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The Impact of Antibody Selection on the Detection of Cardiac Troponin I

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Abstract

Background—Cardiac troponin I (cTnI) is the current standard biomarker for diagnosing acute myocardial infarction and for risk-stratification of acute coronary syndromes in patients. However it remains unclear how the epitope specificity of antibodies in immunoassays influences the detection of various modified forms of cTnI.

Methods—Four mouse anti-human cTnI monoclonal antibodies targeting different regions of human cTnI were chosen for immunoaffinity purification of cTnI from human and swine cardiac tissue. High-resolution intact protein mass spectrometry was employed to assess the comparative performance of these four antibodies in detecting modified forms of cTnI.

Results—Our data revealed that antibody selection significantly impacts the relative protein yield of cTn from immunoaffinity purification. Remarkably, a single amino acid variation in cTnI (G->S) in the epitope region completely abolished the binding between monoclonal antibody 560 and swine cTnI in solution. Moreover, proteolytic degradation around the epitope region severely compromised the detection of proteolytic fragment forms of cTnI by monoclonal antibodies. In contrast, the phosphorylation status near the epitope region did not significantly affect the antibody recognition of cTnI.

Conclusion—Caution needs to be taken in the interpretation of the data produced by immunoassays with monoclonal antibodies against various epitopes of cTnI.

Keywords

cardiac troponin; mass spectrometry; epitope; immunoassays; biomarker; acute myocardial infarction

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1. Introduction

Troponin (Tn) is a heterotrimeric protein complex found in both cardiac and skeletal muscle [1,2]. Tn has 3 subunits and each of them has a unique role in regulating muscle contractility. TnI inhibits the actomyosin ATPase activity; TnC binds to Ca^{2+} ; TnT attaches the Tn complex to tropomyosin and anchors it on thin filaments [1]. Expression of cardiac isoforms of TnI and TnT (cTnI and cTnT) is unique to the cardiac tissue and differs significantly from the skeletal isoforms. cTnI and cTnT are released into the general circulation following myocardial injury, which can be easily and rapidly detected in serum by an enzyme-linked immunosorbent assay (ELISA)[3,4]. Thus cTnI and cTnT are considered to be gold standard biomarkers for diagnosing acute cardiac injury in patients [5–8]. However, currently available commercial ELISA kits from different vendors can give variable results in sensitivity, specificity and selectivity, in part because of the lack of standardization of antibodies that are directed at distinct epitopes on cTnI in addition to the varying quality and specificity provided by the manufacturers [4,9–11]. Moreover, cTn is known to be subject to extensive post-translational modifications (PTMs), notably phosphorylation and proteolysis [7,12–14]. Once released into the bloodstream, cTn circulates in various forms and complexes, including phosphorylated and proteolytic forms, TnI-TnC binary and TnT-TnI-TnC ternary complex [4,12,15]. However it remains unclear how the epitope specificity of the antibody influences the detection of these various modified forms and complexes of cTnI.

Herein we aim to perform a systematic evaluation of the potential impact of antibody epitope specificity on the detection of cTnI employing immunoaffinity chromatography and high-resolution intact protein mass spectrometry (MS). Top-down intact protein-based MS can provide comprehensive analysis of protein modifications including PTMs (e.g. phosphorylation, proteolysis, acetylation) and sequence variants (i.e. mutant, alternatively spliced isoforms, amino acid polymorphisms) [16–26] and protein complexes [27]. In contrast to the conventional peptide-based bottom-up MS approach where the proteins are digested into many small peptides for MS analysis resulting in a limited understanding of the protein modification state, the top-down MS approach directly analyzes intact proteins, providing a “birds’ eye” view of the protein and revealing all types of modifications present *in vivo* without *a priori* knowledge [17–19,28]. Moreover, since the ionization efficiency of intact proteins is much less affected by the presence of modifying groups in comparison with peptides, top-down MS provides quantitative determination of the relative abundance of modified protein forms [22,24,29–30].

