Identification and Characterization of Cardiac Troponin T Fragments in Serum of Patients Suffering from Acute Myocardial Infarction

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Identification and Characterization of Cardiac Troponin T Fragments in Serum of Patients Suffering from Acute Myocardial Infarction

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BACKGROUND: Cardiac troponin T (cTnT) is the preferred biomarker for the diagnosis of acute myocardial infarction (AMI). It has been suggested that cTnT is present predominantly in fragmented forms in human serum following AMI. In this study, we have used a targeted mass spectrometry assay and epitope mapping using Western blotting to confirm this hypothesis.

METHODS: cTnT was captured from the serum of 12 patients diagnosed with AMI using an immunoprecipitation technique employing the M11.7 catcher antibody and fractionated with SDS-PAGE. Coomassie-stained bands of 4 patients at 37, 29, and 16 kDa were excised from the gel, digested with trypsin, and analyzed on a Q Exactive instrument set on targeted Selected Ion Monitoring mode with data-dependent tandem mass spectrometry (MS/MS) for identification. Western blotting employing 3 different antibodies was used for epitope mapping.

RESULTS: Ten cTnT peptides of interest were targeted. By using MS/MS, all of these peptides were identified in the 37-kDa, intact, cTnT band. In the 29- and 16-kDa fragment bands, 8 and 4 cTnT-specific peptides were identified, respectively. Some of these peptides were “semitryptic,” meaning that their C-termini were not formed by trypsin cleavage. The C-termini of these semitryptic peptides represent the C-terminal end of the cTnT molecules present in these bands. These results were confirmed independently by epitope mapping.

CONCLUSIONS: Using LC-MS, we have succeeded in positively identifying the 29- and 16-kDa fragment bands as cTnT-derived products. The amino acid sequences of the 29- and 16-kDa fragments are Ser79-Trp297 and Ser79-Gln199, respectively.

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Cardiac troponin T (cTnT)3 and I (cTnI) have been firmly established as the gold standard biomarkers for the diagnosis of acute myocardial infarction (AMI) (1–3). Owing to patent restrictions, the commercially available cTnT assay by Roche Diagnostics is still the only clinical cTnT assay on the market and is actively being used in 51% of hospitals in Europe (4). The assay works according to the sandwich principle, in which a detector (M7) and catcher (M11.7) antibody are allowed to bind to cTnT, forming a sandwich complex. Since both antibodies bind closely to one another in the center of the cTnT molecule, the assay is able to detect all cTnT isoforms, protein complexes, protein fragments, and peptides containing the epitope sequences corresponding to these antibodies. However, the affinity of the Roche antibodies for the different cTnT molecules may vary. Consequently, any molecular heterogeneity of cTnT may have an impact on the performance of the cTnT assay; therefore, it is important to know whether or not different immunoreactive molecules exist in the serum or plasma of patients suffering from AMI.

In previously published papers, Michielsen et al. (5) and Cardinaels et al. (6) used Western blotting and gel filtration chromatography (GFC) to show that cTnT was primarily present in fragmented forms in human serum. These fragments were detected using the antibodies from the Roche assay (6). A consistent pattern frequently observed in these studies was the appearance of intact cTnT at 37 kDa, a primary cTnT fragment band at 29 kDa, and several smaller secondary fragments between 15 and...
20 kDa. Intact cTnT was only observed in a small percentage (approximately 20%) of patients in these studies. This is in contrast to the findings of Fahie-Wilson and Bates et al., who were unable to identify cTnT fragments and argued that cTnT circulates only in the intact and complexed form (7, 8). The primary detection method of all of these different studies was based without exception on immunoreactivity with the Roche M7 detector antibody in combination with a separation technique. It has been suggested that the appearance of cTnT fragments in these patients should be verified with mass spectrometry (9).

Recently, we have developed a gel-based targeted mass spectrometry assay to identify molecular changes in cTnT (10). We validated the assay with an in vitro experiment where purified human intact cTnT was spiked in a human serum matrix. Incubation at 37 °C resulted in fragmentation similar to that seen in AMI-patients. With tandem mass spectrometry (MS/MS), these fragments were identified as cTnT-derived products. In addition, we showed using relative quantification that it is possible to pinpoint specific cTnT peptides that are differentially present within the different protein bands.

In this study, this targeted mass spectrometry assay is used to identify the observed immunoreactive bands in the serum of patients diagnosed with AMI. Regions of cTnT that are cleaved off are identified using a combination of Western blotting and mass spectrometry. The identification of semitryptic peptides allowed for the direct pinpointing of cleavage sites of cTnT within these samples.

