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Strong transthyretin immunostaining, potential pitfall in cardiac amyloid typing

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Abstract

Although systemic amyloidosis commonly presents with renal disease, cardiac involvement usually determines the patient's prognosis. Cardiac involvement is seen in AL and transthyretin amyloidosis. Distinguishing between these is critical because prognosis and treatment differ. Our study demonstrates the unreliability of transthyretin immunostaining in subtyping cardiac amyloid. Between January 2003 and August 2010, we retrieved 229 native endomyocardial biopsies, 24 had amyloid. Immunohistochemistry for kappa, lambda, transthyretin and serum amyloid A protein were performed on formalin fixed paraffin-embedded sections. Staining was graded as weak (trace to 1+); or strong (2 to 3+). Mass spectrometry (MS) based proteomic typing of microdissected amyloid material was performed on selected cases. Fifteen of the patients had monoclonal gammopathy/plasma cell dyscrasia with cardiac amyloid. Eight of them (53%) showed strong transthyretin staining in the cardiac amyloid deposits. MS was performed in five out of these eight biopsies, and all five revealed AL type amyloid. Two of these five AL amyloid biopsies did not even have concomitant strong staining for the appropriate light chain. Among the 15 cases with plasma cell dyscrasia, only seven biopsies showed strong staining for the corresponding monoclonal light chain.

Strong false positive immunostaining for transthyretin in cardiac amyloid is a potential pitfall, augmented by the frequent lack of staining for immunoglobulin light chains. Therefore, the presence of amyloid in the cardiac biopsy should prompt a search for plasma cell dyscrasia irrespective of transthyretin staining. Confirmation with MS should be sought particularly if there is any discrepancy between kappa/lambda staining and serum immunofixation results.

Keywords

cardiac amyloid; transthyretin; immunohistochemistry

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Introduction

Although systemic amyloidosis often presents first with kidney disease (proteinuria and renal dysfunction), cardiac involvement often determines the patient's prognosis. It is the most serious complication in patients with systemic amyloidosis and the cause of death in more than half of all cases.^{4, 14} Systemic amyloidosis can be classified into three broad types :- AL (monoclonal light chain) amyloid; AA amyloid; and familial forms of amyloid. It is known that the prevalence of different types of amyloidosis varies with the geographic location. However, what is less known is that different organs may have a different predilection for the various amyloid types. In kidney biopsies, AL type amyloid is most common, followed by AA type amyloid (depends on the geographic location). Hereditary forms of amyloid although rare, do involve the kidney; amongst these, fibrinogen A α chain derived amyloid is the most common. Transthyretin amyloid rarely affects the kidney. According to published studies, cardiac amyloidosis is of the AL type in 54% of the cases and of transthyretin type in 42% of the cases.^{2, 7} AA type amyloid is rarely seen in the heart.^{4, 5} Transthyretin amyloid in the heart can be either senile cardiac amyloidosis (due to acquired changes in the wild-type transthyretin molecule), or familial form due to inherited mutations in transthyretin. Therefore, in native heart endomyocardial biopsies with amyloidosis, classifying the amyloid into AL type versus transthyretin type is critical. AL type amyloidosis (affecting the heart) is an aggressive disease. Median survival rate has been reported to be 13 months without treatment and can be extended to 17 months with chemotherapy.⁴ Cardiac involvement denotes a poor prognosis, regardless of the method of treatment. Median survival rate from the onset of congestive heart failure is only 6 months.^{3, 4} Senile transthyretin amyloidosis carries the most favorable prognosis, with a nearly 5-year median survival rate even in the presence of heart failure.⁴ Transthyretin mutations are associated with familial amyloidotic polyneuropathy, especially in endemic areas. In addition, they can cause cardiac amyloidosis as well.¹⁵ The prognosis in familial transthyretin amyloidosis does depend on the type of mutation and severity of cardiac involvement.^{3, 6, 15} Importantly, specific treatment strategies including stem cell transplant have become the standard of care in AL type amyloid. No known effective treatment is available for senile or familial transthyretin amyloid, other than cardiac transplantation. Immunostaining is the first step in classifying amyloid and this method is known to have pitfalls due to weak staining, non-specific staining and sometimes lack of staining^{1, 5, 9, 11, 13}, paving the way for new proteomic techniques like liquid chromatography and tandem mass spectrometry (LC MS/MS).^{11, 16} Most of the previous studies dealing with immunostaining for amyloid subtyping have been performed on kidney biopsies. However the amyloid types involving the heart and the related pitfalls in staining provide a different challenge for the surgical pathologist. The aims of this study were to assess the prevalence of AL versus transthyretin amyloid subtypes in endomyocardial biopsies and to highlight the potentially misleading results of transthyretin staining in combination with kappa and lambda light chain staining.

