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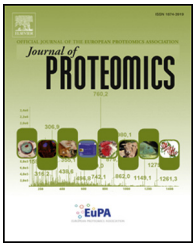

MRM validation of targeted nonglycosylated peptides from N-glycoprotein biomarkers using direct trypsin digestion of undepleted human plasma

Ju Yeon Lee[a,b,1], *Jin Young Kim*[a,1], *Mi Hee Cheon*[a,c], *Gun Wook Park*[a,c], *Yeong Hee Ahn*[a], *Myeong Hee Moon*[b], *Jong Shin Yoo*[a,c,*]

[a]*Division of Mass Spectrometry, Korea Basic Science Institute, 804-1 Yangcheong-Ri, Ochang-eup, Cheongwon-Gun 363-883, Republic of Korea*
[b]*Department of Chemistry, Yonsei University, Seoul 120-749, Republic of Korea*
[c]*Graduate School of Science and Technology, Chungnam National University, Daejeon 305-333, Republic of Korea*

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ABSTRACT

A rapid, simple, and reproducible MRM-based validation method for serological glycoprotein biomarkers in clinical use was developed by targeting the nonglycosylated tryptic peptides adjacent to N-glycosylation sites. Since changes in protein glycosylation are known to be associated with a variety of diseases, glycoproteins have been major targets in biomarker discovery. We previously found that nonglycosylated tryptic peptides adjacent to N-glycosylation sites differed in concentration between normal and hepatocellular carcinoma (HCC) plasma due to differences in steric hindrance of the glycan moiety in N-glycoproteins to tryptic digestion (Lee et al., 2011). To increase the feasibility and applicability of clinical validation of biomarker candidates (nonglycosylated tryptic peptides), we developed a method to effectively monitor nonglycosylated tryptic peptides from a large number of plasma samples and to reduce the total analysis time with maximizing the effect of steric hindrance by the glycans during digestion of glycoproteins. The AUC values of targeted nonglycosylated tryptic peptides were excellent (0.955 for GQYCYELDEK, 0.880 for FEDGVLDPDYPR and 0.907 for TEDTIFLR), indicating that these could be effective biomarkers for hepatocellular carcinoma. This method provides the necessary throughput required to validate glycoprotein biomarkers, as well as quantitative accuracy for human plasma analysis, and should be amenable to clinical use.

Biological significance
Difficulties in verifying and validating putative protein biomarkers are often caused by complex sample preparation procedures required to determine their concentrations in a large number of plasma samples. To solve the difficulties, we developed MRM-based protein biomarker assays that greatly reduce complex, time-consuming, and less reproducible sample pretreatment steps in plasma for clinical implementation.

Abbreviations: MRM, multiple reaction monitoring; ROC, receiver operating characteristic; AUC, areas under the curve; G6PD, glucose-6-phosphate dehydrogenase; HCC, hepatocellular carcinoma.

* *Corresponding author at:* Division of Mass Spectrometry, Korea Basic Science Institute, 804-1 Yangcheong-Ri, Ochang-Myun, Cheongwon-Gun 363-883, Republic of Korea. Tel.: +82 43 240 5150; fax: +82 43 240 5159.
E-mail address: jongshin@kbsi.re.kr (J.S. Yoo).

[1] These authors contributed equally.

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First, we used undepleted human plasma samples without any enrichment procedures. Using nanoLC/MS/MS, we targeted nonglycosylated tryptic peptides adjacent to N-linked glycosylation sites in N-linked glycoprotein biomarkers, which could be detected in human plasma samples without depleting highly abundant proteins.
Second, human plasma proteins were digested with trypsin without reduction and alkylation procedures to minimize sample preparation.
Third, trypsin digestion times were shortened so as to obtain reproducible results with maximization of the steric hindrance effect of the glycans during enzyme digestion.
Finally, this rapid and simple sample preparation method was applied to validate targeted nonglycosylated tryptic peptides as liver cancer biomarker candidates for diagnosis in 40 normal and 41 hepatocellular carcinoma (HCC) human plasma samples.
This strategy provided the necessary throughput required to monitor protein biomarkers, as well as quantitative accuracy in human plasma analysis. From biomarker discovery to clinical implementation, our method will provide a biomarker study platform that is suitable for clinical deployment, and can be applied to high-throughput approaches.

1. Introduction

Glycosylation is a post-translational modification of proteins, and glycosylated proteins play roles in protein folding [2], sorting, degradation, and secretion. Apweiler et al. predicted that more than half of proteins are glycosylated in mammals [3]. The glycans attached to glycoproteins play important roles in stabilizing structural proteins, cell–cell adhesion and signal transduction [4–6]. Changes in the glycoform distribution and glycoprotein abundances are associated with a variety of diseases, including cancer [7–10] and neurodegenerative diseases [11]. Therefore, glycoproteins have been studied as disease biomarkers. CEA [12], a marker of colorectal cancer, CA-125 [13], a marker of ovarian cancer, and PSA [14], a marker of prostate cancer, are all glycoproteins.

Although liquid chromatography–mass spectrometry (LC–MS) techniques are commonly used for protein analysis, glycoprotein analysis in complex samples such as plasma remains difficult. This may be because 1) novel glycoprotein biomarkers are in very low abundance in human plasma, 2) the signal intensities of glycosylated peptides are lower than those of nonglycopeptides and can be suppressed by other abundant peptides [15,16], 3) glycan structures are heterogeneous, and 4) the currently available database and search tools are unsatisfactory for the identification of glycosylated peptides.

To address the low sensitivity, glycoproteins/glycopeptides are often enriched using lectins [17–24], hydrazide chemistry [25–27], HILIC [28–33] separation, or other methods [34,35]. These enrichment methods facilitate the discovery of novel and low-abundance glycoprotein biomarkers by decreasing the sample complexity and increasing the concentrations of glycoproteins/glycopeptides. However, these enrichment processes increase the analysis time and reduce reproducibility and recovery because of the complex procedures required, including sample loading, washing, and elution. Specially, Calvano et al. [36]. reported the poor reproducibility of multi-lectin enrichment for tryptic digested glycosylated peptides in human serum. Only 13 N-glycosylated peptides of the 65 N-glycosylated peptides were identified in all three replicates.

For early disease diagnosis, blood plasma is an important sample that contains a large number of proteins secreted from tissues, and can easily be obtained from patients. However, plasma is a highly complex matrix that represents a dynamic range of protein concentrations exceeding ten orders of magnitude [37], which makes proteomic analysis a formidable task. Although the depletion of highly abundant proteins with immunoaffinity columns and/or protein fractionation have been used for plasma biomarker discovery, these procedures are not suitable for validation or clinical assays with a large number of samples. Moreover, the additional cost, complexity, and time of analysis may reduce the reproducibility and throughput potential.

