이학석사 학위논문

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Determination of SRM peptide transitions by multiplexed product-ion scan mode

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Approved by thesis committee:

Professor_____ Chairperson
Professor_____ Vice Chairperson
Professor_____

Abstract

Determination of SRM peptide transitions by multiplexed product-ion scan mode

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Selected reaction monitoring (SRM) is a targeted proteomics approach that provides high through-put, sensitive and accurate quantification of proteins in complex biological samples. A key prerequisite to successful SRM assay is precise selection and optimization of SRM peptide transitions of target proteins of interest. SRM peptide transitions can be determined based on their MS/MS spectra utilizing proteomics profiling data or various SRM peptide transition prediction tools. Although *in-silico* predictions for the SRM peptide transitions is most commonly used approach, reliable SRM peptide transitions selected from actual experiment are desired. Here

we demonstrated two modes of Triple Quadrupole masss pectrometry (QqQ-MS) todetermine SRM peptide transitions using peptide profiling data information acquired from Linear Ion-Trap mass spectrometry (LIT-MS). These approaches outperform determination of SRM peptide transitions in a multiplexing manner and can complement to the widely used *in-silico* SRM peptide transition prediction method. With the predetermined SRM peptide transitions of key oxidative phosphorylation (OXPHOS) proteins in mitochondria, we performed SRM assay to demonstrate the reliability of the SRM peptide transitions for the detection of target peptides in biological sample matrices in human skeletal muscle tissue sample.

Key words: Selected Reaction Monitoring (SRM), Linear Ion-trap mass spectrometry (LIT-MS), Triple Quadrupole mass spectrometry (QqQ-MS), SRM transition, Oxidative phosphorylation (OXPHOS).
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List of Abbreviations

ACN Acetonitrile, CH3CN

CE Collisioin energy

CV Coefficient of variation

DTT Dithiothreitol

ELASA Enzyme-linked immunosorbent assay

FA Formic acid, HCO2H

FV Fragmentor voltage

FWHM Full width at half maximum

IAA Iodoacetamide

LC-MS/MS Liquid chromatography and tandem mass spectrometry

LIT Linear ion trap

LTQ Linear ion trap quadrupole

MS Mass spectrometry

MS/MS Tandem mass spectrometry

mtDNA Mitochondrial DNA

NaCl Sodium chloride

nuDNA Nuclear DNA

OXPHOS Oxidative phosphorylation

Q1 Precursor ion

Q3 Product ion

QqQ Triple quadrupole

SIS Stable isotope labeled standard

SRM Selected reaction monitoring

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1. Introduction

With the advent of proteomic technologies, a large number of potential biomarker candidates of various diseases have been reported in the literature (McDonald and Yates III 2002). Triaging the clinical utility of those biomarker candidates, rigorous validation methods/technologies are needed for worth of further clinical validation process. Conventional immunoaffinity-based assays, such as western-blot or enzyme-linked immunosorbent assay (ELISA) have been widely employed for biomarker validation (Ellington, Kullo et al. 2010). However, the antibody-based assay is limited by the availability of high-quality of antibody reagents, and it has challenges for multiplex analysis due to the cross-reactivity and interference issues between antibodies and target proteins (Ellington, Kullo et al. 2010, Whiteaker, Lin et al. 2011).

Mass spectrometry (MS)-based quantitative proteomics have been rapidly evolved as an alternative approach for the biomarker discovery and validation (McDonald and Yates III 2002, Ong and Mann 2005, Schiess, Wollscheid et al. 2009). Tandem mass spectrometry (MS/MS) experiment generates a set of peptide fragment ions in which the specific pairs of mass to charge (m/z) values associated with the peptide precursor, and the fragment ions can uniquely represent the target proteins of interest. These peptide signature ions can be preferentially selected and quantitatively monitored through three sequential precursor/product ion's m/z filter systems via triple quadrupole (QqQ) LC-MS instrumentation referred to as selected ion monitoring (SRM) (Schiess, Wollscheid et al. 2009, Gallien, Duriez et al. 2011). Sensitive, selective, and through-put

analytical features of SRM has therefore become an ideal quantitative analytical technique in the clinical proteomics (Anderson and Hunter 2006). More importantly, the targeted SRM approach can be multiplexed which simultaneously measures hundreds of target proteins within a single experiment (Picotti, Rinner et al. 2009).

A key step for successfully quantitative SRM assays is the determination of accurate and precise peptide precursor ion (Q1) and its fragment ions (Q3) (SRM peptide transitions) (Gerber, Rush et al. 2003, Kirkpatrick, Hathaway et al. 2006). The selection of SRM peptide transitions can be achieved as follows; 1) by utilizing public MS/MS spectral library databases (Deutsch, Lam et al. 2008, Prakash, Tomazela et al. 2009), 2) using computational *in-silico* proteotypic peptide prediction tools by the physico-chemical properties of SRM target peptides (Tang, Arnold et al. 2006, Unwin, Griffiths et al. 2009, MacLean, Tomazela et al. 2010), 3) using experimentally acquired LC-MS/MS profiling datasets (Kim, Kim et al. 2009, Tang, Beer et al. 2011) and/or using stable isotope labeled standard (SIS) synthetic peptides to confirm pre-determined peptide transitions. Although *in-silico* design of SRM peptide transitions has been very useful for SRM assay, detection of SRM peptide transitions is not always warrant in the given biological sample matrices. The SIS is the most effective way to determine ideal Q1 and Q3 ions, however, this approach is uneconomical for the purpose of initial screening of SRM peptide transitions (Tang, Beer et al. 2011, Zhi, Wang et al. 2011), and efficiency for the detection of targeted peptide transitions with SIS is usually less than 40%.

In this study, we demonstrated an alternative method to determine SRM peptide transitions by a combination of Linear Ion-Trap/Triple Quadruple (LIT/QqQ) data acquisition system for the selection of

accurate peptide transitions. Peptide transitions were determined by; 1) performing QqQ full MS/MS scan mode and comparing MS/MS spectral patterns between LIT and QqQ, 2) performing SRM with the most four intense Q3 product ions (unbiased SRM scan mode) from the LIT MS/MS scan. In the second approach, we first selected Q1 and several Q3 ions of target peptides from their LIT MS/MS spectra, and then performed SRM assay to confirm identity of the peptides using co-eluted chromatographic patterns of all the selected peptide transitions, and correlation of each transition's chromatographic peak areas and matching their ion intensities with their corresponding ions appeared on the LIT MS/MS spectra. Our approach demonstrates the efficient measurement, especially the low-abundant target proteins which are not detected by the QqQ full MS/MS scan mode. We employed the approaches to determine SRM peptide transitions of 16 oxidative phosphorylation (OXPHOS) proteins which are known be involved in mitochondrial dysfunction (Wagner, Kitami et al. 2008, Calvo and Mootha 2010). We have demonstrated successful measurement of mitochondrial genome (mtDNA)-encoded 6 proteins and nuclear genome (nuDNA)-encoded 10 proteins in the human skeletal muscle tissue sample (Wagner, Kitami et al. 2008, Calvo and Mootha 2010).

