Micro-ribonucleic acid-binding site variants of type 2 diabetes candidate loci predispose to gestational diabetes mellitus in Chinese Han women

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Keywords
Gestational diabetes mellitus, Type 2 diabetes, Variants

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J Diabetes Investig 2018; 9: 1196–1202
doi: 10.1111/jdi.12803

ABSTRACT

Aims/Introduction: Emerging evidence has suggested that the genetic background of gestational diabetes mellitus (GDM) was analogous to type 2 diabetes mellitus. In contrast to type 2 diabetes mellitus, the genetic studies for GDM were limited. Accordingly, the aim of the present study was to extensively explore the influence of micro-ribonucleic acid-binding single-nucleotide polymorphisms (SNPs) in type 2 diabetes mellitus candidate loci on GDM susceptibility in Chinese.

Materials and Methods: A total of 839 GDM patients and 900 controls were enrolled. Six micro-ribonucleic acid-binding SNPs were selected from 30 type 2 diabetes susceptibility loci and genotyped using TaqMan allelic discrimination assays.

Results: The minor allele of three SNPs, PAX4 rs712699 (OR 1.366, 95% confidence interval 1.021–1.828, P = 0.036), KCNB1 rs1051295 (OR 1.579, 95% confidence interval 1.172–2.128, P = 0.003) and MFN2 rs1042842 (OR 1.398, 95% confidence interval 1.050–1.862, P = 0.022) were identified to significantly confer higher a risk of GDM in the additive model. The association between rs1051295 and increased fasting plasma glucose (b = 0.006, P = 0.008), 3-h oral glucose tolerance test plasma glucose (b = 0.058, P = 0.025) and homeostatic model assessment of insulin resistance (b = 0.065, P = 0.017) was also shown. Rs1042842 was correlated with higher 3-h oral glucose tolerance test plasma glucose (b = 0.056, P = 0.028). However, no significant correlation between the other included SNPs (LPIN1 rs1050800, VPS26A rs1802295 and NLRP3 rs10802502) and GDM susceptibility were observed.

Conclusions: The present findings showed that micro-ribonucleic acid-binding SNPs in type 2 diabetes mellitus candidate loci were also associated with GDM susceptibility, which further highlighted the similar genetic basis underlying GDM and type 2 diabetes mellitus.

INTRODUCTION

Gestational diabetes mellitus (GDM) has become a major public health problem owing to its increased prevalence, and its associated short- and long-term complications for the mother and her offspring. Similar to type 2 diabetes mellitus, GDM is a multifactorial disease induced by genetics interacting with environmental factors, and genetic factors are known to be a major determinant. Accumulating evidence shows that the genetic architecture underlying GDM and type 2 diabetes mellitus overlap considerably. First, GDM and type 2 diabetes mellitus share similar pathophysologies characterized by increased insulin resistance and impaired islet β-cell function. Second, GDM is a strong risk factor for developing type 2 diabetes mellitus in later life, and women with a family history of type 2 diabetes mellitus are more likely to develop GDM. Additionally, some known type 2 diabetes mellitus risk variants
been identified to contribute to GDM susceptibility. In contrast to type 2 diabetes mellitus, the genetic basis for GDM remains poorly defined, and the limited GDM genetic studies only center on exploring the association with common variants of type 2 diabetes mellitus candidate loci.

Micro-ribonucleic acids (miRNAs) are small, non-coding RNAs of approximately 22 nucleotides that regulate >60% of protein-coding genes at the post-transcriptional level, indicating that miRNAs hold critical roles in a multitude of biological pathways. miRNAs bind to the 3′ untranslated region (3′UTR) of target messenger RNAs (mRNAs) and act by translation repression or mRNA degradation. Emerging evidence has shown that single-nucleotide polymorphisms (SNPs) in the miRNA binding site (miR-binding SNPs) conferred a higher risk of a variety of diseases by disturbing the interaction between miRNAs and targeted mRNAs. Furthermore, our previous study had first shown that several miR-binding SNPs of reported GDM candidate loci were associated with GDM susceptibility in Chinese Han pregnant women.

