Original Paper

Early Second-Trimester Peptidomic Identification of Serum Peptides for Potential Prediction of Gestational Diabetes Mellitus

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Key Words

GDM • Peptide • Serum • Prediction

Abstract

Background/Aims: Early screening and diagnosis is important for minimizing gestational adverse outcomes. Routine screening of gestational diabetes mellitus (GDM) at 24–28 weeks with 75 g oral glucose challenge test (OGCT) leaves limited time for intervention and prevention. This study aims to analyze maternal serum peptides in the early second-trimester for prediction of gestational diabetes mellitus (GDM). **Methods:** Serum samples were collected from 16–18-week pregnant women that visited Nanjing Maternity and Child Health Care Hospital from April to August 2015. According to gestational outcome with or without GDM in late pregnancy, 200 of serum samples from GDM mothers and controls were randomly divided into two subgroups. Peptidomic identification of serum peptides was performed by combining ultrafiltration and liquid chromatography-tandem mass spectrometry (LC-MS/MS) to investigate the differentially-expressed peptides between two groups. **Results:** A total of 297 identified peptides, originating from 228 proteins, were significantly differentially expressed in the GDM group compared with control. These precursor proteins may play critical roles in cell death of cortical neurons, elongation of cellular protrusions, and stabilization of microtubules. Major networks identified included those involving lipid metabolism, molecular transport and small molecule biochemistry. **Conclusion:** We provide for the first time a validated peptidome profile of early second-trimester serum in normal and GDM mothers, and we investigated the potential serum biomarkers for GDM. We concluded that 297 peptides could serve as potential biomarkers for GDM.

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Introduction

Gestational diabetes mellitus (GDM) is defined as a condition in which carbohydrate intolerance develops during pregnancy [1]. As rates of obesity and diabetes have increased rapidly in recent years, the prevalence of GDM is also increasing and is now estimated at 1.7%-11.6 % [2, 3]. The main adverse impact of GDM on pregnancy includes pregnancy-induced hypertension, fetal macrosomia, shoulder dystocia or birth injury, and premature delivery. Evidence also exists that women diagnosed with GDM in pregnancy are at a higher future risk of developing type 2 diabetes, metabolic complications, and cardiovascular diseases [4–6].

Currently, there is no consensus as to the ideal screening method for GDM. The two competing approaches are the “2-step and the “1-step” approaches. The 2-step approach begins at about 24 to 28 weeks’ gestation with an oral glucose challenge test (OGCT). This is followed by a 3-hour OGTT. The 1-step approach consists of a 2-hour OGTT only. Screening method and prevalence of GDM varies by geography. Body mass index (BMI), maternal age, and certain features of medical history are measures that must be changed with risk factors. However, all these measures are relatively weak as predictors [7, 8]. A method that can more effectively select women at high risk of GDM would be beneficial, especially if it was implemented early in pregnancy. Early intervention, such as diet, medication, and exercise, could avoid the development of GDM or avoid adverse maternal and fetal sequelae. To that end, we examined potential first-trimester serum markers for the prediction of subsequent GDM.

An area of increasing interest in the field of biomarker discovery is peptidomics, defined as the systematic qualitative and quantitative analysis of peptides in biological samples [9–11]. Evidence is accumulating to suggest that endogenous peptides play critical roles in the cardiovascular, endocrine, nervous, respiratory, inflammatory and reproductive systems [12, 13]. However, few studies have investigated the relationship between peptidome profiles in serum and early diagnosis of GDM.

Serum peptides can act as biomarkers, providing valuable information regarding diagnoses and disease states. In this study, we test the hypothesis that serum peptides might serve as potential biomarkers for predicting GDM in early pregnancy.

Materials and Methods

Ethics Statement

This study was approved by the Ethics Committee of the Gynecology and Obstetrics Hospital Affiliated to Nanjing Medical University (ethics committee approval number: NJFY-2015-18) and conducted in accordance with the Declaration of Helsinki. All subjects gave written informed consent prior to the start of the study. Sample collection was performed in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national).