2. Material and methods

2.1 Reagents

HPLC grade acetonitrile (ACN) and water were purchased from J.T Baker (Phillipsburg, NJ). Formic acid (FA), urea, dithiothreitol (DTT), iodoacetamide (IAA), ammonium bicarbonate, and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO). Sep-pak C18 cartridges were from Waters (Milford, MA, USA). Sequencing grade modified porcine trypsin was obtained from Promega (Madison, WI). Synthetic peptides including cytochrome b (DVNYGWIIR), NADH dehydrogenase 1 beta subcomplex, 5 (GDGPWYYYETI), and NADH dehydrogenase 2 (WAIIEEFTK) were from Anygen (Gwangju, Korea).

2.2 Isolation of mitochondrial proteins

Human skeletal muscle tissues were homogenized in mitochondria isolation buffer (250mM sucrose, 25mM Tris-HCl Ph7.4, 1mM EDTA). Subsequently, the lysate was centrifuged at 1,000 g to separate nuclear and cytosolic fractions, the cytosolic fraction was further centrifuged at 10,000 g at 4°C to pellet mitochondria. The isolated mitochondria were further purified through a 10%~40% percoll gradient by centrifugation at 20,000 rpm in a Beckman SW41 rotor for 120 min. The mitochondrial layer in the gradient was removed and washed twice and stored at -70°C for assays.

2.3 Immunoblot Analysis

Mitochondria samples were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Whatman, dassel, Germany). Blocking was performed by 5% skim milk in 0.1% Tween 20, Tris-buffered saline (0.1% T-TBS) for 1h at room temperature. The membrane was incubated with anti-Enolase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-PPAP (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-Prohibitin (NeoMarkers, Premont, CA), anti-LaminB (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing three times with 0.1% T-TBS, the membrane was incubated with the secondary antibody (HRP-conjugated) for 1h at room temperature, and wash three times with 0.1% T-TBS. The signals were detected using SuperSignal West Pico chemiluminescent substrate (Pierce, IL, USA).

2.4 Protein digestion

Digestion of isolated proteins was performed by the Filter Aided Sample Preparation (FASP) method as previously described (Wi and sacute 2009). Briefly, 50 µg of proteins were solublized in 30 µL SDT lysis buffer consisting of 100mM Tris/HCl pH 7.5, 4% SDS, and 100 mM dithiothreitol (DTT) and heated at 95°C for 5 minutes. The solubilized proteins were denaturated with 200 µL UA buffer (8 M urea in 0.1 M Tris/HCl pH 8.5) and loaded onto the 30k filtration unit (Millipore, Ireland) and centrifuged at 14,000 g for 20 minutes. The concentrate was resuspened with 200 µL UA buffer and centrifuged again. Proteins were alkylated with 50 mM IAA at room temperature for 30 minutes incubation in the dark and followed by centrifugation for 15 minutes. Next, 200 µL of UA buffer was added to the filter and centrifuged at 14,000 g for 15 minutes twice, and then 200 µL of 100 mM ammonium bicarbonate were added and

centrifuged under the same condition three times. Proteins were digested with 1 μg sequencing grade modified porcine trypsin in 50 mM ammonium bicarbonate overnight at 37°C. Peptides were eluted with 500 mM NaCl and then desalted using Sep-pak C18 cartridges. Desalted peptides were lyophilized under a Centrivap concentrator (Labconco, Kansas City, MO) and stored at -80°C until use.

2.5 Mass spectrometric analysis and database search

Peptides were resuspended in 50 μL Solvent A (2% ACN, 0.1% FA in water), and 2 μL of the peptide sample was analyzed in triplicate on a LTQ-Velos mass spectrometer (Thermo Fisher Scientific) equipped with nano-HPLC system (Easy nLC, Thermo Fisher Scientific, San Jose, CA). Peptides were separated on an in-house packed micro-capillary Michrom Magic^{TM} C_{18}AQcolumn (75 μm inner diameter, 12 cm length) with a linear gradient of 2 - 38% Solvent B (98% ACN, 0.1% FA in water) for 90 min at a flow rate of 300 nL/min. The spray voltage was set to 1.9 kV and the temperature of the heated capillary was set to 325°C. The LTQ-Velos was operated by data-dependent mode with one survey MS scan at the mass range 400 - 1400 *m/z* and followed by five MS/MS scans using normalized collision energy of 35% and a dynamic exclusion time of 30seconds. Collected MS/MS raw files were converted to mzXML files through the Trans Proteomic pipeline (TPP) (version4.4). The peptides were assigned by using SEQUEST algorithm (Thermo Fisher Scientific, version v.27, rev.11) against the SWISS-PROT database (SWISS-PROT.human.v3.14) by Sorcerer^{TM}. All searches were carried out with semi-tryptic and allowing two missed cleavages. Precursor ion and fragment ion mass tolerance were set to

1.5 and 0.5 Da, respectively. Carbamidomethylation of cystein was considered as the fixed modification and oxidation of methionine as variable modification. Peptide identifications were accepted if they could be established at greater than 95% probability as specified by the Peptide Prophet algorithm (Keller, Nesvizhskii et al. 2002, Nesvizhskii, Keller et al. 2003).

2.6 Selection of SRM peptide transitions

Peptides were analyzed on an Agilent 6430 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) coupled with an Agilent 1200 nano HPLC interfaced with the HPLC-chip spray system. The peptides were resuspended in solvent A (0.1% FA in water) and separated on the HPLC-chip column, ZORBAX 300SB C18 (5 μm, 160 nL enrichment column and 75 μm × 150 mm analytical column) with a linear gradient of 0 - 40% solvent B (0.1% FA in ACN) for 60 min at a flow rate of 300 nL/min. The spray voltage was set to 1.9 kV and the temperature of nitrogen drying gas was set to 325°C in the positive ion mode. SRM peptide transitions were determined using two scan modes; **Product ion scan:** The product ion scan analysis was performed with the following steps; 1) input precursor ion mass to MassHunter Acquisition Analysis software (version B. 4.0, Agilent Technologies), 2) fragmentor voltage (FV) was fixed to 135 V and collision energy (CE) was set to correlation of each target peptide's precursor mass (500−600 m/z → 12−17 V, 600−700 m/z → 18−22 V, 700−800 m/z → 23−30 V, 800 ≤ m/z → 31−35 V), 3) MS/MS scan range was set from 400 to 2000 m/z depending on the precursor ion mass and charge states and the scan time was set to 500ms, 4) both MS and MS/MS ions were monitored with a unit resolution mass window (0.7 FWHM) in Q1

and Q3. **Unbiased Q3-ion monitoring:** The unbiased Q3-ion monitoring assay was performed using SRM mode with the following steps; 1) most intense four fragment y and/or b ions were selected from LTQ MS/MS spectra of target peptides, 2) both fragmentor voltage and collision energy were set with the same values used for the full MS/MS scan method, 3) dwell time of each transition was set to 20 ms and SRM Q1/Q3 transitions were monitored with a unit resolution mass window (0.7 FWHM), 4) chromatographic elution profiles of each transition ion and estimation of chromatographic peak area were carried out using MassHunter Quantitative Analysis software (version B. 4.0, Agilent Technologies).

