Analysis of Urinary Proteome Modifications in Patients with Different Glycated Hemoglobin Levels

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Abstract

Diabetes represents a major global public health challenge, where early diagnosis and timely intervention are of paramount importance. Glycated hemoglobin serves as a biomarker for glycemic management, with its levels demonstrating a continuous association with diabetes risk. This study conducted a comparative analysis of urinary proteome modifications between two patient groups with distinct glycated hemoglobin levels ([6.4$±0.7]±$1.6]%) and a healthy control cohort, identifying 1,954 and 5,545 differentially modified peptides, respectively. In these two groups, differentially modified peptides exhibiting presence-to-absence or absence-to-presence alterations accounted for 48.8% and 86.5%, respectively. Furthermore, random grouping tests revealed that at least 90.6% and 94.1% of the differentially modified peptides in the two groups were not randomly generated. Collectively, these findings demonstrate that urinary proteome modifications can comprehensively and systematically reflect changes associated with elevated glycated hemoglobin levels, with different glycated hemoglobin levels corresponding to distinct modification signatures. This suggests that urinary proteome modifications hold potential for reflecting glycated hemoglobin status, thereby opening new avenues for research into the early diagnosis of diabetes.

Full Text

Analysis of Urinary Proteome Modifications in Patients with Different Glycated Hemoglobin A1c Levels

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Abstract: Diabetes, a major global public health concern, requires early diagnosis and timely intervention. Glycated hemoglobin A1c (HbA1c) serves as a biomarker of glycemic management, with its levels showing a continuous relationship with the risk of developing diabetes. In this study, urinary proteome modifications were compared between each of the two patient groups with different HbA1c levels ([6.4$±0.7]±$1.6]%) and healthy controls. A total of 1,954 and 5,545 differentially modified peptides were identified in the two groups, respectively. Within each group, differentially modified peptides exhibiting changes from presence to absence or vice versa accounted for 48.8% and 86.5%, respectively. Additionally, results from the randomized grouping test indicated that at least 90.6% and 94.1% of these differentially modified peptides in each group were not randomly generated. In conclusion, urinary proteome modifications comprehensively and systematically reflect changes associated with elevated HbA1c levels, with distinct modification profiles corresponding to different HbA1c levels. These findings suggest that urinary proteome modifications have the potential to reflect HbA1c levels and offer a new perspective for research on the early diagnosis of diabetes.

Keywords: urine; proteomics; modifications; glycated hemoglobin A1c; diabetes mellitus; biomarker

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1 Introduction

Diabetes represents a major global public health challenge, with an estimated prevalence of 10.5% (536.6 million people) among individuals aged 20–79 years in 2021, projected to increase to 12.2% (783.2 million) by 2045 [1]. Research indicates that compared with non-diabetic populations, diabetic patients face significantly elevated mortality risks from cardiovascular disease, cancer, chronic obstructive pulmonary disease, and other conditions, severely impacting quality of life [2]. Consequently, early diagnosis and timely intervention for diabetes are critically important.

Biomarkers are monitorable changes associated with physiological or pathophysiological processes that play vital roles in disease diagnosis, treatment, and prognosis. Glycated hemoglobin A1c (HbA1c) reflects average blood glucose levels over the preceding 2–3 months and serves as a biomarker for diabetes [3]. The International Expert Committee recommends using HbA1c $\geq$ 6.5% as a diagnostic criterion for diabetes [4]. Moreover, diabetes risk based on HbA1c levels follows a continuous distribution, with individual risk progressively increasing as

HbA1c approaches the diagnostic threshold. Notably, individuals with HbA1c $\geq$ 6% but $<$ 6.5% may represent the subpopulation at highest risk for progression to diabetes [4].

Urine, as an ultrafiltrate of blood, is not subject to strict homeostatic regulation and can accommodate and accumulate more extensive changes without harming the organism, thereby reflecting changes across all organs and systems earlier and more sensitively. This makes urine an excellent source for biomarker discovery [5]. Leveraging these advantages of urine for comprehensive, systematic, and sensitive reflection of physiological status, we investigated whether differences among patients with varying HbA1c levels could be characterized through urinary proteome modifications. This study explores urinary proteome modification differences in patients with different HbA1c levels to open a new avenue for research on early diabetes diagnosis.