Materials and Methods

During the period from January 2003 to August 2010, we examined 229 native endomyocardial biopsies. Congo red stain is routinely performed on every native heart

biopsy. Biopsies of twenty-four patients were diagnosed as cardiac amyloidosis. All of the biopsies with amyloid were immunostained for kappa and lambda light chains, transthyretin and serum amyloid A protein. Immunostaining for transthyretin (or prealbumin) was routinely performed from 2006 onwards. Biopsies performed before 2006, were retrospectively stained for transthyretin for the purpose of this study. Staining for all four antibodies was performed on formalin-fixed paraffin embedded tissue sections using immunoperoxidase method (see below under Antibodies and staining protocol). The staining of the amyloid deposits was graded as weak (trace to 1+); or strong (2 to 3+). On five of the cases, (Case no. 16, 17, 18, 19 and 20 in Table 1) LC MS/MS (liquid chromatography tandem mass spectrometry) confirmation of amyloid type was performed at the Mayo Clinic, Rochester, MN. On one of the biopsies (Case no. 8, before Mayo Clinic was accepting specimens for LC MS/MS), the paraffin block was sent to the laboratory of Dr. Alan Solomon at Knoxville, TN for mass spectrometry analysis¹⁰ but results on this biopsy were inconclusive because of the small amount of tissue remaining in the paraffin block.

Antibodies and staining protocol

Immunoperoxidase staining was performed on formalin-fixed paraffin embedded tissue sections. Antibody for transthyretin (prealbumin) is a rabbit polyclonal antibody (Dako; A0002), used at a dilution of 1:1200 with Dako's LSAB detection kit. Immunoperoxidase staining for kappa and lambda light chains was performed using mouse monoclonal antibodies from DAKO (Carpinteria, CA) at a dilution of 1:8 and 1:16 respectively. All immunoperoxidase staining was performed on the DAKO Autostainer. The control tissue used for transthyretin staining was an autopsy case of cardiac amyloid.

Liquid chromatography and tandem mass spectrometry (LC MS/MS)

Paraffin block, along with one hematoxylin eosin stained slide and Congo red slide were sent to Dr. Ahmet Dogan at the Mayo Clinic, Rochester, MN. The mass spectrometry analysis was performed according to published protocols.¹⁷

Results

Patients ranged from 57 to 80 years, fifteen were males and nine were females. Three of the patients were of African American ethnicity; the remaining were of Caucasian descent. Fifteen out of twenty-four patients showed serologic evidence of monoclonal gammopathy; ten patients with monoclonal lambda light chains and four patients with monoclonal kappa light chains and one had IgG heavy chains (Table 1). Seven patients had overt myeloma, one patient had Waldenström's macroglobulinemia. One patient (Case no. 8) had histiocytic sarcoma of the head and neck. He had IgG heavy chain gene rearrangement but no free monoclonal light chains in the serum or urine. The amyloid in this case stained for IgG heavy chain (specifically for IgG1); staining for kappa and lambda light chains was weak. The small tissue left in the paraffin block was inadequate for mass spectrometric analysis. Therefore, the nature of this amyloid could not be confirmed by LC MS/MS but the patient did show favorable response of his tumor to chemotherapy. For the purpose of this study, he was included among the patients with monoclonal gammopathy.