2.7 SRM analysis of OXPHOS proteins

Fifty micrograms of the peptides isolated from human skeletal muscle tissue sample were dissolved in 50 µL Solvent A (0.1% FA in water), and 2 µg of the peptides were used for each SRM analysis. The peptides were separated on the HPLC-chip column, ZORBAX 300SB C18 (5 µm, 160 nL enrichment column and 75 µm × 150 mm analytical column). The peptides were eluted using a linear gradient of 0 - 40% solvent B (0.1% FA in ACN) for 70 min at a flow rate of 300 nL/min directly into an Agilent 6430 triple quadrupole mass spectrometer coupled with an Agilent 1200 nano HPLC interfaced with the HPLC-chip spray system. The electrospray ion source was operated in the positive ionization mode with a of nitrogen drying gas temperature of 325°C. Dwell time of each transition was set to 20 ms, and SRM Q1/Q3 transitions were monitored with a unit resolution mass window (0.7 FWHM). The multiplexing SRM assay was performed in duplicate with 36 transitions including 17 SRM target peptides which were determined from both full MS/MS scan

and unbiased Q3-ion monitoring modes. The integration of peak areas for SRM transitions were calculated using MassHunter Quantitative Analysis software (version B. 4.0, Agilent Technologies). The coefficient of variation (% CV) of the integrated peak area for external standard peptide was measured by the ratio of the standard deviation to the mean from measurements. Each integrated peak area of SRM transition was normalized with the external standard peptide to compensate for the systematic bias.

3. Results

3.1 Determination of SRM peptide transitions

Several criteria for the selection of SRM peptide transitions representing target proteins of interests have been well documented (Sherman, McKay et al. 2009, Bianco and Bessant 2010). A typical approach is based on *in-silico* predictions of the SRM peptide transitions followed by optimization of the predicted peptide transitions using SIS. Our approach is utilizing experimental data to identify potential SRM peptides and their transitions using the combination of Linear Ion-Trap/TripleQuadruple (LIT/QqQ) mass spectrometries. Overall workflow for the determination of SRM peptide transitions is shown in Figure 1. A set of SRM peptide candidates for the target proteins are selected from the LIT profiling data following the general selection criteria, such as detectability and uniqueness to the target proteins, the length of peptides (10−20 amino acids) with no modifications, and the charge states that can be detected within the *m/z* scan window (Brusniak, Kwok et al. 2011).

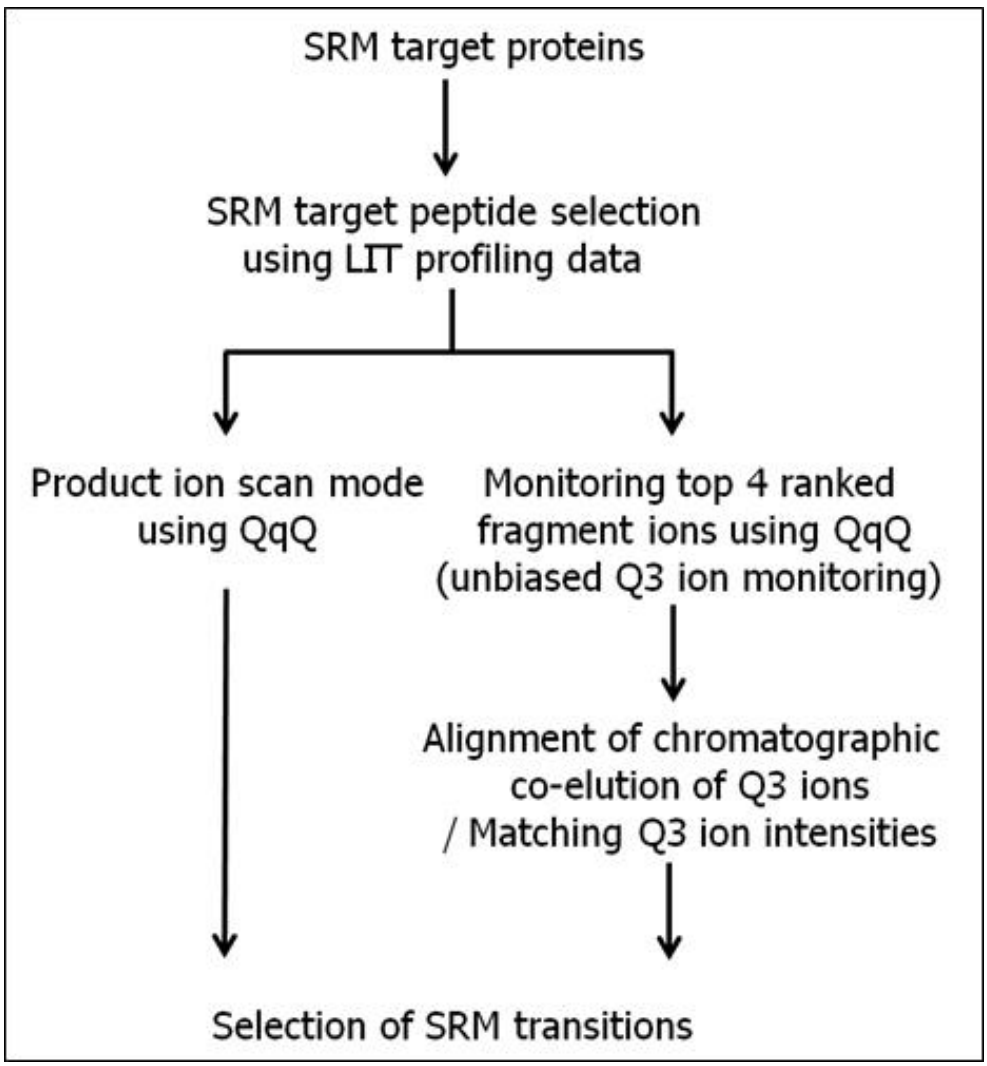

Figure 1. Workflow for determination of SRM peptide transitions

The SRM target proteins were selected based on the previously identified mitochondrial OXPHOS proteins (Wagner, Kitami et al. 2008, Calvo and Mootha 2010) and SRM target peptides were selected by LIT profiling data. Next, the transitions of target peptides were determined using 2 independent methods: the product ion scan mode

using QqQ and the unbiased Q3 ion monitoring. As the unbiased Q3 ion monitoring, all transitions were confirmed by co-elution patterns and their signal intensity orders.

Selected SRM peptide candidates were confirmed their detectability on the QqQ-MS by acquiring full MS/MS spectra by comparing the MS/MS fragmentation patterns with the LIT-MS. As displayed in Figure 2A, MS/MS spectra of GLDPYNVLAPK (1186.6, $MH2^+$) from both MS platforms were mirrored, showing that all fragment ions and their abundances are consistent. Similar peptide fragmentation patterns and ion intensities of other peptide candidates were observed; EDPNLVPSISNK (1312.6, $MH2^+$) (Fig.2B), AANNGALPPDLSYIVR (1670.8, $MH2^+$) (Fig.2C), and LFDYFPK (929.4, $MH2^+$) (Fig.2D). Top three most abundant ions of each peptide were selected for SRM peptide transitions as summarized in Table 1.

