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Analysis of Amyloid in Medullary Thyroid Carcinoma by Mass Spectrometry-Based Proteomic Analysis

Lori A. Erickson [1] · Julie A. Vrana [1] · Jason Theis [1] · Michael Rivera [1] ·
Ricardo V. Lloyd [1] · Ellen McPhail [1] · Jun Zhang [1]

Abstract Amyloid is a characteristic histologic feature in medullary thyroid carcinomas (MTC). We utilized a novel mass spectrometry-based proteomic analysis to determine if we could identify specific proteins associated with amyloid in MTC. We studied 9 MTC (1 multiple endocrine neoplasia type 2A, 1 familial MTC, and 7 sporadic). Laser microdissection was utilized to sample the amyloid which was then trypsin digested and evaluated by liquid chromatography electrospray tandem MS (LC-MS/MS) which identified the presence of amyloidogenic proteins in all cases of MTC. High levels of calcitonin were identified in all 9 cases of MTC. Secretogranin-1 was identified in 6 of 9 MTC. Calcitonin gene-related peptide was identified in 4 of 9 cases of MTC. LC-MS/MS proteomic analysis provides a rapid, highly specific, and sensitive method for identification of the specific type of amyloid in these endocrine tumors. This approach may allow classification of different forms of endocrine amyloid present in neuroendocrine tumors.

Keywords Amyloid · Endocrine · Medullary thyroid carcinoma · Calcitonin

Introduction

Amyloid is a characteristic histologic feature in endocrine tumors such as medullary thyroid carcinomas (MTC). Calci-tonin has been described as a component of amyloid in MTC. Although amyloid can be readily detected by histochemical stains such as Congo red, the specific amyloid present in tumors is difficult to determine. Recently, mass spectrometry-based proteomic analysis has become increasingly used to subtype amyloid in a variety of clinical settings.

This technique was developed as subtyping of amyloid by the more common method of immunohistochemistry can be difficult due to high background staining due to serum contamination, epitope loss due to formalin fixation causing protein cross-linking, and lack of antibodies for all amyloid subtypes [1, 2]. The technique combines specific sampling by laser microdissec-tion and tandem mass spectrometry-based proteomic analysis [1]. Proteins are isolated from the amyloid deposits, extracted, digested with trypsin, and then analyzed by liquid chromatography tandem mass spectrometry [2]. The resulting mass spectrometry spectra are compared with canonical protein sequence data-bases and filtered and identified with post-processing software indicating the specific peptides and associated proteins [2].

Mass spectrometry-based proteomic analysis has been used to subtype amyloid in subcutaneous fat aspirates identifying vari-able gene usage in immunoglobulin light chains and variant pep-tides in hereditary amyloidosis [3]. This technique has also been used to diagnose and type kidney diseases with amyloid includ-ing uncommon and familial forms which are typed based on specific amyloidogenic proteins and immunoglobulins in im-mune complex and complement factors in complement-mediated glomerulonephritis [4]. In patients with monoclonal gammopathy and cardiac amyloid in which blood and urine studies for monoclonal proteins are inadequate to classify amy-loid type, this technique can be used to classify amyloid on endomyocardial biopsies [5]. This technique has not been used to study amyloid in endocrine tumors. We utilized a mass spectrometry-based proteomic analysis to determine if we could identify specific proteins associated with amyloid in MTC.

✉ Lori A. Erickson
erickson.lori@mayo.edu

[1] Department of Laboratory Medicine and Pathology, Mayo Clinic, 200 First Street SW, Rochester, MN 55901, USA

Materials and Methods

Nine MTC were studied. One case was from a patient with multiple endocrine neoplasia type 2A, one case was from a patient with familial MTC, and seven cases were sporadic MTC. Hematoxylin and eosin-stained sections were evaluated for verification of histologic diagnosis. Clinical information was obtained by chart review.