In this study, we have evaluated four mouse anti-human cTnI monoclonal antibodies (mAbs) targeting four distinct epitopes of cTnI to assess their comparative performance in detecting various forms of cTnI. In addition to the detection of cTn from human samples, we have elected to evaluate these mAbs for their detection of swine cTn since the swine heart failure model has been used widely in cardiovascular research due to the similarity between human and swine hearts in size, anatomy, function and coronary artery distribution [31,32]. Our results have shown that the epitope specificity significantly affects the immunoaffinity binding between cTnI and mAbs yielding different amount of cTn from the four mAbs columns. Remarkably, a single amino acid variation in swine cTnI in comparison to human cTnI (human G89 -> swine S90) completely abolished its binding to the mAb 560 that targets human cTnI residues 83–93. Moreover, degradation of cTnI complicates the accurate detection of cTnI by mAbs [13, 33]. Our data show that the proteolytically degraded product of cTnI with both N- and C- terminal truncation failed to be detected by mAbs P4-14G5 that targets human cTnI residues 1–23. In contrast, the phosphorylation status of cTnI did not significantly affect mAbs’ recognition of cTnI. Taken together, our results suggest that caution needs to be taken in the interpretation of data produced by immunoassays with

mAbs against different epitopes of cTnI. Arguably it may become necessary to utilize sequential immunoassays involving multiple antibodies for capture and detection of cTnI in its various modified forms and complexes [7,34].

2. Materials and Methods

2.1 Chemicals and reagents

All reagents were from Sigma Chemical Co (St Louis, MO) unless noted otherwise. Complete protease and phosphatase inhibitor cocktail tablets were purchased from Roche Diagnostics Corporation (Indianapolis, IN). All solutions were prepared in Milli-Q water (Millipore Corporation, Billerica, MA). The pre-packed immunoaffinity columns with mouse anti-human cTnI mAbs (P4-14G5, 560, M46 and MF4) immobilized on CNBr - activated Sepharose CL4B were from Hytest Ltd., Turku, Finland (Figure 1). The characteristics of mAbs including the concentrations (mg/ml) are listed in Table 1.

2.2 Tissue samples

Human left ventricular cardiac tissue samples were obtained from post-mortem donors at the University of Wisconsin Hospital and Clinics as approved by the Institutional Review Board of the University of Wisconsin. Swine left ventricular cardiac tissue samples were obtained from juvenile Yorkshire domestic pigs as approved by the University of Wisconsin Animal Care and Use Committee. The excised heart tissue was immediately frozen in liquid nitrogen and stored in a -80 °C freezer.

2.3 Immunoaffinity purification

The whole cardiac troponin complex was immunoaffinity purified as described previously [21–24, 35–36]. Briefly, approximately 1.0 g of cardiac tissue was homogenized in tissue wash buffer (NaH₂PO₄ 500 mmol/l, Na₂HPO₄ 100 mmol/l, MgCl₂ 100 mmol/l, EGTA 100 mmol/l, NaCl 0.1 mol/l, Triton X-100 1%, DTT 5 mmol/l, protease and phosphatase inhibitor cocktail tablet, PMSF 1 mmol/l, Leupeptin, 2 µg/ml, pH 7.4) using a Polytron electric homogenizer for 15 s on ice. The homogenate was centrifuged at 8,000 rpm for 12 minutes at 4 °C; the supernatant was discarded and the pellet was resuspended in 6 ml of protein extraction buffer (0.7 M LiCl, 25 mmol/l Tris, 5 mmol/l EGTA, 0.1 mmol/l CaCl₂, 5 mmol/l DTT, 1 mmol/l PMSF, 2 µg/ml leupeptin, and 0.75 mg/ml protease and phosphatase inhibitor cocktail, pH 8.0) and protein extraction was performed with agitation on a nutator at 4 °C for 50 min. The sample was then centrifuged at 13,200 rpm for 10 min and the supernatant was collected. The collected supernatant was further centrifuged at 55,000 rpm (Beckman L-55 ultracentrifuge, Beckman Coulter, Fullerton, CA) for 50 minutes to completely remove tissue debris before affinity chromatography purification. The supernatant was incubated with approximately 400 µL of CNBr-activated Sepharose CL-4B conjugated with a mouse antihuman cTnI mAbs (Hytest Ltd., Finland) for 30 min at 4 °C to ensure complete binding of cTn to the mAbs. After washing the column with 1.6 ml of extraction buffer, the bound troponin was eluted with 100 mmol/l glycine at pH 2 into 6 0.4 ml fractions and neutralized immediately with 40 µL of 1M MOPS (pH 9). Fractions were analyzed by 15% SDS-PAGE gels stained with Coomassie Blue (Figs. 2A, 3A).