Subjects

Serum samples were collected from 16- to 18-week pregnant women that visited the Nanjing Maternity and Child Health Care Hospital from April to August 2015. Clinical data and outcomes of GDM women and their paired controls were collected until delivery. All study subjects were healthy and showed no significant differences in age, body mass index (BMI), ethnicity, and gestational stage at the time of blood collection (Table 1). Exclusion criteria for both groups were as follows: hypertensive disorders; history of diabetes prior to conception; obesity; smoking; chemical dependency; use of assisted reproductive technology; multiple gestation; fetal congenital anomalies; and any other confounding pathologies, including hyperthyroidism and hypothyroidism.

Subjects were divided into two groups based on the diagnostic criteria of OGTT recommended by the International Association of the Diabetes in Pregnancy Study Group (IADPSG) at 24–28 weeks: healthy pregnant women and patients with GDM. GDM was diagnosed if fasting glucose ≥ 5.1 mmol/L, or if the 1

h result ≥ 10.0 mmol/L or the 2 h result ≥ 8.5 mmol/L. The remaining women were placed in the healthy group.

Serum sample collection, preparation, and ultrafiltration

Blood samples were obtained at 16 to 18 weeks of gestation at the time of screening for Down's syndrome. Samples were centrifuged at 4000 rpm at 4° C for 10 min to separate them into serum and cellular fractions, followed by centrifugation at 12000 rpm for 15 min to completely remove cell debris.

After thawing on ice, the samples were centrifuged at 12000 rpm for 15 min at 4° C. Then, 20% (v/v) Acetonitrile (CAN) was added to the supernatants, and the samples were briefly vortexed and incubated for 20 min at room temperature. Molecular weight cut-off (MWCO) filters (Millipore, USA) of 10 kDa were washed with 0.5 mL water. The samples were passed through the filters according to the manufacturer's recommendations. The flow-through containing peptides was recovered and lyophilized. Protein concentrations were determined using the bicinchoninic acid method (BCA; Pierce, Rockford, IL, USA).

Peptide desalting and multiplexed isobaric tandem mass tag (TMT) labeling

Samples containing 100 μ g peptides were reduced with 10 mM DTT at 60°C for 1 h, alkylated with 55 mM iodoacetamide (IAA) for 45 min at room temperature, then desalted and dried in vacuo (Speed Vac, Eppendorf). AF peptides were labeled with isobaric tags based on total peptide amount at room temperature for 1 h. TMT labeling was performed according to manufacturer's instructions (TMT 6-plex Label Reagent, Thermo Scientific). TMT results were generated from analysis of isobaric tag combinations. Three normal control AF samples were labeled with reagents 126, 127 and 128, and three VSD AF samples were labeled with reagents 129, 130 and 131.

Mass spectrometry analysis, peptide identification and quantification

Peptides were analyzed by nanoflow LC (Easy-nLC, Thermo

Fisher Scientific Inc., San Jose, CA) combined with an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher Scientific Inc.). Reverse-phase separation of peptides was performed using buffer A (2% ACN, 0.5% acetic acid) and buffer B (80% ACN, 0.5% acetic acid) with a gradient. Eluted peptides were electrosprayed at 1.8 kV into the mass spectrometer which was configured to collect high resolution ($R = 60,000$ at m/z 400) broadband mass spectra (m/z 375-1800) using the lock mass feature for the polydimethylcyclsiloxane ion generated in the electrospray process (m/z 445.12002). The 20 most abundant peptide molecular ions dynamically determined from the MS scan were selected for tandem MS using a relative collision-induced dissociation (CID) energy of 35%. The most intense product ion from the MS2 step was selected for higher energy collision-induced dissociation (HCD) MS3 fragmentation.

Extracted MS/MS spectra were searched against the database containing 20,194 human protein sequences (released May 2015) using in-house PEAKS software (version 7.0, Bioinformatics Solutions, Waterloo, Canada). The fusion target-decoy approach was used for estimation of the false discovery rate (FDR) and was set at $\leq 1\%$ ($-10\log P \geq 20.0$) at both protein and peptide levels. Peptides were considered positively identified only if peptide peaks were present in at least two spectra per sample.