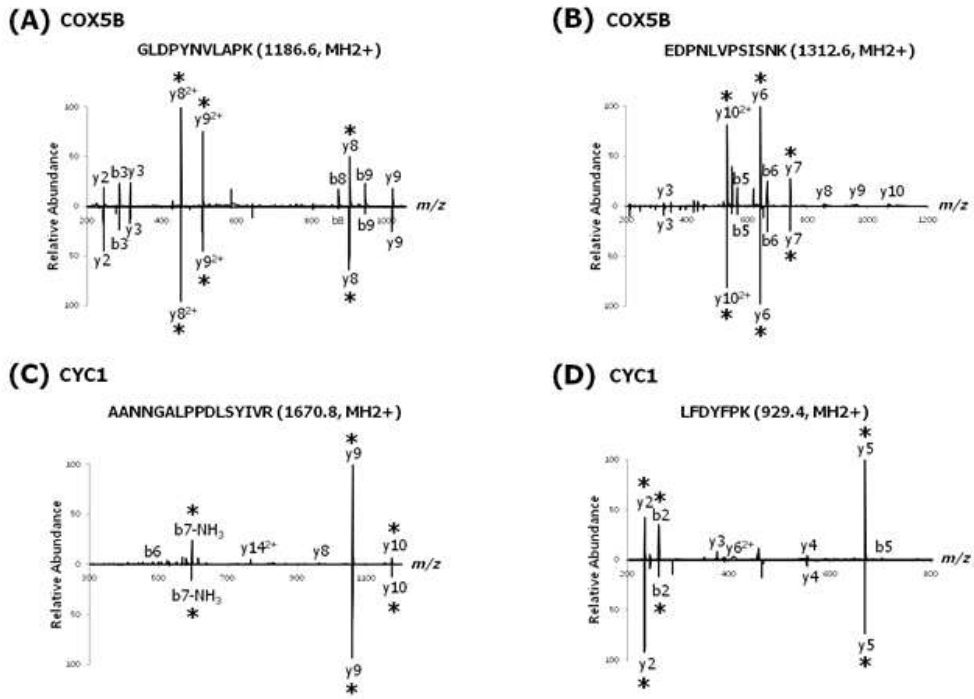

Figure 2. Comparison of peptide MS/MS fragmentation patterns between LIT and QqQ

SRM peptide candidates selected from LIT profiling data were analyzed in MS/MS scan mode with the same study sample. MS/MS spectra from the LIT profiling data (top) and collected from the product ion scan mode by QqQ (bottom) are mirrored. MS/MS spectra are of GLDPYNVLAPK (1186.6, MH2+) (A) and EDPNLVPSISNK (1312.6, MH2+) (B) of COX5B protein. MS/MS spectra are of AANNGALPPDLSYIVR (1670.8, MH2+) (C) and LFDYFPK (929.4, MH2+) (D) of CYC1 protein. All of the corresponding y and b ions are assigned. Selected Q3 ions of each SRM peptide candidate are marked (*).

Table 1. Top three most abundant SRM peptide transitions

Protein name	Peptide sequence	Q1 mass	Charge state	Three most intense Q3 mass
Cytochrome c oxidase subunit 5B	GLDPYNVLAPK	1186.6	2	451.2 ($y8^{2+}$)
(COX5B)				901.3 (y8)
				508.9 ($y9^{2+}$)
	EDPNLVPSISNK	1312.6	2	465.4 (y6)
				535.0 ($y10^{2+}$)
				744.6 (y7)
Cytochrome c-1	AANNGALPPDLSYIVR	1670.8	2	1059.5 (y9)
(CYC1)				595.2 (b7-NH_3)
				1172.6 (y10)
	LFDYFPK	929.4	2	244.1 (y2)
				668.9 (y5)
				261.2 (b2)

In this full MS/MS scan mode and spectral pattern matching method, we encountered difficulties when the MS/MS spectral quality was poor. As an alternative approach, selected fragment ions were blindly monitored by product-ion scan mode in the QqQ MS, which is referring as unbiased Q3-ion monitoring (Fig.1). In this approach, we selected the most intense ions from the LIT MS/MS profiling data including at least one minor intense ion to observe dynamic changes of chromatographic elution profiles. As shown in Figure 3, four fragment ions (y7, y8, y6, and $y10^{2+}$) of target peptide, GDGPWYYYETIDK, monitored by the unbiased Q3-ion monitoring using the QqQ-MS. All the selected ions were eluted at the same chromatographic retention times and their integrated chromatographic peak area were well correlated with the order of ion intensities observed in the LIT-MS profiling data (Fig.3A). Using the same process, we identified potential SRM peptide transitions of selected peptides, DVNYGWIIR (Fig.3B), WAIIEEFTK (Fig.3C), and TISQHQISTSIITSTQK (Fig.3D), which are summarized in Table2.

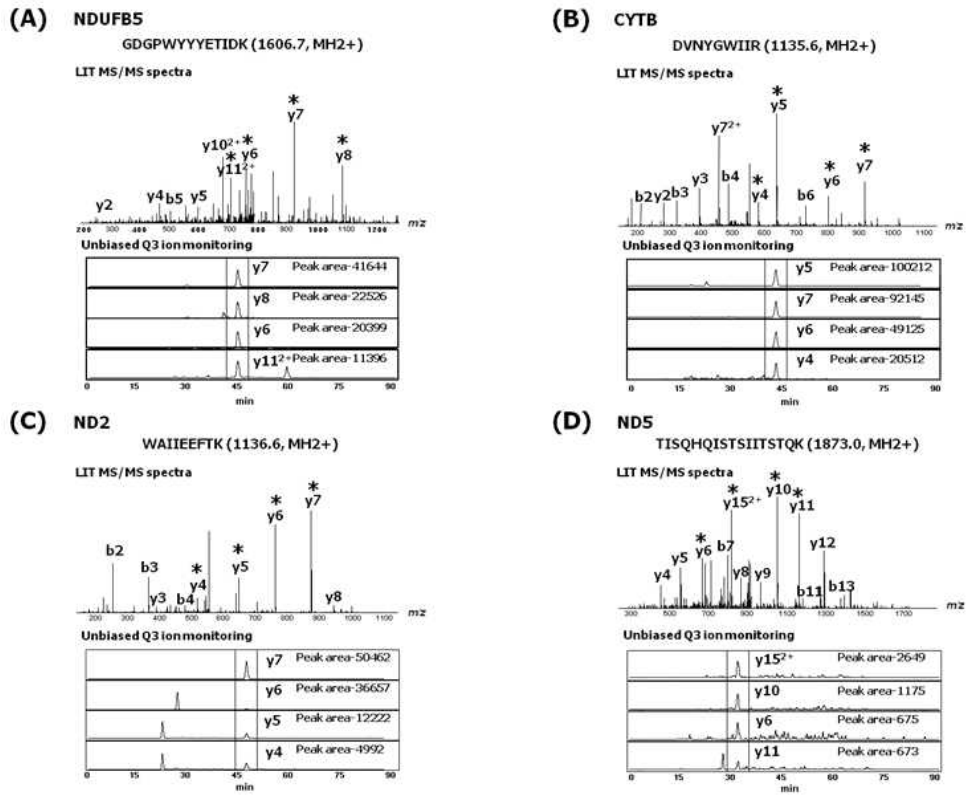

Figure 3. Identification of SRM peptide transitions using the unbiased Q3-ion monitoring

