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ORIGINAL ARTICLE

Sensitive and rapid assessment of amyloid by oligothiophene fluorescence in subcutaneous fat tissue

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Abstract

Systemic amyloidosis (SA) is often diagnosed late. Combining clinical and biochemical biomarkers is necessary for raising suspicion of disease. Fine needle aspiration (FNA) of subcutaneous fat enables SA detection by Congo red staining. The luminescent conjugated probe heptameric formic thiophene acetic acid (h-FTAA) is a sensitive alternative to Congo red-staining of tissue samples. Our objective was to compare h-FTAA fluorescence with the Congo red stain for amyloid detection in FNA-obtained fat tissue. Herein, we studied samples from 57 patients with established SA (19 with AA, 20 with AL, and 18 with ATTR) and 17 age-matched controls (34–75 years). Positivity for h-FTAA was graded according to a Congo red-based grading scale ranging from 0 to 4+. Amyloid grading by both methods correlated strongly ($r = 0.87$). Here h-FTAA was positive in 53 of 54 Congo red-positive cases (sensitivity 98%) and h-FTAA was negative in 7 of 17 Congo red-negative controls (specificity 41%), but was also positive for 3 Congo red-negative SA cases. We conclude that h-FTAA fluorescence is more sensitive than Congo red staining in this small exploratory study of fat tissue samples, implicating potential sensitivity for prodromal amyloidosis, but is less specific for clinical amyloidosis defined by Congo red positivity. Given its simplicity h-FTAA staining may therefore be the most appropriate method for rapid screening of fat tissue samples but should presently treat grade 1+ as only suggestive, whereas 2+ or higher as positive for amyloidosis. Parallel assessment of h-FTAA and Congo red staining appears highly promising for clinical applications.

Abbreviations: AA: amyloid A; AL: amyloid light-chain; ATTR: amyloid transthyretin; h-FTAA: heptameric formic thiophene acetic acid; FNA: fine needle aspiration; LCO: luminescent conjugated oligothiophene; SA: systemic amyloidosis; TTR: transthyretin

Keywords

AA amyloidosis, amyloid light chain, hyper spectral imaging, systemic amyloidosis, transthyretin

History

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Introduction

Many chronic degenerative diseases are caused by the accumulation of misfolded proteins in the form of protein aggregates. Protein deposits known as amyloid form ordered aggregates with fibrillar morphology and β -sheet rich conformations of the constituent protein molecules [1,2]. The amyloid diseases are characterized by extracellular deposits in numerous tissues and organs [3,4]. Some 30 different precursor proteins that have been recognized to form human disease associated amyloids *in vivo* [5]. Accurate detection of amyloid deposits is a necessary method for precision of diagnoses of aggregation proteopathies. Preferred technologies are based on biopsy material or – for a number of systemic amyloidoses – can be done in peripheral fat

tissues. Fine needle aspiration (FNA) of subcutaneous abdominal fat tissue is a modestly invasive, accurate and rapid method that allows for assaying rather wide portions of tissue for systemic amyloidosis (SA) of AA, AL and ATTR types by Congo red staining [6,7].

Congo red staining is the established gold standard method for amyloid diagnosis and has been used for decades [8–10]. However, the use of Congo red also has several pitfalls and un-experienced examiners might reach false-positive or false-negative results [11]. Especially the latter can be hampering for patients due to currently available treatment modalities or for stratification of patients for clinical trials depending on amyloid-positive biopsy. Herein, small amounts of amyloid or a sub-set of protein aggregates not formally classified as amyloid, may remain undetected [12,13], which unfortunately would be expected early in the disease, when treatment is most efficient. In this regard, several efforts have been made to improve, complement or substitute the Congo red staining. Luminescent conjugated oligothiophenes (LCOs) have been

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applied as a novel class of fluorescent amyloid binding probes [14–19]. In comparison with conventional ligands based on Thioflavins or Congo red, LCOs have shown to identify a broader sub-set of disease associated protein deposits and heterogenic populations of protein aggregates [12,13]. In recent presentations, we described the ease of use and sensitive application of the LCO h-FTAA (heptameric formic thiophene acetic acid) for detection of amyloid in tissue sections [12,19–22].

Herein, we report the usefulness of the oligothiophene, h-FTAA, for the detection of amyloid deposits in abdominal fat smears from patients with systemic amyloidoses in direct comparison with Congo red. The study was performed on the basis of the previously established Congo red grading protocol to assess if h-FTAA fluorescence also would respond to various abundances of amyloid in fat tissue. We demonstrate that h-FTAA represents a new, promising, rapid and hypersensitive tool that can aid in the early clinical diagnosis of systemic amyloidoses.