Relative quantification of the serum peptidome was performed using the TMT labeling approach in the PEAKS Q module. Feature detection was performed separately on each sample using the expectation-maximization algorithm. Features of identical peptides from different samples were aligned using the high-performance retention time alignment algorithm [14]. Peptides and proteins were considered to be significantly altered between serum samples when the p-value was < 0.05 and the fold change was > 1.5 . Identified results were included during the last step of TMT labeling quantification.

Table 1. Clinical characteristics of control and GDM group

Variable	Control	GDM	p value
Maternal age (years)	27.45 $\pm$ 2.52	27.70 $\pm$ 2.18	0.37
Ethnicity	Han	Han	-
Gravidity	1.70 $\pm$ 0.86	1.65 $\pm$ 0.88	0.87
Parity	1	1	-
Prepregnancy BMI (kg/m ²)	21.48 $\pm$ 1.38	22.015 $\pm$ 1.32	0.21
Gestational stage at the time of blood collection	16.55 $\pm$ 0.76	16.65 $\pm$ 0.81	0.47
OGTT, fasting (mmol/L)	4.37 $\pm$ 0.36	4.92 $\pm$ 0.44	0.001
OGTT, 1 h (mmol/L)	8.82 $\pm$ 0.58	9.60 $\pm$ 0.86	0.004
OGTT, 2 h (mmol/L)	7.81 $\pm$ 0.43	8.42 $\pm$ 0.58	0.003
HbA1c (%)	4.94 $\pm$ 0.50	5.19 $\pm$ 0.47	0.09

Bioinformatics analysis

The isoelectric point (pI) of each peptide was calculated using the pI/Mw online tool (http://web.expasy.org/compute_pi/). Pathway and gene ontology (GO) analyses (<http://geneontology.org>) were carried to determine potential physiological functions. GO results included three categories; cellular component, biological process and molecular function. The threshold of significance was defined by the P-value and FDR. To further explore the significance of differentially-expressed peptides and their precursor proteins, they were imported into the Ingenuity Pathway Analysis (IPA) Software v7.1 (Ingenuity Systems, Mountain View, CA) for pathway analysis as described previously [15]. This software can also be used for biochemical, biologic, and molecular functions. The identified proteins were mapped to associated network functions that were generated from the Ingenuity Systems Knowledge Base. The MEROPS database (<http://merops.sanger.ac.uk>) was used to seek out candidate enzymes for given cleavage sites [16].

Statistical Analysis

SPSS 15.0 software (SPSS Inc, Chicago, IL). The t-test was used to compare values between control and GDM groups. A p value < 0.05 was considered to indicate statistical significance.

Results

Identification of various peptides in serum

The peptides from serum were directly analyzed by LC-MS/MS. A total of 297 peptides that were significantly differentially expressed (fold change > 1.5) are listed in Supplement 1. The molecular weight (MW) and isoelectric point (pI) distribution characteristics of these peptides are shown in Fig. 1. The MW vs. pI distribution of the identified peptides is shown in Fig. 1C. The MWs of all identified peptides were below 3 kDa, confirming that isolated peptides were of adequate purity.

Bioinformatics analysis of precursors of differentially expressed peptides

A total of 228 precursors of identified peptides were analysis using the PANTHER database to identify canonical pathways, diseases and biological functions and networks. A total of 37 statistically significant canonical

Fig. 1. Distributions of identified peptides. (A) Distribution of molecular weights (MW) of all peptides. (B) Distribution of isoelectric points (pI) of all peptides. (C) Scatter plot of MW versus pI distribution of the peptides. The data in all panels include all the peptides identified in both control and GDM sample groups.

Fig. 2. Diseases, biological functions, and networks of precursors of identified peptides determined by IPA. Proteins in red are upregulated, proteins in green are downregulated, and color intensity is proportional to the magnitude of the expression change between control and GDM groups. The blue dotted line indicates suppression, the yellow dotted line reflects an inconsistency with downstream molecules, and the gray line signifies that no prediction was made. The dotted lines suggest an indirect relationship.

pathways were identified, of which FXR/RXR activation, the intrinsic prothrombin activation pathway, dendritic cell maturation, the ILK signaling pathway, and the AMPK signaling pathway all had a positive z-score (Suppl. Fig S1 - For all supplemental material see www.karger.com/10.1159/000495538/).