SRM peptide candidates selected from LIT profiling data were analyzed in unbiased Q3-ion monitoring with the same study sample. Selected top 4 highly abundant y fragment ions in the MS/MS spectra obtained from LIT profiling data (top). Chromatographic elution profiles of selected fragment ions generated by SRM-triggered scan mode using the QqQ-MS platform (bottom). MS/MS spectra are of GDGPWYYYETIDK (1606.7, MH2+) (A) of NDUFB5 protein and DVNYGWIIR (1135.6, MH2+) (B) of CYTB protein. MS/MS spectra are of WAIIEEFTK (1136.6, MH2+) (C) of ND2 protein and TISQHQISTSIITSTQK (1873.0, MH2+) (D) of ND5 protein. All of the

corresponding y and b ions are assigned. Selected Q3 ions of each SRM peptide candidate are marked (*).

Table 2. Selected SRM transitions using unbiased Q3-ion monitoring

Protein name	Peptide sequence	Q1 mass	Charge state	Four most intense Q3 mass	Retention time (min)	Integrated peak area
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial	GDGPWYYYETIDK	1606.7	2	931.4 (y7)	43.43	41644
(NDUFB5)				1094.5 (y8)	43.40	22526
				768.4 (y6)	43.45	20399
				717.8 ($y11^{2+}$)	43.53	11396
Cytochrome b	DVNYGWIIR	1135.6	2	644.4 (y5)	44.89	100212
(CYTB)				921.5 (y7)	44.78	92145
				807.5 (y6)	44.69	49125
				587.4 (y4)	44.92	20512
NADH-ubiquinone oxidoreductase chain 2	WAIIEEFTK	1136.6	2	879.5 (y7)	48.12	50462
(ND2)				766.4 (y6)	48.10	36657
				653.3 (y5)	48.17	12222
				524.3 (y4)	48.10	4992
NADH-ubiquinone oxidoreductase chain 5	TISQHQISTSIITSTQK	1873.0	2	829.9 ($y15^{2+}$)	31.43	2649
(ND5)				1065.6 (y10)	31.49	1175
				677.4 (y6)	31.41	675
				1178.7 (y11)	31.50	673

3.2 Verification of the unbiased Q3 ion monitoring with synthetic peptides

To confirm the identified SRM peptide transitions using the unbiased SRM approach, we used synthetic peptides. Three synthetic peptides (GDGPWYYYETIDK, DVNYGWIIR, and WAIIEEFTK) were analyzed by the MS/MS scan mode in the QqQ MS and showed similar peptide fragmentation patterns and ion abundances to the native peptides in the LIT MS (Fig.4). We further analyzed the synthetic peptides by the unbiased Q3-ion monitoring approach. All the ions were eluted at the same chromatographic time and their integrated peak areas were well correlated with their ion intensities showing on the LT-MS data (Fig.5). We further confirmed the target peptides in the complex peptide mixtures of mitochondrial proteins isolated from human skeletal muscle tissue sample, showing that the predetermined SRM peptide transitions were detected using the unbiased SRM approach.

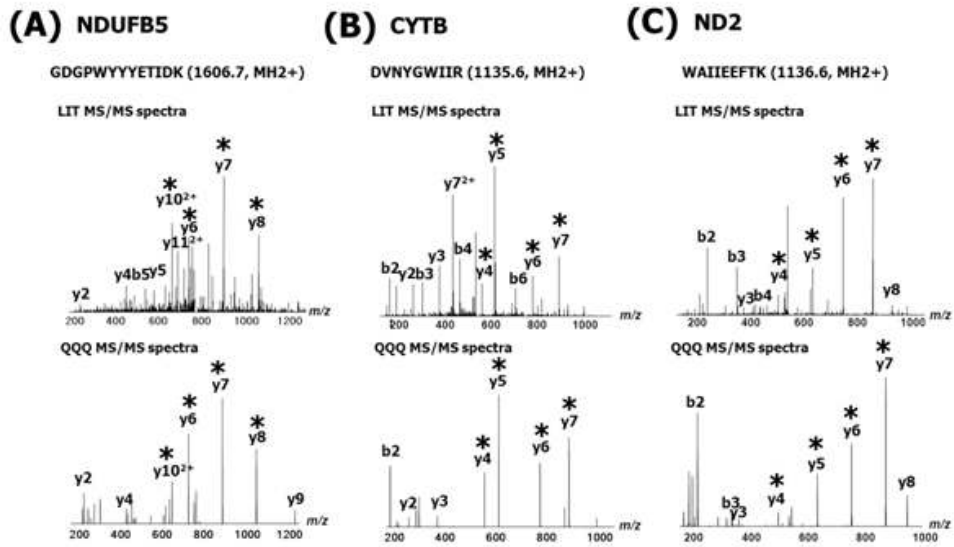

Figure 4. MS/MS fragmentation patterns between native peptides and synthetic peptides

MS/MS spectra from LIT profiling data with native peptides (top) and collected from QqQ full MS/MS scan mode data with synthetic peptides (bottom) are mirrored. MS/MS spectra are of GDGPWYYYETIDK (1606.7, MH2+) (A) of NDUFB5 protein and DVNYGWIIR (1135.6, MH2+) (B) of CYTB protein and WAIIEEFTK (1136.6, MH2+) (C) of ND2 protein. All of the corresponding y and b ions are assigned. Selected Q3 ions of each SRM peptide candidate are marked (*).