To quantify the relative protein yield from each mAb column, we took into consideration of proteins eluted in each of the 6 fractions and combined equal volume of each elution fraction for SDS-PAGE analysis (Figs. 2B, 3B). For a fair comparison, the combined elution fractions from all four columns were run on the same piece of gel with protein molecular weight markers loaded as a control (data not shown). ImageJ software downloaded from NIH website (<http://rsb.info.nih.gov/ij/>) was used to quantify the gel density to estimate the relative yield of cTnI, cTnT and cTnC from the SDS-PAGE stained with Coomassie Blue.

Peak intensities were normalized against the protein molecular weight marker band at 25 kDa. Given the differences among the mAbs concentration in Sepharose (Table 1), we further normalized the cTn yield to mAbs concentration. The final results were presented as relative yield normalized to cTnI purified with column P4-14G5 (Figs. 2B, 3B).

2.4 Mass spectrometry

Immunoaffinity purified cTn complexes were separated and desalted using an offline reversed phase protein microtrap (Michrom Bioresources, Inc., CA), with a 3 step reverse phase gradient elution method, first with 1% acetic acid in 30:70 methanol/water, followed by 1% acetic acid in 50:50 methanol/water, and then 1% acetic acid in 75:25 methanol/water. Samples were analyzed using a linear trap/Fourier transform Ion cyclotron resonance (FTICR) hybrid mass spectrometer (LTQ FT Ultra, Thermo Scientific Inc., Bremen, Germany) equipped with an automated chip-based nano electrospray (ESI) source (Triversa NanoMate, Advion BioSciences, Ithaca, NY) as described previously [21–24]. The resolving power of the FTICR mass analyzer was typically set at 200,000 at m/z 400. Quantitative analysis of the MS data was performed as described previously [21–24]. Briefly, the integrated peak heights of the top five isotopomers were used to calculate the relative abundance of each protein form. The percentages of mono- or bis-phosphorylated cTnI were defined as the summed abundance of mono-(p cTnI) or bis-phosphorylated cTnI (pp cTnI) species (including phosphorylated degradation products of cTnI), respectively, over the entire cTnI population. The total amount of cTnI phosphorylation (P_{total} , mol Pi/mol of cTnI) was defined as the summed abundances of all phosphorylated (including both mono-, and bis-phosphorylated) cTnI species over the summed abundances of the entire cTnI population $P_{total} = (\%p\text{cTnI} + 2 \times \%pp\text{cTnI})/100$ [22].

2.5 Statistical analysis

Data were expressed as mean $\pm$ SD. Student's t -tests were performed between groups to evaluate for statistical significance of variance. Differences among means were considered significant at $p < 0.05$.

3. Results

3.1 Differential enrichment of cTn from four mAbs against various epitopes in cTnI

We selected four mouse anti-human cTnI mAbs from Hytest that target different regions of cTnI to be evaluated in this study (Table 1 and Fig. 1) and compared the mAbs' performance in detecting the cTn complex from both swine and human heart tissues. Swine cTnI (ACCESSION NP_001092069, 210 residues) is 93% identical to human cTnI (NP_000354, 210 residues) with the most variable amino acids (aa) at the N-terminal region of the protein. The sequence of each epitope was aligned between human and swine cTnI with aa variations highlighted in Figure 1B. The mAb P4-14G5 that is derived from aa residues 1–23 at the N-terminal is unique for the cardiac form as the first 30 aa residues of the N-terminal are absent in the skeletal muscle TnI isoform. The targeted sequences of the mAbs P4-14G5 and 560 vary by five and one amino acid(s) between human and swine cTnI, respectively. In contrast, mAbs M46 and MF4 target identical sequences in both human and swine cTnI.

