A New Strategy for Early Diagnosis of Type 2 Diabetes by Standard-Free, Label-Free LC-MS/MS Quantification of Glycated Peptides

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The early diagnosis of diabetes, one of the top three chronic incurable diseases, is becoming increasingly important. Here, we investigated the applicability of an 18O-labeling technique for the development of a standard-free, label-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the early diagnosis of type 2 diabetes mellitus (T2DM). Rather than attempting to identify quantitative differences in proteins as biomarkers, glycation of the highest abundance protein in human plasma, human serum albumin (HSA), was monitored through quantitative analysis of HSA characteristic peptides using the 18O-labeling technique. Eight glucose-sensitive peptides and one glucose-insensitive peptide were discovered. The glucose-insensitive peptide served as the internal standard, and a standard-free, label-free LC-MS/MS method was developed. This method was then used to select putative biomarkers for T2DM in a clinical trial with 389 human plasma samples. As a result, three of the eight glucose-sensitive peptides (FKDLGEENFK, LDELRDEGK, and KVPVSTPTLVEVSIR) were selected and could be used as potential biomarkers for the early diagnosis of T2DM. Diabetes 62:3936–3942, 2013

Diabetes is a group of metabolic diseases characterized by high blood glucose levels, resulting from the body not producing enough insulin or the cells not responding to the insulin produced (1). Among the 3 main types of diabetes (type 1, type 2, and gestational diabetes mellitus), type 2 diabetes mellitus (T2DM) is mainly characterized by insulin resistance, which is different from type 1 diabetes mellitus (T1DM) that results from the body’s failure to produce insulin (1). Although diabetes has been controlled since insulin became available, it is still considered incurable and poses serious threats to human health (2). Reports have suggested that the hyperglycemic condition of patients with diabetes may be greatly alleviated or even reversed if it could be controlled at an early stage of diabetes (3). Thus, early detection and diagnosis of diabetes and prediabetes are becoming increasingly important in the treatment and prevention of diabetes (4).

Diabetes is currently diagnosed by recurrent or persistent hyperglycemia (5). In an effort to identify novel biomarkers for diabetes, research has shown that neither plasma glucose nor glycated hemoglobin (HbA1c) levels are able to be used in the early detection of diabetes (6–9). Plasma glucose is easily affected by patients’ lifestyle (e.g., food intake and body condition). HbA1c levels have a poor quantitative relationship with the accumulation of glycation and low sensitivity.

Therefore, additional biomarkers are being explored, including C-reactive protein (10), alanine aminotransferase (11), triglycerides (12), and plasminogen activator inhibitor (13). ELISA has been widely used in the detection of diabetes biomarkers (14–18). ELISA has high sensitivity because of the high catalytic efficiency of enzymes but requires knowledge of biomarkers and their corresponding antibodies. The concentration of serum advanced glycation end products (AGEs) is also recognized as a marker for monitoring the treatment of diabetes complications and can usually be detected by florescence (19). Besides these protein-level biomarkers, biomarkers on the peptide level, such as C-peptide, are becoming increasingly popular (20). Although a wide variety of biomarkers for diabetes exist, the sensitivities, feasibilities, and capabilities of these methods to detect diabetes at an early stage are limited.

With high sensitivity and specificity, mass spectrometry (MS)-based quantitative proteomics methods, including absolute and relative quantitative proteomics, have been applied in diabetes pathogenesis and biomarker explorations (21,22). Absolute quantitative proteomics uses a standard curve to quantify target proteins. A candidate diabetes biomarker, C-reactive protein, has been quantified using MS-based methods both with and without an affinity removal system (23,24). In addition, relative quantitative proteomics has also been used in diabetes biomarker discovery and mechanism studies. Combined with isotope-labeling techniques, Zhang et al. (25) demonstrated that S-nitrosation might be involved in the pathophysiology of T2DM. Different glycation states of hemoglobin have also been monitored in vitro with the glycation isotope–labeling technique (26). Label-free techniques have also been used in the discovery of five biomarker candidates for T1DM. After six highly abundant plasma proteins were removed, liquid chromatography (LC)-MS was used in the identification and quantification of plasma proteins. Relative quantification was performed based on a mass spectra intensity global normalization procedure (27). In addition, Zhang and colleagues (28–30) developed a bottom-up proteomics method that applied boronate affinity chromatography and electron transfer dissociation tandem MS (MS/MS) analyses in the study of glycated proteins and peptides. They recently reported the identification of 7,749 unique glycated peptides through a similar process after removing 12 highly abundant plasma proteins (31). In our laboratory, we previously identified a series of AGE-precursors and AGEs after
comparing glycated BSA peptides with unmodified BSA peptides (32).

