

Original Article

C4d deposits and a new diagnostic modality for amyloidosis

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Abstract: Amyloidosis is characterized by the deposition of misfolded protein in various organs. The diagnosis of amyloidosis is based on the detection of amyloid deposits by Congo red stain under polarized microscopy. This study was done to investigate C4d deposition in amyloidosis and to determine whether C4d staining can be used as a new diagnostic tool for amyloidosis. This retrospective study included 32 patients who underwent biopsy at our medical center. The samples were stained by Congo red and C4d immunohistochemical stain, respectively. The biopsy samples from 18 patients who had been finally diagnosed with amyloidosis were included as the amyloidosis group, and 14 patients diagnosed with fibrosis were included as the control group. C4d was detected in 17 of 18 amyloidosis patients. On the other hand, in the control group, C4d deposit was not observed in any patient diagnosed with fibrosis. C4d immunohistochemical staining can be a highly useful modality for the diagnosis of amyloidosis. Furthermore, C4d can be applied as a tool for the differential diagnosis of amyloidosis and fibrosis.

Keywords: Amyloidosis, C4d, complement, diagnosis, congo red

Introduction

Amyloidosis is characterized by the deposition of a pathologic protein substance in between cells. Amyloid deposition appears in various tissues and organs and has a variety of clinical manifestations. Identification of this substance in a biopsy specimen is crucial for diagnosing amyloidosis. On light microscopy, amyloid appears as an amorphous, eosinophilic extracellular substance, producing pressure atrophy on adjacent cells. Amyloidosis ultimately leads to destruction of tissues and progressive disease [1].

Recent advances in the treatment of amyloidosis emphasize the importance of an early diagnosis of amyloid. Congo red stain is the most widely used method as it is a sensitive diagnostic method, but a more specific method is the detection of the apple-green birefringence of an amyloid deposit by polarized microscopy [1, 2]. However, caution is advised regarding “over interpreting” collagen as amyloid deposit [1]. The differentiation of amyloid from other hya-

line deposits, such as collagen or fibrin, is essential and it can be performed by various histochemical techniques, but there is no standard strategy.

Research on the complement system as related to amyloidosis is very little. It is reported that the deposition of C1q and other complement components such as C3, C4, C5 and C9 as well as immunoglobulins is observed in cutaneous amyloidosis [3, 4]. Researchers suggest that the local activation of complement via the classical pathway plays a role in the pathogenesis of amyloidosis [3, 4].

The development of a new strategy is needed for regular review and for the creation of standards for the correct and early diagnosis of amyloidosis. Therefore, we undertook an immunohistochemical study of C4d depositions in 32 cases of biopsy specimens. The objective of this study was to determine whether C4d staining can be used as a new modality for the diagnosis of amyloidosis, compared to conventional Congo red staining under polarized microscopy,

Table 1. The results of Congo red stain under light and polarized microscopy and C4d immunohistochemistry

		CR on LM (%)	CR under PM (%)	C4d (%)
Amyloidosis (n=18)	Positive	18 (100)	18 (100)	17 (94.4)
	Negative	0 (0)	0 (0)	1 (5.6)
Fibrosis (n=14)	Positive	5 (35.7)	0 (0)	0 (0)
	Negative	9 (64.3)	14 (100)	14 (100)

CR, Congo red; LM, light microscopy; PM, polarized microscopy.

and whether it can be used as a useful method of differentiating amyloid and collagen deposits.

Materials and methods

Material

This retrospective study enrolled 18 cases of amyloidosis and 14 cases of fibrosis diagnosed at biopsy specimen. The diagnosis of amyloidosis was based on the histological assessment of the biopsy specimen by light microscopy using hematoxylin and eosin stain and Congo red stain, and Congo red stain under polarized microscopy. The definite diagnosis of amyloidosis was based on positive apple green birefringence in Congo red stained section under polarized microscopy.

Congo red staining

For Congo red stain, formalin-fixed paraffin-embedded tissue blocks were cut into 6- μ m-thick sections, deparaffinized in xylene, rehydrated, and then drowned in alkaline solution (alcohol 80%, sodium chloride 2%, sodium hydroxide 1%) for 20 min. After then, the slides were incubated with alkaline Congo red stain for 60 min and counterstained with hematoxylin. The positive Congo red stain was considered that orange red deposition in light microscopy and apple-green birefringence under polarized microscopy.