Out of the 15 biopsies from patients with monoclonal gammopathy, eight biopsies showed strong (2 to 3+) positivity for transthyretin in the amyloid deposits (case no. 8, 9, 12, 16, 17, 18, 19 and 20). Out of these eight biopsies with strong transthyretin staining, four did have concomitant strong (2 to 3+) staining for the appropriate light chain (Case no. 9, 12, 17 and 20), but the remaining four biopsies (Case no. 8, 16, 18 and 19) did not, (Fig.2A, B, C and D). These results are shown in Table 2. LCM MS/MS confirmation of AL type amyloid was obtained on Cases 16, 17, 18, 19 and 20. On case no. 8, mass spectrometry was attempted but tissue was inadequate.

As shown in Table 2, among the nine patients with cardiac amyloid and without proven plasma cell dyscrasia, seven biopsies did show strong transthyretin staining as expected, one showed weak staining and one did not have diagnostic tissue left. Also, three biopsies showed strong staining for one of the light chains (concomitant with transthyretin staining). Two patients were confirmed as having familial form of transthyretin amyloid (Ala 60 mutation confirmed by blood test performed at the laboratory of Prof. PN Hawkins, National Amyloidosis Center Royal Free Hospital, London, UK). The remaining seven patients were treated as senile transthyretin amyloidosis (Table 3).

The sensitivity, specificity, and the predictive values of positive and negative staining for transthyretin and immunoglobulin light chains are shown in Table 4.

Discussion

Cardiac amyloidosis is most commonly of the AL type or transthyretin type.⁷ Our study confirms this prevalence pattern. AA type amyloid is uncommon in the US and rare hereditary forms including fibrinogen A α chain amyloid, apolipoprotein AI and AII amyloid, gelsolin, lysozyme and so on have not been reported in the heart. Transthyretin amyloid can be either senile cardiac amyloidosis due to amyloidogenic wild-type transthyretin; or familial/hereditary amyloid due to inherited transthyretin mutations.^{3, 4, 6, 7, 15} Although the age of presentation for both AL and transthyretin amyloidosis is similar (5th to 6th decade), the disease prognosis differs greatly. AL type amyloidosis is an aggressive disease with poor long term patient outcome. On the other hand senile cardiac amyloidosis is a more indolent disease. In familial type transthyretin amyloidosis, prognosis may vary according to the type of mutation. Therefore on endomyocardial biopsies with amyloid, the critical step is to be able to differentiate between AL type amyloid and transthyretin type amyloid.

Immunostaining is the most readily available technique in the hands of a surgical pathologist for subtyping amyloid. However it is known that immunostaining of amyloid is fraught with problems. These include: i) false negative results due to weak staining or even lack of staining, often seen with commercial antibodies to kappa and lambda light chains^{11, 16} due to presence of truncated light chains in AL type amyloid deposits; ii) false positive results due to non-specific immunostaining of the amyloid deposits. Strong diffuse homogeneous staining of the amyloid deposits for any of the precursor proteins would usually be interpreted as a positive result by the pathologist. However, we want to draw attention to the fact that even when the staining is strong and diffuse, it can be non-specific (false positive).

This is illustrated in our study by the strong distinct (false positive) transthyretin staining of amyloid deposits in several of the endomyocardial biopsies when the amyloid is AL type.

We found 8/15 such biopsies with strong false positive transthyretin staining among patients with plasma cell dyscrasia and monoclonal gammopathy, in our retrospective study. Five of the biopsies were sent for mass spectrometric analysis and were confirmed as AL type amyloid. Some of these patients did not have a diagnosis of monoclonal gammopathy at the time of the cardiac biopsy, but were subsequently worked up for it and were found to have a plasma cell dyscrasia. It would have been ideal if all these cases were tested by LC MS/MS for confirmation, but the technique is expensive, and this being a retrospective study, the results would not have improved patient care at this point. Also, endomyocardial biopsies are small and much of the tissue may have been cut away from the paraffin block by the time the diagnosis of amyloid was made. We found a positive predictive value for transthyretin immunostaining of only 46.6% in our study.