The SDS-PAGE analysis of the immunoaffinity purified human cTn from all four mAb columns revealed the detection of the cTn heterotrimeric protein complex (Fig. 2A). The 3 major bands representing the 3 subunits, cTnT, cTnI, and cTnC, appeared in some but not all of the 6 elution fractions. This indicates that each mAb was able to recognize and bind human cTnI but the reactivity between mAbs and their respective epitopes might be somewhat varied as the cTn complex was noted to be eluted in different elution fractions.

Interestingly, we observe the highest concentration of cTn in the E1 elution fraction from the P4-14G5 column in comparison to the E2 elution fraction from the MF4 column. The gel band density of cTnT is comparable to cTnI. The cTnC gel band is discernably lighter than those of cTnT and cTnI as expected due to the weak Coomassie blue staining of cTnC that has been reported previously [37,38].

We then quantified the relative protein yield of cTn from each of the four affinity columns after taking into consideration all 6 eluted fractions and the concentration of mAb with proper normalization factors as described in the method section. The normalized relative protein yields for human cTnI from mAbs columns, P4-14G5, 560, M46 and MF4, are 1.00 ± 0.18 , 0.66 ± 0.07 , 0.48 ± 0.07 , and 2.15 ± 0.22 , respectively (Fig. 2B). The normalized relative protein yields from mAbs P4-14G5, 560, M46 and MF4 columns are 1.17 ± 0.15 , 0.81 ± 0.11 , 0.58 ± 0.11 , and 2.67 ± 0.55 for human cTnT, and 0.37 ± 0.11 , 0.19 ± 0.02 , 0.12 ± 0.02 , and 0.86 ± 0.23 for human cTnC, respectively. The amount of cTnI and cTnT enriched by MF4 was significantly higher than P4-14G5, 560 and M46 ($p < 0.05$). This implicates that the reactivity of mAbs with their respective epitopes might be in the order of MF4 $\gg$ P4-14G5 $>$ 560 $>$ M46 for human cTn.

The SDS-PAGE analysis of the immunoaffinity purified swine cTn from all four mAb columns revealed similar results as from human cTn, except that mAb 560 failed to enrich any detectable amount of cTn from swine cardiac tissue (Fig. 3A). We performed multiple independent affinity purification experiments using tissue samples from 3 different swine hearts and all yielded similar results confirming the absence of Tn from the mAb 560 column. Thus, a single amino acid difference in the mAb 560 epitope region (Fig. 1B, human G89 $\rightarrow$ swine S90) abolished swine cTnI affinity binding to the mAb 560 in solution. Overall, the normalized relative protein yield from P4-14G5, M46 and MF4 are 1.00 ± 0.18 , 0.64 ± 0.09 , and 2.33 ± 0.80 , for swine cTnI, 1.40 ± 0.10 , 0.91 ± 0.05 , and 3.08 ± 0.43 for swine cTnT, and 0.26 ± 0.06 , 0.17 ± 0.03 , and 0.60 ± 0.21 for swine cTnC, respectively. These results suggest that the epitope specificity of the mAb does appear to appreciably impact the yield of cTnI and cTnT enriched from swine cardiac tissues presumably due to the varied binding affinity. The amount of cTnI and cTnT enriched by MF4 was significantly higher than P4-14G5 and M46 ($p < 0.05$). Conceivably, the reactivity of mAbs with their respective epitopes might be in an order of MF4 $\gg$ P4-14G5 $>$ M46 $\gg$ 560 for swine cTn.