Nonenzymatic glycation of proteins, also known as the Maillard or browning reaction, has recently attracted increased attention in proteomics research due to its clinical relevance to diabetes and related complications (33,34). As a nonenzymatic reaction, the Maillard reaction (glycation) does not have chemical selectivity. Therefore, higher abundance proteins have more opportunity to be modified by glucose in the plasma. As a result, quantification of glycation levels on high-abundance proteins by MS analysis is much more sensitive than testing plasma glucose to determine the presence of diabetes.

In this work, we developed a standard-free, label-free MS-based proteomics method for the early diagnosis of T2DM. The highest-abundance protein in human plasma (−62%), human serum albumin (HSA), served as a glycation collector and was monitored through quantitative analysis of HSA characteristic peptides. This strategy is different from the traditional concept that almost all functional proteins are low-abundance proteins and, thus, that biomarkers for diseases should be screened from low-abundance proteins. Moreover, the use of the highest-abundance protein as the target makes the quantification accurate because glycation of high-abundance proteins is dominant in vivo, and the use of high-abundance protein as a target protein can drastically improve the sensitivity of the MS analysis by reducing detection errors. In addition, another reason peptide biomarkers were preferred in our study rather than protein biomarkers was because proteins in vivo could undergo several types of post-translational modifications, such as ubiquitination, phosphorylation, glycosylation, methylation, and acetylation (35). Each post-translational modification can possibly change the molecular weight of the target protein, which leads to difficulties in MS analysis.

RESEARCH DESIGN AND METHODS

Study design. Figure 1 shows a complete workflow of the experiment. First, 18O-labeling, a convenient proteomics technique (36), was used to screen glucose-sensitive and glucose-insensitive peptides within HSA characteristic peptides. Next, a glucose-insensitive peptide was selected as the internal standard, and glucose-sensitive peptides underwent further screening as biomarker candidates. With the glucose-insensitive peptide as the internal standard, a standard-free quantitative method was then applied in the discovery of glucose-sensitive and glucose-insensitive peptides within HSA characteristic peptides. This strategy is different from the traditional concept that almost all functional proteins are low-abundance proteins and, thus, that biomarkers for diseases should be screened from low-abundance proteins. Moreover, the use of the highest-abundance protein as the target makes the quantification accurate because glycation of high-abundance proteins is dominant in vivo, and the use of high-abundance protein as a target protein can drastically improve the sensitivity of the MS analysis by reducing detection errors. In addition, another reason peptide biomarkers were preferred in our study rather than protein biomarkers was because proteins in vivo could undergo several types of post-translational modifications, such as ubiquitination, phosphorylation, glycosylation, methylation, and acetylation (35). Each post-translational modification can possibly change the molecular weight of the target protein, which leads to difficulties in MS analysis.

HPLC/electrospray ion trap MS. The same Agilent 1100 series HPLC system was coupled with an Agilent MS trap for qualitative analysis. The same chromatographic and ESI conditions were used. MS and MS/MS analyses were performed on favorable fragmentations to double- and triple-charged ions. Ion trap MS also had a mass window of 300–1,800 amu was used for the TOF MS instrument.

FIG. 1. Schematic workflow of the experiments.
Clinical sample collection. Clinical samples for the T2DM, IGT, and NGT groups were provided by the Beijing Institute of Technology Hospital, Beijing, China. During 2009–2010, upon request, 389 volunteers provided blood samples with questionnaires about their characteristics, including basic characteristics (age, sex, and BMI), family history of diabetes, and symptoms of early diabetes. Biochemical examinations of their blood samples were also performed and recorded. This clinical study was conducted in accordance with the requirements of medical ethics.

RESULTS

The participants who provided blood samples were classified into 3 groups: T2DM, IGT, and NGT, as described in the RESEARCH DESIGN AND METHODS. Table 1 lists the basic characteristics of the study participants. As expected, patients classified into the T2DM group had a higher BMI and a higher probability of having a family history of diabetes than patients classified into the NGT group. Furthermore, patients in the T2DM group were more inclined to feel that they had symptoms of diabetes and its complications than patients in the NGT group. Biochemical examinations showed that patients in the T2DM group had significantly higher glucose, cholesterol, alanine aminotransferase, triglyceride, and LDL levels than patients in the NGT group.