C4d immunohistochemical staining

The staining of C4d was performed by immunohistochemical staining. Paraffin embedded tissues were cut into 4- μ m-thick sections and deparaffinized. The sections underwent immunohistochemical staining using anti-human C4d polyclonal antibodies as the primary antibody (Biomedica, Vienna, Austria). The C4d

staining was considered to be positive if an amorphous staining pattern was present at the same site of the Congo red stained area. All the sections from biopsy specimens were reviewed without any clinical information.

Results

The tissues were obtained from various sites including small intestine, large intestine, gallbladder, heart, larynx, nasal mucosa, lymph node, and lung. The section of selected cases showed amorphous hyalinized fibrosis in hematoxylin and eosin stain on light microscopy. The results of Congo red stain under light and polarized microscopy and C4d immunohistochemistry were summarized at **Table 1**.

On light microscopic examination of Congo red stained sections, all amyloidosis group had orange red stained deposition (**Figure 1A**). On other hand, 9 cases of fibrosis group showed negative result, but 5 cases showed weak positivity on Congo red stain (**Figure 1D**).

On polarized microscopic examination of Congo red stained sections, amyloidosis group showed apple-green colored birefringence (**Figure 1B**) and fibrosis group showed no birefringence detection (**Figure 1E**).

C4d was detected in 17 of 18 patients with amyloidosis (**Figure 2**). The C4d positive location is correlated with Congo red positive lesions (**Figure 1C**). No C4d deposits were observed in any fibrosis group specimen. Five cases of fibrosis group, presented false positives in Congo red stain on light microscopic examination, showed negative C4d immunohistochemical staining (**Figure 1F**). C4d immunoreactivity of amyloidosis was well correlated with polarized microscopic examination of Congo red stain.

Discussion

Amyloidoses are disorders of diverse etiology in which deposits of abnormally folded proteins share distinctive staining properties and fibrillar ultrastructural appearance [5-7]. Recent advances in the treatment of systemic amyloidoses have occurred and the importance of an early diagnosis of amyloid in making a correct