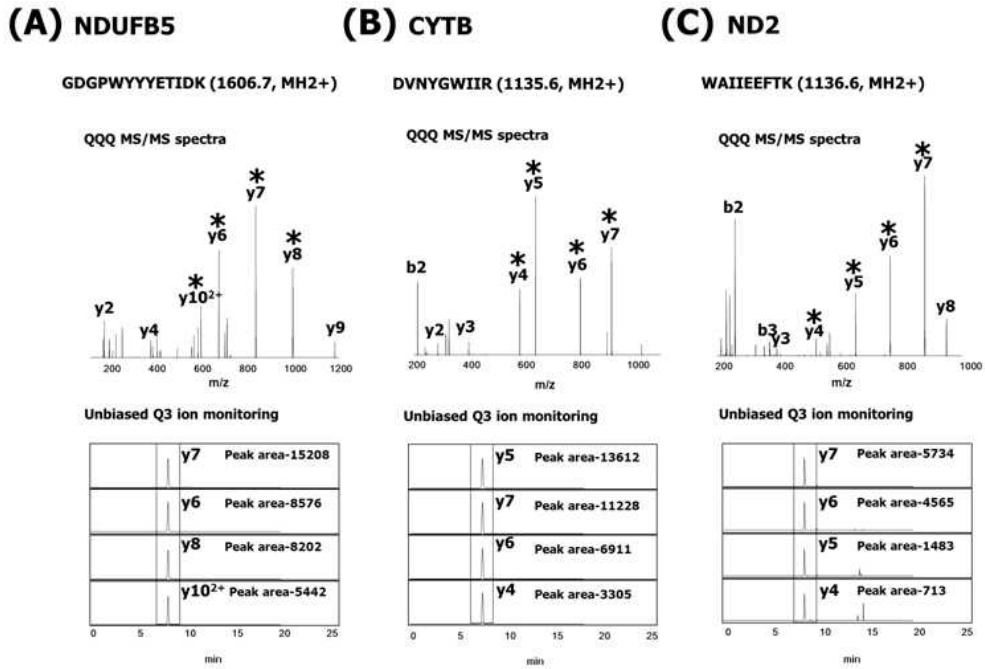

Figure 5. Validation of SRM peptide transitions with synthetic peptide

Three synthetic peptides of SRM peptide candidates were analyzed in unbiased Q3 ion monitoring. Selected top 4 highly abundant y fragment ions in the MS/MS spectra obtained from QqQ full MS/MS scan data (top). Chromatographic elution profiles of selected fragment ions generated by unbiased Q3 ion monitoring mode using the QqQ-MS platform (bottom). MS/MS spectra are of GDGPWYYYETIDK (1606.7, MH2+) (A) of NDUFB5 protein, DVNYGWIIR (1135.6, MH2+) (B) of CYTB protein and WAIIEEFTK (1136.6, MH2+) (C) of ND2 protein. All of the corresponding y and b ions are assigned. Selected Q3 ions of each SRM peptide candidate are marked (*).

3.3 SRM analysis of 16 mitochondrial proteins with the predetermined SRM transitions

With the selected SRM peptide transitions of target proteins (Table 3), we performed the SRM analysis of 16 mitochondrial OXPHOS proteins in the human skeletal muscle tissue. Western blotting analyses have confirmed that the mitochondria were intact, and that mitochondrial fractions of the muscle tissue samples exhibited markers of NDUFB8 (Complex I), SDHB (Complex II), UQCR2 (Complex III), COX5B (Complex IV), and ATP5A1 (Complex V). Figure 7A is a chromatographic trace of SRM peptide transitions of the tissue sample and the inserted Figure 7A illustrates the chromatographic responses of the external peptides varying the concentrations of angiotensin I and bradykinin while kept angiotensin I constant in the same study sample. Relative abundance of each peptide transition was estimated by integrating peak area followed by normalization with the signal intensity of external standard peptide transitions, in which both bradykinin (m/z, 354.3/555.3) and angiotensin I (m/z, 433.0/513.3) showed a good linear correlation (R^2 = 0.992, 0.986 for bradykinin and angiotensin I, respectively) as shown in Figure7B and 7C. The reproducibility of SRM assay representing target OXPHOS proteins is summarized in Table 4. Most target proteins were measured with reliable CV value (CV $\leq$ 20%) in triple replicates.

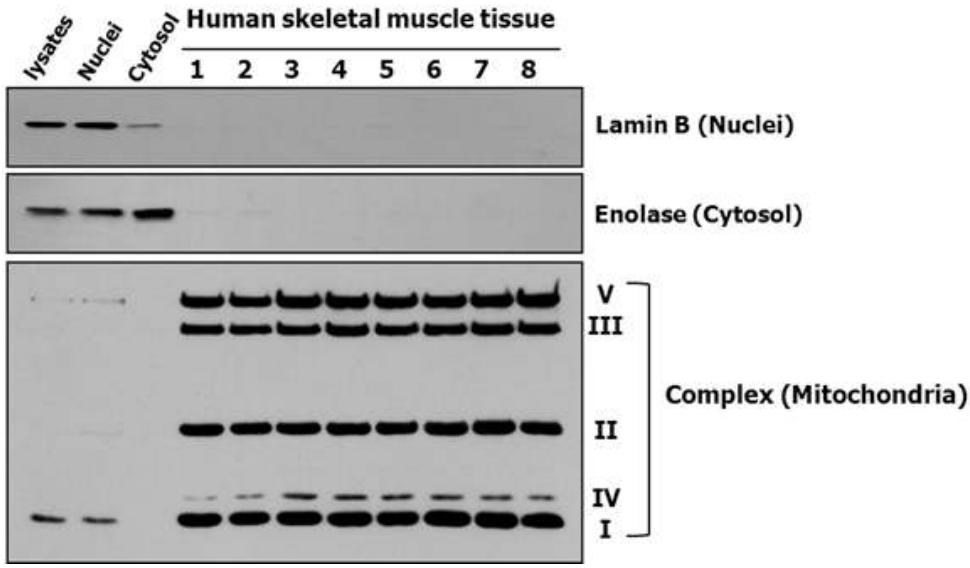

Figure 6. Isolation of mitochondrial proteins from human skeletal tissue samples

Each fraction was analyzed by western blot with anti-Lamin B, anti-Enolase, anti-Mitochondria Complex Coctail (Complex I of NDUFB8, Complex II of SDHB, Complex III of UQCR2, Complex IV of COX5B, and Complex V of ATP5A1).

