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Variation of IgG N-linked glycosylation profile in diabetic retinopathy

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Abstract

Background: The relationship of IgG glycosylation with diabetes and diabetic nephropathy has been reported, but its role in diabetic retinopathy (DR) remains unclear. We aimed to investigate and validate the association of IgG glycosylation with DR.

Methods: We analyzed the IgG N-linked glycosylation profile and primarily selected candidate glycans by Lasso in the discovery population. The findings were validated in the replication population using a binary logistics model. The association between the significant glycosylation panel and clinical features was illustrated with Spearman's coefficient. The results were confirmed by sensitivity analyses.

Results: Among 16 selected glycan candidates using Lasso, 2 IgG glycans (GP15, GP20) and 2 derived traits (IGP32, IGP54) were identified and validated to be significantly associated with DR ($P<0.05$), and the combined adjusted ORs were 0.587, 0.613, 1.970, and 0.593, respectively. The glycosylation panel showed a weak correlation with clinical features, except for age. In addition, the results remained consistent when the subjects with prediabetes were excluded from the controls, and the adjusted ORs were 0.677, 0.738, 1.597, and 0.678 in the whole population. Furthermore, in the 1:3 rematched population, a significant association was observed, apart from GP20.

Conclusions: The IgG glycosylation profile, reflecting an aging and proinflammatory status, was significantly associated with DR. The variation in IgG glycome deserves more attention in diabetic complications.

Highlights:

1. IgG glycosylation variation was associated with diabetic retinopathy, in which GP15, GP20, and IGP54 showed a negative trend and IGP32 positivity.

2. The significant glycosylation panel, reflecting an aging and proinflammatory status, may capture a specific biological aspect and become a novel biomarker and drug target of DR.

Keywords: diabetic retinopathy; glycosylation; IgG

Instruction

Type 2 diabetes, characterized by abnormal glycometabolism and impaired insulin function, has become a serious challenge to global health. Type 2 diabetes accounts for approximately 90% of diabetes worldwide, and it is estimated that 171 million people were diagnosed with diabetes in 2000; this number is expected to reach 366 million by 2030 [1]. People living with type 2 diabetes have a higher risk of developing health-threatening complications, such as diabetic retinopathy (DR). Approximately one-third of individuals with diabetes have different degrees of retinal impairment, and DR has become the leading cause of blindness in the working-age population as a common microcardiovascular outcome [2-3]. In recent years, the incidence of prediabetes, characterized by impaired glucose tolerance and/or impaired fasting glucose, has also increased. People with prediabetes possess a potential risk of future development of type 2 diabetes and then progression to DR [4]. However, the etiological mechanism of the aggravation of diabetes status remains unclear, and potential biological targets related to the onset of DR are urgently needed.

Glycometabolism is influenced by the interplay of genetic and environmental factors [5], among which glycosylation is one of the dynamic and posttranscriptional modifications. The glycans attached to proteins or lipids regulate crucial biological functions, including cellular membrane recognition and molecular pathway effects [6]. Variations in IgG glycans have been widely investigated, and covalently attached glycans are reported to affect IgG stability, half-life, trafficking, solubility, and interaction with other proteins and the balance between proinflammatory or anti-inflammatory effects

[7-8]. Bisecting GlcNac and complex N-glycan structures exert a proinflammatory effect, while the addition of galactose, sialic acid or fucose has an anti-inflammatory effect [9]. Recently, variations in IgG glycans have emerged as potential biomarkers and therapeutic targets of various metabolic diseases, such as aging [10], dyslipidemia [11], immune disease [12], type 2 diabetes [13-14] and diabetic nephropathy [9]. In fact, type 2 diabetes is accompanied by glucose metabolic disorder and imbalanced regulation of inflammation. Moreover, IgG glycosylation profiles interact with risk factors for type 2 diabetes, such as obesity [15], blood pressure [16-17] and fasting blood glucose (FBG) [18]. Therefore, it is rational to infer that a specific IgG glycosylation pattern plays an important role in the pathological process of DR. Lemmers et al. [13] and our team [14] identified the differential IgG glycans between the diabetes population and healthy controls. Singh et al. [9] reported that IgG glycosylation patterns were associated with the course of kidney function in type 2 diabetes. However, the biological effect of the IgG glycosylation profile on the development of DR remains unclear.