Recently, Borchers et al. have published several studies [38–40] on MRM-based high-throughput assays designed for clinical utility with undepleted and non-enriched human plasma for the verification and validation of protein biomarkers. The MRM-based assay was used for the quantitation of 67 putative cardiovascular disease biomarkers with 135 stable isotope-labeled peptide standards in tryptic digests of whole plasma in a 30-min assay. Inter-assay variability over three separate days resulted in CVs of <20% for 109 of 135 assays [38]. Another assay for 27 cancer biomarkers, including insulin-like growth factor 1, also showed good signal stability and a median inter-day CV of 6.1% for the overall platform [39]. The authors reported an optimal analytical workflow for biomarker validation or clinical assays in plasma that did not require complex sample preparation (such as depletion), which can reduce analytical reproducibility.

Our previous study [1] detailed the development and validation of a simple and practical MS-based proteomics technique for the discovery of biomarkers in human plasma, targeting nonglycosylated tryptic peptides from N-glycoproteins. Since glycan moieties in N-glycoproteins significantly affect the efficiency of proteolytic digestion if an enzymatically active amino acid is adjacent to the N-glycosylation site, proteolytic digestion results in quantitatively different peptide products in accordance with the degree of glycosylation. Based on this concept, several nonglycosylated tryptic peptides adjacent to N-glycosylation sites from glycoproteins were discovered as biomarkers using label-free LC/MS-based proteomics technology, and were quantitatively validated using a multiple reaction monitoring (MRM) method.

On the base of the previous MRM method, we required high-throughput, quantitatively precise, and reproducible assays to

validate biomarker candidates and for clinical implementation. Here, we present a rapid and simple MS-based validation method with more clinical applicability. First, we used undepleted human plasma samples without any depletion procedures. Second, human plasma proteins were digested with trypsin without reduction and alkylation procedures. Third, trypsin digestion times were shortened so as to obtain reproducible results during digestion. Finally, this method was applied for targeted nonglycosylated tryptic peptides as liver cancer biomarker candidates to validate in normal and hepatocellular carcinoma (HCC) human plasma samples.

2. Experimental

2.1. Materials

Glucose-6-phosphate dehydrogenase (G6PD) standard protein (source: yeast), dithiothreitol (DTT), iodoacetamide (IAA), and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Trypsin for protein digestion was obtained from Promega (Madison, WI). HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, NJ). Water was deionized through a Millipore system prior to use (Millipore, Eugene, OR). The stable isotope-labeled peptides GQYCYEL*DEK, FEDGVLDPDYP*R, TEDTIFL*R, and NTVISVFGASGDL*AK (isotopically labeled [^{13}C and ^{15}N] at amino acid sites marked with asterisks) were obtained from Anygen Co. (Kwangju, Korea).

2.2. Plasma sample preparation

Plasma samples were obtained with informed consent and in accordance with IRB guidelines from Yonsei University College of Medicine (Seoul, Korea).

Plasma samples from each healthy donor and each HCC cancer patient (sample information in Supplemental Table 1) were divided into four equal-volume bags with an appropriate concentration of K_2EDTA. Each aliquot was frozen and stored at −80 °C until use.

2.3. Digestion of human plasma samples for nanoLC/MRM analysis

To optimize the trypsin treatment time, the desalted plasma solution was treated with trypsin (trypsin:proteins = 1:10 (w:w)) at 37 °C for periods of 30 min, 1 h, 2 h, 4 h, 6 h, or overnight (16 h), without the depletion of abundant proteins and the reduction of DTT reagent. The digested solutions were made up to 3% formic acid to quench the trypsin digestion. Then, three stable isotope-labeled heavy standard peptides for three target nonglycosylated tryptic peptides adjacent to N-glycosylation sites were spiked equally into all digested plasma samples. The solutions were dried using a SpeedVac, and the dried samples were re-dissolved in an aqueous solution of 0.1% formic acid prior to LC/MRM quantification.

To validate targeted nonglycosylated tryptic peptides as biomarkers in 40 normal and 41 HCC plasma samples, plasma samples were desalted by centrifugal filtration using 10,000-Da MWCO (molecular weight cutoff) Vivaspin filters (VS0602; Sartorius) without depleting the six most abundant proteins. Aliquots (25 μg) of plasma samples that had been quantitatively analyzed by Bradford protein assays were diluted with 100 mM Tris–HCl buffer (pH 8.00). An internal standard protein of G6PD was prepared in 100 mM Tris–HCl buffer (pH 8.00), and spiked equally into all plasma samples (G6PD protein 500 fmol to plasma proteins 1 μg) following digestion with trypsin at 37 °C for 4 h without reductive reagents. The digested solutions were made up to 3% formic acid to quench the trypsin digestion. Then, three stable isotope-labeled heavy peptides (the amounts shown in Table 1) for three targeted nonglycosylated tryptic peptides adjacent to N-glycosylation sites, and one stable isotope-labeled heavy peptide (the amounts shown in Table 1) for the peptide of the internal standard protein G6PD were spiked equally into all digested plasma samples, including the digested internal standard G6PD. The final solutions were dried using a SpeedVac, and were re-dissolved in an aqueous solution of 0.1% formic acid prior to MRM quantification.

2.4. MRM quantification by online nanoLC–MS: minimization and reproducibility of trypsin treatment time, and validation of targeted nonglycosylated tryptic peptides

All nanoLC/MRM experiments were performed on an Agilent 1200 Series nanopump system and an Agilent 6430 Triple Quad LC/MS system connected to an Agilent Chip Cube LC/MS interface. The LC system consisted of a capillary pump for sample loading, a nanoflow pump for nanoflow separation, and a microwell-plate autosampler equipped with a thermostat. The HPLC microfluidics chip configuration consisted of a 160-nL enrichment column and a 75 μm × 150 mm analytical column (Zorbax 300SB-C_{18}, 5-μm particles). Mobile phases A and B were composed of 97% water/3% acetonitrile (ACN) with 0.1% formic acid and 10% water/90% ACN with 0.1% formic acid, respectively. Sample loading onto the enrichment column was performed at 4 μL/min with 0.1% formic acid in water using a capillary pump. An LC gradient was started at 3% mobile phase B, with a 400 nL/min flow rate for the nanoflow pump and ramped to 10% B for 3 min, 12% B for 5 min, 30% B for 34 min, 45% B for 3 min, 70% B for 4 min, and 100% B for 5 min. The 100% mobile phase B was descended to 3% B for 1 min. 3% mobile phase B was maintained for 5 min.