3.2 The effect of antibody selection on the detection of cTnI phosphorylation

To further investigate whether these mAbs targeting different epitope regions in cTnI showed a bias toward un-phosphorylated versus phosphorylated cTnI, the purified cTn complex was separated and desalted via an offline reversed phase HPLC and subjected to high-resolution ESI/FTICR MS analysis. In the human samples, all four mAb columns enriched both un-phosphorylated and mono-phosphorylated cTnI at comparable efficiencies (Fig. 4A). The quantification of the un-, mono-, and bis-phosphorylated cTnI and total phosphorylated human cTnI from the four mAb columns are presented in Figure 4B–C. The relative abundance of un-phosphorylated cTnI were $65.7 \pm 5.2\%$, $60.5 \pm 8.0\%$, $57.9 \pm 10.5\%$, and $54.4 \pm 10.8\%$ from P4-14G5, 560, M46 and MF4, respectively. The relative abundances of mono-phosphorylated cTnI were $24.1 \pm 3.3\%$, $26.5 \pm 4.6\%$, $28.4 \pm 5.1\%$, $28.6 \pm 5.7\%$; and $9.3 \pm 3.8\%$, $13.1 \pm 3.5\%$, $13.8 \pm 6.0\%$, $17.0 \pm 5.6\%$ for bis-phosphorylated cTnI purified by P4-14G5, 560, M46, and MF4, respectively. The total phosphorylated levels of cTnI (P_{total}) were 0.45 ± 0.04 , 0.51 ± 0.10 , 0.53 ± 0.15 , and 0.59 ± 0.15 for 14G5, 560, M46, and MF4, respectively. Although P4-14G5 seemed to enrich slightly less amount of phosphorylated cTnI than MF4, 560 and M46, no statistical significance was observed between the four mAb columns ($p > 0.05$, $n=3$).

The MS spectra of swine samples revealed that cTnI is present in its un-, mono-, and bis-phosphorylated forms purified from mAb columns P4-14G5, M46, and MF4 (Fig. 5). No cTnI peaks were observed in the mAb 560 enriched samples suggesting that the mAb 560 did not bind any cTnI molecules, which is consistent with the SDS-PAGE result (Fig. 3A). The quantification of the un-, mono-, and bis-phosphorylated cTnI and total phosphorylated cTnI from the four mAb columns are presented in Figure 5B–C. The relative abundances of un-phosphorylated cTnI are $20.1 \pm 2.8\%$, $9.5 \pm 3.6\%$, and $15.9 \pm 1.8\%$; $43.8 \pm 3.7\%$, $34.7 \pm 5.9\%$, and $37.1 \pm 1.6\%$ for mono-phosphorylated cTnI; $36.1 \pm 6.4\%$, $55.8 \pm 8.2\%$, and $47.0 \pm 0.3\%$ for bis-phosphorylated cTnI for P4-14G5, M46, and MF4, respectively. The total phosphorylated levels of cTnI (P_{total}) were 1.22 ± 0.15 , 1.43 ± 0.11 , and 1.32 ± 0.02 for P4-14G5, M46, and MF4, respectively. Although the average total cTnI phosphorylation level enriched from P4-14G5 appeared to be slightly lower than M46 and MF4, no statistical significance was observed between group comparisons of all four mAb columns ($p > 0.05$, $n=3$).

3.3 The impact of antibody selection on the detection of cTnI proteolytic fragments

To evaluate the potential impact of epitope specificity on the enrichment of proteolytic fragments, we performed a comprehensive analysis of high-resolution ESI/FTICR MS data on human cTnI. C-terminally truncated forms of cTnI (I, residues 1–209aa; II, 1–207aa; III, 1–206aa, IV, 1–205aa) are present in the spectra of human cTnI purified from all four mAb columns evaluated. The cTnI truncated form cTnI (III) was the most abundant isoform in these human postmortem samples (Fig. 6) whereas full-length (I) and the other C-terminally truncated forms, [II] and [IV], were present as minor components. The four mAbs showed slightly different enrichment of the C-terminally truncated forms of cTnI (II, III and IV). A proteolytic truncated form of cTnI involving both N- and C-terminal truncations, cTnI Y[25–206]K, is present in mAbs 560, M46 and MF4, but not in mAb P4-14G5 columns. This is conceivable since mAb P4-14G5 only targets the N-terminal 1–23 aa residues in cTnI. Thus, antibody selection does affect the detection of proteolytically degraded truncated cTnI forms.