In this study, we aimed to investigate the association of IgG glycosylation with the onset of DR to identify early glycome biomarkers related to DR.

Methods

Study design and population

In 2015, 54 subjects with new-onset DR and 108 controls (22 prediabetes and 86 diabetes) from the Beijing health management cohort were enrolled in this study as the discovery population. Subsequently, 54 cases of DR and 108 controls (18 prediabetes and 90 diabetes) were recruited in 2016 as the replication population. The controls were

matched 1:2 according to age, sex and body mass index (BMI). The Beijing health management cohort, established since January 2008, involves participants aged ≥18 years for etiological and risk factor research of metabolism-related diseases [19]. All participants in this cohort underwent physical and biochemical examinations, and the plasma samples were separated from the fasting blood and stored at -80°C.

The following inclusion criteria were required: (1) signing informed consent prior to enrollment; (2) at least 18 years old; (3) confirmed diagnosis of type 2 diabetes; and (4) fundus photography data available for diagnosing DR. The exclusion criteria were as follows: (1) history of type 1 diabetes; (2) participants with diabetic nephropathy or diabetic foot; and (3) history of mental illness, infectious disease, cardiovascular diseases, stroke, liver disease, renal failure, cancer or autoimmune diseases. Participants with new-onset DR were included as cases, while participants with type 2 diabetes without DR and other complications were included as controls. This study was conducted following the Declaration of Helsinki and approved by the Capital Medical University Ethics Committee (approval number 2020SY031).

IgG glycosylation experiment

The glycosylation experiment and analysis comprised four major parts: IgG protein isolation and purification from plasma; N-linked glycan release and fluorescence labeling; glycan quantitative detection; and direct glycan and derived trait computation, as described previously [20-21]. In brief, IgG protein was obtained from 2 ml plasma using 96-well protein G monolithic plates; the N-linked glycans were released from the purified IgG protein using PNGase F and fluorescently labeled using 2-AB; the direct

glycans were quantitatively measured using an ultra-performance liquid chromatography platform (Waters, America).

Finally, 24 direct glycan peaks (GPs) were detected and quantitatively expressed as percentages of the total integrated peak area. In addition, 54 glycan traits (IGPs) were derived to reflect the proportion of specific modifications, such as galactosylation, sialylation, bisecting *N*-acetylglucosamine (GlcNAc), core fucosylation and mannose. Detailed information on each GP and IGP is shown in **Appendix Table A.1**. The amounts of GP and IGP were normalized and log-transformed, and the batch size was considered and corrected before analysis.

Covariates

Demographic characteristics such as age, sex and current smoking were obtained at baseline by questionnaires. BMI was defined as weight (in kilograms)/height^2 (in meters squared) and divided into <25 and ≥ 25. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) are presented as the mean of two measures on the right arm using a sphygmomanometer after resting for at least 10 min. High blood pressure (HBP) was defined as SBP ≥ 140 or DBP ≥ 90 accordingly. Fasting blood glucose (FBG) was measured after overnight fasting, and postprandial blood glucose (PBG) was measured 2 hours after the beginning of meals using the glucose oxidase-peroxidase method (Mind Bioengineering Co. Ltd., Shanghai, China). HbA1c was considered to reflect the glucose metabolism status in the past three months. Triglycerides, total cholesterol (TC), high-density lipoprotein cholesterol (HDL cholesterol, HDLC), and low-density lipoprotein cholesterol (LDL cholesterol, LDLC) were measured with an

Olympus Automatic Biochemical Analyzer (Hitachi 747; Tokyo, Japan). The estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI 2009) serum creatinine equation [22].