Materials and Methods

An expanded materials and methods section is available in the online Supplemental Material (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue2).

PATIENT SERUM SAMPLES AND QUALITY CONTROL

Patients diagnosed with ST-segment elevation myocardial infarction (STEMI) were included over a period of 1 year when at least 1 routinely analyzed serum sample had a cTnT concentration of >8000 ng/L. According to these criteria, 14 patients were included and all related serum samples were collected and stored at −80 °C until analysis. Two patients were later excluded because fewer than 3 serum samples were available. Pooled human serum ([cTnT] <14 ng/L) obtained from healthy volunteers was used as a negative QC. As a positive QC, cTnT purified from human heart tissue was purchased from Hytest. In addition, a synthetic peptide standard consisting of the targeted peptides (synthesized by Pepscan with an average purity of >95%) was used as an instrument QC. Handling of all serum samples was in accordance with the code for proper secondary use of human tissue in the Netherlands (www.fmwv.nl).

Peptide and epitope sequence numbering throughout this article is based on the canonical human cTnT protein species (cTnT-1, P45379). Peptide sequences with a bold type, lowercase m represent peptides with an oxidized methionine.

cTnT CONCENTRATION MEASUREMENTS

cTnT concentration was measured with the 5th-generation (high-sensitivity) cTnT-STAT assay on the Cobas® 6000 instrument (Roche Diagnostics). The assay has a limit of blank of 3 ng/L, a limit of detection (LOD) of 5 ng/L, and a limit of quantification of 13 ng/L. The linear measuring range is 3.00–10000 ng/L.

IMMUNOPRECIPITATION AND WESTERN BLOTTING

Immunoprecipitation of cTnT, fractionation with SDS-PAGE, and the subsequent detection of cTnT with different antibodies using Western blot was performed on patient serum samples, and on the positive and negative QCs as described previously (10, 11). All experiments were done in duplicate, where one gel was used for Western blotting and the other for Coomassie staining and mass spectrometry.

MASS SPECTROMETRIC ANALYSIS

Sample work-up, mass spectrometry and data analysis were performed as previously described (10). In brief, individual gel bands at 37, 29, and 16 kDa were carefully excised from the gel and digested with mass spectrometry grade trypsin gold (Promega). Quantitative LC-MS/MS measurements were performed on a Q Exactive hybrid quadrupole-Orbitrap mass spectrometer, connected to an ultra-high-performance liquid chromatography (UHPLC) Dionex Ultimate 3000 (both by Thermo Fisher Scientific). Table 1 shows all specific settings, including target m/z, of the employed mass spectrometry method.

DATA ANALYSIS

Product ion spectra were searched against the human UniProtKB/Swiss-Prot database (dated November 13, 2013, with a total of 39 690 entries) using the search engine SEQUEST with Proteome Discoverer, version 1.8. Quantification was performed by calculating the area under the curve (AUC) of the monoisotopic (M+0) precursor ions from the extracted ion chromatograms (XIC) using Skyline v.2.6 (12, 13). A precursor/reference (P/R) ratio was calculated for each targeted ion in each sample as described previously (10). The P/R ratio is defined as the AUC of a specific precursor ion (P) divided by the AUC of a reference ion (R) within the same sample. Resulting P/R ratios were then normalized with respect to the highest ratio within each single experiment.
DATA ACCESSIBILITY

The mass spectrometry data (.raw files and .msf files) associated with this research are accessible at the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (14) with the data set identifier PXD004660.

Table 1. Liquid chromatography and mass spectrometry settings for the t-SIM/dd-MS/MSa assay.

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<th>Parameter Setting</th>
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<th>Polarity</th>
<th>Target m/z and chargeb</th>
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a t-SIM/dd-MS/MS, targeted SIM with data-dependent MS/MS.

b A lowercase, bold type m indicates an oxidized methionine.

c This peptide was not included in the synthetic peptide standard.

d FWHM, full width at half maximum.

e HCD, higher-energy collisional dissociation.

Results

FRAGMENTATION PATTERN ANALYSIS USING WESTERN BLOTTING

The specificity of the custom-designed antibodies was first validated using purified skeletal (sk)TnT and the negative QC. These antibodies are specific for cTnT and show no cross-reaction with skTnT (see Supplemental Fig. 1 in the online Data Supplement). Furthermore, online Supplemental Fig. 2 in the online Data Supplement shows that both antibodies do not nonspecifically bind to proteins present in serum and that the LODs of the N- and C-terminal antibodies, expressed as absolute amount of cTnT, are 100 and 50 pg, respectively.