Electrospray ionization was performed with a spray voltage of 1800, and a drying gas temperature of 325 °C (2.5 L/min) was set for the MS instrument. To perform MRM (multiple reaction monitoring) analysis, the optimum transition ions and collision energy conditions for each peptide were determined using Optimizer software in conjunction with the Agilent 6430 Triple Quad LC/MS system. Each peptide was injected into a HPLC-microfluidics chip and analyzed using transition ions containing all possible b- and y-series fragment ions, while the collision energy (CE) was ramping. Using this approach, the three most intense fragment ions as three transition ions were selected, and the CE voltages maximizing the generation of each transition ion for each peptide were determined. All transitions of the corresponding peptides were acquired in the dynamic MRM method. Three transitions for each peptide were selected and monitored. First, precursor ions with a specific mass were transmitted from Q1 to the collision cell for fragmentation. Three fragment ions were then transmitted through Q3, yielding the signals used for quantification [41]. Q1 (MS1) and Q3 (MS2)

Table 1 – MRM conditions of target nonglycosylated peptides and stable isotope-labeled standard peptides. Asterisk indicates isotopically labeled sites.

No.	Sequence	Q1 (m/z, charge)	Q3 (m/z, +1)	Collision energy	Protein	Isotope (ΔDa)	Femtomole [a]
1	FEDGVLDPDYPR	711.831 (+2)	647.314 (y5)	20	Vitronectin (targeted peptide)		
			762.341 (y6)	20			
			875.425 (y7)	20			
	FEDGVLDPDYP*R	714.838 (+2)	653.328 (y5)	20	Isotope-labeled standard	P: 13C5, 15N (6.014)	50
			768.355 (y6)	20			
			881.439 (y7)	20			
2	GQYCYELDEK	624.266 (+2)	796.372 (y6)	18	Vitronectin (targeted peptide)		
			899.381 (y7)	16			
			1062.444 (y8)	16			
	GQYCYEL*DEK	627.775 (+2)	803.389 (y6)	18	Isotope-labeled standard	L: 13C6, 15N (7.017)	250
			906.398 (y7)	16			
			1069.461 (y8)	16			
3	TEDTIFLR	497.764 (+2)	435.271 (y3)	13	Alpha-1 acid glycoprotein 1 (targeted peptide)		
			649.403 (y5)	17			
			764.430 (y)	13			
	TEDTIFL*R	501.272 (+2)	442.288 (y3)	13	Isotope-labeled standard	L: 13C6, 15N (7.017)	25
			656.420 (y5)	17			
			771.447 (y6)	13			
4	NTVISVFGASGDLAK	739.896 (+2)	718.372 (y8)	16	Glucose-6-phosphate dehydrogenase (internal standard)		
			865.441 (y9)	15			
			1051.541 (y11)	22			
	NTVISVFGASGDL*AK	743.405 (+2)	725.390 (y8)	16	Isotope-labeled standard	L: 13C6, 15N (7.017)	500
			872.458 (y9)	15			
			1058.558 (y11)	22			

[a] The amounts of the stable isotope-labeled standard (heavy) peptides spiked in each 1 μg plasma sample.

were kept at 0.7 m/z FWHM (full width at half maximum) during the LC/MRM analyses.

2.5. *Identification and quantification for each peptide in MRM analysis*

All plasma samples were subjected to five repeat analyses by HPLC-microfluidics chip LC/MRM/triple quadrupole mass spectrometric analyses. Mass Hunter Quantitative Analysis Software (Agilent) was used for data analysis. The most intense peak (quantifier) of the three transition ions was used for quantitative analysis. The other two transition ions were used for the identification of each peptide. To identify with interference-free for each light and heavy peptide, the ratio of the first qualifier (the second intense transition of the three transitions) to the quantifier (the most intense transition of the three transitions) was calculated because the intensity of the first qualifier is higher than that of the second qualifier. The qualifier to quantifier ratio from light peptides was compared with that from heavy peptides to measure the quantitatively average accuracy of each peptide from total experiments (calibration curve, minimization and reproducibility of trypsin treatment time (for peptide TEDTIFLR, 0.5-h, 1-h, 2-h normal and all HCC data were excluded because of non-detection or low intensity) and validation (for peptide TEDTIFLR, all HCC data were excluded because of low intensity)). The quantitatively average accuracy of total experiments (more than 90%; 93.23% for GQYCYELDEK, 96.99% for FEDGVLDPDYPR, 95.83% for TEDTIFLR and 100% for NTVISVFGASGDLAK) was within 25%. The average precision was below median CV 25% in each experiment set (calibration curve, minimization and reproducibility of trypsin treatment time, and validation). Data that resulted in an average of three to five detection events of the five trials were accepted.

All of the data were used for quantification analysis because more than 90% from all the data measured by HPLC-microfluidics chip LC/MRM/triple quadrupole mass spectrometric analyses were interference-free. For quantitative analysis, the quantifier of the light peptide was compared with that of the heavy peptide. Data that resulted in an average light to heavy ratio of three to five detection events of the five measurements were accepted, and their CV (coefficient of variation) values were below 25%.

2.6. *Statistical analyses*

MRM results of targeted peptides from plasma samples were compared statistically using MedCalc (ver. 12.5.0). The diagnostic accuracy of each peptide from the candidate biomarkers was evaluated using receiver operating characteristic (ROC) curve analyses. The area under the curve (AUC) was reported at the 95% confidence interval (CI), and the sensitivity and specificity were also determined.

3. Results and discussion

Difficulties in verifying and validating putative protein biomarkers are often caused by complex sample preparation procedures required to determine their concentrations in a large number of plasma samples. Here, we focused on the development of MRM-based protein biomarker assays in plasma for clinical implementation, highlighting the need for rapid and

simple sample preparation without complex, time-consuming, and less reproducible sample pretreatment steps.

3.1. Selection of targeted nonglycosylated tryptic peptides and detection from undepleted human plasma samples

Depletion of major plasma proteins is one of the most promising approaches to utilize low-abundance biomarkers, as it can improve the depth of proteome analysis by 1–2 orders of magnitude [42–45]. However, the depletion procedure diminishes sample throughput, increases the %CV, and increases the cost and time of the assay. Thus, depletion may not be the best choice to include in the analytical workflow at the biomarker validation stage.