The specific methods of laser microdissection/mass spectrometry have been published previously [1]. Briefly, formalin-fixed paraffin-embedded tissue sections cut at 10 μm were stained with Congo red, and fluorescent light was used to identify areas of Congo red positivity (Fig. 1). In each case, Congo red-positive amyloid plaques were microdissected from the MTC. Four amyloid specimens from each case were evaluated. The collected tissue then was trypsin digested into peptides, reduced with dithiothreitol, and separated by nanoflow liquid chromatography–electrospray tandem mass spectrometry (LC-MS/MS) using a ThermoFinnigan LTQ Orbitrap Hybrid Mass Spectrometer (Thermo Electron, Bremen, Germany) coupled to an Eksigent nanoLC-2D HPLC system (Eksigent, Dublin, CA, USA). The resulting LC-MS/MS data was correlated to theoretical fragmentation patterns of tryptic peptide sequences from the Swissprot database using Scaffold (Mascot, Sequest, and X!Tandem search algorithms). Peptide identifications were accepted if they could be established at greater than 90.0 % probability, and protein identifications were accepted if they could be established at greater than 90.0 % probability and contain at least two identified spectra. The identified proteins were subsequently examined for the presence or absence of amyloid-related peptides.

Results

The clinical and pathologic findings are presented in Table 1. The patients ranged in age from 22 to 76 years of age, and 4 were female and 5 male. Of the 9 patients, 7 had sporadic MTC, one had familial MTC, and one had MTC in the setting of multiple endocrine neoplasia type 2A. Six of the nine had lymph node metastases at the time of diagnosis. Follow-up ranged from 2 to 12 years. LC MS/MS identified the presence of amyloidogenic proteins in all 9 cases of MTC. All 9 cases had high levels of amyloid signature proteins apolipoprotein A-IV and apolipoprotein E, and 5 of 9 also had serum amyloid P, indicating the material analyzed was amyloid. The correlation between these signature proteins and Congo red amyloid positivity was recently confirmed by Vrana et al. [3]. A mutation database search was also performed on the data from these 9 MTC, and no mutation was identified in the amyloid signature proteins [2].

High levels of calcitonin protein were identified in all cases of MTC. Katacalcin was also identified in all samples. A few peptides of secretogranin-1 were identified in 6 of 9 MTC, and a few peptides of calcitonin gene -peptide-beta (CGRP) were identified in 4 of 9 MTC.

Discussion

Amyloid is a characteristic feature of medullary thyroid carcinoma. Early descriptions of MTC include amorphous material that was identified as amyloid. By electron microscopy, amyloid fibrils have been identified in MTC, and amyloid was confirmed to be a product of the MTC cells by in vitro culture

Fig. 1 a Sporadic medullary thyroid with amyloid carcinoma in a 65-year-old male (H&E, 10×). b Sporadic medullary thyroid carcinoma with amyloid in a 66-year-old male (H&E, 10×). c Medullary thyroid carcinoma with amyloid in a 61-year-old male with multiple endocrine neoplasia type 2A. d Congo red apple green birefringence with polarization in medullary thyroid carcinoma from a 61-year-old male with multiple endocrine neoplasia type 2A
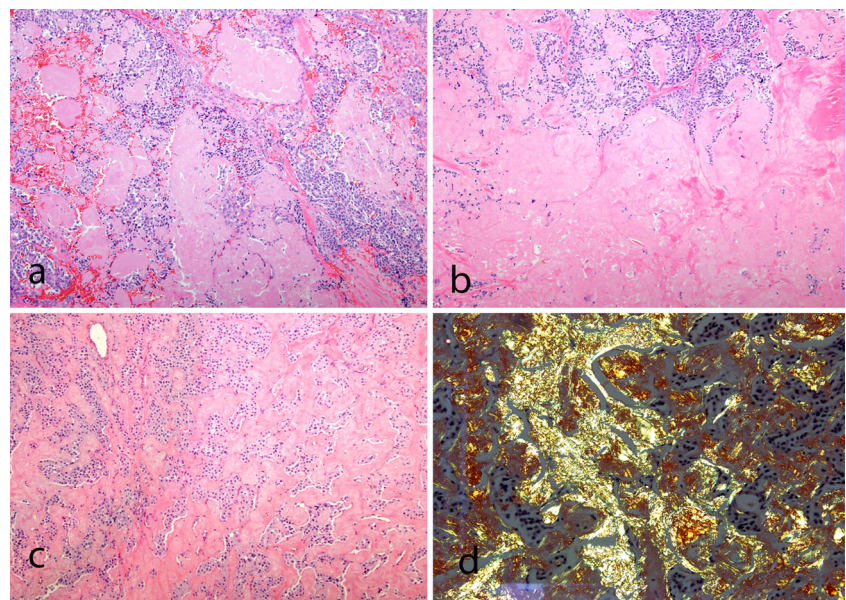
Table 1 Medullary thyroid carcinomas studied and amyloid peptide identified