A total of 197 statistically significant disease categories were identified, the most prominent of which were cell death of cortical neurons, elongation of cellular protrusions, and stabilization of microtubules. We arranged 12 differentially expressed protein nodes around these three categories (Fig. 2).

IPA was also used to analyze the molecular networks associated with main disease category. The principal networks identified were lipid metabolism, molecular transport and small molecule biochemistry. Lipid metabolism consisted of six parts: concentration of sterol, transport of phospholipid, quantity of vitamin A, clearance of lipid, clearance of triacylglycerol, and efflux of phospholipid. Eleven of 13 proteins were associated with sterol concentration, four are associated with phospholipid transport, and three were associated with lipid clearance, clearance of triacylglycerol and efflux of phospholipid (Fig. 3).

Putative biomarker peptides in the onset of GDM

All differentially expressed peptides in glucose and fat metabolism were analyzed. For example, Table 2 presents the peptides whose precursor proteins were associated with the concentration of D-glucose, including the amino acid sequences, amino acids with PTM, peptide location, and domain/region description. Fig. 4 shows the network of these precursor proteins.

Random validation of putative biomarker peptides using LC-MS/MS

To validate the differential expression levels of the putative biomarker peptides, we collected serum samples from 30 normal pregnant women and 30 women with GDM (GDM group) at 16–18 weeks of gestation. Five peptides, which were dramatically highly expressed or exclusively expressed in the GDM group, were randomly chosen and synthesized. Four peptides were identified by LC-MS/MS, and their expression levels were consistent with those obtained in the primary results (Fig. 5, Table 4).

Fig. 3. Network of interacting proteins and modules of functionally related proteins involved in the top disease and biological function subcategories of the lipid metabolism category. Proteins in red are upregulated, proteins in green are downregulated, and the color intensity is proportional to the magnitude of the expression change between control and GDM groups. The blue dotted line indicates suppression, the yellow dotted line indicates an inconsistency with downstream molecules and the gray line signifies that no prediction was made. The dotted lines suggest an indirect relationship.

Table 2. Identified differentially expressed peptides within the functional domains of D-glucose-related proteins, and their post-translational modifications (PTM)

Precursor protein	Peptides	Peptide location	Doman/region description	Fold change (GDM/Control)
APOA4	DKTVSLPELEQQQEQQEQQQ	365–377		2.477
	EELRRSLAP	217–225	AA approximate tandem repeats	5.837
C3	KTVAVRTLDP	940–949		1.821
CAMKK2	RPAPGG	217–222	RP domain	–2.855
EP300	AALGLPYQVN	468–478		4.330
IRS2	RTYSLTT	574–580		5.801
ITGB2	KGYPI	123–127	VWFA	–2.740
MAGEL2	ATATTQEASKTSVE	864–877		–2.054
	LSSEPGSSLPVVSEVASVSP	981–1001		1.631
MLXIPL	TAGSNNPCLTQ	520–530		–2.118
PARG	KWLGTP	452–457	A-domain	–2.735
PKD1	FPASPGATLVGP	269–280	WSC, PKD 1	–3.610
	TNQTVLIRS	2352–2360	REJ	–3.152
	EAATCAGPG	151–159	LRRCT	2.109
	EAGGCALNFGPRGSST	2306–2322	REJ	3.961
RNF213	YLLVLTKN	3041–3048	RING-type zinc finger domain	–3.971

Fig. 4. Network of D-glucose concentration. Red indicates upregulated proteins, green indicates downregulated proteins, and the color intensity is proportional to the magnitude of the expression change between the control and GDM groups. The blue dotted line indicates suppression, the yellow dotted line reflects an inconsistency with downstream molecules, and the gray line signifies that no prediction was made. Solid lines suggest a direct relationship, while the dotted lines suggest an indirect relationship.