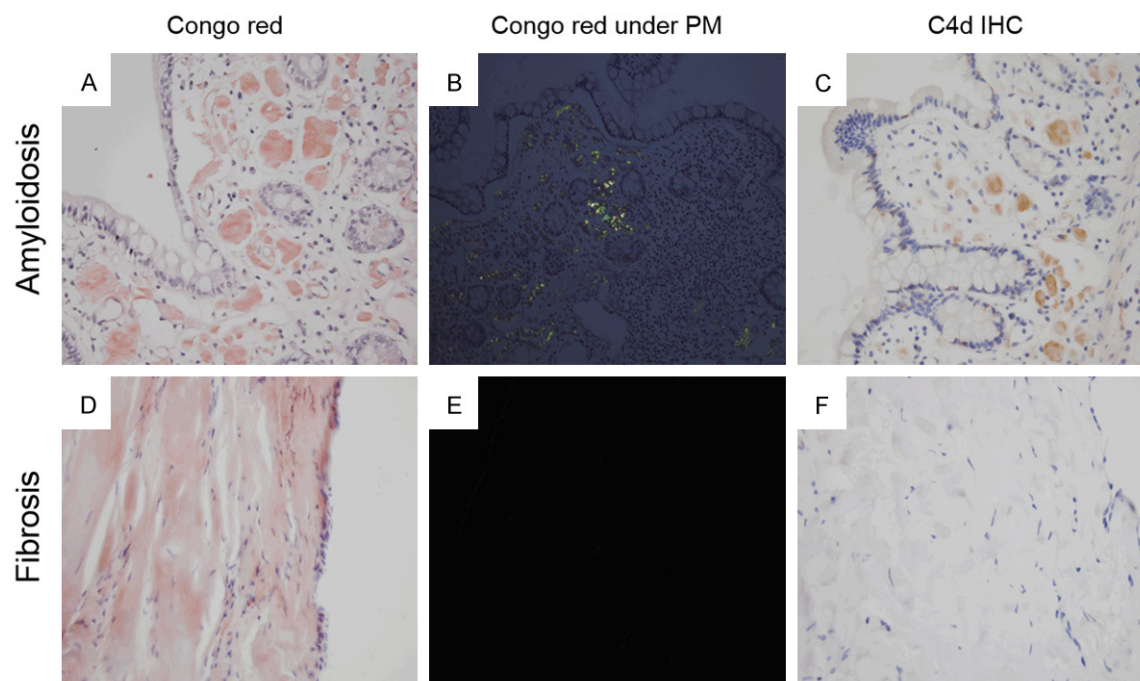


Figure 1. The comparison between amyloidosis in the ileum (A-C) and fibrosis in the valve of heart (D-F). On the Congo red stains, both amyloid deposits (A) and hyaline deposits in fibrosis (D) are observed as orange red on the light microscopy. However, under polarized microscopy, birefringent amyloid deposits are only observed in amyloidosis (B). There is no birefringence in fibrosis (E). As in polarized microscopy, C4d immunohistochemical staining presents positive staining on amyloidosis (C) but negative staining on fibrosis (F). (PM, polarized microscopy; IHC, immunohistochemistry).

diagnosis has been recognized [6, 8-13]. Thus, currently, the challenge is to detect amyloid early and correctly [1]. The diagnosis of amyloidosis is based on the detection of deposits in tissues [6, 7]. Currently, Congo red stain continues to be the gold standard for detection of amyloid deposits [6, 7].

In this study, we aimed to evaluate the role of C4d immunohistochemical staining for the diagnosis of amyloidosis through differentiating amyloid and collagen deposits. In our study, C4d staining proved to be a very useful modality for detecting amyloid deposits and distinguishing them from collagen deposits. Compared with Congo red stain under polarized microscopy, 17 (94.4%) of 18 amyloidosis patient were positive on C4d immunohistochemical staining. Therefore, we could validate C4d immunohistochemical staining as a specific method for differentiating amyloidosis and fibrosis on light microscopy.

As amyloidosis ultimately leads to destruction of tissues and progressive disease [1], caution

is advised regarding overinterpreting collagen as amyloid [1]. Furthermore, small deposits, in particular in thinner sections, may not be apparent in bright light [1]. Importantly, the bright field appearance in itself is not diagnostic [1]. Congo red-stained slides must be examined under polarized light and only the presence of apple-green birefringent deposits is considered diagnostic of amyloid [14]. Five cases of the control group showed weak positivity on Congo red stain on light microscopy, and showed no deposits with green birefringence under polarized microscopy. Additionally, there was no deposition of C4d on immunohistochemical staining.

C4d is a degraded product of C4, and it is generated during the activation of the classical pathway of the complement system mediated by antibodies. Once produced, the C4d binds covalently to tissue components at the activation site, and is, therefore, a biomarker of complement activation [15]. Recently, the detection of C4d has served a critical role in deciding the likelihood of rejection of a transplanted kidney.