Outcomes

Prediabetes and diabetes were defined by endocrinologists according to the American Diabetes Association standards [23] as follows: (1) prediabetes: 7.0>FBG>6.1 mmol/L, or 11.1>PBG≥7.8 mmol/L and (2) diabetes: FBG≥7.0 mmol/L, PBG≥11.1 mmol/L, regular use of antidiabetic drugs, or history of diabetes.

Patients with DR were diagnosed by ophthalmologists according to the International Clinical Diabetic Retinopathy Disease Severity Scale [24]. Retinopathy could be divided into four severities according to mydriatic fundus examination by fundus photography: mild nonproliferative DR; moderate nonproliferative DR; severe nonproliferative DR; and proliferative DR. The typical clinical appearance of mild and moderate nonproliferative DR includes microarteoma, retinal hemorrhage, and venous loop. The appearance of severe nonproliferative DR includes venous beading, venous reduplication, multiple blot hemorrhages, and intraretinal microvascular abnormalities. The appearance of proliferative DR includes neovascularization and vitreous or retinal hemorrhage [25]. The appearance of different stages of DR is shown in **Appendix Figure A.1.** All participants in the BHMC cohort underwent physical examinations, involving fundus photography examination, annually. Participants with new-onset DR, that is, mild or moderate nonproliferative DR, were recruited in this study.

Statistical analysis

Continuous variables adhering to the normal distribution are represented as the mean ± standard deviation (SD), and the differences between groups were tested by independent Student's t tests; otherwise, the interquartile range (P_{25} - P_{75}) was used, and the differences between groups were explored by Mann-Whitney U tests. Categorical variables are presented as n (%), and the differences were tested by chi-squared tests. Box plots were used to show the distribution of IgG glycans and traits between groups. The controls were matched by propensity score method. The Lasso algorithm and adjusted binary logistics model were used to identify the IgG glycans and traits associated with the onset of DR in both the discovery and replication populations. Age, sex, BMI, smoking status, blood pressure, glucose, lipids and eGFR were considered potential confounding factors. Furthermore, the association between the significant IgG glycosylation panel and clinical features was illustrated with Spearman's coefficient. In addition, sensitivity analyses were performed in the following two situations: subjects with prediabetes were excluded from the controls to assess the association between diabetes and DR, and the controls were rematched 1:3 and analyzed. The sensitivity analyses were based on the whole population.

All reported *P* values were two-tailed, and $P<0.05$ was considered significant. All analyses presented above were performed using R software (version 3.6.3).

Results

Demographic and clinical characteristics

In the discovery population, the mean age of this population was 61 (range from 37 to 93), involving 131 males (80.9%). In the replication population, the mean age of this

population was 60 (range from 27 to 88), involving 135 males (83.3%). The demographic characteristics were similar between the discovery and replication populations. Additionally, there were no significant differences in smoking status, HBP, TG, HDLC, or eGFR between the DR group and the controls, while TC declined in the DR group. Detailed information is shown in **Table 1**.

Associations of IgG glycosylation and DR

In the discovery population, 9 glycans (GP2-GP5, GP11, GP12, GP15, GP16, and GP20) and 7 derived traits (IGP32, IGP40, IGP43, IGP44, IGP52, IGP54, and IGP61) were primarily selected by the Lasso algorithm. Then, GP15, GP20, IGP32, and IGP54 were validated for the replication population both in the unadjusted and adjusted models, assuming that the glycosylation panel was associated with DR. The distribution of the discriminative panel is shown in **Figure 1**, and the adjusted ORs in the combined population were 0.587, 0.613, 1.970, and 0.593, respectively, as shown in **Table 2**. The detailed distribution of all these glycans and traits is shown in **Appendix Table A.2**. The glycosylation panel showed a weak correlation with clinical features in the discovery and replication populations, as the Spearman's coefficients were less than 0.3 for BMI, SBP, FBG, PBG, HbA1c, TG, LDLC, TC, and HDLC, as shown in **Figure 2**. The glycan panel showed a significant correlation with age, DBP and eGFR. The glycosylation panel may capture a specific aspect of the biological status.