Materials and methods

Patient samples

Fifty-seven patients with established SA were selected for the study, comprising 19 with AA, 20 with AL, and 18 with ATTR type. In addition, 17 age-matched controls (34–75 years) were studied, suffering from a variety of comparable diseases, such as heart failure, kidney failure, polyneuropathy, inflammation, and carrier of a transthyretin (TTR) mutation (Table 1). Abdominal fat was collected using the FNA procedure as shown in an instruction video (www.amyloid.nl) and described previously [6]. The study was approved by the local ethics committee and all patients and controls gave their informed consent.

Congo red staining

Samples were stained with Congo red dye according to the method described by Puchtler et al. and graded from 0 to 4+ as described previously [7,10]. A semi-quantitative grading scale was used: 0 (negative, no apple-green birefringence detectable), 1+ (minute, <1% of surface area), 2+ (little,

Table 1. Details of patients of the study with Congo red grades and h-FTAA grades.

Patient number	Congo red grade	hFTAA grade	Sex	Age (yrs)	Amyloid type*	Comment
1	1	1	f	41	AA	FMF
2	0	1	m	52	AA	FMF
3	1	1	m	66	AA	Spondylarthropathy
4	1	1	f	70	AA	RA
5	1	3	f	45	AA	M. Whipple
6	1	1	f	63	AA	RA
7	2	2	m	66	AA	Psoriatic Arthritis
8	2	2	f	22	AA	Chronic infections
9	2	3	f	59	AA	RA
10	2	3	f	56	AA	RA

(continued)

Table 1. Continued

Patient number	Congo red grade	hFTAA grade	Sex	Age (yrs)	Amyloid type*	Comment
11	2	4	f	69	AA	RA
12	3	3	f	65	AA	Decubitus
13	3	4	m	23	AA	FMF
14	3	4	f	25	AA	Chronic infections
15	3	3	f	46	AA	FMF
16	4	3	m	34	AA	cystic fibrosis
17	4	4	m	28	AA	FMF
18	4	4	f	64	AA	RA
19	4	4	f	69	AA	RA
20	0	1	f	67	AL	MM
21	1	2	m	75	AL	MGUS
22	1	1	m	67	AL	MGUS
23	1	1	f	77	AL	MGUS
24	1	2	m	55	AL	MGUS
25	1	2	m	71	AL	MGUS
26	2	2	f	51	AL	MM
27	2	2	f	53	AL	MM
28	2	3	m	71	AL	MGUS
29	2	3	m	68	AL	MGUS
30	3	2	m	72	AL	MGUS
31	3	3	m	55	AL	MM
32	3	4	m	67	AL	MGUS
33	3	4	m	61	AL	MM
34	3	3	m	75	AL	NHL
35	4	3	f	73	AL	MGUS
36	4	4	f	61	AL	MGUS
37	4	4	m	76	AL	MM
38	4	4	m	63	AL	MM
39	4	4	m	58	AL	MGUS
40	0	1	m	65	ATTR	Wild type
41	1	1	f	31	ATTR	Y114C
42	1	1	m	52	ATTR	V71A
43	1	2	m	47	ATTR	V71A
44	1	2	f	58	ATTR	V30M
45	1	0	m	54	ATTR	V71A
46	2	1	m	56	ATTR	Y114C
47	2	2	m	65	ATTR	G47E
48	2	2	f	67	ATTR	Y114C
49	2	2	m	37	ATTR	V71A
50	3	3	f	36	ATTR	V30M
51	3	3	m	37	ATTR	V71A
52	3	3	m	74	ATTR	V30M
53	3	4	f	60	ATTR	Y114C
54	4	3	m	60	ATTR	V30M
55	4	4	f	42	ATTR	V30M
56	4	4	f	66	ATTR	V122I
57	4	3	f	65	ATTR	Y114C
58	0	0	f	75	ctrl	Localized amyloid
59	0	0	f	34	ctrl	Carrier TTR V71A
60	0	1	f	63	ctrl	Carrier TTR V30M
61	0	1	f	63	ctrl	RA
62	0	0	f	45	ctrl	Healthy Control
63	0	0	f	64	ctrl	Polyneuropathy
64	0	0	f	56	ctrl	MCTD
65	0	0	m	67	ctrl	Polyneuropathy
66	0	0	f	51	ctrl	RA
67	0	1	m	59	ctrl	Healthy Control
68	0	1	m	45	ctrl	Healthy Control
69	0	1	m	62	ctrl	Polyneuropathy
70	0	1	m	53	ctrl	Cirrhosis
71	0	1	m	75	ctrl	Pericarditis
72	0	1	f	47	ctrl	Healthy Control
73	0	2	f	64	ctrl	Polyneuropathy
74	0	2	f	69	ctrl	Healthy Control