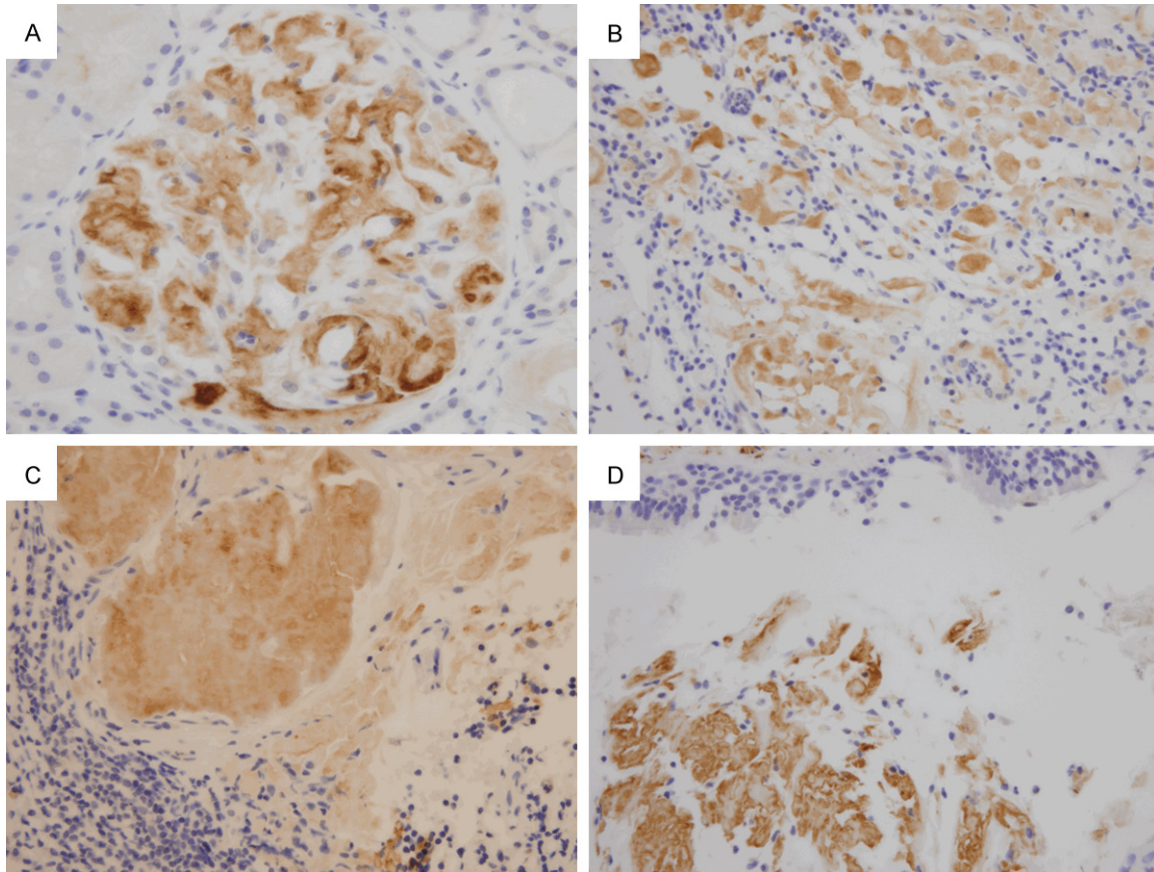


Figure 2. Positive C4d immunohistochemical staining in various tissues of amyloidosis. Amyloid deposits are observed in the kidney (A), ileum (B), larynx (C), and lung (D), respectively. The amyloid deposits are observed as green birefringence in Congo red staining under polarized microscopy.

However, very few studies have analysed complement deposition in amyloidosis. In cutaneous amyloidosis, Danno et al showed that the deposition of complement components and antibodies occurs. In our study, 17 of 18 cases diagnosed as amyloidosis showed C4d deposition in various organs (**Figure 2**). This result is in agreement with the hypothesis that the local activation of complement via the classical pathway may play a role in the pathogenesis of amyloidosis [3, 4].

The interpretation of C4d immunohistochemical staining and the diagnosing of amyloidosis should be done with caution and with consideration of the limitations. Firstly, interpretation of C4d staining must be done in the context of Congo red positivity. Congo red staining should be examined not only to confirm a suspicion of amyloid deposits but also to rule out the possible presence of early deposits, which are otherwise inconspicuous in hematoxylin-eosin or

other special stains [1]. In this study on amyloidosis patients, the C4d deposits are located in the site where amyloid deposits are proven to exist and synchronized by Congo red stain. Secondly, because deposits of amyloid are frequently very focal and irregularly distributed in tissue sections, multiple sections may need to be examined [6]. Thirdly, the relationship between subtypes of amyloidosis and C4d was not evaluated in this study.

In summary, this is the first study on amyloidosis performed by C4d immunohistochemical staining. Although Congo red staining is currently the gold standard for detection of amyloid, our study shows that C4d immunohistochemical staining can be a highly useful modality for the diagnosis of amyloidosis. Besides, C4d staining can be applied as a tool for the differential diagnosis of amyloidosis and fibrosis. However, the interpretation of the C4d staining must be based on the identification of

amyloid protein within the deposits and should be done with caution and with a full awareness of its limitations [1].

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Disclosure of conflict of interest

None.

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