Considering GDM shares a similar genetic predisposition with type 2 diabetes mellitus and combined with our previous findings, the present study broadened the scope of screening and focused on type 2 diabetes mellitus candidate loci that had been confirmed in the Chinese Han population and had not been studied in our previous research. We aimed to intensively investigate the association between miR-binding SNPs of type 2 diabetes mellitus candidate loci and the risk of GDM, which would further improve our knowledge about the role of miR-binding SNPs in GDM pathogenesis, and provide additional insights into the similar genetic basis underlying GDM and type 2 diabetes mellitus.

**METHODS**

**Ethics statement**
The ethics committee of Peking Union Medical College Hospital approved the study protocol. The study was carried out in accordance with the principles of the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013). Written informed consent was obtained from each participant.

**Study participants**
The recruitment flow of study participants has been previously reported. In brief, the study participants consisted of 900 controls and 839 GDM patients who were recruited between January 2006 and June 2011. All pregnant women who had not been previously diagnosed with diabetes underwent a 50-g glucose challenge test at 24–28 weeks-of-gestation. Individuals with a positive result (1-h plasma glucose levels ≥ 7.8 mmol/L) were then followed by a 3-h, 100-g oral glucose tolerance test (OGTT). The diagnosis of GDM was in accordance with the American Diabetes Association criteria.

We classified participants with negative glucose challenge test results or normal glucose tolerance tests as the control group. The exclusion criteria were severe pregnancy complications, taking medicines affecting glucose metabolism and diabetes diagnosed before pregnancy.

**Clinical and laboratory analysis**
Clinical data including weight before pregnancy, height and blood pressure were collected at 24–28 weeks-of-gestation by a trained physician. The measurement methods and instruments for plasma glucose and serum insulin levels have been reported previously. Homeostatic model assessments of islet β-cell function (HOMA-β) and basal insulin resistance (HOMA-IR) were evaluated. The calculation formulas were as follows: HOMA-β = fasting serum insulin (mU/L) × 20 / (fasting plasma glucose in mmol/L – 3.5), HOMA-IR = fasting serum insulin (mU/L) × fasting plasma glucose (mmol/L) / 22.5.

**SNP selection and genotyping**
The flow diagram of SNP selection is shown in Figure 1. We selected 28 type 2 diabetes mellitus susceptibility genes that were identified by a genome-wide association study and confirmed in the Chinese Han population as candidate genes. These genes were as follows: KCNB1, GIPR, MADD, CRY2, NLRP3, PAX4, GRK5, RASGRF1, BDNF, EXT2, MEF2, ADRB3, JAZF1, EXT2, VPS26A, DUSP9, ARHGEF11, POU2F1, FGF21, LPIN1, CSL2, HSPA5, TP53INP1, CAMK1D, SPRY2, G6PC2, HNF4A and GLI3 (Table S1 shows the reference of these genes). The other detailed information about the process of SNP selection has been described in our previous study.

**Figure 1** | Flow diagram of single-nucleotide polymorphism (SNP) selection. 3′UTR, 3′ untranslated region; CHB, Chinese Han in Beijing; GWAS, genome-wide association study; MAF, minor allele frequency; miRNA, micro-ribonucleic acid; NCBI dbSNP, National Center for Biotechnology Information SNP database; T2DM, type 2 diabetes mellitus.
Finally, six potential miRNA-binding site SNPs that were predicted to be located in the same miRNA-binding site by at least two different types of software were included in the present study. TaqMan allelic discrimination assays were applied for SNP genotyping. Each 384-well plate included four blank samples to ensure the genotyping quality. In addition, we carried out replicate assays and Sanger sequencing to further confirm the genotyping accuracy. The results of replicate assays and Sanger sequencing were completely consistent with the original analysis. The genotyping success rate of six SNPs was 97.87%, 95.63%, 97.35%, 99.37%, 98.50% and 99.31% for rs712699, rs1051295, rs1042842, rs1050800, rs1802295 and rs10802502, respectively. Sequence validation of SNPs is supplied in Figure S1.

