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Quantification of CSF cystatin C using liquid chromatography tandem mass spectrometry

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ABSTRACT

Background: Cystatin C (CST3), a ubiquitously expressed cysteine protease inhibitor, is implicated in several neurological diseases. Here, we have developed an accurate CST3 measurement system based on liquid chromatography tandem mass spectrometry (LC-MS/MS).

Methods: LC-MS/MS based measurement for CSF CST3 was validated by determination of assay precision, accuracy and recovery. The values were compared with those measured by immunoassay. Glycosylation of CST3 in CSF was analyzed by Western blotting and lectin blotting.

Results: Measuring standard CST3 by LC-MS/MS produced a linear standard curve that correlated with assigned values ($r^2 = 0.99$). Both intra- and inter-assay variation was < 10%. Although showed a correlation, the average CST3 concentration measured by LC-MS/MS was significantly higher than that of immunoassay. Western blotting showed the presence of a 25 KDa species along with CST3 monomer (14 KDa) in CSF. The volume of 25 KDa species was decreased by deglycosylation. Lectin blotting revealed a 25 KDa glycosylated protein in sialidase-treated CSF, which was decreased by deglycosylation. However, deglycosylation did not alter CST3 concentration measured by immunoassay.

Conclusions: Our results suggest that LC-MS/MS-based CST3 measurement is a robust method with higher detection ability. Such method could be useful for the diagnosis and monitoring of neurological diseases.

1. Introduction

Cystatin C (CST3) is a 13-kDa protein that consists of 120 amino acids encoded by a 7.3-kb gene located on chromosome 20 [1]. It is produced by all nucleated cells and localized mainly in the lysosome. However, it also demonstrated to be a secreted protein. CST3 functions as a potent endogenous inhibitor of lysosomal cysteine proteases including cathepsin B, D and L, and thereby modulate various intra- and extracellular events including vascular remodeling and inflammation through regulating the balance of protease activity. Hence, the level of serum CST3 was found to change in a variety of disease condition including chronic kidney disease and coronary heart disease. In the central nervous system, CST3 expresses in choroid plexus, leptomeningeal cells, glial cells and neurons in the state of health and diseases [2–5]. Importantly, CST3 was found to be associated with several neurodegenerative diseases. The connection of CST3 with neurodegenerative diseases was suggested by its co-localization with deposited amyloid β peptide in amyloid-laden vascular walls, in senile plaque cores of amyloid in the brains of Alzheimer's disease (AD) patients, and CST3 immunoreactivity was decreased in the spinal cord neurons in amyotrophic lateral sclerosis [6–8]. It is reported that CST3 concentration is decreased in the CSF of AD and amyotrophic lateral sclerosis patients [9–11]. Hence, CST3 could be a good target for the diagnosis and therapy of neurodegenerative diseases.

Serum CST3 concentration is proposed as a useful marker to assess glomerular filtration rate (GFR) and renal function because it is freely filtered, almost reabsorbed and degraded but not secreted by proximal tubular cells [12,13]. Its level has been found 5 times higher in the cerebrospinal fluid (CSF) than in the plasma [14]. Our previous studies demonstrated that CST3 concentration was decreased and cathepsins

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activities were increased in the CSF of patients with cerebral amyloid angiopathy, CNS inflammation and leptomeningeal metastasis [15–18]. These findings are indicating the inhibiting role of CSTS for cathepsins in the CSF and also, the measurement of CST3 levels in the CSF might have a diagnostic value for neurological diseases.

In laboratory setting, immunoassay methods are mainly employed for CST3 measurements. Immunoassay based methods have several drawbacks including nonspecific target binding, alteration of assay sensitivity depending on samples, and variation of specificity depending on the antibody used for the assay. Hence, there could be variation of CST3 measurement depending on the laboratory setting, source and lot of antibody and sample source.

Recently, the use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is thought to be an interesting alternative method in routine clinical laboratory uses. This system has significant advantages over immunoassay like requirement of very small amounts of samples, almost no effects of source of samples, ability to identify any modification of target molecules, and possible identification and accurate quantification of many targets in a sample during a single run. Since LC-MS/MS measurement has a merit not to be affected by non-specific binding of antibodies seen in enzyme-linked immunosorbent assay (ELISA) method, we tried to establish CST3 measurement in the CSF with LC-MS/MS to further evaluate differences of CST3 levels in the disease conditions.

2. Materials and methods

2.1. Chemicals and reagents

LC-MS grade methanol (MeOH), acetonitrile (ACN), and water were purchased from Sigma Aldrich (St. Louis, MO, USA). Glu1-fibrinopeptide B (glufib) was purchased from Sigma Aldrich. Formic acid, and all the other solvents used in this study are HPLC grade, and were obtained from Wako Pure Chemical Industries (Osaka, Japan). Water was further purified using a Millipore Milli-Q UV plus and Ultra-Pure Water System (Tokyo, Japan). Other chemicals were purchased from standard sources and were of the highest quality available. Standard CST3, extracted from human urine, was purchased from Calbiochem (La Jolla, CA, USA). Peptides used to quantify CST3 were purchased from Thermo Fisher Scientific Inc. (Waltham, MA USA). Artificial CSF (aCSF) was purchased from Otsuka Pharmaceutical factory Inc. (Tokushima, Japan).