We analyzed undepleted human plasma samples without enrichment. We first considered four nonglycosylated peptides as targets (GQYCYELDEK from vitronectin, carbamidomethylated at the cysteine; TEDTIFLR from alpha-1 acid glycoprotein 1; TINPAVDHCCK from afamin, carbamidomethylated at the cysteine; and ENFLFLTPDCK from Kininogen-1, carbamidomethylated at the cysteine) based on our previous results [1]. Two peptides, TINPAVDHCCK and ENFLFLTPDCK, were excluded as they contain cysteine, which forms disulfide bonds in a protein. FEDGVLDPDYPR from vitronectin was added for comparison with the GQYCYELDEK peptide from the same protein. All three target peptides are located adjacent to N-glycosylation sites and were identified in the nanoLC–MS/MS analysis of undepleted plasma samples with high MASCOT search scores, as they originate from abundant glycoproteins in human plasma (Supplemental Table 2).

3.2. Optimization of trypsin treatment time

Masking of cleavage sites in proteins plays important roles in the stoichiometry of peptide production generated during proteolysis. Our study was based on the hypothesis that the glycan moieties attached at N-glycosylation sites in a protein affect proteolytic digestion by masking cleavage sites, and can generate different levels of peptides according to the degree of glycosylation.

To increase the glycan masking effect, we needed to digest proteins in their native state. Thus, we directly added trypsin to plasma sample solutions of 1 μg/μL without performing reduction and alkylation. All target nonglycosylated tryptic peptides were detected with stable isotope-labeled peptides in the MRM analysis and the interference-free of all data was confirmed in plasma (refer to "experimental" part). The three most intense fragment ions were selected as optimum transition ions and collision energy conditions were optimized for each peptide using Optimizer software in conjunction with the Agilent 6430 Triple Quad LC/MS system (Table 1). Chromatograms of three transition ions of the stable-isotope labeled heavy peptides for each targeted nonglycosylated peptide are shown in Supplemental Fig. 1.

The calibration curves of each targeted nonglycosylated peptide in the tryptic digested plasma (1 μg) showed strong linearity (R^2 = 0.9908 for FEDGVLDPDYPR in the range of 10 to 2000 fmol, R^2 = 0.9971 for GQYCYELDEK in the range of 10 to 2000 fmol, R^2 = 0.9958 for TEDTIFLR in the range of 1 to 2000 fmol, Supplemental Fig. 2). Supplemental Table 3 shows the %CV (all data were below 20%) of the heavy/light ratio of targeted nonglycosylated tryptic peptides for each calibration concentration in a 1 μg plasma digest without depletion, reduction, and alkylation.

The accessibility of the enzyme to cleavage sites increases as the protein structure is degraded by digestion. We therefore optimized the trypsin treatment time to maximize the difference between normal and HCC plasma samples.

Although proteome samples are generally digested for at least 16 h to ensure complete trypsin digestion, we reduced the trypsin treatment time while still providing reproducibility for quantitative analysis of nonglycosylated tryptic peptides adjacent to N-glycosylation sites. One normal and one HCC plasma sample were desalted and concentrated by 10-kDa MWCO membrane filters, then, equivalent amounts of trypsin were added directly to each plasma sample without depletion, reduction, and alkylation.

To quantitatively compare the results of nanoLC/MRM analyses for the three targeted nonglycosylated peptides (GQYCYELDEK, FEDGVLDPDYPR, and TEDTIFLR) in plasma samples subjected to different trypsin treatment times (0.5 h, 1 h, 2 h, 4 h, 6 h, and 16 h), each stable isotope-labeled standard peptide (heavy) of the three selected native nonglycosylated tryptic peptides (light) was spiked to equal levels into each plasma digest. NanoLC/MRM analyses were performed using triple quadrupole mass spectrometry connected to an Agilent Chip Cube LC/MS interface.

The quantitative differences of the three targeted nonglycosylated peptides between the normal and the HCC plasma samples were monitored as a function of the trypsin treatment time (0.5 h, 1 h, 2 h, 4 h, 6 h, 16 h). The MRM chromatograms used for quantitation of three target peptides according to the trypsin treatment time are shown in Supplemental Fig. 3. Data resulting in detection events over three of five trials (within a CV <25% except peptide TEDTIFLR of 6-h tryptic digested normal plasma) were considered as differentiating between normal and diseased (HCC) samples. The %CV of light/heavy ratios of targeted nonglycosylated tryptic peptides by nanoLC/MRM analyses in normal and HCC plasma samples according to the trypsin treatment time is shown in Supplemental Table 4.

3.2.1. FEDGVLDPDYPR and GQYCYELDEK for vitronectin

Fig. 1-1 shows the ratio of the peptide FEDGVLDPDYPR to a heavy-isotope labeled standard at different trypsin treatment times. In all experiments, normal plasma resulted in FEDGVLDPDYPR at higher concentrations than in HCC plasma. In normal plasma, vitronectin rapidly generated the peptide FEDGVLDPDYPR at short trypsin treatment times. The rate of production of the peptide FEDGVLDPDYPR slowed after 4 h of trypsin treatment. However, in HCC plasma, in which vitronectin is expected to be modified with the more bulky glycan moiety, FEDGVLDPDYPR was generated at lower rates, most likely due to greater steric hindrance by the bulky glycan.

Differences in the concentrations of FEDGVLDPDYPR in normal and HCC plasma peaked at 4 h and then decreased as the trypsin treatment continued. As discussed above, this may occur because the masking effects on cleavage sites by glycan may be weakened gradually as the glycoprotein is enzymatically digested.