Statistical analysis
Statistical analysis was carried out using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The χ²-test was used to assess the Hardy–Weinberg equilibrium of genotype distribution for each SNP in control groups. Skewed distribution variables (age, pre-pregnancy body mass index, parity, fasting plasma glucose and insulin, HOMA-β and HOMA-IR) are expressed as the median and interquartile range (IQR), and comparisons between GDM and controls were carried out using non-parametric tests. The association between SNPs and the risk of GDM was evaluated by multivariate logistic regression analysis adjusting for age in three genetic models. Multiple linear regression with adjustment for age was applied to determine the effect of the minor alleles of each SNP on glucose metabolism traits (including plasma glucose, HOMA-β and HOMA-IR), assuming an additive model of inheritance. The regression coefficients (b) and 95% confidence interval (CI) were calculated. Before linear regression analysis, HOMA-IR and HOMA-β were lognormally transformed to nearly normal distribution.

RESULTS
The median age of GDM patients was 32.0 years (IQR 30.0–35.0 years), which was older than the control group (median 31.0, IQR 28.0–40.0; P < 0.05). GDM patients presented with higher fasting glucose, 1-h OGTT glucose, 2-h OGTT glucose and 3-h OGTT glucose than controls. Furthermore, GDM patients showed higher insulin resistance and lower islet function. However, pre-pregnancy body mass index was not significantly different between the GDM and control groups, as well as parity (median 1 [IQR 0–3] vs 1 [IQR 0–3]). Detailed information on the clinical characteristics is supplied in Table S2.

The six selected SNPs, PAX4 rs712699, KCNB1 rs1051295, MFN2 rs1042842, LPIN1 rs1050800, VPS26A rs1802295 and NLRP3 rs10802502, were predicted to be located in the binding site of hsa-miR-223, hsa-miR-216a, hsa-miR-183, hsa-miR-96, hsa-miR-510 and hsa-miR-548, respectively. The minor allele frequency of each SNP in the present study was similar to the data supplied by the 1,000 Genome Browser. The genotype distribution of these SNPs was in accordance with the Hardy–Weinberg equilibrium in the control groups (P > 0.05). Table 1 shows detailed information on the six SNPs.

| SNP       | Gene       | Main function               | Min/ Maj | Chr. position | MAF (CHB)† | Sample MAF | Predicted binding miRNA | Algorithm          | P    |
|-----------|------------|-----------------------------|----------|---------------|------------|------------|--------------------------|-------------------|------|
| rs712699  | PAX4       | Pancreatic development      | A/G      | Chr.7:127250597 | 0.356      | 0.403      | hsa-miR-223              | TargetScan, MiRDB | 0.122|
| rs1051295 | KCNB1      | Insulin secretion           | C/T      | Chr.20:47988905  | 0.444      | 0.413      | hsa-miR-216a             | PolymiRTS, TargetScan | 0.485|
| rs1042842 | MFN2       | Mitochondrial fusion        | A/G      | Chr.1:12071680  | 0.376      | 0.397      | hsa-miR-183              | PolymiRTS, TargetScan | 0.116|
| rs1050800 | LPIN1      | Adipocyte differentiation   | T/C      | Chr.2:11965814  | 0.211      | 0.256      | hsa-miR-96               | PolymiRTS, TargetScan | 0.727|
| rs1802295 | VPS26A     | Transport proteins          | T/C      | Chr.10:70931474 | 0.108      | 0.116      | hsa-miR-510              | PolymiRTS, MiRDB    | 0.861|
| rs10802502| NLRP3      | Regulate inflammation       | C/T      | Chr.1:247612295 | 0.423      | 0.484      | hsa-miR-548              | PolymiRTS, TargetScan | 0.074|

†From 1,000-genome database. CHB, Chinese Han in Beijing, Chr., chromosome; MAF, frequency of minor allele; Maj, major allele; Min, minor allele; miRNA, micro-ribonucleic acid.
OR of 1.224 (95% CI 1.066–1.405, P = 0.004). In addition, the pregnant women carrying the T allele of LPIN1 rs1050800 had a nominally significant 1.169-fold risk of GDM (95% CI 1.000–1.354, P = 0.049) after adjustment for age compared with the women harboring the C allele.