2.1 Urine Sample Information and Mass Spectrometry Parameters

The mass spectrometry data used in this study were derived from three published studies [6–8], two of which involved patient groups with different HbA1c levels: one group at (6.4$±0.7)±$1.6)%. Each patient group was compared with healthy control samples, designated as Group A comparison and Group B comparison, respectively. All mass spectrometry data were acquired in Data Dependent Acquisition (DDA) mode. Detailed sample information and mass spectrometry parameters are presented in Table 1 .

Table 1 Sample Information, Processing Methods, and Mass Spectrometry Parameters

Parameter	Group A [6]	Group B
	Elevated HbA1c (n=5)	Healthy individual group (n=5)
HbA1c% (Average±SD)	6.4$±0.7|5.6±4.4|8.6±1.6|5.1±1.6|Age(Average±SD)|68±3|56±4.4|51±$16	
Reduction and alkylation methods	DTT/IAA	DTT/IAA
Type of protease	Trypsin	Trypsin
Ultra-High-Performance nanoflow liquid chromatography system	EASY-nLC 1000 ultrahigh-pressure system (Thermo Fisher Scientific)	Ultimate 3000 nano Liquid Chromatograph (Thermo Scientific)
Mass spectrometer	Q Exactive (Thermo Fisher Scientific, Inc. Bremen, Germany)	Q Exactive HF-X (Thermo Scientific)

Parameter	Group A [6]	Group B
Trap column	2 cm × 75 μm Acclaim Pepmap 100 column	C18 PepMap100, 300 μm × 5 mm, 5 μm, 100 Å (Thermo Scientific)
Analytical column	12.5 cm × 75 μm NTCC-360 home-made column (75 μm internal diameter) with C18 packing resins (1.8 m particle size, 100 Å pore size, Dikma Technologies, USA)	20 cm capillary 75 μm$×$10 cm, 5 μm (New Objective, MA)
Mobile phase A	0.1% formic acid	0.1% formic acid in 2% acetonitrile
Mobile phase B	0.1% formic acid in acetonitrile	0.1% formic acid in 90% acetonitrile
Flow rate	300 nL/min	300 nL/min
Gradient elution time	120 min	65 min
Gradient elution program	Linear gradient of 2% phase B to 35% phase B	0~12 min, 5~10% phase B; 12~50 min, 10~26% phase B; 50~60 min, 26~45% phase B; 60~61 min, 45~80% phase B; 61~65 min, 80% phase B
The spray voltage	2.0 kV	2.1 kV
MS1 resolution	60,000	70,000
MS2 resolution	15,000	17,500

2.2 Database Searching and Data Processing

Proteome modification information was obtained using pFind Studio software (version 3.2.2, Institute of Computing Technology, Chinese Academy of Sciences) for label-free quantitative analysis of the mass spectrometry data. The target search database was the Homo sapiens database downloaded from UniProt (updated to July 2025). Search parameters included: instrument type HCD-FTMS, trypsin digestion, maximum of 2 missed cleavages allowed per peptide, and precursor ion mass and fragment tolerance both set to $±$20 ppm. To identify comprehensive modifications, an Open Search strategy was employed. Filtering criteria were: FDR < 1% at spectrum, peptide, and protein levels. A Python script "pFind_{{{protein}}*{{contrast}}}*{script}.py"was used to extract sample information (Total_{{{spec}}_{{num}}}@pep) from the pFind Studio analysis results [14,15].

2.3 Data Analysis

Modified peptide spectrum counts were compared between each of the two patient groups with different HbA1c levels and the healthy control group. Differentially modified peptides were screened based on the criteria of fold change (FC) ≥ 1.5 or ≤ 0.67 and two-tailed unpaired t-test analysis with $p < 0.05$.

3.1 Identification of Differentially Modified Peptides

Using a label-free quantitative proteomics approach, experimental data were obtained through LC-MS/MS analysis. Following open-pFind searching, peptide spectrum count information was acquired for each sample, including the proteins in which peptides were located and the types of modifications contained within the peptides. Modified peptides with intra-group reproducibility $\geq 50\%$ in both the elevated HbA1c group and healthy control group were selected, and the union was taken.

