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Probing the use of fluorescence spectroscopy as a novel diagnostic tool in patients with rheumatoid arthritis: Applicability in the detection of secondary amyloidosis

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

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Summary

Background: Secondary amyloidosis is a frequently reported complication of rheumatoid arthritis. Currently, accepted diagnostic protocols for secondary amyloidosis involve histopathological and histochemical examinations of collected tissue specimens. The purpose of the current report was to evaluate the value of fluorescence spectroscopy as a supplementary tool in the diagnosis of secondary amyloidosis.

Material/Methods: Tissue specimens were collected from abdominal folds, gingiva or rectal mucosa of 99 patients affected with rheumatoid arthritis. Tissue samples were subjected to preliminary clinical observations, histopathological examinations and laboratory tests. These procedures were used to subdivide tissue samples into either amyloid-containing or amyloid-free control subgroups. All collected tissue samples were examined with the use of a designated spectrofluorometer and fluorescence spectral images were generated.

Results: It was found that fluorescence spectra for amyloid-containing tissues were typically characterized by a double emittance peak. In contrast, amyloid-free samples were characterized by fluorescence spectra with a single λmax value. Specimen collection site, age and sex did not appear to influence the morphology of electromagnetic spectra, which were generated for both amyloid-containing and amyloid-free tissue samples. The sensitivity of the fluorometric approach was ~78% and the specificity was 100%. Possible shortcomings of the technique may be due to the limit of detection of the instrument used.

Conclusions: Fluorescence spectroscopy may potentially be used as an effective, instantaneous and low-cost diagnostic tool for suspected secondary amyloidosis in patients affected with rheumatoid arthritis.

key words: **secondary amyloidosis • rheumatoid arthritis • fluorescence spectroscopy**

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Background

Secondary amyloidosis (AA) can occur during the dynamic progression of chronic rheumatic diseases such as rheumatoid arthritis (RA), juvenile idiopathic arthritis (JIA), ankylosing spondylitis and psoriatic arthritis [1–5].

Despite recent progress in the treatment of AA, the prognosis of this condition remains relatively poor. The prospective disease progression of AA often depends on a number of factors such as the presence of comorbidities and the degree of organ involvement pathology. It is generally accepted that the early diagnosis of AA may influence both disease prognosis and progression. The clinical condition of RA patients with advanced AA is usually poor and the possibility of effective intensive treatment in such patients diminishes over time [6]. In addition, a significant proportion of patients with RA display pathological amyloid deposits [2]. Due to these issues, clinical screening on a regular basis and at a relatively early period of disease progression remains essential in overcoming AA-associated clinical complications.

Laboratory tests and imaging techniques are generally used for the clinical diagnosis of suspected amyloidosis. Histopathological examinations still remain the accepted 'gold standard'. Amyloid deposits are generally detected with the use of the Congo Red Dye test and subsequent viewing of the treated specimen under a polarized light microscope. Amyloid deposits in the treated tissue sample display optical birefringence and appear green in color under plane-polarized light [7].

Over the previous 2 decades progress has been made in clinical laboratory tests as well as diagnostic imaging techniques such as ultrasound, MRI and CT. Nonetheless, the early diagnosis of connective tissue disorders (CTD) and other diseases remain problematic, most probably due to the sensitivity of available instruments. Fluorescence spectroscopy is a technique previously used for the detection of cancerous tumors, delineating the efficacy of surgical excision of tumour growths, diagnostic assessment of human cutaneous melanoma, and ultrastructural detection of morphological changes in connective tissue [8–10]. Compared to conventional diagnostic approaches, fluorescence spectroscopy is characterized by relatively high precision, efficacy, efficiency and cost-effectiveness. It was with this in mind that an alternative fluoroscopy-based modality for the detection of AA was developed and its applicability and generalizability within a clinical setting were investigated.

Based on prior diagnostic research experience [10] and preliminary findings obtained within our own research group [11], a decision was made to conduct an extensive systematic analysis of fluorescence spectra of different tissue samples in which the presence or absence of amyloid deposits were previously confirmed. In so doing, the possibility of using fluorescence spectroscopy in diagnosing secondary amyloidosis was investigated.

Material and Methods

Patients affected with RA (n=99) between 39 and 65 years of age were examined (Table 1). The cohort study group was divided into 2 separate subgroups. The first subgroup consisted of patients in whom the diagnosis of AA was confirmed by clinical observations, histopathological examinations and laboratory tests. The second cohort study subgroup consisted of study subjects in whom the intracorporeal presence of AA was excluded via the use of the same 'gold standard' diagnostic methodologies. Demographics related to sex and age are also given in Table 1.

