant. These results justify further study of screening for hereditary variants in patients with apparent AL, and highlight the need for practical techniques for identifying fibrils extracted from tissue.

INTRODUCTION

In the report of the first autologous stem cell transplantation (SCT) trial for systemic AL (Ig light-chain) amyloidosis (AL), the authors wrote that patients with hereditary amyloidosis "are never candidates for dose-intensive melphalan."[1] In hereditary amyloid, the mutant protein is often hepatic in origin and the standard treatment is liver transplant not high-dose chemotherapy. [2,3,4] In AL, the precursor protein is an immunoglobulin light chain produced by clonal plasma cells and standard treatment is cytoreductive chemotherapy. [5,6,7]

The issue of misdiagnosis of AL has been raised by several investigators.[8,9] The national amyloidosis center in the United Kingdom (UK) reported that, of 350 patients thought to have AL, 10% had hereditary variants instead, including patients who had understandably failed SCT. The investigators noted that hereditary variants have variable penetrance making family history an ineffective screen, and that the immunohistochemical (IHC) staining techniques used to type tissue amyloid as derived from Ig light chains were unreliable, although IHC staining for non-Ig amyloid-forming proteins such as transthyretin (TTR) or fibrinogen Aα may be useful.[9] The lack of reliable staining for all types of amyloid limits the utility of IHC approaches.[10]

Most hereditary variants are due to point-mutations causing amino acid replacements. In the series of 350 patients from the UK, the identification of hereditary variants was based upon polymerase chain reaction (PCR) amplification and sequencing of potentially mutated genes.[9] Obviously the issue of diagnostic confidence would be moot if the amyloid protein in each case could be extracted easily from tissue and evaluated for identity.[11] Currently such techniques are not practical because, given the variability of deposits, obtaining enough tissue for protein extraction or for immunogold electronmicroscopy is problematic.[11,12] In this study we asked how often patients with symptomatic amyloidosis might have both a monoclonal gammopathy and a

hereditary variant, employing a screening algorithm based on patterns of presentation of hereditary and AL amyloidosis. Our results support the need for routine DNA-based screening and for new methods for typing amyloid.

PATIENTS, MATERIALS AND METHODS

Patients and Screening

Between 06/01/2002 and 08/01/2005 patients referred for assessment of systemic amyloidosis were evaluated for monoclonal gammopathy and organ-involvement with amyloid as previously described.[13,14,15] Approval was obtained from the institutional review board of Memorial Sloan-Kettering Cancer Center for these studies and patients gave written informed consent. We screened all patients in the following categories for hereditary variants whether or not they had a monoclonal gammopathy: (1) African-Americans were screened for the presence of a mutant transthyretin; the Val122Ile variant of TTR occurs in 4% of African-Americans;[16] (2) patients with dominant peripheral nervous system involvement were screened for the variants in Table 1; peripheral neuropathy is a common presentation of AL amyloidosis and several hereditary variants;[13] (3) patients with isolated renal amyloidosis and no amyloid in the bone marrow were screened for the fibrinogen Aα variant that occurred in 5% of UK patients, all with renal but not marrow amyloid;[9] and (4) patients sent for hereditary screening or with a biopsy reporting amyloidosis were screened for all variants in Table 1 and tested for a monoclonal gammopathy; some were re-biopsied.

Polymerase chain reaction (PCR) assays

Genomic DNA was extracted from mononuclear cells as previously described.[17] Primer pairs were designed for transthyretin, apolipoprotein AI, apolipoprotein AII, fibrinogen Aα, and lysozyme (Table 1). [3,18,19,20,21] PCR amplicons were sequenced at our core facility and results

scanned with Chromas Version 1.45 (Griffith University, Queensland, Australia) and evaluated by BLAST (Genbank).