Case	Tumor characteristics and outcome	Amyloid peptide[a]	Peptides at low levels of uncertain significance[b]
22-year-old female	Familial MTC. Bilateral (3.2 and 1.1 cm) tumors, 1 of 4 perithyroidal LN involved. Alive without disease, 5.2 years FU.	Calcitonin	Secretogranin-1 (2 of 4)
65-year-old male	Sporadic MTC. 5.5 cm tumor, 18 of 45 neck and axillary LN involved. Developed recurrence in the thyroid bed and lung, died of disease, 2.4 years FU.	Calcitonin	
51-year-old female	Sporadic MTC. 2.5 cm tumor with 1 of 15 central compartment LN positive. Alive with disease, 2 years FU.	Calcitonin	Secretogranin-1 (4 of 4), CGRP (3 of 4)
53-year-old male	Sporadic MTC. 3 cm tumor, 2 of 2 central and 1 of 3 midline LN positive. Died without disease, 11 years FU.	Calcitonin	Secretogranin-1 (2 of 4), CGRP (3 of 4)
50-year-old male	Sporadic MTC. 3.3 cm tumor. Central compartment LN negative. Alive without disease, 6.5 years FU.	Calcitonin	Secretogranin-1 (2 of 4)
74-year-old female	Sporadic MTC. 1.6 cm tumor, 2 of 8 perithyroid and 11 of 19 neck LN positive. Alive without disease, 12 years FU.	Calcitonin	Secretogranin-1 (4 of 4), CGRP (4 of 4)
61-year-old male	Multiple endocrine neoplasia type 2A. Bilateral (4.4 cm, 4.2 cm) tumors, 1 of 15 LN positive. Developed liver, LN, bone metastases; died of disease, 3.8 years FU.	Calcitonin	Secretogranin-1 (3 of 4), CGRP (1 of 4)
66-year-old male	Sporadic MTC. 2.2 cm tumor. LN negative. Alive without disease, 13.7 years FU.	Calcitonin	Secretogranin-1 (3 of 4), CGRP (3 of 4)
76-year-old female	Sporadic MTC. 3.3 cm tumor, LN negative. Alive without disease, 10 years FU.	Calcitonin	

MTC medullary thyroid carcinoma, *LN* lymph node, *FU* follow-up, *CGRP* calcitonin gene-related peptide-beta

[a] Calcitonin peptides were detected at high levels in all 4 of 4 specimens of amyloid from all 9 medullary thyroid carcinomas studied

[b] Few peptides of each were detected among subset (*n* of 4) of the 4 specimens per case and of unknown significance to the amyloid

of medullary carcinoma cells [6]. Others have shown that the amyloid fibrils in MTC could not be distinguished from other forms of amyloid occurring naturally or experimentally [7]. Basement membranes were observed between tumor cells and stroma but were usually absent where MTC cells were contiguous with amyloid [6]. The formation of amyloid is predominantly extracellular in MTC [7]. Secretory granules have been identified closely related to the amyloid of MTC, suggesting that the granules may have a role in the formation of the amyloid [8].

Medullary thyroid carcinoma cells contain calcitonin, and calcitonin has been identified in the amyloid stroma by immunohistochemistry and immunofluorescence [9, 10]. Amyloid fibrils from two MTC demonstrated AMCT (amyloid medullary carcinoma of the thyroid) protein to be the major protein in MTC amyloid fibrils [10]. Two different components of amyloid were referred to as the P component which was immunologically homogeneous, and the AMCT protein, which was "immunologically distinct and did not react with various antisera against known amyloid fibril proteins" [10]. The degraded MTC amyloid fibrils and the isolated AMCT protein reacted with antiserum raised against the MTC amyloid proteins but not with other amyloid proteins [10]. Sequence analysis identified the portion of the AMCT protein to be calcitonin, but the molecular weight of the AMCT protein was larger than calcitonin and thought to possibly be a prohormone of calcitonin [10]. A subsequent study directly evaluated the

component of MTC after denaturation of amyloid utilizing matrix-assisted laser desorption ionization-time of flight mass spectrometry analysis [11]. Rather than the alternatively processed prohormone of calcitonin as was suggested by prior authors [10], Khurana et al. [11] reported "full-length calcitonin is the sole constituent of amyloid in MTC." The authors noted it would be "much better to perform MS/MS analysis to say with complete authority that calcitonin is involved in amyloid formation in MTC tissue" and attempted MS/MS but were not able to obtain satisfactory results which they attributed most likely due to insufficient sample size [11].