Tissue specimens were collected from various sites, including adipose tissue from the abdominal fold, rectal mucosa or gingiva. Following collection, all tissue samples were subjected to histopathological examinations. To this end, collected tissue specimens were preserved in 10% formalin, processed and paraffin-embedded. A microtome was used to slice tissue samples to a thickness of ~2–6 μm. Each slice was subsequently transferred to a microscope slide, stained with Congo red and observed under plane-polarized light with the use of a designated light microscope. In all cases, immunohistochemical examinations were performed on each tissue sample with the EnVision™ (DAKO) kit, which employed the following monoclonal antibodies: amyloid A component; amyloid P component; transthyretin; and kappa and lambda light chains.

With regards to specimen preparation for fluorescence spectroscopy, each paraffin-embedded biopsied tissue sample was characterized by strong autofluorescence due to the presence of paraffin. As a result, an additional step was necessary to suppress the possible autofluorescence signal via deparaffinization. To this end, each collected tissue sample was transferred to a xylene-containing tissue bath and then dried by gradual heating. The treated tissue sample was then transferred to a microscope slide for observation via fluorescence spectroscopy. Prior to performing the fluorescence spectroscopy, each collected tissue sample was independently coded such that the investigator was blinded without knowledge of the code-breaking procedures. In so doing, experimental bias was minimized during this crucial experimental step. A Hitachi F-2500 spectrofluorometer was used to generate fluorescence spectra. The excitatory light source was transmitted at a wavelength of 250 nm. Emitted fluorescence spectra were obtained between 260 nm and 450 nm with a scanning speed of 1500 nm/min and a spectral resolution of 2.5 nm. Since the microscope used glass slides that were impermeable to UV light, specially constructed spectrofluorometer sample holders were used to facilitate detection of the fluorescence signal at the superior surface of the sample. In addition, the incident light beam was transmitted at a 60° angle to the surface of the tissue sample and the reflected fluorescence signal was detected perpendicularly to the sample tissue surface. This experimental configuration was found to optimize the fluorescence signal by maximizing the transmission of the generated fluorescence beam to the filter and photodiode array detector. Since fluorescence intensity is influenced by the size of the examined sample, values obtained for the generation of spectral images were normalized for this parameter. Fluorescence spectra were eventually generated by the collation of all spectra for the amyloidosis-affected and control groups by obtaining average and standard deviation values at each particular emitted fluorescence wavelength. In so doing, a direct comparison of the obtained spectra between these 2 separate subgroups was obtained.

Table 1. Distribution of the collected tissue samples as well as the sensitivity and specificity of the fluorescence spectroscopic technique which was used in the current study.

Affected with AA?*	Collection site	Gender**	n***	N#	Total##	Age	Sensitivity (%)	Specificity (%)
No###	Adipose tissue from abdominal fold	F	17	22	40	51.48±5.71	–	100
		M	5				–	100
	Rectal mucosa	F	8	13		49.93±7.35	–	100
		M	5				–	100
	Gingiva	F	4	5		51.00±6.14	–	100
		M	1				–	100
Yes	Adipose tissue from abdominal fold	F	19	30	59	55.99±4.98	78.95	–
		M	11				72.73	–
	Rectal mucosa	F	14	21		49.71±6.03	78.57	–
		M	7				71.43	–
	Gingiva	F	6	8		54.63±3.34	83.33	–
		M	2				100.00	–

* AA designates secondary amyloidosis; ** F and M designate female and male patients, respectively; *** designates the proportion of male or female patients; # designates total number of patients; ## designates the total number of patients per each category; ### indicative of the control group; '±' symbols indicate mean and standard deviation values.

Following fluorescence spectroscopy, tissue samples were subjected to histopathological examinations to determine whether sample preparation for fluorescence spectroscopy had the potential to damage the biopsied tissue in any way.

Results

Figure 1 illustrates typical sample spectra obtained from the amyloid-containing and amyloid-free control groups. For this set of results, the influence of anatomical collection sites was not taken into consideration. For the amyloid-containing tissue samples, the generated fluorescence spectra were characterized by a maximum emission peak at ~340 nm, with a shoulder region at ~280 nm. With regards to the amyloid-free control group tissue samples, the spectral image was typified by a single λmax value at approximately 340 nm, with no shoulder region. The presence of a shoulder emission region at ~280 nm at the quantum limit region of the fluorescence spectrum for amyloid-containing tissue samples was accepted as a preliminary diagnostic criterion for secondary amyloidosis.