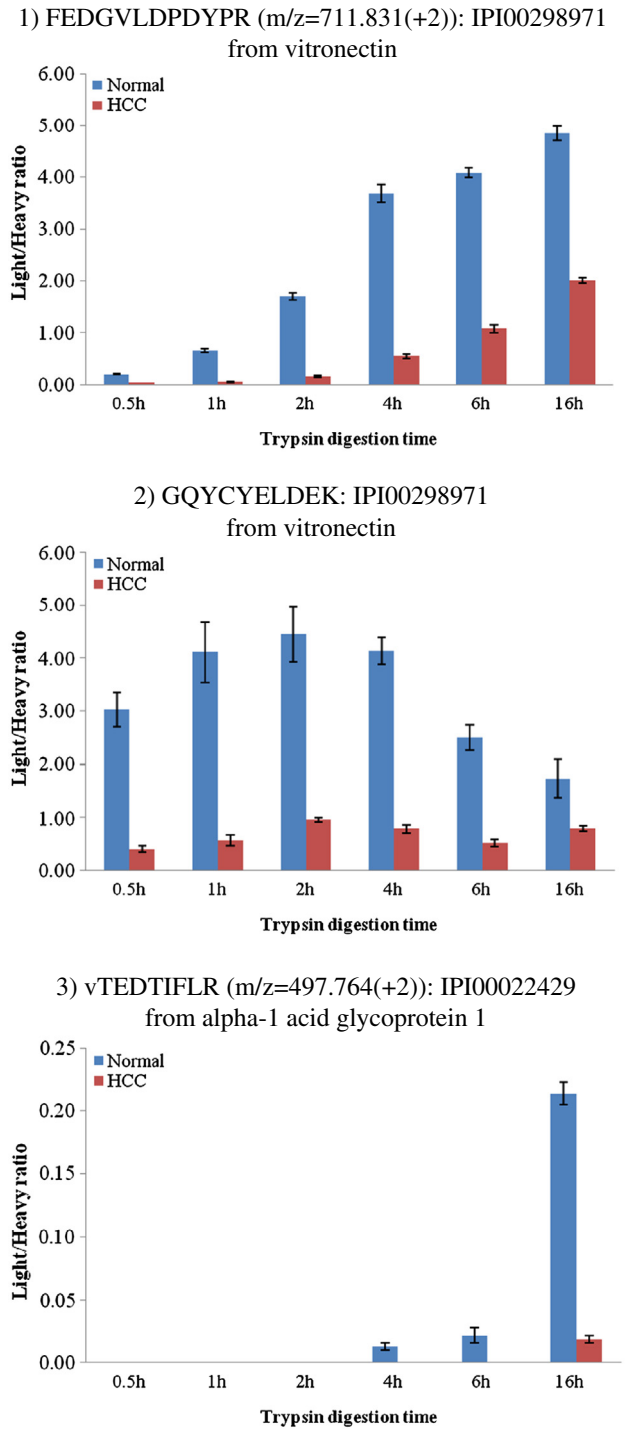


Fig. 1 – Light to heavy ratios of nonglycosylated tryptic peptides between normal and HCC human plasma samples, according to trypsin treatment time.

For the peptide GQYCYELDEK, normal plasma also resulted in GQYCYELDEK at higher concentrations than in HCC plasma in all experiments. However, Fig. 1-2 shows different tends than Fig. 1-1. The generation of GQYCYELDEK in normal plasma slowed after 4 h of trypsin treatment. This may be because the peptide GQYCYELDEK is additionally decomposed or modified by other events such as oxidation at cysteine residue after 4 h of digestion. For example, since this peptide contains a free cysteine, it may form disulfide bond with other peptides containing free cysteine [46]. Recently, Proc et al. [47] divided 45 proteins into 4 groups according to their digestion profiles. GQYCYELDEK belongs to a group that shows a maximum signal within 4 h of digestion, after which the signal decreases. In HCC plasma, the GQYCYELDEK concentrations increased at lower rates than in normal plasma, and may decrease after 16 h of trypsin treatment (similar to normal cases). The differences in the concentrations of GQYCYELDEK between normal and HCC plasmas were highest between 1 and 4 h of trypsin treatment.

3.2.2. *TEDTIFLR for alpha-1 acid glycoprotein*

Fig. 1-3 shows the quantitative differences observed for the peptide TEDTIFLR. This peptide was not detected after 0.5, 1, or 2 h of trypsin treatment by nanoLC/MRM analyses. However, it was detected in normal plasma after 4 h and in HCC after 16 h of trypsin treatment.

To maximize the glycan steric hindrance effect and the resulting quantitative differences in all targeted nonglycosylated peptides adjacent to N-glycosylation sites in glycoproteins between normal and diseased (HCC) plasma samples, we selected a 4-h trypsin treatment for limited digestion. This trypsin treatment time may be different according to target peptides because tryptic peptides could be generated at different speeds during digestion [47].

3.3. *Reproducibility of the 4-h trypsin treatment method*

The reproducibility of the trypsin treatment is an important factor when target proteins are quantified by peptide composition [47]. A non-reproducible trypsin treatment can cause errors in MS-based protein assays through peptide quantitation. Thus, the reproducibility of the 4-h trypsin treatment method was examined and compared with that of the 16-h trypsin treatment (conventional overnight digestion) method. The reproducibility of the three targeted nonglycosylated peptides was evaluated using a median intra-day %CV of five repeat experiments in one day and by a median inter-day %CV of three repeat experiments over three different days using nanoLC/MRM analyses (Table 2). One normal and one HCC undepleted plasma sample were directly digested with trypsin without reduction and alkylation. These digested samples were analyzed by nanoLC/MRM after

Table 2 – Coefficient of variation (CV) percentages of heavy/light ratios for targeted nonglycosylated tryptic peptides from intra-day and inter-day experiments in complex human plasma digests (1 μg, one normal and one HCC plasma sample). Each value was calculated as the average of three or five measurements among five measurements from MRM analyses, using an Agilent 6430 Triple Quad LC/MS system connected to an Agilent Chip Cube LC/MS interface.

No.	Peptide	Median inter-set %CV of light/heavy ratio			
		Intra-day		Inter-day	
		4 h	16 h	4 h	16 h
1	FEDGVLDPDYPR	4.48	3.64	5.58	3.35
2	GQYCYELDEK	8.08	12.58	7.29	10.17
3	TEDTIFLR	5.02	9.77	13.54	8.14

spiking of the stable isotope-labeled standard peptide (heavy) for each target peptide.

The results of both the 4-h and 16-h trypsin treatment methods were equally acceptable (Table 2). The median intra-day %CV of the light to heavy ratios for the three targeted nonglycosylated peptides ranged from 4.48 to 8.08% for the 4-h trypsin treatment, and 3.64 to 12.58% for the 16-h trypsin treatment. The median inter-day %CV values of the light to heavy ratio for the target nonglycosylated peptides were 5.58 to 13.54% with the 4-h trypsin treatment, and 3.35 to 10.17% with the 16-h trypsin treatment. The 4-h trypsin treatment method was as reproducible as the 16-h trypsin treatment method.

The average ratios of the target nonglycosylated peptides in normal and HCC plasmas in intra-day (five repeats in one day) and inter-day (three repeats in three days) experiments are shown for each peptide with error bars in Fig. 2. The y axis represents the ratio (light/heavy) of the quantified target peptides in normal and HCC plasmas. The average ratios of the two targeted nonglycosylated peptides (GQYCYELDEK and FEDGVLDPDYPR) in normal and HCC plasma samples were over 5 in all 4-h trypsin treatments, but were only about 3 in the 16-h trypsin treatment. The %CV of the normal to HCC ratios for the intra-day and inter-day experiments also indicated that the 4-h trypsin treatment was comparable to the 16-h trypsin treatment (Fig. 2).