The cTnT concentration profile and Western blots of a patient showing all commonly observed bands is depicted in Fig. 1. Fig. 1A shows the concentration profile of cTnT. Figs. 1B, 1C, and 1D show Western blots of serum samples from this same patient probed with the Roche M7, the Medimabs N-terminal, and the Medimabs C-terminal antibodies, respectively. It is apparent that the N-terminal antibody only stains the 37-kDa, intact, protein band (Fig. 1C). The C-terminal antibody stains the intact protein and the 29-kDa, primary fragment, band (Fig. 1D). The secondary fragments at 15–20 kDa are only visible with the M7 detector antibody as employed in the clinical assay (Fig. 1B). Only the 37-kDa, intact, band is present in the positive QC samples, indicating that the degradation is independent from the methodology used. All Western blot images from the other 11 patients support these observations and are available in the online Supplemental Material.

VALIDATION OF THE MASS SPECTROMETRY ASSAY

The LC-MS assay used in this study was established previously and was validated extensively (10, 15). Two peptides that were targeted in the validation study have been omitted in this study because their CVs were deemed too high. The CVs of all targeted peptides in the validation study were /H11021/23% except for the elongated N-terminal peptide 53AEED. .PKPR78 which had a CV of 55%. This peptide was included because of its location on the N-terminus of cTnT which was considered crucial for this study. All targets showed good linearity (r2 > 0.95), except for the 291AKVTGRW297 peptide (r2 = 0.60). The LOD of each peptide (expressed as serum equivalent of immunoreactive cTnT in ng/L) was also determined in the validation study and was lower than the cTnT concentration in our samples. Furthermore, we showed that all targeted peptides were specific for cTnT (with the notable exception of 95VDFDDIHR102, which is also present in skTnT).

Identification and Characterization of cTnT in Serum

Clinical Chemistry 63:2 (2017) 565

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MASS SPECTROMETRIC ANALYSIS OF FRAGMENTS

Samples from patient nos. 6 (n = 3), 7 (n = 4), 10 (n = 4), and 12 (n = 4) were selected for mass spectrometric identification of the excised bands based on the cTnT concentrations in the available samples. Since only a few samples had the intact cTnT band visible at the position of 37 kDa on the Western blot, bands in gels at this position were considered to be identified as intact cTnT when the characteristic N-terminal peptide 64EADG...78PKPR was identified. For patients in which no 37-kDa band could be included according to this criterion, comparisons were made with a positive QC sample (purified human cTnT spiked in PBS).

Fig. 2 shows the relative abundance of each targeted peptide (expressed as normalized P/R ratio) in the positive QC samples (top) and in each of the 4 patients. In the positive QC samples, all targeted peptides are present except for the 2 peptides 187ALSN...GYIQ and 291AKVTGRW, which are semitryptic. Semitryptic peptides are peptides of which 1 of the 2 termini (in these cases: the C-terminus) is not formed by trypsin cleavage, and can be used to identify proteolytic cleavage sites by proteases other than trypsin. Since all other targeted peptides are found in high abundance, in view of the Western blot results (Fig. 1), the positive QC can be identified as intact human cTnT. Supplemental Fig. 3 in the online Data Supplement shows the annotated chromatogram of the targeted peptides in the positive QC sample.

The 37-kDa protein band was detected as cTnT in 3 of the 12 patients using Western blot, but could only be detected as cTnT in 3 of the 12 patients using Western blot, but could only be
identified as intact cTnT in 3 samples belonging to patient No. 10 using MS. Although patient nos. 7 and 12 also had a faint 37-kDa protein band visible on the Western blots (see the Supplemental Material in the online Data Supplement), the amount of cTnT in those bands proved to be insufficient to identify the N-terminal peptides that are characteristic of intact cTnT. For this reason, only the intact, 37-kDa band measurements of patient no. 10 are shown.

When considering the identification of the primary (29 kDa) and secondary (16 kDa) fragment bands, it was apparent that the 2 N-terminal peptides...
were absent in both of them (Fig. 2). This was consistent in all samples of all 4 patients. The peptide 79SFMP...VPPK88, which lies directly adjacent to the absent peptides, was identified in all samples. Moreover, in the intact and primary fragment samples, all C-terminal peptides (up to and including the semitryptic 291AKVTGRW297 peptide) were detected. Interestingly, this semitryptic peptide was not found in the positive QC sample (Fig. 2). This may mean that the last amino acid of the cTnT protein, Lys-298, while present in the purified cTnT standard, was absent in the intact and primary fragment bands in serum.