2.2. Sample collection and ethical procedures

Cerebrospinal fluid (CSF) was collected from 27 subjects by lumbar puncture at the Department of Neurology, Shimane University Hospital. The Ethics Committees of the Shimane University Hospital approved the collection and analysis of the CSF samples. All patients or their relatives provided written informed consent to be included in this study.

2.3. Analysis of CST3 amino acid sequence for trypsin-digested peptide fragments and glycosylation sites

To determine trypsin-digested peptide fragment, human CST3 amino acid sequence was analyzed using a web-based enzyme analysis tool (http://web.expasy.org/peptide_mass/), which predicted 11 trypsin-digested peptide fragments with molecular weight above 500 Da. Then molecular weight-matched peptide fragments of CST3 were identified in MS scan of trypsin-digested standard CST3, and further verified by MS/MS analysis. To determine potential glycosylation site(s) in CST3, amino acid sequence was analyzed using web-based glycosylation identification tools (http://www.cbs.dtu.dk/services/NetNGlyc/, http://www.cbs.dtu.dk/services/NetOGlyc/ and http://www.imtech.res.in/raghava/glycoep/submit.html).

2.4. Sample preparation for LC-MS/MS: proteolytic digestion and peptide purification

Standard CST3 and CSF samples (n = 17) were trypsin-digested using Sequencing Grade Modified Trypsin (Promega, Leiden, The Netherlands). Forty microliters of CST3 standard (0.5 mg/ml in aCSF) or CSF was mixed with 6.25 µl of a solution of 0.1 M Tris-HCl (pH 8) and 8 M urea, and denatured for 1 h at 65 °C. The samples were incubated with 1.2 µl of 1 M dithiothreitol (DTT) at 55 °C for 1 h with shaking, then cooled to room temperature and alkylated with 1.6 µl of 550 mM iodoacetamide (IAA) for 1 h with shaking. After alkylation, 42.15 µl water, 4 µl of 1 M ammonium bicarbonate, and 4.8 µl of 100 µg/ml trypsin were added to the sample to make a final volume of 100 µl, sonicated and incubated at 37 °C for 24 h. Digestion was terminated by adding 200 µl of trifluoroacetic acid (TFA) at final concentration of 0.1%. After mixing by vortex, the mixture was applied to a Sep-Pak C18 Plus Light cartridge (55–105 µm particle, Waters corporation, MA, USA) pre-treated with 1.5 ml of 0.5% TFA in 70% ACN. Then the cartridge was washed with 2 ml of 0.5% TFA. The digested peptides were eluted with 1 ml of 0.5% TFA in 70% ACN. After evaporation to dryness, the digested peptide was dissolved in 100 µl of 0.1% formic acid (FA) aqueous solution, and 10 µl aliquot of the sample was subjected to LC-MS/MS analysis.

2.5. Mass spectrometry LC-MS/MS

Samples of peptide solutions resulting from digestion of CST3 and glufib were analyzed using a LCMS-8030 (Shimadzu Co., Kyoto, Japan).

LC was carried out on an InertSustain Guard column (1.5 mm × 10 mm i.d.) followed by an InertSustain C18 column (3 µm, 2.1 mm × 100 mm i.d., GL Science Inc., Tokyo, Japan). Ten microliter of samples were injected, and eluted with binary solvent gradient of 0.1% FA aqueous solution (solvent A) and 0.1% FA in ACN (solvent B) at a flow rate of 0.2 ml/min. Separation was conducted at 40°C according to the following gradient program: 0–5 min, 18%(B) to 30%(B); 5–5.1 min, 30%(B) to 90%(B); 5.1–10 min, 90%(B); 10–10.1 min, 90(B) to 18%(B); 10.1–15 min, 18%(B).

The mass spectrometer was operated in electrospray positive ionization mode, the desolvation line temperature was 250 °C and heat block temperature was 400 °C, drying gas flow was 15.0 l/min and nebulizer gas flow was 3.0 l/min. The transitions and collision energies for the analytes are given in Table 1. Three peptides were selected for qualitative marker peptide of CST3 (Fig. 1). Transitions were monitored in multiple reaction monitoring (MRM) mode with a dwell time of 0.1 s. Glufib was used as an internal standard. Peptide peak areas were

Table 1
MRM transitions and collision energies for tryptic peptides of CST3 and glufib.

Analyte	Peptide sequence	Mass (Da)	Precursor ion (m/z)	Product ion (m/z)	Collision energy (eV)
Peptides1	ALDFAVGEYNK	1226.6051	613.6	86.15/157.10	54.0/34.0
Peptides2	ALQVVR	685.4355	685.3	100.90/122.25/184.95	55.0/48.0/50/0
Peptides3	QIVAGVNYFLDVELGR	1792.9592	896.2	474.9	21
Glufib	EGVNDNEEGFFSAR	1570.6768	785.7	72.15/120.1/186.9	52.0/55.0/45.0