*Ctrl defined as Congo red grade 0 without additional positive biopsy. MGUS, monoclonal gammaopathy of undetermined significance; MM, multiple myeloma; FMF, familial Mediterranean Fever; RA, rheumatoid arthritis; NHL, non-Hodgkin lymphoma.

between 1% and 10%), 3+ (moderate, between 10% and 60%), and 4+ (abundant, >60%).

h-FTAA staining and fluorescence imaging

Air-dried FNA fat-tissue smears on objective glass were stained rehydrated in 100mM Tris-HCl pH 8.5 buffer. Staining was performed with a diluted staining solution containing 600nM h-FTAA. Washing was performed with 10mM Tris-HCl pH 8.5. Mounting of the slide was done in DAKO fluorescence cover medium (Dako Sweden AB, Stockholm, Sweden) and the slides were left for 24h prior to microscopy. Fluorescence images and spectra were recorded with a Leica DM6000 B fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a SpectraCube[®] spectral camera module and the acquisition software SpectraView (version 5.0.0.1) (Applied Spectral Imaging Ltd, Migdal Ha-Emek, Israel) using a Prior Lumen200 (Prior Scientific Ltd, Cambridge, UK) as light source and filter cube 436/20 (LP460) [C 140 879] (Chroma Technology Corporation, Bellows Falls, VT).

h-FTAA grading according to Congo red grading

One training session was performed using five typical examples of Congo red birefringence graded from 0 to 4+ [7]. Five young healthy controls (aged between 24 and 27 years) were used as additional Congo red-negative samples. Blinded study samples (2–3 smears from each case) of cases previously already graded using Congo red were now graded

using the same h-FTAA staining and fluorescence set up as used for the training reference scale. Two observers assessed the stained smears, either at the microscope or from digital images.

Statistical methods

The Spearman rank correlation was used for analyzing the relation between the two methods.

Results

Following a staining procedure previously established for surgical biopsies from patients with SA, we used FNA-acquired fat tissue by making fat smears on glass slides [12]. Samples were analyzed using a fluorescence microscope using 436-nm excitation light with long pass emission filter allowing full fluorescence detection between 455 and 750 nm. The procedure is schematically described in Figure 1. With this setup, autofluorescence and h-FTAA-stained amyloid, respectively, can clearly be distinguished by eye. Assessment of h-FTAA positivity was used for an open training set applying samples previously established in a scoring system by Congo red between 0 and 4+. Images were obtained using a spectral camera to document the specimens and to allow distinguishing autofluorescence from the tissue versus h-FTAA-positive amyloid aggregates (Figure 1). Amyloid-containing abdominal fat tissue stained with h-FTAA and excited at 436 nm showed two major components colored blue and yellow, respectively, in the fluorescence micrographs

Figure 1. Procedure for assessment of amyloid in adipose tissue fat smears by fluorescence microscopy. A requirement for analysis is excitation by 436 nm and a microscope equipped with a long pass emission filter to distinguish the blue autofluorescence signal from fat from the amyloid signal of h-FTAA as shown in the spectrum. The hyperspectral fluorescence micrograph of h-FTAA stained smear by h-FTAA is representing a Congo red grade 2+ image from the training set.

Figure 2. Fluorescence micrographs from the open training set of fat biopsy smears stained with h-FTAA and scored according Congo red grade 0–4+. Top two rows show Congo red in bright field (CR BF) respective cross-polarized mode (CR $\times$ Pol), bottom three rows shows representative distribution of h-FTAA stained amyloid by fluorescence.