In an environment with high glucose concentrations, glycation will occur on certain sites of HSA (i.e., lysines, arginines, and the N-terminal free amine), which are called glycation-sensitive sites. Peptides with glucose-sensitive sites were potential glucose-sensitive peptides, whereas peptides without glucose-sensitive sites were potential glucose-insensitive peptides. With increasing degrees of glycation, three types of peptides were expected after HSA was digested to peptides: 1) glucose-insensitive peptides, 2) glucose-modified glucose-sensitive peptides, and 3) unmodified glucose-sensitive peptides. In this study, type 1 and type 3 peptides were quantitatively analyzed and used for diagnosis of T2DM.

The $^{18}$O-labeling proteomics technique was first used in vitro samples to search for type 1 and type 3 peptides. Within glucose-insensitive peptides (summarized in Supplementary Table 1), the peptide AAFTECCQAADKAACLLPK ($m/z = 977.4$ and retention time of 28.7 min) was chosen as the internal standard peptide. As shown in Fig. 2, the peak area ratio ($^{18}$O-to-$^{16}$O) of peptide AAFTECCQAADKAACLLPK was close to 1 ($0.961 \pm 0.077$; Fig. 2A) in 72 MS runs of 36 samples, where 4 different glucose concentrations were used for incubations of 10, 20, or 30 days. After glycated HSA samples were digested with trypsin, 57 unmodified peptide sequences with usable sequence coverage of 83% in total were matched in the database search query by exporting their MS/MS data to Mascot (Supplementary Table 2). Similar results have been reported (36).

### TABLE 1

Basic characteristics of individuals who participated in this clinical study

| Characteristics                                      | T2DM group | IGT group | NGT group |
|------------------------------------------------------|------------|-----------|-----------|
| $n$                                                   | 73         | 63        | 253       |
| Basic characteristics                                 |            |           |           |
| Age (years)                                          | 56 ± 16    | 52 ± 13   | 48 ± 13   |
| Sex (n)                                              | Male 39    | 33        | 132       |
|                                                     | Female 34  | 30        | 121       |
| BMI (kg/m²)                                          | **29.2 ± 2.5** | 25.6 ± 1.6 | 26.0 ± 1.7 |
| Family history of diabetes (%)                       | **42.0**   | 36.0      | 18.0      |
| Symptoms (%)                                         |            |           |           |
| Fatigue                                              | 34.2       | 30.1      | 10.7      |
| Rapid decline in visual acuity                       | 49.3       | 31.7      | 12.5      |
| Acral itch, pain, or numbness                        | 26.1       | 6.3       | 5.1       |
| Lower-extremity edema                                | 4.1        | 7.9       | 5.9       |
| Prone to infection                                   | 8.2        | 4.7       | 1.2       |
| Red face                                             | 2.7        | 1.6       | 2.0       |
| Simplex                                              | 1.4        | 3.2       | 1.2       |
| Cutaneous xanthoma                                   | 2.7        | 3.2       | 2.0       |
| Repeated infections on vulva or prostate             | 6.8        | 4.0       | 2.8       |
| Urinary tract infection                              | 8.2        | 4.0       | 3.2       |
| Easy to have hypoglycemia                            | 2.7        | 1.6       | 1.2       |
| Gastrointestinal disorders                           | 5.4        | 4.0       | 2.0       |
| Lose weight suddenly                                 | 2.7        | 3.2       | 1.6       |
| Diagnostic tests                                     |            |           |           |
| Alanine aminotransferase (U/L)                       | 31 ± 16    | 23 ± 4    | 18 ± 5    |
| Blood urea nitrogen (mmol/L)                         | 4.5 ± 0.8  | 4.9 ± 1.4 | 4.7 ± 1.5 |
| Creatinine (μmol/L)                                  | 76 ± 16    | 80 ± 15   | 77 ± 14   |
| Uric acid (μmol/L)                                   | **298.2 ± 83.4** | 282.9 ± 48.2 | 279.4 ± 48.2 |
| Glucose (mmol/L)                                     | **10.8 ± 4.5** | 7.2 ± 0.8 | 5.2 ± 0.5 |
| Triglyceride (mmol/L)                                | **2.36 ± 1.55** | 1.88 ± 1.60 | 1.87 ± 1.62 |
| Cholesterol (mmol/L)                                 | **5.5 ± 0.9** | 4.9 ± 1.1 | 4.7 ± 0.9 |
| HDL (mmol/L)                                         | 1.5 ± 0.5  | 1.4 ± 0.4 | 1.4 ± 0.3 |
| LDL (mmol/L)                                         | **3.3 ± 0.6** | 2.7 ± 0.6 | 2.4 ± 0.5 |
| Hemoglobin                                           | All negative | All negative | All negative |