4. Discussion

We compared the performance of four mAbs targeting different epitopes of cTnI in immunoaffinity purification and further analyzed the purified proteins by high-resolution MS. Our data showed that the epitope selection affects the mAb recognition of cTnI. The four mouse anti-human cTnI mAbs used in this study target different epitope regions of human cTnI, with P4-14G5 targeting the N-terminus (residues 1–23), 560 (residues 83–93) and M46 (residues 130–145) in the central regions, and MF4 close to the C-terminus (residues 190–196). Whereas all four mAbs could enrich human cTnI, mAb 560 targeting human residues 83–93 failed to bind swine cTnI. Remarkably, only a single amino acid difference (G89->S90) between the human and swine cTnI completely abolished swine cTnI recognition by mAb 560 in the presence of Ca^{2+} in solution. This suggests that the mAb 560 could not bind to swine cTnI in its native complex form. cTnI residues 80–89 are located between two helices which are known to be less conserved, have an extended structure and interact with cTnT forming a hydrophobic core [1]. The change from nonpolar glycine to polar serine may potentially alter the folding of the two helices and the interaction with cTnT and prohibit adequate access of the mAb 560 to the swine cTnI.

Here, the mAbs P4-14G5, and M46 showed similar enrichment for swine cTnI. The mAb MF4, which targets epitope near the C-terminus, gave the highest cTnI yield in both species. It is reasonable to believe that the C-terminus of cTnI lacks direct interaction with other molecules and therefore makes it easily accessible for mAbs [1]. Therefore, to obtain the highest yield from affinity purification, the mAb MF4 is the best choice among these four

mAbs for both human and swine cardiac cTn complex enrichment. Given that cTnI is released into the circulation of patients of acute myocardial infarction predominantly in its complex form, it is crucial to use antibodies which can recognize not only free cTnI but also cTnI complexed with other cTn subunits [15].

Phosphorylation of cTnI is critical for modulation of contractility and Ca^{2+} sensitivity of cardiac filaments. This phosphorylated form of cTnI is also present in the serum after cardiac injury. One concern in cTnI immunoassays is whether phosphorylation of cTnI may cause structural and conformational changes thus affecting cTnI recognition by mAbs [12]. cTnI is well-known to be phosphorylated by protein kinase A (PKA), protein kinase C and other kinases at Ser22/23 [21–22,24,39–40]. Therefore we have performed a side-by-side comparison of mAb P4-14G5 which targets cTnI residues 1-23 together with other mAbs against other epitopes in the central or C-terminal of cTnI. The high-resolution MS data of the cTnI immunoaffinity purified from four mAbs enriched both un-phosphorylated and phosphorylated cTnI from human heart tissues. The phosphorylation levels in human cTnI detected by the four mAbs shows slight variations, but overall no statistical differences were observed among group comparisons between the four columns (Fig. 4). From swine cTnI, the mAb 560 did not recognize swine cTnI in its complex form (Fig. 5). Also noted was that the mAbs M46 and MF4 appeared to enrich slightly more phosphorylated cTnI than mAb P4-14G5 but with no statistical significance. This suggests that the binding between mAb and cTnI are minimally affected by the phosphorylation state, consistent with our previous findings [21,24] and the manufacture product description from Hytest.