Sensitivity analysis

Sensitivity analyses were performed on the whole population due to the relatively small sample size. On the one hand, 40 subjects with prediabetes were excluded from the

controls; 176 with diabetes and 108 with DR were included in the analysis. As shown in **Figure 3**, GP15 (OR: 0.68; 95% CI: 0.50-0.91), GP20 (OR: 0.74; 95% CI: 0.54-0.91), IGP32 (OR: 1.60; 95% CI: 1.11 -2.38), and IGP54 (OR: 0.68; 95% CI: 0.50-0.91) remained significantly associated with DR in the multivariate model. On the other hand, the control population was rematched 1:3. Then, GP15 (OR: 0.77; 95% CI: 0.65-0.89), IGP32 (OR: 1.88; 95% CI: 1.33-2.24), and IGP54 (OR: 0.72; 95% CI: 0.58-0.91) remained significantly associated with DR, while GP20 (OR: 0.90; 95% CI: 0.67-1.02) changed to an insignificant positive correlation.

Discussion

In this study, we investigated the relationship between the IgG glycosylation profile and DR in two matched populations. The panel of GP15, GP20, IGP32, and IGP54 was validated to be associated with DR. The IgG glycosylation panel showed a weak correlation with common clinical features, including BMI, blood pressure, glucose and lipids, which were also risk factors for diabetes and DR. The results suggested that the IgG glycosylation panel, reflecting aging and proinflammatory effects, may capture a specific aspect of the physiological state. We proposed that the specific variation in the IgG glycosylation profile, independent of common clinical factors, played an important role in the pathological process of DR. Moreover, the GP15, GP20, IGP32, and IGP54 panels could be potential biomarkers and novel targets, which could contribute to the early prevention and intervention of DR.

Both genetic and environmental factors affect the incidence and development of diabetes and its complications, and the glycosylation of IgG proteins is one of the most

common posttranslational modifications and is involved in almost all physiological processes, such as signaling pathways, cellular immunity, and the mutual recognition of proteins [26]. Variations in IgG glycosylation profiles, reflecting both genetic and environmental characteristics [27], are reported to be associated with various diseases, especially autoimmune diseases and chronic metabolic and inflammatory diseases [28-30]. In fact, both glycometabolism disorders and impaired immunologic function are involved in the pathophysiological process of diabetes and DR. In this study, we found that GP15, GP20, and IGP54 were negatively associated with the onset of DR, while IGP32 showed a positive association. The variation in the IgG glycosylation pattern was in accordance with a decrease in digalactosylated biantennary glycan with bisecting GlcNAc and core fucose (GP15), digalactosylated monosialylated biantennary with core and antennary fucose (GP20), digalactosylated biantennary glycan with core fucose structures in total neutral IgG glycans (IGP54) and an increase in disialylation of fucosylated digalactosylated structures with bisecting GlcNAc (IGP32).

The results above were largely consistent with previous studies of IgG glycosylation profiles of type 2 diabetes, diabetic complications and related risk factors. Previous studies have reported that complex glycan structures with bisecting GlcNAc were highly associated with abnormal glucose metabolism, reflecting a body status of proinflammation [13, 31-32]. IgG proteins are sensitive to biological inflammatory stress, and variations in IgG glycans can reverse their anti-inflammatory function [33-34]. Therefore, the substantially increased proportion of complex glycan structures, such as disialylation of fucosylated digalactosylated structures with bisecting GlcNAc, may be