Only centrally located cTnT peptides could be detected in the secondary fragment. Interestingly, in these samples, a double peak was observed in the extracted ion chromatogram (XIC) of the 187ALSN...YIQK200 peptide (Fig. 3A, right panel). This double peak was absent in the XIC of the intact cTnT bands (Fig. 3A, left panel) and in the primary fragment bands (not shown) of all analyzed samples. MS/MS measurements identified the leftmost peak as the targeted peptide (187ALSN...YIQK200) itself and the rightmost peak as the isobaric peptide 186KALS...GYIQ199 (Fig. 3A, inserts), containing both a missed cleavage and a semitryptic end. The formation of a semitryptic peptide at this abundance is a rare occurrence and allowed only in specific cases (like in the C-terminal end of a protein) (16). Missed cleavages, however, are relatively common and can be the result of incomplete digestion or reduced affinity for the target substrate (17). We hypothesized

**Fig. 3.** Extracted ion chromatograms (XIC) of the ALSNMMHFGGYIQ(K) peptides of an AMI patient.

(A) XIC at m/z 532.93. Left panel shows a single peak in the 37-kDa band, the right panel shows a double peak in the 16-kDa fragment band.

(B) XIC at m/z 734.84. Left panel shows only noise in the 37-kDa band, the right panel shows a peak in the 16-kDa fragment band. Insets show MS/MS product ion spectra identifying each peptide.
that this semitryptic peptide might represent the C-terminal cleavage site of the secondary fragment. If this were the case, the semitryptic peptide without the missed cleavage (187ALSN...GYIQ199) should also be present in these samples. Fig. 3B shows that 187ALSN...GYIQ199 is indeed present in the secondary cTnT fragment (right panel) and absent in intact cTnT (left panel) and the primary fragments (not shown). This was confirmed in all analyzed samples, as well as the positive QC (Fig. 2).

Discussion

While the fragmentation of cTnI in blood is well recognized (18) and likely influences assay harmonization (19), fragmentation of cTnT is subject to an ongoing debate. However, it was reported in 1998, that cTnT may also be degraded in the serum of AMI patients (20), which was subsequently confirmed by Western blotting in 2000 (21). Over the years, this effect has been reproduced in various other studies using multiple antibodies specific to cTnT (5, 6). It has also been shown that the spiking of purified intact cTnT in the serum of healthy individuals and incubation at 37 °C for prolonged periods of time induces the fragmentation of cTnT in a similar pattern (10, 22). Furthermore, in vitro studies employing neonatal rat cardiomyocytes (23) and immortalized mouse atrial cardiomyocytes (24) show that cTnT fragmentation products can be formed intracellularly when subjected to metabolic inhibition or ischemic conditions. Lastly, a recently performed study showed similar cTnT fragments in heart tissue of deceased individuals resulting from AMI and other conditions (25). All of these studies repeatedly and consistently show that cTnT can be present in the intact form (37 kDa), a primary fragment (29 kDa), and several secondary fragments (15–20 kDa) in the serum of patients suffering from AMI. In addition to these forms of free cTnT, troponin was found to be present in its complex form (26).

In the present study, we have used a combination of Western blotting and mass spectrometry to identify the nature of these fragments. At least 4 proteotypic cTnT peptides have been detected in all fragment samples. A proteotypic peptide is a peptide that is observable by MS and is unique for a specific protein or protein isoform (27, 28). After the identification of these peptides with tandem-MS, we can now state with certainty that the bands visible on the Western blots are indeed cTnT-derived products. It is a common practice in discovery-based proteomics that at least 2 proteotypic peptides need to be identified in a sample to reliably identify a specific protein (29). This is not necessarily the case with quantitative proteomics, where often single “quantotypic” peptides are targeted and quantified absolutely for each protein of interest (30). Great care should be taken when interpreting such quantification results as peptide selection merely based on the optimal mass spectrometric response may not always be representative. We have shown here, with relative quantification of the selected ion monitoring (SIM) measurements, that the 29-kDa fragment is an N-terminally cleaved degradation product of cTnT. The 16-kDa secondary fragment is further degraded at the C-terminus and is most likely cleaved between Glu-199 and Lys-200. This interpretation is summarized in Fig. 4. With this knowledge, the absolute amount of these different fragments can now be determined by the direct digestion of proteins in serum—for example, using a strategy based on so-called “proteolytic signature peptides” (31).