In the Group A comparison, a total of 6,833 modified peptides were identified. Based on the criteria of FC ≥ 1.5 or ≤ 0.67 and two-tailed unpaired t-test analysis with $p < 0.05$, 1,954 differentially modified peptides were identified in the mildly elevated HbA1c group compared with the healthy control group. Among these, 942 showed presence-to-absence changes (identified in more than half of healthy control samples but absent in all elevated HbA1c samples), while 11 showed absence-to-presence changes (identified in more than half of elevated HbA1c samples but absent in all healthy controls). Collectively, 48.8% of differentially modified peptides exhibited presence-to-absence or absence-to-presence changes. Detailed information for all differentially modified peptides is provided in Supplementary File 1, including peptide sequences, modification types, and the proteins in which the differentially modified peptides were located.

In the Group B comparison, a total of 8,162 modified peptides were identified. Using the same criteria, 5,545 differentially modified peptides were identified in the elevated HbA1c group compared with the healthy control group. Among these, 778 showed presence-to-absence changes and 4,017 showed absence-to-presence changes, accounting for 86.5% of all differentially modified peptides. Detailed information is provided in Supplementary File 2.

To determine the likelihood of random generation of the identified differentially modified peptides, randomized grouping validation was performed on the total modified peptides from both groups. For Group A, 10 samples were shuffled and randomly combined into two new groups, yielding 126 possible combinations. Differential screening based on the same criteria (FC ≥ 1.5 or ≤ 0.67, $p < 0.05$) produced an average of 183.7 differentially modified peptides, indicating that at least 90.6% of differentially modified peptides in Group A were not randomly

generated. For Group B, 14 samples were shuffled and randomly combined into two new groups, yielding 3,003 possible combinations. Using the same criteria, an average of 327.2 differentially modified peptides were generated, indicating that at least 94.1% of differentially modified peptides in Group B were not randomly generated.

3.2 Analysis of Commonly Identified Differentially Modified Peptides Between Two Groups

Across the Group A and Group B comparisons, 602 differentially modified peptides were commonly identified, potentially reflecting shared changes associated with elevated HbA1c levels. Among these, 15 differentially modified peptides showed consistent presence-to-absence or absence-to-presence changes in both groups, demonstrating consistent and significant alteration patterns (Table 2). Detailed information for all commonly identified differentially modified peptides is provided in Supplementary File 3, including peptide sequences, modification types, and the proteins in which the differentially modified peptides were located.

Table 2 Differentially Modified Peptides Showing Consistent Presence-to-Absence or Absence-to-Presence Changes in Both Groups

UniProt ID	Peptide	Modification	FC (Group A)	FC (Group B)
P01834	SGTASVVCLLNNFYPR	1,Amino[Y]	Presence-to-absence	Presence-to-absence
P02768	MPCAEDYLSVVLNQLCVLHEK	1,Oxidation[M];16,Carbamidomethyl[C]	Presence-to-absence	Presence-to-absence
P02790	EWFWDLATGTVK	1,Oxidation[M]	Presence-to-absence	Presence-to-absence
Q9HCU0	HLVSTEFEVVTPEGSVAAVQPQGR	2,Carbamidomethyl[C]	Presence-to-absence	Presence-to-absence
O60494	NLNCVWIIACPVNK	4,Carbamidomethyl[C]	Presence-to-absence	Presence-to-absence
P02760	VVAQGVGIPEGSHITMCDIP	1,Oxidation[M];16,Carbamidomethyl[C]	Presence-to-absence	Presence-to-absence
Q96NY8	LPCFYR	3,Carbamidomethyl[C]	Presence-to-absence	Presence-to-absence
Q14982	GILSCEASAVCPMAEFQWFKPLC	5,Carbamidomethyl[C]	Presence-to-absence	Presence-to-absence
P02768	HPYFYAPELLFFAKR	1,Acetyl[AnyN-term]	Presence-to-absence	Presence-to-absence

UniProt ID	Peptide	Modification	FC (Group A)	FC (Group B)
P01876	VFPLSLCST[illegible]	7,Carbamidomethyl[C],Carbamyl[AnyN-term]	Absence-to-presence	Absence-to-presence
P02768	MPCAEDYL[illegible]	18,Carbamidomethyl[C]	Absence-to-presence	Absence-to-presence
P12109	DTTPLNVL[illegible]	9,Carbamidomethyl[C]	Absence-to-presence	Absence-to-presence
Q14624	ERRLDYQE[illegible]	17,Carbamidomethyl[C]	Absence-to-presence	Absence-to-presence
Q14624	HRQGPVNL[illegible]	0,Carbamyl[AnyN-term]	Absence-to-presence	Absence-to-presence
P01876/P01877	VAAEDWK	0,Carbamyl[AnyN-term]	Absence-to-presence	Absence-to-presence