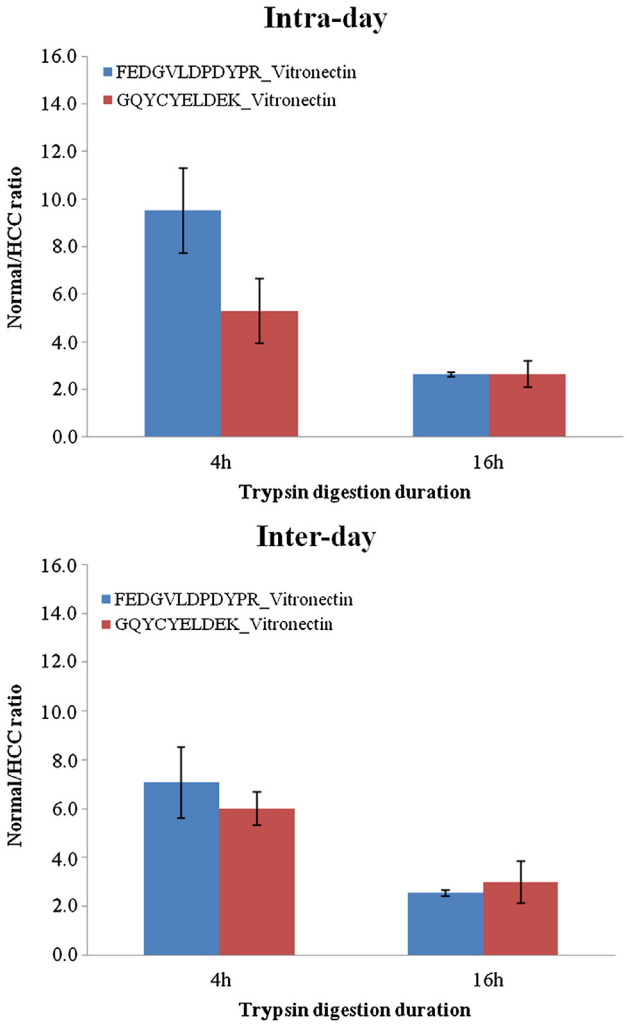


Fig. 2 – Reproducibility of five repeat experiments in one day (intra-day) and of three repeat experiments over three days (inter-day) for target nonglycosylated peptides in undepleted human plasma samples subjected to 4 or 16 h of trypsin treatment without reduction or alkylation.

For the peptide FEDGVLDPDYPR from vitronectin, the %CV values of the normal to HCC ratio were 18.69%/20.05% for intra-/inter-day tests of the 4-h trypsin treatment, and 3.00%/5.12% for intra-/inter-day tests of the 16-h trypsin treatment. For the peptide GQYCYELDEK from vitronectin, the %CV of the normal to HCC ratio for intra-day and inter-day tests of the 4-h trypsin treatment was also within an acceptable range for quantitative analysis (26.00%/11.08% intra-/inter-day 4-h trypsin treatment %CV; 21.11%/28.90% intra-/inter-day 16-h trypsin treatment %CV) (Supplemental Tables 5 and 6).

The peptide TEDTIFLR was detected from normal plasma treated with trypsin for 4 h and 16 h, but not from HCC plasma treated with trypsin for 4 h. Because this peptide was not detected in HCC plasma, the %CV of the normal to HCC ratio for TEDTIFLR from the 4-h trypsin treatment was not calculated. The median %CV of the light to heavy ratio from intra-day and inter-day experiments in normal plasma (Supplemental Tables 7 and 8) was reasonable for quantitative analysis. Based on these data, this peptide should be detected in only normal plasma after 4 h of trypsin treatment.

The 16-h trypsin digestion method showed better reproducibility. However, the 4-h trypsin treatment yielded larger quantitative differences that reveal differences between the two groups (normal and HCC) than the 16-h treatment, with acceptable %CV (<25) at shorter times (five LC/MRM measurements' coefficient variations (CVs) of light to heavy ratio are below 25%). For all peptides, the %CV of the light to heavy ratios and the %CV of the normal to HCC ratios of the target peptides in each intra-day and inter-day experiment according to the trypsin treatment time are shown in Supplemental Tables 5, 6, 7 and 8. NanoLC/MRM chromatograms of the three target peptides are shown in Supplemental Figs. 4 and 5.

3.4. *Validation of target nonglycosylated peptides adjacent to N-glycosylation sites in human plasma*

The three target nonglycosylated peptides adjacent to N-glycosylation sites in plasma samples were monitored using MS-based MRM assays with triple quadrupole mass spectrometry using our fast sample preparation method in 40 normal and 41 HCC plasma samples (sample information in Supplemental Table 1). As an internal standard protein, G6PD (glucose-6-phosphate dehydrogenase of yeast) was spiked equally into all plasma samples prior to trypsin digestion to evaluate technical errors during sample preparation. All stable isotope-labeled standard peptides (heavy) were equally spiked into plasma digests (Table 1) and were monitored using nanoLC–MRM assays. The average values of three to five detections from five measurements with CVs of 25% were accepted. Supplemental Table 9 shows the %CV of light to heavy ratios of target peptides from 40 normal and 41 HCC plasma samples. All peak area values from the four tryptic peptides were above the limit of quantification(S/N ratio >3), except for the peptide TEDTIFLR

values from 12 HCC plasma samples due to S/N ratio <3. These results are marked with double asterisks in Supplemental Table 9.

The internal standard protein G6PD was added equally to all plasma samples. The light to heavy ratio of its tryptic peptide NTVISVFGASGDLAK was theoretically expected to be the same in all samples. But, actually, the ratio from each plasma sample includes the technical error. Therefore, the average ratio was calculated from the light to heavy ratio of NTVISVFGASGDLAK from eighty-one plasma samples (normal 40 and HCC 41). The percentage of each sample was obtained from this average ratio. Data outside the range of acceptable technical error (70% to 130%) for NTVISVFGASGDLAK from G6PD were excluded. We hypothesize that trypsin digestion did not proceed normally in the excluded samples.

Supplemental Table 10 shows the average light to heavy ratio from five repeat measurements of the peptide NTVISVFGASGDLAK in each plasma sample and the average ratio for all of the plasma samples. In addition, the percentage values were calculated by dividing the ratios of 81 plasma samples (40 normal and 41 HCC samples) with the total average (0.58). A total of 78 samples showed acceptable results (70% to 130%), suggesting that 96% of the undepleted plasma samples were reproducibly digested by trypsin in 4 h without reduction and alkylation procedures within tolerance of 30%.