induced by biological inflammation in the process of DR occurrence. In addition, the decreased proportion of galactosylation, accompanied by a decreased percentage of sialylation as sialic acids were attached to galactose, is thought to strengthen the complement-dependent cytotoxicity (CDC) effect of IgG [35-36]. The presence of bisecting GlcNAc and lack of core or antennary fucose are thought to strengthen the antibody-dependent cell-mediated cytotoxicity (ADCC) effect of IgG [37]. Both the CDC and ADCC effects of IgG were reported to switch its anti-inflammatory role to a proinflammatory role. Consistently, Dotz et al. [32] found decreased alpha 2,3-linked sialylation of plasma protein in type 2 diabetes. Lemmers et al. [13] reported an IgG glycosylation pattern of decreased galactosylation, sialylation, and fucosylation structures and increased bisecting GlcNac structures associated with type 2 diabetes based on a European population. Furthermore, we found that the IgG glycosylation profile was associated with DR in this study. The panel was related to an overall decrease in digalactosylated fucosylated structures with and without GlcNAc, with monosialytion or without sialic acid. Moreover, the structures of bisecting GlcNac and disialylation appeared to exert synergetic effects in DR.

The strength of our study was that we analyzed the variation in IgG glycosylation profiles and identified the glycans and traits associated with DR for the first time. To date, FBG, PBG and insulin resistance indices have been applied in the diagnosis and intervention of DR, and it is of great importance to discover more metabolic biomarkers and potential targets for the prevention and intervention of DR. Additionally, we proposed that IgG glycosylation, reflecting aging and proinflammatory status, may

capture a specific pathological aspect of health. However, the results should be interpreted in the context of some limitations. First, the sample size was relatively small, although the logistic regression of a binary response variable (DR or not) on a continuous variable (e.g., IGP32) with a sample size of 162 observations achieves 82.8% power at a 0.05 significance level to detect a change in the probability of DR onset when IGP32 increases by one unit. This change corresponds to an odds ratio of 1.970, as shown in the multivariate model. We could not claim a causal association due to the lack of prospective follow-up. The biological mechanism of IgG glycosylation profiles in DR or other diabetic complications warrants further investigation at the animal or cell level. Second, this study only involved subjects with new-onset DR. We failed to illustrate whether the IgG glycosylation pattern was associated with the severity of DR. Third, diabetes duration data were unavailable in this study. The diabetes duration between the DR group and the control may be different.

In general, the IgG glycosylation profile, reflecting aging and proinflammatory status, was validated to be associated with DR. Variations in IgG glycans and traits could be novel biomarkers and potential drug targets for DR and other diabetic complications.

List of abbreviations

DR: Diabetic retinopathy; **BMI**: Body mass index; **FBG**: Fasting blood glucose; **PBG**: Postprandial blood glucose; **SBP**: Systolic blood pressure; **DBP**: Diastolic blood pressure; **HBP**: High blood pressure; **TC**: Total cholesterol; **HDLC**: High-density lipoprotein cholesterol;

LDLC: Low-density lipoprotein cholesterol; **GP**: Glycan peak;

ADCC: Antibody-dependent cell-mediated cytotoxicity;

CDC: Complement-dependent cytotoxicity.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committees of Capital Medical University. All participants gave informed consent to participate before participating. The number/ID of the approval was 2020SY031.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1

Characteristics of participants in the discovery and replication populations.