Immunohistochemical Staining for Transthyretin

Tissue sections stained for the identification of amyloidosis by Congo red dichroism were also stained for transthyretin in patients with both a monoclonal gammopathy and hereditary TTR variants and in patients suspected of hereditary disease or senile cardiac amyloidosis, a diagnosis that requires TTR-positive endomyocardial amyloid and wildtype TTR gene sequence.[22]

Polyclonal rabbit anti-human transthyretin from DAKO (Carpenteria, CA) was used with citric acid pH 6.00 at a 1:2000 dilution. Slides were incubated with the primary antibody overnight at 4^{o} C in a humidity chamber. Secondary antibody at 1:500 dilution in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was applied for 1 hour at room temperature then washed in PBS. Peroxidase-conjugated tertiary antibody from DAKO at 1:500 in 1% BSA/PBS was applied for 45-60 minutes at room temperature then washed. Slides were transferred to a diaminobenzidine bath for 5-15 minutes, washed in tap water, then counterstained using Harris modified hematoxylin, decolorized with 1% acid alcohol in ammonia water, dehydrated three times in 95% ethanol, 100% ethanol and xylene for two minutes each, and cover-slipped with mounting media.

Amyloid deposits were TTR-positive if the TTR immunostain specifically demonstrated concordance with the Congo red stained section; that is, if the areas of amyloid by Congo red dichroisim were TTR-positive and the Congo red-negative areas were TTR-negative.

RESULTS AND DISCUSSION

One hundred and seventy-eight consecutive patients were evaluated for amyloidosis and the diagnosis confirmed in 96%, most of whom were symptomatic (Table 2). In an attempt to determine how often both a monoclonal gammopathy and a hereditary variant occurred in the same patient, patients in four categories were screened and 1 patient with both proteins was identified in three of the four categories. They represented 6% of those screened and 2% of symptomatic patients. One was African-American, one had peripheral neuropathy, and the third was referred for evaluation of hereditary amyloidosis and was diagnosed with Durie-Salmon stage I multiple myeloma. All had *bona fide* monoclonal gammopathies not faint bands on urine immunofixation and all had a variant TTR (Table 2). TTR staining indicated that in two cases amyloid was likely due to variant TTR and in one was not (Table 2).

The amyloidoses are diseases caused by protein misfolding.[23,24] The most common type encountered by hematologists is due to monoclonal immunoglobulin light chains (AL) and can be difficult to diagnose and treat in a timely fashion.[13] The median survival even with the most aggressive therapy is less than 5 years.[6] Diagnostic confidence is critical in order to plan therapy, and hereditary and senile cardiac variants are not treated with cytotoxic therapies or SCT.

Our results speak to the need for reliable tools to aid in the diagnosis and management of patients with amyloidosis. Combined with the recent report from the UK, the data indicate that the issue of diagnostic confidence is important because one patient can have both a monoclonal gammopathy and a hereditary variant, representing two possible sources of amyloid-forming

proteins.[9] It should also be recalled that the incidence of MGUS increases with age and that hereditary variants in the USA usually present in older patients. Both MGUS and Val122Ile mutant TTR are also more common in African-Americans.

Given the implications for patients and their families if AL is misdiagnosed and a hereditary mutation not identified, we agree with our colleagues in the UK that all new cases of amyloidosis should be screened for both AL and hereditary variants.[23] The PCR technique we used is reliable and easily implemented in human genetics laboratories. In addition, innovative and improved techniques are needed for typing amyloid from tissue biopsies.[10,11,12] Finally, the issue of diagnostic confidence becomes even more critical as new therapies are evaluated for both AL and hereditary disease.[8,25]

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Table 1. Genes, primers, PCR conditions and amplicon sizes

GENE (exon)	PRIMERS (5' to 3') *Forward* Reverse	ANNEALLING TEMPERATURE, CYCLES	AMPLICON SIZE (bp)
TRANSTHYRETIN[1] (2)	*gtaattcttgtttcgctccaga* gataaaaccaagtcctgtggg	58° C, 35	271
TRANSTHYRETIN (3)	*ggtgtattactttgccatgcc* ctcgaaggtctgtatactcac	58° C, 35	226
TRANSTHYRETIN (4)	*tcatgtgtgtcatctgtcacg* gttaaagtggaatgaaaagtgc	58° C, 35	340
APOLIPOPROTEIN AI[2] (2)	*caccctcagggagccaggctcgg* taggtgaggactcggccagtctgg	65° C, 35	255
APOLIPOPROTEIN AI (3)	*cagccctcaacccttctgtctcacc* cagatgcgtgcgcagcgcgtccaca	65° C, 35	392
APOLIPOPROTEIN AI (3)	*agctgcaagagaagctgagcccact* aacgtttattctgagcaccgggaag	65° C, 35	370
FIBRINOGEN Aα[3]	*tgatgaagctgccttcttcga* ctcatctgccattttatagctc	58° C, 35	277
APOLIPOPROTEIN AII[4] (3)	*tgctgtggacccagctga* gaacccttgccctgaga	60° C, 35	217
APOLIPOPROTEIN AII (4)	*ctaatcccctcaccta* ggaagacaatggtctg	45° C, 35	161
LYSOZYME[5] (2)	*agtcacttagtgttgctgttt* accagattggtcaaatattag	54 °C, 35	242