Our data also showed that epitope specificity affects the detection of proteolytically truncated forms of cTnI. Multiple degraded cTnI isoforms were observed on MS spectra as the human cardiac tissue was obtained from postmortem samples. The 4 mAbs showed slightly different enrichment of the C-terminally truncated forms of cTnI (Fig. 6). More significantly, the proteolytic fragment Y[25–206]K with the truncation of both the first 24 residues of the N-terminal and the final 3 residues at the C-terminal could not be detected by mAb P4-14G5. It has been of a major concern that once cTnI is released into the blood, proteolysis occurs and may complicate the accurate detection of cTnI [13], especially in the case that the epitope region is cleaved during the proteolytic degradation. Indeed, our data confirmed that the mAb P4-14G5 targeting the N-terminal epitope (1-23aa) cannot detect proteolytic fragments with the truncation of the N-terminal residues. This poses a serious problem for clinical labs since the mAb P4-14G5 targeting the cardiac-specific N-terminus is the most commonly used mAb in ELISA assays. The N- and C- terminal regions of cTnI are known to be highly susceptible to proteolytic degradation in comparison to the central region between aa residues 30 and 110 which demonstrated higher stability possibly due to protection conferred by the interaction with cTnC [33]. cTnI degradation products are commonly detected in the serum of patients after myocardial injury [12] and in the myocardium of patients undergoing coronary artery bypass surgery [41]. Hence, it is critically important to develop new strategies using multiple antibodies with distinct epitopes to improve the accuracy and coverage of multiple modified forms of cTnI [7]. Recently, Van Eyk and co-workers developed a qualitative cTnI assay which used sequential application of one capture antibody but multiple detector antibodies without an antibody-stripping step [34]. This enables real-time interrogation of five different epitopes of cTnI with each immunodetection done independently but in a single sequential assay. This diffraction-based immunoassay successfully characterized cTnI and determined whether cTnI was degraded at the N- and/or C-terminus or phosphorylated or in its cTn complex (cTnI-cTnC-cTnT). Importantly, the sequential qualitative assay can capture various modification forms of cTnI and direct detection of the cTn complex [34], which, arguably, can afford a more accurate detection of cTnI in the serum.

5. Conclusion

We have evaluated the potential impact of antibody selection on cTnI detection using immunoaffinity chromatography and high-resolution MS. Four mouse anti-human cTnI mAbs that target different epitope regions of human cTnI were chosen for immunopurification of cTn from both human and swine cardiac tissue. Overall, our data indicate that the epitope specificity of a mAb can have a significant impact on the enrichment and detection of cTnI. Remarkably, a single amino acid difference (G89->S90) in the epitope region of human cTnI (83-93) nearly obliterated the binding between swine cTnI and mAb 560. Moreover, proteolytic degradation around the epitope region severely compromised the detection of cTnI fragments by mAbs. In contrast, mAb and cTnI interactions are minimally affected by the presence of phosphorylation around the epitope region. Hence, caution needs to be taken in the interpretation of the data produced by immunoassays with antibodies against different epitopes. Conceivably, an ideal assay should detect all circulating forms of cTnI in the bloodstream involving multiple antibodies in sequential immunoassays for more accurate detection of cTnI in clinical diagnostics.

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Abbreviations

cTnT,I, C	cardiac troponin T, I, C
mAbs	monoclonal antibodies
aa	amino acid
PTMs	post-translational modifications
FTICR	Fourier transform ion cyclotron resonance

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Highlights

- We report the potential impact of antibody selection on cardiac troponin I (cTnI) detection using immunoaffinity chromatography and high-resolution intact protein mass spectrometry.
- Our data revealed that antibody epitope specificity significantly impacts the relative protein yield of cTn from immunoaffinity purification.
- Remarkably, a single amino acid variation in cTnI (G->S) in the epitope region completely abolished the binding between monoclonal antibody 560 and swine cTnI.
- Proteolytic degradation around the epitope region severely compromised the detection of proteolytic fragment forms of cTnI by monoclonal antibodies.
- The phosphorylation status near the epitope region did not significantly affect the antibody recognition of cTnI.

Figure 1. Schematic representation of cTnI and the epitopes targeted by four mouse anti-human cTnI mAbs (all IgG1) from HyTest
(A) The mAbs, P4-14G5, 560, M46 and MF4, target different regions in human cTnI. (B) The sequence alignments of 4 epitopes in human and swine cTnI. Variations in amino acid sequences between human and swine cTnI are highlighted.