Fig. 5. Validation of four randomly selected peptides. Four peptides that were dramatically highly expressed or exclusively expressed in the GDM group were validated by LC-MS/MS using serum samples from 30 normal controls and 30 GDM women at 16–18 weeks of gestation. A: FSSPNKTG, B: KKLPPAS, C: RPQGT, D: RTYSLTT

Identification of candidate enzymes

We selected “RTYSLTT,” which showed a 10.112-fold higher change in the GDM group compared to the control group, as our target. A search of the peptidase database (MEROPS) identified a number of candidate peptidases that would likely cleave the start and end bonds, also referred to as the K-RT and T-P sites of RTYSLTT (Table 3). This list included peptidases and proteases from different classes (cysteine, aspartic, metallo, serine, and threonine), as well as soluble and membrane-bound forms of the enzyme. According to this list, cathepsin V and cathepsin L could cleave RTYSLTT at both the cleavage sites, suggesting that RTYSLTT may be regulated by a single enzyme.

Discussion

Human serum is a rich source of endogenous peptides, many of which may serve as biomarkers. However, relatively few studies have explored the potential of this class of molecules as a source of GDM biomarkers. In this study, we focused on early detection and identification of alterations in peptide profiles between GDM patients and healthy controls.

Gestational diabetes mellitus (GDM) affects an increasing number of pregnant women worldwide. GDM is related to obesity, family history, ethnicity, and environmental factors. Poorly controlled glucose results in adverse outcomes for both mother and fetus. Therefore, early screening and diagnosing at appropriate gestational age is important to minimize adverse outcomes. Routine screening at 24-28 weeks with a glucose challenge test leaves limited time for intervention and prevention. There is evidence that many women manifest hyperglycemia early in pregnancy as a consequence of previously undiagnosed diabetes, or they display higher levels of insulin resistance and reduced capacity to increase insulin secretion, resulting in early decompensation as early onset GDM [17]. Recently several investigators reported early predictors of GDM may include high-sensitivity C-reactive protein [18], sex hormone binding globulin [19], soluble pro-renin receptor [20], glycosylated fibronectin [21] and galanin [22]. However, due to various limitations, these tests are not widely used. Using effective biomarkers for early prediction and screening of GDM, allows detection and treatment in a timely manner.

Genes and proteomics have been studied for years in the search for biological markers for a variety of diseases. However, due to the character of genes and proteomics that are not routinely secreted into biofluids [4], biomarker discovery has proved difficult. Peptidomics, low molecular weight (LMW) peptides and proteolytic fragments of molecular mass [23], are frequently secreted into serum. Therefore, peptidomics is becoming an intense focus of research on serum biomarkers. In these studies, protein mixtures are digested with trypsin, and the resulting peptide mixtures are analyzed by tandem mass spectrometry (LC-MS/MS). Today, many peptides have been found to biological markers for the cancer. For example, plasma fibronectin 1 (FN1) might be a tool for screening and diagnosis of renal cell carcinoma, as plasma levels of FN1 are up-regulated in the early stage of RCC [24].

Table 3. Peptide "RTYSLTT"-degrading enzymes predicted by MEROPS. Cathepsin V and cathepsin L (in bold) were the only enzymes predicted to cleave both "RTYSLTT" cleavage sites

Peptidase	Enzyme class
K-RT cleavage site	
Cathepsin V	Cysteine
Cathepsin L	Cysteine
Cathepsin K	Cysteine
Trypsin 1	Serine
T-P cleavage site	
Polyporopepsin	Aspartic
Necepsin-1	Aspartic
Necepsin-2	Aspartic
Cathepsin V	Cysteine
Cathepsin L	Cysteine
Cathepsin S	Cysteine
Falcpain-3	Cysteine
Cathepsin L3	Cysteine
Calpain-1	Cysteine
Calpain-2	Cysteine
Legumain, animal-type	Cysteine
Hedgehog protein	Cysteine
Matrix metallopeptidase-9	Metallo
Matrix metallopeptidase-3	Metallo
Matrix metallopeptidase-6	Metallo
Endothelin-converting enzyme 1	Metallo
Aminopeptidase P	Metallo
Pro-Hyp dipeptidase	Metallo
Kallikrein-related peptidase 6	Serine
Glutamyl endopeptidase I	Serine
Lactocepin-3	Serine
Keratinase	Serine
Proteasome subunit beta 5c	Threonine
20 S Constitutive proteasome peptidase complex	Threonine
26 S Proteasome peptidase complex	Threonine