	Discovery population			Validation population		
	Controls (n=108)	**DR (n=54)**	**P value**	**Controls (n=108)**	**DR (n=54)**	**P value**
Age (years)†	60.41(12.22)	62.50(11.36)	0.295	59.26(11.85)	60.41(11.10)	0.554
Sex (men)‡	87(80.6)	44(81.5)	>0.999	89(82.4)	46(85.2)	0.823
BMI (≥25 kg/m^2)‡	70(64.8)	35(64.8)	>0.999	80(74.1)	40(74.1)	>0.999
Smoking (yes)‡	40(37.0)	26(48.1)	0.235	49(45.4)	19(35.2)	0.285
FBG (mmol/L)†	7.01[6.38,8.32]	8.00[6.52,10.05]	0.010*	7.25[6.41,8.55]	8.00[6.46,9.68]	0.068
PBG (mmol/L)†	11.00[9.40,13.17]	12.40[10.15,14.55]	0.080	10.80[9.70,13.31]	11.90[9.88,14.40]	0.134
HbA1c (%)†	7.17[6.04, 8.80]	7.20[6.01,9.20]	0.773	7.07[6.32, 8.50]	7.22[6.43,8.41]	0.702
HBP (yes)‡	35(32.4)	18(33.3)	>0.999	37(34.3)	11(20.4)	0.100
TG (mmol/L) §	1.43[1.06,1.88]	1.36[0.88,1.86]	0.423	1.43[0.97,2.06]	1.29[0.90,1.70]	0.123
LDLC (mmol/L) §	2.69[2.15,3.47]	2.71[1.98,3.26]	0.243	2.71[2.15,3.45]	2.25[1.94,3.10]	0.041*
HDLC (mmol/L) §	1.35[1.05,1.58]	1.22[0.95,1.41]	0.050	1.25[1.06,1.45]	1.16[1.03,1.33]	0.101
TC (mmol/L) §	4.55[3.91,5.40]	4.19[3.36,4.97]	0.018*	4.41[3.87,5.25]	3.99[3.22,4.83]	0.004**
eGFR (mL/min per 1.73 m^2) §	104.7[92.8, 119.8]	96.8[90.1, 109.1]	0.064	104.04[90.9, 120.1]	102.4[92.5, 115.4]	0.851

† mean (SD), Student's t test; ‡ numbers of each category (%) are given, chi-squared test; § median (P25 - P75), Mann-Whitney U test.

Table 2

Associations of IgG glycosylation and DR by binary logistics model.

	Discovery Population		Validation Population		Combined Population	
	OR	P value	OR	P value	OR	P value
GP15						
univariate	0.604	0.007 **	0.678	0.033 *	0.633	0.000 ***
multivariate†	0.618	0.009 **	0.535	0.006 **	0.587	0.007 **
GP20						
univariate	0.654	0.016 *	0.640	0.011 *	0.608	0.000 ***
multivariate†	0.649	0.013 *	0.599	0.042 *	0.613	0.031 *
IGP32						
univariate	1.898	0.009 **	1.861	0.010 *	1.995	0.000***
multivariate†	2.130	0.003 **	1.933	0.018 *	1.970	0.012 *
IGP54						
univariate	0.587	0.005 **	0.677	0.033 *	0.635	0.000 ***
multivariate†	0.610	0.008 **	0.557	0.009 **	0.593	0.010 *

† Age, sex, smoking status, BMI, HBP, FBG, PBG, HbA1c, TG, TC, HDLC, LDLC, and eGFR were adjusted in the multivariate model.

Figures:

Figure 1: Distribution boxplot of the IgG glycosylation panel in the discovery and replication populations.

Figure 2: Correlation analysis between the IgG glycosylation panel and clinical features.

A: Plot of Spearman's coefficients in the discovery population;

B: Plot of Spearman's coefficients in the validation population;

C: Plot of Spearman's coefficients in the combined population.

Figure 3: Results of the sensitivity analyses.

Part A: Association of the IgG glycosylation panel with DR after subjects with prediabetes were excluded;

Part B: Association of IgG glycosylation and DR in the 1:3 matched population.

A total of 176 cases of diabetes and 108 cases of DR were included in part A of the analysis;

A total of 324 controls and 108 DR patients were included in part B of the analysis;

Age, sex, smoking status, BMI, HBP, FBG, PBG, HbA1c, TG, TC, HDLC, LDLC, and eGFR were adjusted in the multivariate model.

Supplementary materials:

Appendix Table A.1: Detailed descriptions of the IgG glycans and traits.

Appendix Table A.2: Distribution of all IgG glycans and traits in the discovery and replication populations.

Appendix Figure A.1: The clinical appearances of different stages of retinopathy.