(Figure 1). Autofluorescence from adipocytes and connective tissue showed emission in the blue region with a broad peak centered at 520 nm whereas amyloid fluoresce more intensively in the range 530–620 nm with two prominent peaks at 545 and 585 nm (Figure 1). Amyloid aggregates were observed in a gradual increasing pattern in the training set which was well in accordance with the expectations from the Congo red grades (Figure 2). One could also recognize differences, comparing the same cases, between h-FTAA staining and Congo red staining when both are viewed side by side (Figure 2). It was apparent that the amount of amyloid identified by h-FTAA fluorescence appeared overall larger in comparison with Congo red even when viewed in bright field mode. The difference was striking for Congo red birefringence (crossed polarizers). It has also been discussed that collagen when viewed through crossed polarizers, used for Congo red, can be deceiving due to polarization of white light as seen in Figure 2. Notwithstanding collagen provides a bright gray appearance and is not affording the anomalous color (green) as should be observed for proper Congo red birefringence of amyloid. However, the confounding

birefringence from collagen was not an issue when using h-FTAA fluorescence. Thus, h-FTAA fluorescence displayed much better signal-to-noise ratios than inspection of Congo red-stained sections under cross-polarized light, suggesting that h-FTAA fluorescence may be advantageous for accurate assessment of small amount of amyloid deposition.

The h-FTAA-positive aggregates revealed a rather wide distribution of morphologies and the amount of deposition did not follow a sharp boundary between each step in the correlated CR-grading classifications, e.g. distinguishing Congo red grade 3+ from 4+ was not obvious by either method (Figure 2). Five young controls were included in the open set for establishment of expected background contributions in the h-FTAA spectral range. All five young controls were negative for abundant h-FTAA amyloid fluorescence, but showed minor punctate or diffuse fluorescence regions within the fat tissue. Without further training, we studied 74 blinded samples which were analyzed in analogy to the training set and young control cohort (Table 2). Scoring was performed by analysis in the fluorescence microscope and by digital images (Figure 3) by two independent observers.

Both methods correlated strongly in all 74 blinded samples (Spearman $r=0.87$). Amyloid stained by h-FTAA compared to Congo red-positive samples as standard revealed that the h-FTAA sensitivity was 53/54 (98%). For Congo red-negative controls the h-FTAA was deemed negative for only 7/17

Table 2. Stratified case information and grade values.

No. patients*	Amyloid type	Mean Congo red grade	Mean h-FTAA grade
19 (1; 0)	AA	2.3	2.7
20 (1; 0)	AL	2.4	2.7
18 (1; 1)	ATTR†	2.3	2.3
17 (17; 7)	Controls‡	0	0.7

*Number of patients in each category (Congo red negative; h-FTAA grade 0).
†ATTR variants (wt = 1; V30M = 5; G47E = 1; Y114C = 5; V71A = 5; V122I = 1).
‡Of the controls, one female aged 63 was diagnosed with rheumatoid arthritis and one female aged 63 was a TTR V30M heterozygote carrier. The controls used herein included patients suffering from a variety of comparable diseases, such as heart failure, kidney failure, polyneuropathy and inflammation (Table 1).

(putative specificity 41%). If h-FTAA grade 1+ was also considered to be negative, the negative h-FTAA samples increase to 15/17, resulting in a specificity of 88%. By doing so, the number of positive samples dropped to 44/54, resulting in a sensitivity of 81%. Importantly, it should be noted that h-FTAA was deemed positive for three cases (1 AA, 1 AL, and 1 ATTR) with established SA which were negative with Congo red (Table 1 and Figure 4).

Discussion

In this small exploratory study, we combined routine clinical practice, entailing established amyloid diagnostics, with novel fluorescence research tools for amyloid detection. The new oligothiophene-based fluorescent method correlates strongly with the established Congo red method. This concordance of the two semi-quantitative methods indicates that both methods reveal amyloid deposition, though each in its own way. Limitations were the small size of the study, that the study was performed on the basis of the established Congo red gold standard for amyloid diagnostics, and that only a very

Figure 3. Examples of fluorescence micrographs of blinded fat biopsy smears stained with h-FTAA and graded according to the training set. (A) Graded 0, (B) Grade 1+, (C) Grade 2+, (D) Grade 3+, (E) Grade 4+, (F) Matrix of Congo red grade versus h-FTAA grade for all blinded cases.

Figure 4. Three cases positive for h-FTAA with established amyloidosis which were negative with Congo red and one case positive with Congo red and grade 0 with h-FTAA. Top row shows Congo red stained fat tissue in bright field (CR BF), middle row shows the corresponding sample between crossed polarizers (CR $\times$ Pol). Bottom row shows h-FTAA stained amyloid in fat tissue by fluorescence. (A) Male 65 years with ATTRwt (senile systemic amyloidosis) (Case No. 40 in Table 1), (B) Woman 67 years with AL amyloidosis (Case No. 20 in Table 1), (C) Male 52 years with AA and established FMF (Case No. 2 in Table 1), and (D) Male 54 years with ATTR V71A (Case No. 45 in Table 1).