Table 4. Information of four peptides and precursor proteins (G/C: GDM/Control)

Accession	Protein	Peptide	Fold change (G/C)	P value
P35658	NU214	FSSPNKTG	3.869	<0.001
Q6PFW1	VIP1	KKLPPAS	10.273	<0.001
P32004	L1CAM	RPQGT	#DIV/0!	<0.001
Q9Y4H2	IRS2	RTYSLTT	10.112	<0.001

Peptides are stable cleavage products generated from protein degradation by proteolysis [25]. It has been reported that peptidomic analyses of body fluids yielded disease biomarkers representing cleavage fragments [26]. Different cleavage sites may represent different protease activities, as the specificity of a protease is reflected by the cleavage events it triggers. We found that the proteases cathepsin V and cathepsin L can cleave RTYSLTT at both cleavage sites. Cathepsin V has been reported to be associated with type 1 diabetes [27], and cathepsin L activity can control adipogenesis and glucose tolerance [28].

One of the major challenges in peptidome research is processing and analyzing the large amount of information generated from a particular biological context. In our study, the peptidome was expanded to identify the precursor proteins, which could reveal important information regarding glucose and fat metabolism. For example, the peptide RTYSLTT is a fragment of insulin receptor substrate 2 (IRS-2), whose expression in pancreatic β -cells is essential to regulation of β -cell metabolism and survival. When IRS-2 expression is specifically increased in β -cells, it maintains adequate functional β -cell mass, and thereby forestalls the onset of diabetes [29]. IRS-2 depends on the downstream IRS/PI3-kinase/Akt signaling cascade required for insulin regulation in glucose homeostasis [30]. It has been suggested that IRS-2 plays an important role in glucose homeostasis, particularly in the liver [31]. Araki found that loss of IRS-2 causes diabetes in mice due to β -cell insufficiency and peripheral insulin resistance [32]. GDM is the consequence of the imbalanced insulin secretion that occurs to counteract insulin resistance. Previous studies in insulin-sensitive maternal peripheral tissues have showed altered expression of important proteins involved in insulin signaling in GDM. In skeletal muscle, the IRS-2 protein was more highly expressed and its phosphorylation was dramatically reduced in women with GDM compared with pregnant controls, leading to an alteration in the insulin signaling pathway. The expression of IRS-2 in adipose tissue was increased in women with GDM compared with the controls [33]. Some investigators found a correlation between IRS-2 expression and maternal insulin levels. Fetal insulin had a weak correlation with IRS-2 [34]. In our study, we found that RTYSLTT expression was substantially higher in the GDM group (fold change = 10.112) than in the control group. Whether this peptide was derived from IRS-2 degradation or from newly synthesized material, its differential abundance in early second-trimester serum of GDM women may reflect changes in the expression and function of IRS-2, and consequently be putative bioactive peptides for GDM.

PKD1 or polycystin-1 has been demonstrated to play an essential role in renal tubular morphogenesis. Disruption of its function causes cystogenesis in human autosomal-dominant polycystic kidney disease (ADPKD) [35]. Currently, however, there is no research on the role of PKD1 in diabetes. In the present study, we found four differentially expressed endogenous peptides derived from PKD1 mapped to the functional domains of this protein (Table 2). It is known that cleavage of precursor proteins by proteases generates peptides that may gain specialized functions not ascribed to their precursors, which could reflect the state of a cell under certain physiological conditions or pathological processes [36]. Therefore, it is reasonable to believe that although the functions of these PKD1-derived peptides may not have a strong association with their precursor protein, these peptides may play an important role in the early prediction of GDM.

Our results are the first peptidome profile of the differentially expressed peptides in maternal serum at 16–18 weeks of gestation, which was much earlier than the time of GDM onset. Therefore, a large amount of differentially expressed peptides identified in this study may not be the result of GDM but its cause or concomitant factors involved in its progression. Furthermore, our study provided a background for future research; impaired glucose tolerance and GDM remain a public health priority, and earlier pregnancy identification of at-risk women has the potential to increase the time for intervention and prevention.

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Disclosure Statement

The authors have declared no conflicts of interest.

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