The problem of weak immunoperoxidase staining for kappa and lambda light chains is known, primarily from the literature on renal amyloidosis.^{9,11} We found a sensitivity of just 46.6% in our study for light chain staining in patients with confirmed AL amyloidosis. It can range from as low as 38% to 65%,^{9,11} (in kidney biopsies with amyloid) necessitating a high index of suspicion and better correlation with clinical manifestations, imaging studies and sensitive immunoassays to detect circulating free monoclonal light chains.

Low sensitivity for light chain staining and concomitant false positive staining for transthyretin together, constitute a diagnostic problem. The clinical relevance of this is that AL type amyloid (which is the more aggressive but potentially treatable form of systemic amyloid) can be missed. One may argue that antibodies from different sources may give different staining results. While this is true, the question arises which antibody is ideal for use in routine diagnostic immunohistochemistry. One cannot keep changing them from case to case. The antibody concentration used is somewhat high, but the staining obtained is very specific and restricted to the areas of amyloid. There is no non-specific background staining away from the area of amyloid deposition. Also the control tissue used is an autopsy case of transthyretin amyloid, which is very appropriate. The sensitivity of the transthyretin stain was found to be 87.5% in our study. There are not too many histopathological studies specifically looking at transthyretin immunostaining, but one previous study,⁸ also showed high sensitivity of staining in cardiac amyloid. It has to be emphasized that the specificity of transthyretin immunostaining in cardiac amyloid is low and one has to be aware of the high rate of false positive results.

The cause of the strong nonspecific transthyretin staining in cardiac amyloid is not known. One hypothesis is that the contractile activity of the heart and the disturbance of the normal architecture by amyloid can support the diffusion of serum proteins, such as transthyretin (prealbumin) into the amyloid deposits and these will be detected on immunostaining.⁷ Non-specific staining of amyloid can be seen even in the kidney for immunoreactants like IgG, suggesting that the amyloid material itself is “sticky” and has a tendency to non-specifically trap serum proteins either in vivo or in vitro.¹⁶ Because of the above pitfalls, LC MS/MS has now become a new gold standard for the typing of amyloid, but is still an

expensive test. Still, if there is any discrepancy between the immunofixation test results and tissue light chain staining results, or if there is serologic evidence of monoclonal gammopathy, but strong immunostaining for transthyretin in the amyloid deposits, it is imperative to send the biopsy tissue for amyloid subtyping by LC MS/MS.

In summary, distinguishing AL from transthyretin amyloid in cardiac biopsies is crucial. Immunostaining is still the most commonly used method. However, the pathologist has to be aware of the potential pitfalls. Strong transthyretin staining can occur in AL type amyloid (and often with weak concomitant light chain staining) and this is obviously misleading. Therefore, it is important that after the diagnosis of amyloidosis in an endomyocardial biopsy (or in any other tissue), irrespective of the staining pattern, the patient should undergo workup for monoclonal gammopathy and plasma cell dyscrasia. Serum and urine protein electrophoresis with immunofixation are recommended. If these tests are negative or inconclusive, a more sensitive quantitative free light chain assay is recommended. On the other hand, a coincidental serum monoclonal spike (of unknown significance, MGUS) can simultaneously be present and this by itself does not prove that the amyloid is of the AL type. It should coincide with appropriate light chain staining in the amyloid deposits in tissue. Corresponding monoclonal free light chains in the urine is also important to investigate. It is important that the monoclonal light chain in the serum (and urine) and the monoclonal light chain staining in the tissue amyloid deposits match.