The N-terminal cleavage of cTnT between Arg-78 and Ser-79 has been described previously (6) and is suggested to be caused by µ-calpain cleavage (32). However, it was recently shown that thrombin may also play a role in this process (33). To test this hypothesis, the epitope of the polyclonal N-terminal antibody designed for the Western blots performed in this study was chosen to lie directly adjacent to this hypothesized cleavage site (Fig. 4). Our Western blotting and mass spectrometry results both showed that this targeted region was removed in the 29- and 16-kDa fragments. Another suggested cleavage site, catalyzed by caspase-3, was claimed to lie between Asp-98 and Asp-99 (34). However, our results showed that this site remains intact in all analyzed samples. On the C-terminal side, we had no prior indication on where cleavage would occur (if at all). For this reason, the C-terminal antibody was designed to bind as far toward the C-terminal end of cTnT as possible. Western blotting and mass spectrometry results were in agreement that the C-terminus of cTnT was being cleaved off in the secondary fragments of AMI patients, but that it was still present in the primary fragment band.

Similar results were found in an in vitro experiment where purified cTnT was spiked in a human serum matrix and analyzed with this same MS assay (10). A few notable differences need to be discussed. First and foremost, the semitryptic peptide defining the C-terminal end of the 16-kDa fragment (187ALSN...GYIQ199) was not detected in the in vitro experiment. For this reason, the reference peptide chosen in this study was different from the one used in the validation study, which covered this site. This may suggest that, while similar, the in vitro experiment performed previously (10) was not completely comparable to the various processes occurring in vivo (such as the potential intracellular degradation of troponin). Lastly, in the in vitro experiment, the semitryptic peptide 291AKVTGRW297 was also detected. This was an interesting observation because the same positive QC was used as in the current study,
only then spiked in serum instead of PBS. While being only a minor finding, this observation may indicate that the last lysine residue of cTnT is very unstable and gets cleaved off when cTnT comes into contact with serum.

Recommended calibrators and QC samples used for both the Roche 4th- and 5th-generation cTnT immunoassays in principle consist of intact recombinant cTnT in a serum-like matrix (Troponin T hs STAT product insert, Roche). As demonstrated in this study, this recombinant form does not correspond to the native form of cTnT in the serum of AMI patients. Moreover, as the affinity of the antibodies in the immunoassay for intact and degraded cTnT can be different, this noncorrespondence between cTnT forms in calibrators/QC samples vs AMI patient samples may lead to an over- or underestimation of the “true” cTnT concentration in serum. In addition, it has been suggested that different underlying pathologies may result in a different fragmentation pattern (6, 35). For example, recent results from our laboratory suggest that chronically increased cTnT [such as is the case in patients with end stage renal disease (ESRD)] only circulates in the secondary fragment form (36). Targeting the C-terminal end of cTnT in a future assay might therefore be useful to differentiate between acute and chronically increased cTnT concentrations.

As the LOD of our mass spectrometry assay is relatively high (equivalent to a cTnT concentration of 1000–8000 ng/L, depending on the target peptide) (10), only a limited number of patients were included with high cTnT concentrations. This is in contrast with cTnT concentrations found in patients with chronic conditions, such as ESRD, which are low in comparison. However, considering the highly consistent and reproducible results found in multiple studies, which are completely in line with the MS results presented here, we made the assumption that the biological mechanism of cTnT degradation is independent of concentration. Future work is needed to further lower the LOD of our MS assay for analysis of the cTnT forms in kidney patients, diabetics, and endurance athletes.

![Fig. 4. Alignment of the intact, primary, and secondary fragments with the theoretical sequence of cTnT. Highlighted in green are the peptides targeted in this study. Highlighted in purple are the binding epitopes of the various antibodies used in this study. Ab, antibody. Highlighted in orange is a 10 amino acid region that is spliced in the adult cTnT isoform.](https://academic.oup.com/clinchem/article/63/2/563/5612852)
In conclusion, with 2 different proteomic approaches (Western blotting and mass spectrometry), we have shown that cTnT in the serum of patients suffering from AMI consists of a heterogeneous mixture of intact cTnT (37 kDa), a primary fragment spanning from Ser-79 to Trp-297 (29 kDa), and several smaller secondary fragments (15–20 kDa) of which the most abundant fragment (16-kDa) spans from Ser-79 to Gln-199.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

References


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