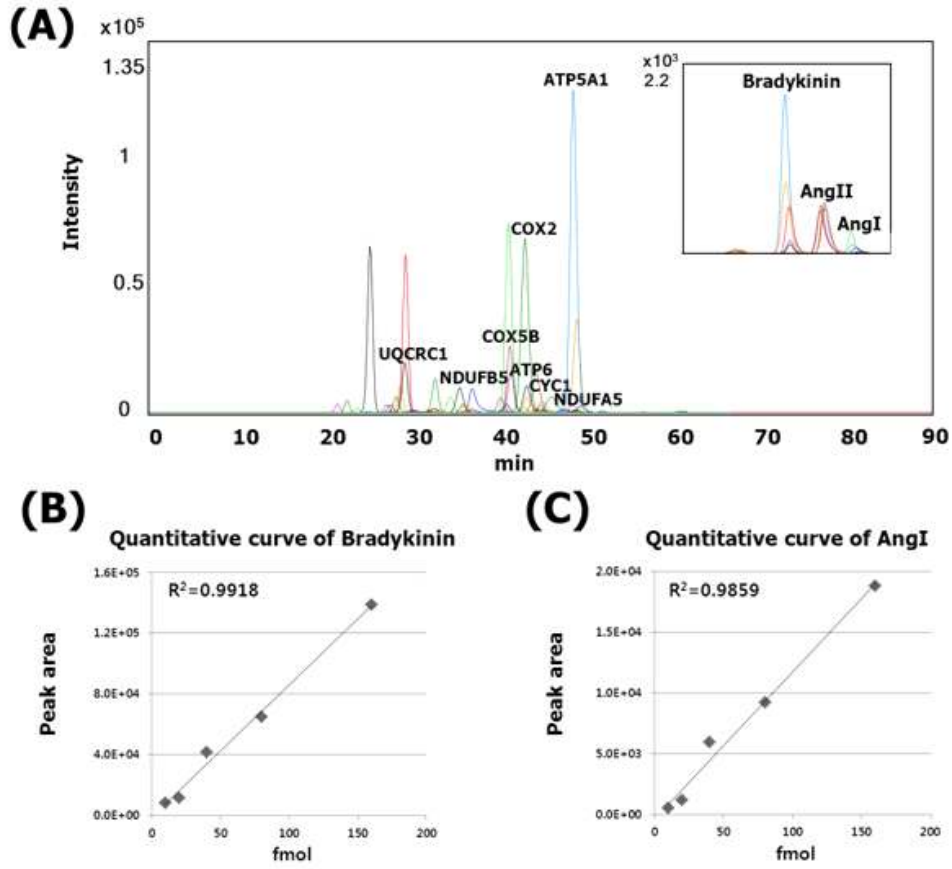

Figure 7. SRM analysis of target proteins

SRM assay was performed with mitochondrial target proteins using three external standard peptides (DRVYIHPFHL of angiotensin I, DRVpYIHPF of angiotensin II and RPPGFSPFR of bradykinin) spiked into the skeletal muscle tissue sample. Five SRM assays were performed at 5 concentration (10, 20, 40, 80, 160 fmole) of two external peptides (angiotensin I and bradykinin) while kept angiotensin II constant (60 fmole) in the same study sample. Figure (A) is a chromatographic profile of transition ions of mitochondrial target peptides including three external standard peptides. It is

apparent that the relative peak area of two peptides transitions correlates well with the peak area of angiotensin II as shown in the inserted figure. The quantitative curves of bradykinin (B) and angiotensin I are drawn. The curve resulted in a linearity of R^2 = 0.9918 of bradykinin and R^2 = 0.9859 of angiotensin I, respectively.

Table 3. Selected transitions of 16 target proteins for multiplexing SRM assay

Protein name	Peptide sequence	Q1 mass	Charge state	Q3 mass	Collision energy (V)
ATP synthase subunit alpha, mitochondrial (ATP5A1)	LELAQAR	892.5	2	650.3 (y5) 537.3 (y4)	10
ATP synthase subunit gamma, mitochondrial (ATP5C1)	LTLTFNR	864.5	2	650.2 (y5) 537.3 (y4)	10
ATP synthase subunit O, mitochondrial (ATP5O)	TDPSILGGMIVR	1258.7	2	521.5 ($y10^{2+}$) 744.9 (y7)	17
ATP synthase subunit a (ATP6)	LITTQQWLIK	1243.7	2	1017.4 (y8) 227.2 (b2)	16
ATP synthase protein 8 (ATP8)	NYNKPWEPK	1175.6	2	656.3 (y5) 784.8 (y6)	16
Cytochrome c oxidase I (COX1)	WLFSTNHK	1032.5	2	733.4 (y6) 586.4 (y5)	13
Cytochrome c oxidase subunit II (COX2)	VVLPIEAPIR	1106.7	2	795.4 (y7) 398.2 ($y7^{2+}$)	13
Cytochrome c oxidase subunit 5B (COX5B)	GLDPYNVLAPK	1186.6	2	451.2 ($y8^{2+}$) 508.9 ($y9^{2+}$)	14
Cytochrome c oxidase subunit 7A2, mitochondrial (COX7A2)	LFQEDDEIPLYLK	1622.8	2	633.4 (y5) 990.6 (y8)	22
Cytochrome c-1 (CYC1)	AANNGALPPDLSYIVR	1670.9	2	1059.5 (y9) 1172.6 (y10)	25
	LFDYFPK	929.5	2	668.9 (y5) 244.1 (y2)	8
Cytochrome b (CYTB)	DVNYGWIIR	1135.6	2	644.4 (y5) 921.5 (y7)	15
NADH-ubiquinone oxidoreductase chain 4 (ND4)	LTLILNPLTK	1125.7	2	685.4 (y6) 911.6 (y8)	13
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 5 (NDUFA5)	ILDVLEEIPK	1168.7	2	942.2 (y8) 728.7 (y6)	16
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5, mitochondrial (NDUFB5)	GDGPWYYYETIDK	1606.7	2	931.4 (y7) 768.4 (y6)	23
Cytochrome b-c1 complex subunit 9 (UQCR10)	LYSLLFR	911.5	2	635.2 (y5) 364.1 (b3)	11
Cytochrome b-c1 complex subunit 1, mitochondrial (UQCRC1)	IAEVDASVVR	1058.6	2	874.4 (y8) 646.4 (y6)	16

Table 4. Reproducibility of SRM assay for OXPHOS proteins

Protein name	Transition (Q1/Q3)	Mean	St. dev	CV
ATP5A1	446.7/650.3	2.79E+06	2.27E+05	8.2%
ATP5C1	432.8/650.2	3.39E+05	1.80E+04	5.3%
ATP5O	629.8/521.5	9.66E+05	4.82E+04	5.0%
ATP6	622.4/1017.6	6.06E+05	3.60E+04	5.9%
ATP8	588.3/656.3	1.65E+04	4.33E+03	26.2%
COX1	516.4/733.4	8.16E+04	2.40E+03	2.9%
COX2	553.9/795.4	4.13E+06	2.32E+05	5.6%
COX5B	593.8/451.2	2.99E+05	2.40E+04	8.0%
COX7A2	811.9/633.4	1.84E+05	4.08E+03	2.2%
CYC1	835.9/1059.6	5.30E+05	6.41E+03	1.2%
	465.2/668.9	9.39E+04	6.73E+03	7.2%
CYTB	568.3/644.4	7.04E+04	1.27E+03	1.8%
ND4	563.4/685.4	1.81E+04	2.83E+03	15.6%
NDUFA5	584.8/942.2	1.36E+05	8.14E+03	6.0%
NDUFB5	803.9/931.4	3.57E+04	1.78E+03	5.0%
UQCR10	456.3/635.2	3.01E+05	1.84E+04	6.1%
UQCRC1	529.8/874.4	4.64E+05	5.02E+04	10.8%
Angiotensin II	376.3/216.0	1.12E+04	7.69E+02	6.8%

4. DISCUSSION

In the course SRM assay development for biomarkers in T2DM patient skeletal muscle tissue, we have employed two modes of QqQ-MS operation coupled with LIT-MS profiling data to identify SRM peptide transitions. In both QqQ-MS operation modes, we took advantage of the similar MS/MS patterns and ion abundances between the two MS systems (LTQ ion-trap and Agilent Triple Quadrupole MS). Although two MS systems have different ionization and detection mechanisms, we have observed that MS/MS patterns and ion abundances are resembled between the two MS systems. Similar phenomena were also observed by Prakash et al. (Prakash, Tomazela et al. 2009). In their development of an automate SRM tool, they developed a scoring algorithm to predict SRM peptide transitions using the spectral data between the LTQ ion-trap and TSQ Quantum MS (Prakash, Tomazela et al. 2009). Our experiment also demonstrates that two MS systems (LTQ ion-trap and Agilent Triple Quadrupole MS) show strong agreement of MS/MS patterns and ion abundances, which provide a reliable way to design SRM peptide transitions.