Supplemental Fig. 6 presents examples of MRM chromatograms for the three targeted nonglycosylated tryptic peptides and one peptide from the internal standard protein G6PD with those of the stable isotope-labeled standard peptides. The MRM analyses were statistically analyzed using MedCalc (ver. 12.5.0). Fig. 3 shows ROC curves and quantitative scatter plots showing the MRM results in the 40 normal and 41 HCC groups. The peptide from the internal standard protein G6PD was quantitatively equivalent in the normal and HCC groups (Fig. 3-1), but the three target nonglycosylated tryptic peptides were present at lower levels in the HCC groups than in the

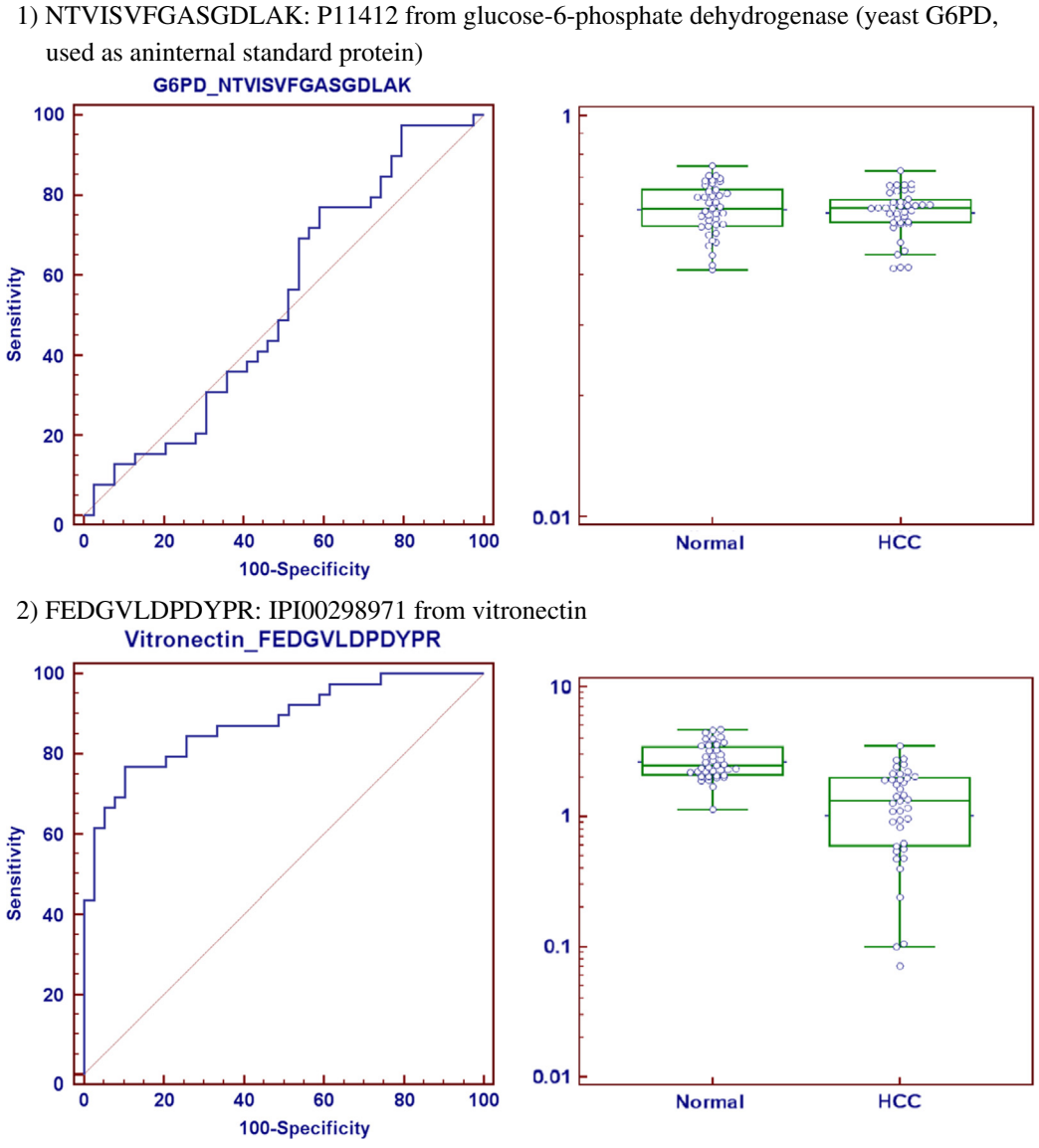
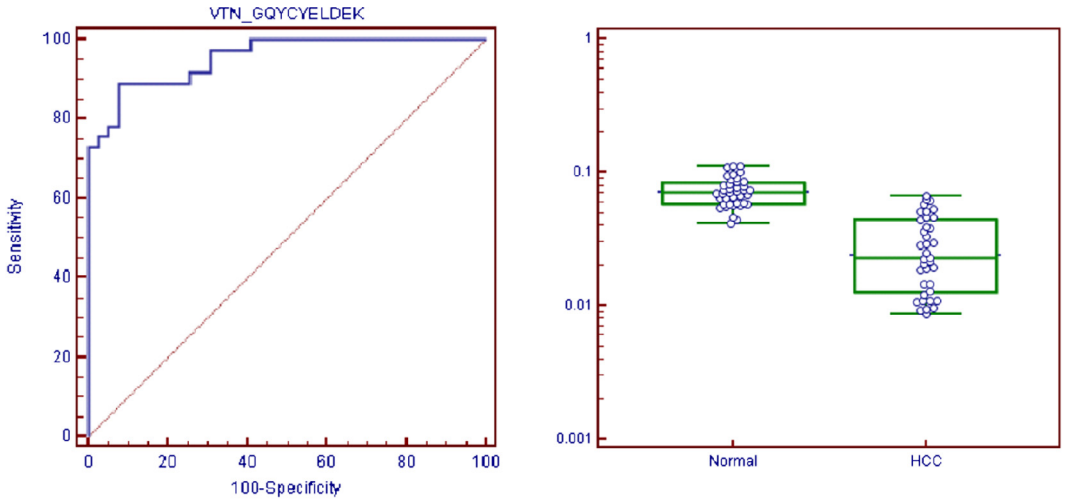


Fig. 3 – ROC curves and scatter plots of one internal standard peptide (from G6PD) and three target nonglycosylated peptides from normal and HCC plasma samples, as determined by MRM quantification using triple-quadrupole mass spectrometry.