Continuous variables are expressed as mean ± SD and categorical variables as indicated. The values in boldface represent significant differences between the T2DM and IGT/NGT groups. *By self-evaluation of volunteers.
As an example, Fig. 2B shows the concentration variation of one of these glucose-sensitive peptides, FKDLGEENFK (m/z = 614.8), with a retention time of 25.5 min. Glycation is dependent on the concentration of glucose and has an accumulation effect. As a result, the concentration of a glucose-sensitive peptide in glycated HSA samples will be a function of the glucose concentration and the incubation duration. Supplementary Table 3 reports the relative intensity (peak area ratio 16O-to-18O of the LC-TOF MS data) variations of selected peptide ions (usually detected in each MS run) in glycated HSA samples. By calculating their statistical correlations with respect to glucose concentration and incubation duration, eight peptides were provisionally recognized as unmodified glucose-sensitive peptides (or type 3 peptides) for HSA (summarized in Table 2) because they exhibited a steady decrease in peak area ratio (significant difference) against increasing glucose concentration and incubation duration. The decreasing ratio among peak area ratios (16O-to-18O) of these glucose-sensitive peptides with different incubation durations is presented in Supplementary Table 4, and the peak area ratios of three peptides with respect to glucose concentration and incubation times is shown in Fig. 3. Detailed information describing statistical correlation values (P values) with respect to glucose concentrations is given in Supplementary Table 5.

Finally, when the standard-free and label-free method was applied to the clinical samples, the amount of three peptide ions (m/z = 537.7, 614.8, and 820.5) of the eight potential biomarkers showed significant differences among the T2DM, IGT, and NGT groups. Figure 4 shows the box plot of these three peptides for the T2DM, IGT, and NGT groups and Supplementary Table 6 provides the corresponding P values. These three peptides (LDELRDEGK, FKDLGEENFK, and KVPQVSTPTLVEVSR) showed significant decreases in their concentrations in the T2DM group compared with the IGT group. Furthermore, there were significant differences (P < 0.01) between both NGT/IGT and IGT/T2DM groups for peptides FKDLGEENFK and KVPQVSTPTLVEVSR, indicating that these three peptides could be used as potential biomarkers for the early diagnosis of T2DM.

In addition, Supplementary Fig. 1 shows the ROC analysis for early diagnosis of T2DM. Peptides FKDLGEENFK and KVPQVSTPTLVEVSR showed excellent sensitivities and specificities between the NGT/IGT groups (Supplementary Fig. 1A) and the NGT/IGT and T2DM groups (Supplementary Fig. 1B). The parameters of the ROC analysis (sensitivity, specificity, area under the curve, and cutoff point) are given in Supplementary Table 7.

DISCUSSION
In this study, we developed a novel method (outlined in Fig. 1) for the detection of glycated peptides as a potential technique for the early diagnosis of T2DM. During the process of 18O-labeling, control HSA was prepared in the presence of an 18O-enriched PBS buffer, whereas glycated HSA was prepared in an 16O-enriched buffer. After incubation and digestion, the corresponding glycated HSA peptide sample was mixed with the control sample in a 1:1 ratio. Supplementary Fig. 2 shows the relative peak area ratios (16O-to-18O) with respect to incubation day and glucose concentration (Conc.) are shown for a glucose-insensitive peptide (AAFTECCQAADKAACLLPK) (A) and a glucose-sensitive peptide (FKDLGEENFK) (B). The error bars are SD values of triplicate.