In the presence of amyloid deposits in the biopsy, irrespective of the immunostaining results, the possibility of AL type amyloid should be first convincingly ruled out by investigating the patient for possible plasma cell dyscrasia. This should be done even if the immunostaining for kappa/lambda light chains is weak/absent and is strong for transthyretin.

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Fig.1.

Defining the intensity of immunostaining from weak to strong. 1A and 1B show weak staining of amyloid. 1A represents 1+ kappa light chain staining in Case no. 16; 1B represents 1+ transthyretin staining in Case no. 23. Fig.1C and 1D show bright staining of amyloid. 1C represents 3+ lambda staining in Case no. 12; 1D represents 3+ transthyretin staining in Case no. 16.

Fig.2.

Biopsy on Case 19 with AL lambda amyloid proven by mass spectrometry. 2A. Congo red positive amyloid deposits surrounding individual myocytes; 2B. Weak staining for lambda light chain; 2C. Weak staining for kappa light chain; 2D. Strong staining for transthyretin.

Table 1

24 patients with cardiac amyloidosis diagnosed on endomyocardial biopsy.

No.	Age (yrs)	ethnicity	Gender	Intensity of Immunostaining				Monoclonal spike in serum and/or urine	Myeloma/MGUS	Final Clinical Diagnosis
				kappa	lambda	AA	TTR			
1	66	W	M	1+	1+	neg	no tissue	Absent	Absent	senile transthyretin amyloidosis.
2	57	W	M	neg	neg	neg	no tissue	IgG lambda	MGUS	AL amyloidosis
3	66	W	M	1+	3+	neg	neg	IgM lambda	Waldenstrom's	AL amyloidosis.
4	66	W	F	2+	trace	neg	3+	Absent	Absent	familial transthyretin amyloid with Ala 60 mutation. Confirmed by blood test.
5	61	B	F	neg	1+	neg	1+	IgG lambda	MGUS	AL amyloid
6	72	W	M	negative	negative	not done	1+	IgG lambda	MGUS	AL amyloidosis involving kidney, cardiac, peripheral nerves
7	80	B	M	1+	1+	neg	3+	Absent	Absent	senile transthyretin amyloidosis
8	59	W	M	1+	1+	neg	3+	IgG heavy chain	Histiocytic sarcoma	IgG heavy chain, but could not be confirmed by LC MS/MS
9	59	W	F	1+ focal	2+	neg	2+	lambda light chains	MGUS	AL amyloidosis
10	68	W	F	1+	neg	neg	3+	Absent	Absent	senile transthyretin amyloidosis
11	77	W	M	2+	trace	serum	3+	Absent	Absent	transthyretin amyloidosis- senile amyloidosis vs. familial - unclear
12	58	W	F	1+	3+	trace	2+	IgM lambda	Myeloma	AL amyloidosis
13	77	W	M	2+	1+	neg	3+	Absent	Absent	familial transthyretin amyloid with Ala 60 mutation. Confirmed by blood test.
14	75	W	M	neg	neg	background	3+	absent	absent	transthyretin amyloidosis- senile amyloidosis vs. familial - unclear
15	80	W	M	1+	neg	neg	3+	absent	Absent	transthyretin amyloidosis- senile amyloidosis vs. familial - unclear
16	69	W	F	1+	neg	background	3+	kappa light chains	Myeloma	AL amyloidosis
17	56	B	F	2+	neg	neg	3+	kappa light chains	Myeloma	AL amyloidosis
18	68	W	F	1+	neg	3+	3+	kappa light chains	Myeloma	AL amyloidosis
19	72	W	M	trace	trace	neg	3+	IgA lambda	Myeloma	AL amyloidosis
20	76	W	M	neg	2+	neg	2+	IgA lambda	Myeloma	AL amyloidosis
21	48	W	M	neg	trace	not done	1+	IgG lambda	MGUS	AL amyloidosis
22	68	W	F	trace	3+	not done	neg	lambda light chains	MGUS	AL amyloidosis
23	72	W	M	neg	1+	background	1+	Absent	Absent	senile transthyretin amyloidosis.