Further investigations focused on analyzing the influence, if any, of anatomical collection site on the spectral images obtained from amyloid-containing and control group tissue samples. For this set of data analysis the following parameters were taken into account: presence or absence of secondary amyloidosis; and anatomical site (abdominal fold, rectal mucosa, and gingiva). In so doing, data was collated within 6 separate subcategories or data permutations. For all amyloid-containing biopsy tissue samples, a single emission maxima at ~350 nm with a shoulder region at ~280 nm were observed. In contrast, all amyloid-free tissue samples were typified by only one λmax at approximately 340 nm. The anatomical tissue site location did not appear to influence the generated spectral image – the images within each amyloid subcategory (amyloid-containing and the amyloid-free groups) were similar. Since the anatomical site did not appear to influence the generated spectral image, all the data was collated, averaged and graphed separately for the amyloid-containing and amyloid-free subgroups (Figure 2). It can be seen that since the 2 resultant spectra do not superimpose at the initial wavelength values of the graph, the 2 spectra can be considered to be significantly different.

Table 1 depicts the sensitivity and specificity of the fluorescence spectroscopy technique used in the current study. The results indicate that the specimen collection site did not appear to influence these parameters. The minor differences observed between each collection site may be due to differences in the number of samples across each subcategory, as well as the inherent heterogenicity of each collected tissue. Furthermore, the reported sensitivity values may be due to the limit of detection of the spectrofluorometer instrument.

Tissue samples were further processed for histopathological examinations and this indicated that the samples used for fluorescence spectroscopy were not damaged in any way and could potentially be used in further processing measures and observations.

Discussion

The results of the current research indicate that amyloid-containing and amyloid-free tissue samples are characterized by significantly different fluorescence spectra. While

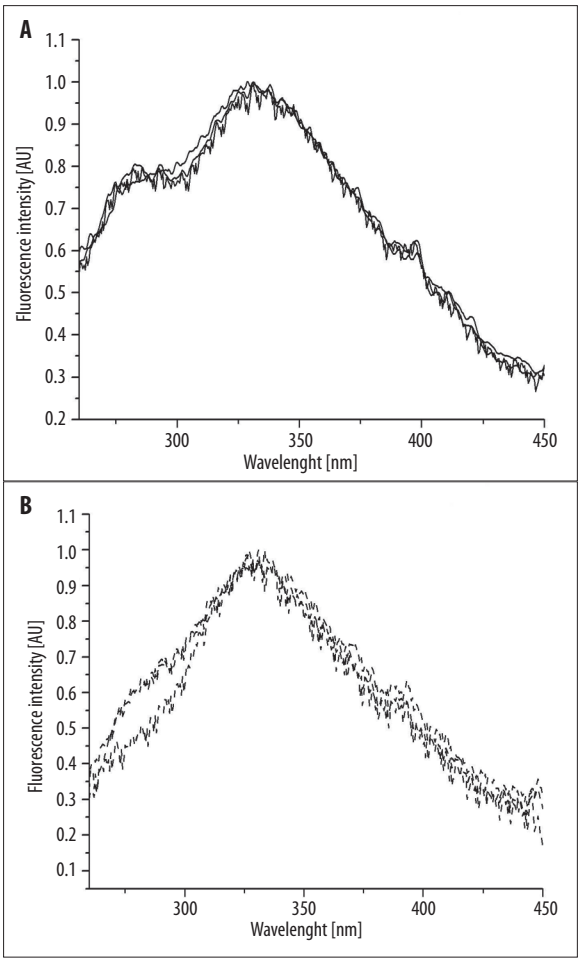

Figure 1. Typical fluorescence spectra which were obtained for (**A**) amyloid-containing specimens and (**B**) amyloid-free (control group) specimens.

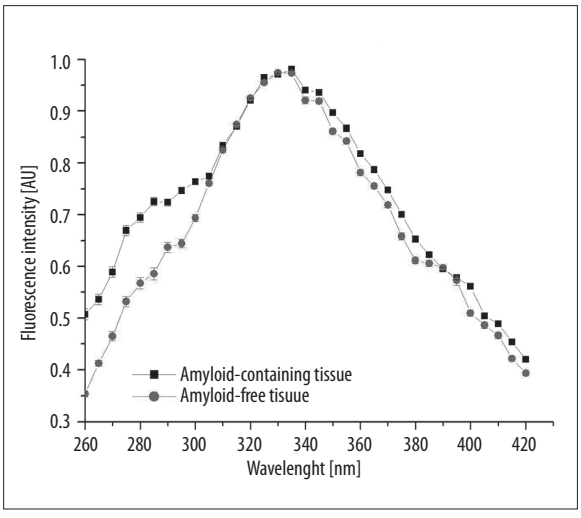

Figure 2. Generated fluorescence spectra for amyloid-containing and amyloid-free control groups. Error bars represent standard error of the mean values for each amyloid subcategory.