[1]The Online Mendelian Inheritance in Man identifier (OMIM ID) for transthyretin (TTR) is +176300. There are over 100 amyloidogenic TTR variants. Common ones include Val30Met, Thr60Ala, Ile84Thr, and Val122Ile (see reference 3).

[2]The OMIM ID for apolipoprotein AI is *107680. Most variants are non-neuropathic and cause cardiac and cutaneous amyloidosis. Common ones include Gly26Arg, Leu60Arg, Trp50Arg, Leu90Pro, Arg173Pro, Leu174Ser, Ala175pro and a deletion variant with a 2-bp insertion at position 60.

[3]The OMIM ID for fibrinogen Aα is +134820. There are at least 3 amyloidogenic variants, Arg554Leu, Glu526Val and a frameshift mutation that causes termination at codon 548.

[4]The OMIM ID for apolipoprotein AII is *107670. The amyloidogenic variants involve stop codon mutations at position 78 to glycine, serine or arginine, with abnormal extension of the protein; these variants are usually non-neuropathic.

[5]The OMIM ID for lysozyme is *153450. Amyloidogenic variants include Ile56Thr, Asp67His, Trp64Arg and Phe57Ile, and are usually non-neuropathic.

Table 2. Patients seen between 6/1/02 and 8/1/05 for assessment of systemic amyloidosis

Feature	Number
Patients	178
Men / Women	110 / 68
Median years old (range)	60 (34-83)
Diagnoses	170
AL	150
ATTR	7
Both MG & hereditary variant identified	3
AA	1
Senile cardiac	2
Plasmacytoma	2
Other	5
Asymptomatic patients	5
ATTR	2
Localized AL in plasmacytomas	2
Localized with cancer	1
Patients with symptoms of organ involvement	165
Dominant organ involvement (n, (%))	
Cardiac	69 (42%)
Renal	50 (30%)
Other	46 (28%)
Subsets screened for hereditary variants	
Patients (% of total)	54 (30%)
African-American With MG = 16, without MG = 4	20
Peripheral neuropathy With MG = 11, without MG = 5	16
Renal without marrow amyloid With MG = 7	7
Other With MG = 1, without MG = 10	11
Patients with both MG & hereditary variant	Monoclonal gammopathy / hereditary variant
46M soft-tissue, nodal and cardiac amyloid (TTR-negative by IHC)	Free λ = 28mg/dl* V122I TTR 28% lambda-restricted plasma cells
59M peripheral neuropathy (TTR-positive by IHC)	Free λ = 3mg/dl* F64L TTR 10% lambda-restricted plasma cells
70W GI involvement (TTR-positive by IHC)	Free λ = 2700mg/dl* T60A TTR 30% lambda-restricted plasma cells

* = with abnormal κ:λ ratio; AA = secondary amyloid due to serum amyloid A protein; AL = light-chain amyloid; ATTR = familial transthyretin amyloidosis; IHC = immunohistochemical staining; MG = monoclonal gammopathy; M = man; TTR = transthyretin; W = woman

REFERENCES

[1] Comenzo RL, Vosburgh E, Falk RH, et al. Dose-intensive melphalan with blood stem-cell support for the treatment of AL amyloidosis: survival and responses in 25 patients. Blood. 1998;91:3662-3670

[2] Merlini G, Bellotti V. Molecular Mechanisms of Amyloidosis. N Engl J Med 2003;349:583-96.

[3] Connors LH, Lim A, Prokaeva, Roskens VA, Costello CE. Tabulation of human transthyretin (TTR) variants, 2003. Amyloid 2003;10:160-184.

[4] Ericzon BG, Larsson M, Herlenius G, et al. Report from the Familial Amyloidotic Polyneuropathy World Transplant Registry (FAPWTR) and the Domino Liver Transplant Registry (DLTR). Amyloid 2003;10(S1):67-76.