No.	Age (yrs)	ethnicity	Gender	Intensity of Immunostaining				Monoclonal spike in serum and/or urine	Myeloma/MGUS	Final Clinical Diagnosis
				kappa	lambda	AA	TTR			
24	63	W	M	neg	neg	neg	neg	kappa light chains	Myeloma	AL amyloidosis

Table 2

TTR and light chain staining in the 24 biopsies.

	MG/PD present (15)	MG/PD absent (9)
Strong TTR staining	8*	7
Weak/absent TTR	7	1 [†]
Strong light chain	7 ⁺	3
Weak/absent light chain staining	8	6

TTR=transthyretin; MG=monoclonal gammopathy; PD=plasma cell dyscrasia.

* Out of the 8 cases, 5 were confirmed to be AL amyloid by LC MS/MS.

[†] One additional biopsy had no diagnostic tissue left;

⁺ One biopsy had IgG heavy chain amyloid, tissue inadequate for LC MS/MS.

Table 3

Treatment approach and followup.

No.	Myeloma/MGUS	Amyloid subtype	Treatment	Date of biopsy	Last follow up	patient outcome
4	Absent	transthyretin	none, expired	4/29/2004	5/25/2004	died
7	Absent	transthyretin	Cardiac cure	4/24/2006	2/3/2008	unknown
10	Absent	transthyretin	Cardiac cure	2/26/2008	9/21/2010	alive
11	Absent	transthyretin	Cardiac cure	4/3/2008	5/14/2008	unknown
13	Absent	transthyretin	Cardiac cure	3/31/2009	9/21/2010	alive
15	Absent	transthyretin	Cardiac cure	7/23/2009	9/3/2009	unknown
1	Absent	tissue inadequate	Cardiac cure	11/5/2003	9/20/2010	alive
23	Absent	transthyretin	none	11/17/2009	11/17/2009	died
14	Absent	transthyretin	no followup	5/5/2009	5/5/2009	unknown
8	Histiocytic sarcoma	IgG	chemotherapy for sarcoma	12/22/2006	9/3/2010	alive
2	MGUS	AL	melfalan, prednisone	1/6/2004	5/29/2004	unknown
3	Waldenstrom's	AL	none, expired	4/21/2004	4/30/2004	died
5	MGUS	AL	none, hospice	11/16/2004	11/23/2004	died
6	MGUS	AL	decadron	10/25/2004	6/17/2005	unknown
9	MGUS	AL	melfalan, dexamethasone	10/17/2007	10/21/2008	died
12	Myeloma	AL	melfalan, dexamethasone	9/25/2008	7/6/2010	alive
16	Myeloma	AL	melfalan, dexamethasone, revlimide	7/28/2009	9/9/2010	alive
17	Myeloma	AL	celcade, dexamethasone	8/24/2009	9/23/2010	alive
18	Myeloma	AL	none, expired	12/28/2009	1/1/2010	died
19	Myeloma	AL	revlimide, dexamethasone	5/20/2010	6/11/2010	died
20	Myeloma	AL	revlimide, dexamethasone	6/18/2010	8/18/2010	died
21	MGUS	AL	autologous stem cell transplant	9/1/2005	9/16/2010	alive
22	MGUS	AL	melfalan, dexamethasone	6/13/2008	8/19/2008	died
24	Myeloma	AL	melfalan, dexamethasone, velcade	12/20/2004	9/21/2010	alive

Table 4

Sensitivity and specificity of transthyretin and light chain immunostaining.

	Transthyretin	Light chains
Sensitivity	7/8 (87.5%)	7/15 (46.6%)
Specificity	6/14 (42.8%)	6/9 (66.6%)
PPV	7/15 (46.6%)	7/10 (70%)
NPV	6/7 (85.7%)	6/14 (42.8%)

PPV=positive predictive value;

NPV=negative predictive value.