amyloid-containing tissue samples displayed fluorescent emission spectra characterized by 2 λmax values, the control-group biopsy specimens were typified by spectral images with 1 emittance peak. Furthermore, when the fluorescence spectra of amyloid-containing tissue samples were analyzed, no detectable influence of collection site on generated spectra was apparent. Such results are encouraging since it may thus be assumed that the inherent autofluorescence of the tissue collection site may be obviated from the final analysis. In so doing, the current fluorometric approach for the detection of AA may possibly be resistant to the variability in collection site types. However, it may be necessary to extend the current research by collecting tissue samples from other anatomical sites in order to delineate the diagnostic rigor of the current approach. In addition to these results, a further analysis indicated that both age and sex of the participating subjects did not appear to influence the generated fluorescence spectra of both amyloid-containing and control group tissue samples.

With regards to the sensitivity of the current technique, amyloid-free tissue samples were always correctly diagnosed. However, while the sensitivity of amyloid-containing tissue samples was relatively high, some margin for error still existed. As a result, in some instances amyloid-containing tissue samples appeared to be amyloid-free, indicative of a false-negative result and a misdiagnosis in practice. This is most probably due to the limit of detection of the detection instrument used in the current study. The diagnostic error was probably due to trace quantities of amyloid deposits in the tissue samples that current conventional approaches could detect, while the resultant emitted fluorescence was below the limit of detection of the fluorometer used in the current study. Work is currently underway to delineate this limitation. To this end, tissue samples will be quantitatively spiked with known amounts of amyloid fibrous protein aggregates, and appropriate calibration curves will be constructed. This may also help assess the applicability of the current approach in the diagnosis of initial onset of AA. Other types of amyloidosis (eg, primary, inherent) may also be investigated to gauge the applicability of the current spectroscopic approach.

To the best of our knowledge, this study represents the first of its kind in which this type of fluorescence spectroscopic approach was used for the detection of amyloid deposits in these tissue types in a relatively large number of subjects. Naiki et al. (1989) used the fluorescent dye thioflavin T1 to determine the presence of amyloid fibrils in mouse liver tissue *in vitro* [12]. Koh et al. (2006) utilized temporal resolution-based fluorescence microscopy to detect isolated β-amyloid peptide deposits [13]. While these reports describe novel approaches, the methodologies generally involve multiple staining steps, are relatively cumbersome and have not always been used for amyloid-containing human tissue samples. As a result, a direct comparative assessment of the current results with those studies may be difficult.

Refinement of the current approach may allow fluorescence spectroscopy to be used as a fast, cost-effective preliminary diagnostic procedure prior to histopathological examination of the same tissue sample. This may help in the early detection of secondary amyloidosis, especially in less well-developed clinical settings. In addition, the current research could be extended to investigate amyloid aggregation processes via the use of time-resolved spectroscopy, since such amyloid structures have been implicated in the development of various pathologies [13].

Conclusions

In conclusion, the results of the current study indicate that fluorescence spectroscopy may potentially be used in the diagnosis of AA. Although current amyloid-detection approaches are characterized by a relatively larger sensitivity index, the relatively positive results of the current study are encouraging. Efforts are currently underway to refine the spectroscopic approach used in this study to develop a fast, economical and user-friendly approach for the almost instantaneous detection of secondary amyloidosis in the general population in different clinical settings.

It is also important that the conduct of such research does not harm the patient. The amount of material received for histopathological examination at time of biopsy is so large that the preparation of additional materials for the study autofluorescence does not present any difficulties, and performing an additional test may in some cases help in diagnosis. Although histopathological examination is the gold standard in the case of amyloidosis, in some cases it is not clear. In such cases, the application of our method can help in determination of correct diagnosis.

Another alternative might be studies using fluorescence spectroscopy without performing biopsy. This type of research is already taking place with the use of infrared spectroscopy in the study of Alzheimer's disease and the study of skin autofluorescence in patients with cardiovascular disease [14,15].

However, much work remains to be done, and at present we cannot say whether this type of research will be possible when searching for deposits of amyloidosis.

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