The results of multiple linear regression analysis adjusted for age (Table 3, Table S4) showed that the minor C allele of rs1051295 was associated with increased fasting plasma glucose (FPG; b = 0.006, P = 0.008), 2-h OGTT plasma glucose (b = 0.056, P = 0.023), 3-h OGTT plasma glucose (b = 0.058, P = 0.025) and HOMA-IR (b = 0.065, P = 0.017). In addition, the A allele of rs1042842 was correlated with higher 3-h OGTT plasma glucose with a per-allele effect of 0.056 mmol/L (P = 0.028). The A allele of rs712699 also had an effect on 2-h OGTT plasma glucose (b = 0.051, P = 0.036). We also observed the minor T allele of rs1050800 was associated with elevated 3-h OGTT plasma glucose (b = 0.061, P = 0.016) and decreased HOMA-β (b = −0.059, P = 0.029). The T allele of rs1802295 had a mild effect on FPG with an increase of 0.05 mmol/L per risk allele (P = 0.040), but the association with mildly decreased HOMA-IR was also found (b = −0.056, P = 0.042).

Table 2 | Genotype distribution and gestational diabetes mellitus susceptibility in additive model

| SNP ID  | Gene    | Model   | Genotypes/minor allele       | GDM               | Controls | OR (95% CI)     | P-value |
|---------|---------|---------|-----------------------------|-------------------|----------|----------------|---------|
| rs712699 | PAX4    | Additive | A/A vs C/C                  | 154               | 293      | 1.366 (1.021–1.828) | 0.036*  |
| rs1051295 | KCNB1  | Additive | C/C vs T/T                  | 149               | 247      | 1.579 (1.172–2.128) | 0.003*  |
| rs1042842 | MFN2   | Additive | A/A vs G/G                  | 153               | 278      | 1.398 (1.050–1.862) | 0.022*  |
| rs1050800 | LPIN1  | Additive | T/T vs C/C                  | 46                | 461      | 1.027 (0.665–1.587) | 0.903   |
| rs1802295 | VPS26A | Additive | T/T vs C/C                  | 12                | 642      | 0.932 (0.411–2.113) | 0.866   |
| rs10802502 | NLRP3  | Additive | C/C vs T/T                  | 195               | 228      | 0.945 (0.716–1.247) | 0.691   |

*Significant P-values (P < 0.05). CI, confidence interval; GDM, gestational diabetes mellitus; OR, odds ratio; SNP, single-nucleotide polymorphism.

DISCUSSION

A growing body of evidence suggests that GDM and type 2 diabetes mellitus share a similar genetic background. In particular, our previous research discovered that miR-binding SNPs played a critical role in the genetic pathogenesis of GDM. Accordingly, in the present study, we extensively investigated the effect of miR-binding SNPs in type 2 diabetes mellitus candidate loci on the risk of GDM in the Chinese Han population. The present findings showed that three SNPs of type 2 diabetes mellitus risk genes were also associated with GDM.

The PAX4 gene functions as a transcription repressor and plays essential roles in islet β-cell development during embryogenesis, and regulating β-cell survival and proliferation during adulthood\cite{16}. PAX4-knockout mice presented with the absence of mature insulin-producing β-cells and hyperplasia of glucagon-producing α-cells\cite{16}. Variants in the PAX4 gene have been found to be associated with several forms of diabetes mellitus. The PAX4 mutation causes maturity-onset diabetes of the young, type 9\cite{17}. Genetic association studies showed that SNPs of PAX4 were linked to both type 1 diabetes mellitus and type 2 diabetes mellitus susceptibility across various ethnic groups\cite{18,19}. Subsequently, rs6467136 near the PAX4 gene and rs10229583 locating downstream of the PAX4 were identified as risk variants for type 2 diabetes mellitus by a genome-wide association study in a Chinese population\cite{20} and a large meta-analysis in an East Asian population\cite{21}, respectively. It is worth mentioning that quite recently an exome-chip association analysis revealed an Asian-specific coding variant rs2233580 of PAX4 associated with type 2 diabetes mellitus in Chinese individuals\cite{22}. Such observations suggested PAX4 was a crucial locus for diabetes mellitus, especially in Asian people. The present study found for the first time that the A allele of PAX4 rs712699 increased the risk of GDM in Chinese Han pregnant women. The A allele was predicted to create a novel hsa-miR-223 binding site, which might impair PAX4 gene expression. It was speculated that the defect had an influence on β-cells differentiation or mature β-cells survival contributing to GDM susceptibility. The possible molecular mechanisms need to be clarified by functional studies in the future.