3.3 Analysis of Differential Modifications

The types and identification frequencies of modifications in differentially modified peptides were statistically analyzed for both groups. Group A comparison identified 160 modification types, while Group B comparison identified 284 modification types. The number of modification types increased with HbA1c level. Detailed information is provided in Supplementary File 4, and the top 11 most frequently identified modification types in each group are shown in Table 3 .

Table 3 Top 11 Most Frequently Identified Differential Modification Types

Group A Modification	Number	Group B Modification	Number
Carbamidomethyl[C]	854	Carbamidomethyl[C]	2,218
Oxidation[M]	195	Carbamyl[AnyN-term]	441
Carbamyl[AnyN-term]	139	Deamidated[N]	279
Deamidated[N]	136	AEBS[Y]	252
GGC	71	Oxidation[M]	249
Gln->pyro-Glu[AnyN-termQ]	57	GGC	204
Pyro-carbamidomethyl[AnyN-termC]	51	Gln->pyro-Glu[AnyN-termQ]	183
Propionamide_{2H}(3)[C]	42	Oxidation[Y]	147
Dehydrated[D]	37	C+12[AnyN-term]	139
Cation_{Ca}[II][E]	35	AEBS[K]	134
Trp->Kynurenin[W]	33	Pyro-carbamidomethyl[AnyN-termC]	130

In both groups, the most prevalent modification type was carbamidomethylation (Carbamidomethyl), an artificial modification generated by the alkylation reagent iodoacetamide (IAA) acting on cysteine residues (Cys, C). This modification accounted for 43.7% of all differential modification identifications in Group A and 40% in Group B. Since the sample processing methods were identical for both the mildly elevated HbA1c group and healthy control group in Group A, no differences should exist if the status of cysteine residues was consistent before processing. However, the results revealed an extremely high frequency of differential identification for this modification, suggesting that the status of cysteine residues may have already differed between the two groups prior to sample processing.

Disulfide bonds formed between cysteine residues through oxidation are essential for maintaining protein stability [9]. Research has shown that in diabetic states, protein disulfide isomerase (PDI) exists predominantly in its reduced form and cannot form disulfide bonds in nascent proteins [10].

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4 Discussion

As a biomarker for glycemic management [11], HbA1c exhibits a continuous distribution in its association with diabetes risk. This study integrated mass spectrometry data from three published studies and systematically explored differences in patients with varying HbA1c levels from the perspective of urinary proteome modifications, comparing two patient groups ([6.4$±0.7]±$1.6]%) with healthy controls.

The results demonstrated that urinary proteome modifications could reflect changes associated with elevated HbA1c levels in both comparisons. Group A comparison identified 1,954 differentially modified peptides, while Group B comparison identified 5,545 differentially modified peptides. Furthermore, the proportions of differentially modified peptides showing presence-to-absence or absence-to-presence changes were 48.8% and 86.5% in Groups A and B, respectively. Both the total number of differentially modified peptides and the proportion of peptides with significant changes increased markedly with HbA1c level. Randomization test results further validated the reliability of these findings, confirming that at least 90.6% and 94.1% of differentially modified peptides in Groups A and B, respectively, were not randomly generated.

Integrating the results from both comparisons, different HbA1c levels correspond to distinct urinary proteome modification profiles, establishing a foundation for using urinary proteome modifications to reflect HbA1c levels. This study has certain limitations: it represents a retrospective data analysis with relatively limited sample sizes, and age differences between healthy controls and elevated HbA1c groups may introduce confounding effects. Subsequent large-scale clinical studies are needed for further validation.

5 Conclusion

This study explored differences among patients with different HbA1c levels from the perspective of urinary proteome modifications. Urinary proteome modifications can comprehensively and systematically reflect changes associated with elevated HbA1c levels, with distinct modification profiles corresponding to different HbA1c levels. These findings suggest that urinary proteome modifications have the potential to reflect HbA1c levels and offer a new perspective for research on the early diagnosis of diabetes.

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Note: Figure translations are in progress. See original paper for figures.