![FIG. 2. The relative peak area ratios (16O-to-18O) with respect to incubation day and glucose concentration (Conc.) are shown for a glucose-insensitive peptide (AAFTECCQAADKAACLLPK) (A) and a glucose-sensitive peptide (FKDLGEENFK) (B). The error bars are SD values of triplicate.](image)

**TABLE 2**

Characteristics of the eight glucose-sensitive peptides identified through MS

| Peptide location | m/z   |Retention time (min) | Charge | Sequence                  | Mascot score |
|-----------------|-------|---------------------|--------|--------------------------|--------------|
| 35–44           | 614.8 |24.1                 | 2      | FKDLGEENFK               | 50           |
| 162–168         | 464.3 |22.9                 | 2      | YLYEIR                   | 31           |
| 206–214         | 537.7 |22.8                 | 2      | LDELRDEGK                | 31           |
| 243–249         | 438.2 |31.5                 | 2      | LSQRFPK                  | 40           |
| 258–264         | 395.2 |19.7                 | 2      | LVTDLTK                  | 41           |
| 348–360         | 812.5 |36.3                 | 2      | DVFLGMFLYEYAR            | 64           |
| 384–396         | 691.8 |25.1                 | 2      | CCAAADPHECYAK            | 53           |
| 438–452         | 820.5 |31.2                 | 2      | KVPQVSTPTLVEVSR          | 53           |

**FIG. 2.** The relative peak area ratios (16O-to-18O) with respect to incubation day and glucose concentration (Conc.) are shown for a glucose-insensitive peptide (AAFTECCQAADKAACLLPK) (A) and a glucose-sensitive peptide (FKDLGEENFK) (B). The error bars are SD values of triplicate.
ratio (v/v), and the mixtures were injected into HPLC/ESI-TOF MS for quantitative analysis. This allowed for the identification of an internal standard peptide and three promising glucose-sensitive peptides, which may have potential application in the early diagnosis of T2DM. Then, following the identification of an internal standard peptide and putative biomarker peptides, a standard-free and label-free method was developed and applied to clinical samples. Thus, instead of using the 18O-labeling technique for final quantification and validation of the data, we performed quantification of the putative biomarker peptides by calculating the peak area ratios of the putative biomarker peptide over the internal standard peptide (m/z = 977.4).

Peptide AAFTECCQAADKAACLLPK (m/z = 977.4 and retention time of 28.7 min) was chosen as the internal standard peptide because its concentration was independent of the concentration of glucose solution and the incubation duration. In addition, this peptide had a stable and strong enough signal (× 10^5 in vitro and × 10^4 in vivo) in the TOF mass spectrum, which would ensure analysis accuracy. Internal standards in quantitative analysis usually have to be exogenously added. However, there are many limits to the selection of an internal standard; for example, the internal standard substance must be completely dissolved in the sample, no interactions must occur between the internal standard and the component to be measured, and the retention time of the two peaks should be close in the chromatogram. We solved this problem in our study by using the above-mentioned peptide, which was cleaved from HSA itself.

In addition, as shown in Supplementary Fig. 2, the glucose-insensitive peptide used as the internal standard (AAFTECCQAADKAACLLPK) was located at the center of the HSA protein. Thus, the location of this peptide within the HSA protein may contribute to its glucose-insensitivity, because it would be difficult for small molecules (such as glucose) to attack the modification site.

As reported in Supplementary Table 1, three glucose-insensitive peptides were obtained from the in vitro study. That the three peptides are insensitive to glucose in the clinical samples (the T2DM group, 73 cases) was also confirmed. Therefore, the peptide (AAFTECCQAADKAACLLPK) was used as an internal standard for the clinical study.

HSA has no unique peptide sequence, and today we know that there are hundreds of different proteins in the human plasma. HSA characteristic peptides are the peptides that are present in HSA but are not the highest...
abundance proteins in human plasma. However, HSA itself is the highest abundance protein in human plasma, and proteins that have similar amino acid sequences to HSA are all albumins. Importantly, studies have demonstrated that no other albumin protein is among the 137 highest abundance proteins in human plasma (22).

In conclusion, this report describes the development of a standard-free and label-free LC-MS/MS method for the discovery of putative biomarkers for T2DM. In vitro glycation of the most abundant protein, HSA, in human blood plasma was monitored through quantitative analysis of HSA characteristic peptides using 18O-labeling techniques. Verified by clinical samples, three peptides (FKDLGEENFK, LDELRDEGK, and KVPQVSTPTLVEVSR) exhibited significant differences between T2DM and NGT groups, and two of these peptides (FKDLGEENFK and KVPQVSTPTLVEVSR) also showed significant differences between both NGT/IGT and IGT/T2DM groups. Our results indicate that these peptides could be putative biomarkers for the early diagnosis of T2DM.

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M.Z. collected data and prepared the manuscript. W.X. was involved in data integration and analysis and also in preparing the manuscript. Y.D. conceived the idea and designed the study. Y.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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