limited set of samples were used for training. With this backdrop in mind the obtained results were surprisingly robust showing h-FTAA sensitivity to be 98% in comparison with Congo red. In agreement with our earlier study on surgical samples from SA patients [12], the higher sensitivity of h-FTAA fluorescence compared to Congo red fluorescence or birefringence was in this study confirmed for fat tissue samples. The high sensitivity is very encouraging but is on the other side challenging for cases with low abundance of amyloid, i.e. distinguishing cases with Congo red grades 0 and 1+ (Figure 3). From these results summarized in Figure 3(F), it was apparent that the blinded grading of amyloid by h-FTAA generated a more consistent amyloid load also for the low grades (0–2+) rather than directly grading the samples based on Congo red (Supplementary Figures S1–S3). While this result was expected it reassured us that we were consistent in the blinded grading of the samples. At first glance h-FTAA seems to reflect a very high number of false-positive results using the Congo red method as gold standard resulting in an apparent specificity of 35%, namely only 7 of 20 Congo red-negative samples. However, in this respect it is of interest to take a closer look at some of the Congo red-negative samples. Initially, five samples with established amyloidosis in other organs were deemed Congo red negative, however reassessment of these samples as guided by h-FTAA fluorescence data resulted in finding

minuscule amounts in two of these by Congo red (Supplementary Figure S4). Hence, three samples remained with established SA but still negative Congo red grades. Hence, the three cases with amyloidosis who had a negative Congo red stain may have (pre-)amyloid in their fat tissue that is below the detection range of the Congo red stain appear to be detected with h-FTAA. Figure 4 shows micrographs from these cases. This finding strongly implicates the potential for detection of prodromal amyloidosis by h-FTAA. It is of note that the single ATTR case (case 40 in Table 1) negative for Congo red in our study was WT TTR, which is known to be difficult to detect in abdominal fat aspiration samples by Congo red [23,24] was positive with h-FTAA. Furthermore, two controls were identified that possibly could have amyloidosis (one V30M TTR carrier and one rheumatoid arthritis patient) showed positivity for h-FTAA (Tables 1 and 2). If we remove these two samples from the analysis the calculated specificity increases to 47%, namely 7 of 15 Congo red-negative control samples. While this is still an unacceptable low specificity and urge us to work on improvement of the detection limit between 0 and 1+. The results may also indicate that amyloid-like deposits in human tissues are more frequent than currently appreciated, at least in the control group used in this study. In this respect, the four controls with polyneuropathy (cases 63, 65, 69, and 73) should have been checked for the possible presence of a TTR mutation, but this

was not done. Of note, there was one ATTR patient (case 45) with a positive Congo red biopsy and deemed grade 0 with h-FTAA stain (Figure 4D).

In conclusion, these results indicate that, as we have seen for surgical biopsies and in disease model systems, h-FTAA is more sensitive than Congo red, but the specificity for clinically established amyloidosis is low. Specificity is higher (88%) if grade 1+ is considered to be negative, at the cost of a lower sensitivity (81%). For practical purposes, we will consider a h-FTAA fluorescence 1+ positive sample highly suggestive for amyloid, but amyloidosis needs to be confirmed by a positive Congo red-stained fat sample or other tissue sample of the patient. Currently, the amyloid field is striving for early diagnostics of amyloidosis. The need is urgent. Accurate treatment of amyloidosis of AA, AL or ATTR type is totally dependent on early accurate diagnosis. Currently, diagnosis is delayed and can in certain cases take several years. Hence, the need for sensitive detection procedures which are rapid and translatable to routine clinical practice is of utmost importance. At present, h-FTAA staining may certainly be used as complement and a guide to Congo Red staining to assess amyloid deposits in fat tissue samples. With further development of user training, sample treatment and spectral analysis [20], we foresee excellent potential for this technology for rapid assessment of amyloid detection in fat tissue. Dual amyloid analysis including Congo red and combination of h-FTAA fluorescence with laser capture and mass spectrometry is projected to be a promising amyloid typing method in the clinic.

Declarations of interest

The authors have no conflicts of interest to declare. Our work was supported by the European Commission FP-7 Health project LUPAS (D. S., P. H., K. P. R. N.), The Swedish Foundation for Strategic Research (K. P. R. N.), the Swedish Research Council (P. H., K. P. R. N.) and the Linköping University Center for Neuroscience (D. S.). K. P. R. N is financed by an ERC Starting Independent Researcher Grant (Project: MUMID) from the European Research Council.

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