By liquid chromatography electrospray tandem MS, we were able to demonstrate calcitonin protein in all 9 cases of amyloid in the MTCs in our study. All four amyloid specimens from each case showed calcitonin protein in all 9 cases of MTC. (Pro)calcitonin is the precursor protein of ACal fibril protein in C-cell thyroid tumors [12]. Our analysis identified peptides mapping to both calcitonin and katacalcin (Fig. 2). Calcitonin and katacalcin were identified in all samples and showed complete coverage. These peptides were independently mapped to the calcitonin precursor entry in the Swiss protein database which is a concatenated sequence containing the signaling peptide (amino acids 1–25), the propeptide (amino acids 25–82), calcitonin protein (amino acids 85–116), and katacalcin protein (amino acids 121–141). Katacalcin is a member of the calcitonin gene family and is a calcium-lowering hormone. Katacalcin flanks calcitonin in the human

Fig. 2 Sequence coverage of the calcitonin precursor identified by laser microdissection mass spectrometry. *Yellow highlights* indicate identified amino acids

CALC_HUMAN (100%), 15,467.8 Da
Calcitonin precursor [Contains: Calcitonin; Katacalcin - Homo sapiens (Human) (P01258)
19 exclusive unique peptides, 27 exclusive unique spectra, 142 total spectra, 110/141 amino acids (78% coverage)

calcitonin precursor—which is cleaved into calcitonin and katacalcin. The amyloid fibril proteins are designated protein A followed by the abbreviated form of the precursor protein [12]. Thus, "A" with suffix of "Cal" or "ACal" for the fibril protein in C-cell thyroid tumors. The full-length sequences for both calcitonin and katacalcin were detected via mass spectrometry indicating the proteins were intact and not truncated. The proteins were also detected in the absence of any variable modification allowance in the bioinformatics software suggesting the proteins lack any significant amidation, glycosylation, or other post-translational modifications.

Calcitonin and katacalcin are not unique as proteins to be identified in the amyloid state. There are approximately 50 disorders associated with misfolding of normal functional peptides and proteins that can result in aggregates such as amyloid [13]. The amyloid state has been described as "generic" as can be adopted by many proteins and peptides and is not associated with a specific amino acid sequence [13]. Proteinaceous deposits in misfolding diseases have amyloid characteristics, but the sequences, native structures, and protein functions do not have obvious similarities [13, 14]. The specific mechanism for calcitonin and katacalcin to be in the amyloid state in some cases of MTC remains unclear. Protein concentrations in these tumors could be associated the amyloid state. Thermodynamic studies have shown that conversion into the amyloid state will take place when its free energy is lower than that of its native state and the stability of the amyloid state is dependent on the protein concentration [13]. Maintenance of solubility, free energy barriers with transitions between states, rates of synthesis and degredation, interactions with chaperones, or other modifications may have a role.

Secretograin-1 was identified in 6 of 9 MTC and calcitonin gene-related peptide in 4 of 9 MTC in our study. The finding of only a few peptides each of secretogranin-1 and calcitonin gene-related peptide in a subset of the cases in our study is unclear. As there were only a few signals of these peptides, their significance to the amyloid remains uncertain. It is possible that they may be associated with the amyloid, but it is also possible that they may be artifact or contaminant as they were seen at low levels and not in every case and not in every sample of each case where it was identified—unlike calcitonin which was identified at high levels in every sample of every case. Secretogranin-1 (chromogranin-B) is a neuroendocrine secretory granule protein and member of the chromogranin/secretogranin family [15]. Calcitonin gene-related peptide-beta (CGRP) was identified in 4 of 9 MTC. This peptide is part of the calcitonin family of peptides. The mitogen-activated protein kinases signaling pathway and cytokines are involved in regulating the CGRP gene [16, 17]. CGRP receptors are identified in many tissues, and CGRP is associated with the cardiovascular system, with pain, vasodilation, cutaneous epithelium, and wound healing, among many other roles [18, 19].

In conclusion, we utilized a novel mass spectrometry-based proteomic analysis and identified calcitonin associated with amyloid in all 4 samples in each of all 9 cases of MTC (1 multiple endocrine neoplasia type 2A, 1 familial MTC, and 7 sporadic MTC) studied. LC-MS/MS proteomic analysis provides a rapid, highly specific, and sensitive method for the analysis of the specific type of amyloid in these tumors. This approach may allow for the classification of different forms of endocrine amyloid present in neuroendocrine tumors.

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