In the first mode of QqQ-MS operation, we verify the identities of target peptides with acquired MS/MS spectra using a product-ion scan mode using only the peptide precursor ion information. We then selected potential SRM peptide transitions based on the fragment ion abundances appeared on the full MS/MS spectra of the QqQ-MS. In the second mode of QqQ-MS operation named as unbiased Q3-ion monitoring approach, we blindly performed SRM with peptide precursor ion and four relatively abundant fragment ion information

obtained from LIT profiling data. Using the product-ion scan mode approach, we were able to determine SRM transitions for 12 target proteins. We have identified additional 4 target proteins using the unbiased Q3-ion monitoring approach because we were not able to get good quality of MS/MS spectra to assign reliable target peptides with the product-ion scan mode. This finding suggests that we can monitor potential Q3 ions reliably using the unbiased Q3-ion monitoring approach, although MS/MS spectral quality is not good to match between the MS systems. The results were further confirmed by synthetic peptides, suggesting the unbiased Q3-ion monitoring is an efficient method to detect potential SRM peptide transitions experimentally when proteins are in low abundance in complex biological sample.

In the quantitative proteomic analysis of biological samples, such as serum, urine, tissue etc, maintaining consistent proteomics sample preparation and LC/MS analysis condition is crucial. In that respect, identifying reproducibly detectible proteolytic peptides representing target proteins of interest in the given biological sample matrices are important in the SRM assay design. Despite invaluable SRM peptide transition information can be obtained using various *in-silico* prediction tools (Tang, Arnold et al. 2006, Unwin, Griffiths et al. 2009, MacLean, Tomazela et al. 2010), detection for *in-silico* predicted peptides experimentally using SIS in the given biological sample is not always warrant (~40%) (Kim et al, personal communication). Bruno et al. also demonstrated that verification of SRM peptides with the discovery profiling data was essential (Lange, Picotti et al. 2008, Bodenmiller et al. 2009). To enhance systematic detection of target peptides, throughout the process (for both profiling experiment and identifying SRM peptide transitions), we used the same experimental

sample which had been processed with the identical proteomics sample preparation to ensure the detection of authentic proteolytic peptides reflecting the given biological conditions. Hence, we have developed a reasonable SRM transition determination strategy that improves the probability of success for SRM analysis with real complex biological samples.

In this study, we have applied multiplexed SRM assay to measure OXPHOS mitochondrial proteins in human skeletal muscle tissue. We selected a set of OXPHOS proteins which had been known to be key regulatory proteins responsible for ATP synthesis (Wallace 1999, Wallace, Brown et al. 2008) and abnormalities of human mitochondrial OXPHOS proteins have been known to be associated with T2DM (Calvo and Mootha 2010). As summarized in Table 4, SRM assay with the selected SRM peptide transitions provide reproducible protein abundance measurements in the triplicate analysis (CV $\leq$ 20%) indicating that our selection of SRM peptide transitions is well representing the target OXPHOS proteins in the complex biological sample. The results also suggest that our SRM method may complement the optimization processes of SRM transitions without SIS.

In conclusion, we have found that our new SRM peptide transition determination strategies (product ion scan and unbiased Q3-ion monitoring approach) are reliable methods for the selection of SRM peptide transitions, which are truly representing the target peptides present in the real-world sample. We also have demonstrated that the approaches are more rapid and sensitive in the determination of SRM peptide transitions. Clearly, the method is a promising tool in the biomarker discovery-validation pipeline.

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6. Abstract in Korean

Selected reaction monitoring (SRM)은 생체 시료 내에서 표적 단백질만을 선별하여 high through-put으로 정확하게 정량하는 프로테오믹스적 분석법이다. 효율적이고 신뢰성 있는 SRM 정량 분석을 위하여, 표적 단백질을 대표하는 펩타이드의 transition 조건을 정확하게 선별하고 확립하는 것은 매우 중요하다. 일반적으로 SRM 펩타이드의 transition을 선정하기 위해서는 프로테오믹스 프로파일링 데이터를 기반으로 한 표적 펩타이드의 MS/MS 스펙트럼을 이용하거나, 다양한 펩타이드 transition 예측 소프트웨어를 이용한다. 현재 가장 보편화 되어있는 SRM transition 선정 방법은 Skyline, MRMaid 등과 같은 예측 소프트웨어를 이용하는 것이다. 그러나 이와 같은 방법을 통해 선정된 transition들은 기본적으로 프로그램을 통해 예측된 가상의 transition들이기 때문에 실제 SRM 정량 분석시 탐지되지 않을 가능성이 존재한다는 단점이 있다. 본 연구에서는 Linear Ion Trap (LIT) 질량분석기를 통해 분석된 실제 샘플의 프로파일링 데이터 정보를 기반으로, Triple Quadrupole (QqQ)의 고감도 SRM mode를 이용하여 SRM 펩타이드 transition을 선정하는 방법을 연구하였다. 이러한 방법은 실제 질량분석기를 통해 얻어진 데이터를 기반으로 transition을 선정하기 때문에, 실제 실험 샘플 내에 존재하는 표적 펩타이드를 신뢰성 있게 탐지할 수 있으며, multiplexing manner를 이용해 한 번의 분석을 통해 다수의 표적 펩타이드 transition들을 선정할 수도 있다. 또한 QqQ의 고감도 SRM mode를 이용하여 transition을 선정함으로써, 기존의 방법들로는 탐지되지 않았던 low-abundant한 표적 단백질의 transition들을 선정하여, 기존에 이용되던 펩타이드 transition 선정 방법들의 한계점을 보완하고 궁극적으로 신뢰성 있는 SRM 정량분석을 수행할 수 있었다. 본 연구에서는 이 분석법을 실제 미토콘드리아의 16개 OXPHOS 표적 단백질들에 적용시켜 보았다. 기존의 분석법을 통해서는 12개 표적 단백질들의 transition만이

선정되었으나, 본 논문에서 제시한 방법을 통해서는 전체 16개 표적 단백질들의 transition들을 모두 선정할 수 있었다. 최종적으로 이렇게 선정된 transition들을 이용하여 정상인 골격근 조직의 미토콘드리아 샘플을 이용해 multiplex-SRM 분석을 3반복 수행하였다. 그 결과, 각 표적 단백질이 재현성 있게 탐지되는 것을 확인할 수 있었다. 이를 통해 본 논문에서 제시하는 SRM transition 선정 방법이 기존 방법들의 한계점을 보완하고, 실제 생체 시료 내에서도 적용가능 하다는 것을 확인할 수 있었다.

Key words: Selected Reaction Monitoring (SRM), Linear Ion-trap mass spectrometry (LIT-MS), Triple Quadrupole mass spectrometry (QqQ-MS), SRM transition, Oxidative phosphorylation (OXPHOS)
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