3) GQYCYELDEK: IPI00298971 from vitronectin



4) TEDTIFLR: IPI00022429 from alpha-1 acid glycoprotein 1

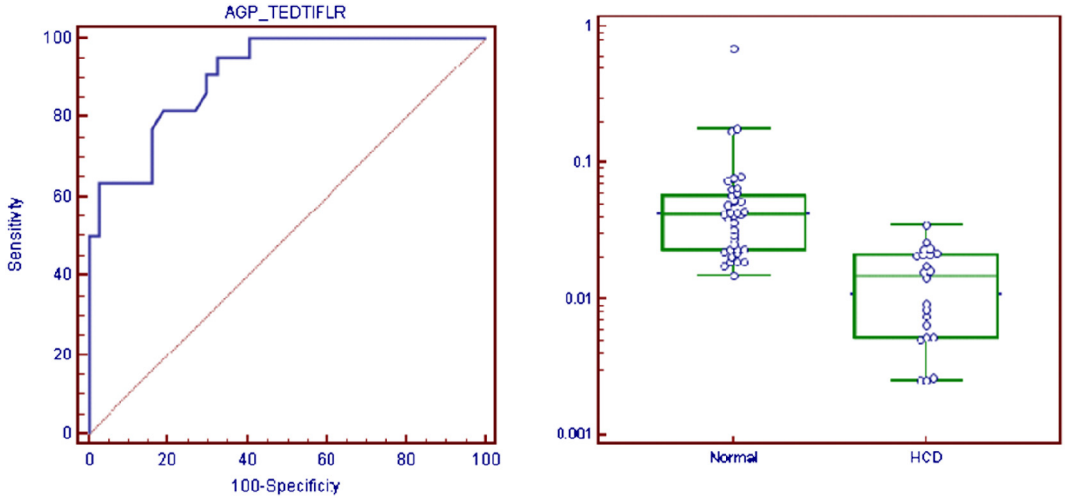


Fig. 3 (*continued*).

normal group. This decreased detection from HCC samples was consistent with findings of a previous study [1]. The sensitivity for the three target peptides ranged from 63.6 to 89.2%, and the specificity ranged from 89.7 to 97.3% at the 95% confidence interval (CI) (Table 3). The AUC values of GQYCYELDEK and FEDGVLDPDYPR from vitronectin and TEDTIFLR from AGP were 0.955, 0.880 and 0.907 respectively. The AUC of the peptide NTVISVFGASGDLAK from the internal standard protein G6PD was 0.535. This result suggests that the levels of this internal standard protein were the same in normal and HCC samples, as expected.

Vitronectin [48–50] and alpha-1 acid glycoprotein [51–53] are proteins well known to be related to HCC disease. Previous studies have reported that changes in the glycosylation in those proteins are related to hepatic diseases. Ogawa and coworkers reported the changes of the sialylation in vitronectin by glycosyltransferases in the analysis of rat plasma for liver regeneration after partial hepatectomy [54–56]. In other reports, the

Table 3 – Area under the ROC curve (AUC) values of one internal standard peptide and three targeted nonglycosylated peptides from normal and HCC plasma samples.

Peptide	# of normal plasma samples	# of HCC plasma samples	AUC in this study	Sensitivity (%)	Specificity (%)
NTVISVFGASGDLAK	39	39	0.535	76.9	41.0
FEDGVLDPDYPR	39	39	0.880	76.9	89.7
GQYCYELDEK	39	37	0.955	89.2	92.3
TEDTIFLR	37	22	0.907	63.6	97.3

levels of the glycopeptides **NGS**LFAFR (N169, N-glycosylation site) and **NIS**DGFDGIPDNVDAALALPAHSYSGR (N242, N-glycosylation site) were increased 1.5 fold in vitronectin from HCC human plasma in 1D LC–MS/MS analysis using iTRAQ labeling [49]. Those two glycopeptides are the nearest N-glycopeptides of our targets, GQYCYELDEK and FEDGVLDPDYPR, respectively.

Asialo alpha-1 acid glycoprotein (AsAGP) as a liver disease diagnostic candidate marker was validated by a solid-phase sandwich assay [51,53]. The AUC values of AsAGP were 0.919 and 0.946 for liver cirrhosis (LC) and HCC, respectively, from human sera (healthy 41, LC 230, HCC 72) [51]. Also, a study by Anderson has reported that glycopeptides with hyperfucose and N-acetylgalactosamine (GalNAc) occurred in AGP from liver disease samples [57].

Recently, reports [38,58,59] on MRM-based high-throughput assays of peptide biomarkers from a large number of plasma samples are greatly increased, raising the possibility for clinical use. They demonstrated that the CVs of target peptides quantified from plasma samples were within 20%. Percy et al. [44] showed intra- and inter-laboratory test results by six common LC/MS platforms using two sample kits (one for the effectiveness of the LC/MRM–MS and the other for that of the entire analytical workflow). The equivalent concentrations for the sample panel of 22 plasma proteins were obtained with CVs of 8% in log concentration regardless of the kit, operator and instrument. Kennedy et al. [60] reported that multiplexed MRM–MS has been successfully applied to monitor targeted proteins in biological specimens as the results of a pilot study designed to test the feasibility of a large-scale MRM assay of target peptides for protein measurements. In the validation of the 645 novel MRM assays from 319 proteins in human breast cancer cell, the median assay precision was 5.4% across three laboratories.

Here, we specially targeted nonglycosylated tryptic peptides adjacent to N-glycosylation sites from abundant glycoproteins in plasma. These nonglycosylated tryptic peptides were detected by MS analysis with higher sensitivity than the corresponding glycopeptides, showing the difference in concentration between normal and HCC plasma samples, which circumvented the need for major protein depletion and/or enrichment procedures. The use of undepleted plasma also provided benefits in terms of high throughput, high reproducibility, and cost effectiveness, and decreased the threshold for translation into clinical fields.

4. Conclusions

We have developed a method to effectively monitor biomarker candidates, nonglycosylated tryptic peptides adjacent to N-glycosylation sites, from a large number of plasma samples. This was applied to validate three nonglycosylated tryptic peptides as liver cancer biomarker candidates in 40 normal and 41 HCC human plasma samples (GQYCYELDEK (from vitronectin), FEDGVLDPDYPR (from vitronectin), and TEDTIFLR (from AGP)). Based on these analyses, the AUC (the area under the ROC (receiver operating characteristic) curve) value of GQYCYELDEK (from vitronectin) was 0.955; that from FEDGVLDPDYPR (from vitronectin) was 0.880; and that from TEDTIFLR (from AGP) 0.907.

Eventually, this method was proven to have considerable benefits for the validation of biomarkers from plasma samples. Since we targeted specific nonglycosylated peptides from glycoproteins that are abundant in human plasma, they are detectable in crude plasma without depletion and/or enrichment procedures. In addition, we reduced the total analysis time by decreasing the time required for trypsin digestion to enhance the steric hindrance effect by glycans. This strategy, therefore, provided the throughput necessary to monitor protein biomarkers, as well as quantitative accuracy in human plasma analysis. In the future, we plan to extend this approach to a large number of clinical cohort studies for translational research for cancer biomarker validation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.01.003.

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