Figure 2. The impact of mAb epitope specificity on immunoaffinity purification of human cTn
 (A) SDS-PAGE analysis of purified human cTn complexes from four immunoaffinity columns. M, protein molecular weight marker; FL, flow through; W, wash step; 1–6, elution fractions 1–6. Representative SDS-PAGE gels from 3 affinity purifications were shown. (B) Quantification of human cTn subunits yield from four affinity columns. Result was presented as relative yield normalized to human cTnI purified with column 14G5. Data are expressed as mean $\pm$ S.D., n=3. *, $p < 0.05$.

Figure 3. The impact of mAb epitope specificity on immunoaffinity purification of swine cTn
 (A) SDS-PAGE analysis of purified swine cTn complexes from four immunoaffinity columns, 14G5, 560, M46, and MF4. M, protein molecular weight marker; FL, flow through; W, wash step; 1–6, elution fractions. Representative SDS-PAGE gels from 3 affinity purifications are shown. (B) Quantification of swine cTn subunits yielded from four affinity columns. Results were presented as relative yield normalized to swine cTnI purified with column 14G5. Data are expressed as mean $\pm$ S.D., $n=3$. *, $p < 0.05$.

Figure 4. Comparison of phosphorylation levels of human cTnI purified from four HyTest immunoaffinity columns

(A) Partial ESI/FTMS spectra from human cTnI immunoaffinity purified with 14G5 (i), 560 (ii), M46 (iii) and MF4 (iv). cTnI (II), residues 1–207; cTnI (III), residues 1–206. (B) Quantitation of relative abundance of un-, mono- and bis-phosphorylated human cTnI as observed in (Ai-Aiv). (C) The total amount of cTnI phosphorylation (P_{total} , Mol Pi/Mol cTnI). Data are expressed as mean $\pm$ S.D., $n=3$. No statistical significance was observed between group comparisons of any of the four mAb columns ($p > 0.05$).

Figure 5. Comparison of phosphorylation of swine cTnI purified from four immunoaffinity columns

(A) Partial ESI/FTMS spectra from swine cTnI immunoaffinity purified with 14G5 (i), 560 (ii), M46 (iii) and MF4 (iv). cTnI (I), residues 1–209. (B) Quantitation of relative abundance of un-, mono- and bis-phosphorylated swine cTnI as observed in (Ai-Aiv). (C) The total amount of cTnI phosphorylation (P_{total} , Mol Pi/Mol cTnI). Data are expressed as mean $\pm$ S.D. $n=3$. No statistical significance was observed between group comparisons of any of the four mAb columns ($p > 0.05$).

Figure 6. The impact of epitope selection on the detection of human cTnI proteolytic fragments High-resolution ESI/FTMS analysis of human cTnI immunoaffinity purified from four affinity columns, (A) 14G5, (B) 560, (C) M46 and (D) MF4, respectively. Partial FTMS spectra were shown with the peaks corresponding to different modifications and proteolytic fragments. Roman numerals (I–IV) refer to C-terminal truncated isoforms of human cTnI (I, 1–209aa; II, 1–207aa; III, 1–206aa; IV, 1–205aa). Subscript *p* stands for mono-phosphorylation. cTnI Y[25–206]K is a proteolytic truncated form of cTnI involving both C- and N-terminal truncations, which are present in (B) 560, (C) M46 and (D) MF4, but not in (A) P4-14G5. The proteolytic fragments of full-length cTnI (I, 1–209 aa), cTnI (II, 1–207 aa); cTnI (III, 1–206 aa), cTnI (IV, 1–205 aa) and cTnI Y[25–206]K are highlighted. The dashed arrow indicates the absence of the proteolytic fragment of cTnI Y[25–206]K in (A).

Table 1

Characteristics of the four Hytest immunoaffinity columns with mouse anti-human cTnI mAb immobilized to CNBr-activated sepharose CL-4B.

name	mAb Clone	Epitope	mAb concentration mg/ml
14G5	P4-14G5	1–23 aa	7.4
560	560	83–93 aa	11
M46	M46	130–145 aa	12
MF4	MF4	190–196 aa	5