[5] Comenzo RL, Gertz MA. Autologous stem cell transplantation for primary systemic amyloidosis. Blood 2002;99:4276-82

[6] Skinner M, Sanchorawala V, Seldin DC, et al. High-Dose Melphalan and Autologous Stem-Cell Transplantation in Patients with AL Amyloidosis: An 8-Year Study. Ann Intern Med. 2004;140:85-93.

[7] Gertz MA, Lacy MQ, Dispenzieri A, et al. Stem Cell Transplantation for the Management of Primary Systemic Amyloidosis. Am J Med. 2002;113:549–555.

[8] Anesi E, Palladini G, Perfetti V, et al. Therapeutic advances demand accurate typing of amyloid deposits. Am J Med 2001;111:243-44.

[9] Lachmann HJ, Booth DR, Booth SE, et al. Misdiagnosis of hereditary amyloidosis as AL (primary) amyloidosis. N Engl J Med 2002;346:1786-91.

[10] Murphy CL, Eulitz M, Hrncic R, et al. Chemical Typing of Amyloid Protein Contained in Formalin-Fixed Paraffin-Embedded Biopsy Specimens. Am J Clin Pathol 2001;116:135-142

[11] Yazaki M, Liepnieks JJ, Callaghan J, et al. Chemical characterization of a lambda I amyloid protein isolated from formalin-fixed and paraffin-embedded tissue sections. Amyloid 2004;11:50–55.

[12] Arbustini E, Morbini P, Verga L, et al. Light and electron microscopy immunohistochemical characterization of amyloid deposits. Amyloid 1997;4:157-70.

[13] Falk RH, Comenzo RL, Skinner M. The Systemic Amyloidoses. New Engl J Med 1997;337:898-909.

[14] Cohen A, Zhou P, Xiao Q, et al. Systemic AL amyloidosis due to non-Hodgkin's lymphoma: an unusual clinicopathologic association. Br J Haematol 2004;124:309–314.

[15] Comenzo RL, Zhang Y, Martinez C, Osman K, Herrera GA. The tropism of organ involvement in primary systemic amyloidosis: contributions of Ig V_L germ line gene use and clonal plasma cell burden. Blood 2001;98:714-20.

[16] Jacobson DR, Pastore RD, Yaghoubian R, et al. Variant-sequence transthyretin (isoleucine 122) in late-onset cardiac amyloidosis in black Americans. N Engl J Med 1997;336:466-73.

[17] Zhou P, Zhang Y, Martinez C, et al. Melphalan-mobilized blood stem cell components contain minimal clonotypic myeloma cell contamination. Blood 2003;102:477-479.

[18] Benson MD, Liepnieks J, Uemichi T, Wheeler G, Correa R. Hereditary renal amyloidosis associated with a mutant fibrinogen a-chain. Nature Gen 1993;3:252–255.

[19] Booth DR, Tan SY, Booth SE, et al. Hereditary Hepatic and Systemic Amyloidosis Caused by a New Deletion/Insertion Mutation in the Apolipoprotein AI Gene. J Clin Invest 1996. 97:2714–2721.

[20] Pepys MB, Hawkins PN, Booth DR, et al. Human lysozyme gene mutations cause hereditary systemic amyloidosis. Nature 1993;362:553-7.

[21] Benson MD, Liepnieks JJ, Yazaki M, Yamashita T, Hamidi Asl K, Guenther B, Kluve-Beckerman B. A new human hereditary amyloidosis: the result of a stop-codon mutation in the apolipoprotein AII gene. Genomics 2001; 72: 272-7.

[22] Persey MR, Booth DR, Booth SE, et al. Fibril in senile systemic amyloidosis is derived from normal transthyretin. Proc Natl Acad Sci USA 1990, 87:2843–2845

[23] Pepys MB. Amyloidosis. Annu Rev Med 2006;57:1–19

[24] Wetzel R. Domain stability in immunoglobulin light chain deposition disorders. Adv Protein Chem 1997;50:183-242

[25] Hrncic R, Wall J, Wolfenbarger DA, et al. Antibody-mediated resolution of light chain-associated amyloid deposits. Am J Pathol 2000;157:1239-46.