The KCNB1 gene encoded the voltage-dependent K+ (Kv) channel isofrom Kv2.1, and was highly enriched in islet β-cells. In the process of insulin secretion, Kv2.1 mediated the repolarization of pancreatic β-cells action potential to control the duration of depolarization\cite{23}. Kv2.1-null mice had improved glucose tolerance with reduced fasting glucose\cite{24}. Pancreatic β-cells from Kv2.1-null mice showed greater glucose-stimulated insulin secretion, and pharmacological inhibition of Kv2.1 enhanced glucose-stimulated insulin secretion\cite{25}. The present study showed that the carriers of the rs1051295-CC genotype in KCNB1 had a higher risk of developing GDM, and the minor C allele was associated with elevated FPG, 3-h OGTT plasma glucose and HOMA-IR. Given the T allele of rs1051295 was predicted to be located in the hsa-miR-216a binding site, we supposed that the presence of the minor C allele might block the interaction and increase KCNB1 expression, thereby contributing to the glucose metabolism disorder. Certainly, the biological mechanisms of miR-216a and rs1051295 in the GDM pathogenesis need to be elucidated in the future. Interestingly, the significant association between rs1051295 and type 2 diabetes mellitus susceptibility and decreased insulin sensitivity were also discovered in the Chinese population, but the risk
Table 3  Association between minor allele and glucose metabolism-related quantitative traits

| SNP ID | Gene   | Minor allele | Fasting plasma glucose (n = 1,413) | HOMA-R (n = 1,413) | HOMA-B (n = 1,413) |
|--------|--------|--------------|-----------------------------------|------------------|-------------------|
| rs712999| PAX4   | A            | -0.019 (0.06, 0.003)              | 0.014            | 0.002             |
| rs1051295| KCNB1  | C            | 0.036 (0.075, 0.019)              | 0.611            | 0.616             |
| rs1042842| MFN2   | A            | 0.029 (0.013, 0.049)              | 0.318            | 0.318             |
| rs1050800| LPIN1  | C            | -0.019 (0.001, 0.034)            | 0.017            | 0.017             |

*P-values < 0.05. CI, confidence interval; HOMA-R, homeostatic model assessments of insulin resistance; HOMA-B, homeostatic model assessments of beta-cell function. HOMA-IR, homeostatic model assessments of insulin resistance; OGTT, oral glucose tolerance test; OR, odds ratio; SNP, single-nucleotide polymorphism.

The present study first identified that the A allele of rs1802295 in the MFN-2 gene was significantly associated with GDM and increased 3-h OGTT plasma glucose. The significant association might be explained by the repression of the MFN-2 gene due to the binding between the hsa-miR-183 and rs1042842 A allele. Nevertheless, functional studies are warrant to confirm the speculation.

The LPIN1 gene, which was primarily expressed in skeletal muscle and adipose tissue, was discovered as the causative gene in the fatty liver dystrophy mouse. Except for its important roles in adipocyte differentiation and lipid metabolism, recent studies showed that the LPIN1 gene was also expressed in pancreatic β-cells and implicated in glucose metabolism. Furthermore, SNPs and a rare haplotype of the LPIN1 gene were reported to be significantly associated with type 2 diabetes mellitus. So far, the relationship between variants of the LPIN1 gene and GDM has not been investigated. In the present study, we found that the T allele of rs1050800-LPIN1 was not associated with GDM susceptibility, but was significantly correlated with increased fasting glucose and decreased HOMA-β. It was possible that the disturbed interaction between rs1050800 and predicted binding miRNA (hsa-miR-96) decreased LPIN1 expression, contributing to the observed association with glucose metabolism traits. In parallel, a strong negative correlation was shown between lipin mRNA levels in human adipose tissue and fasting glucose. In contrast, miR-96 was shown to play a key role in insulin exocytosis modulating the plasma glucose homeostasis. However, the real influence of rs1050800 on LPIN1 expression has not been verified.
observed that the T allele of rs1802295 was not associated with GDM susceptibility, only a significant association with higher FPG and decreased HOMA-IR were indicated. Consistent with the present findings, rs1802295 was recently reported to have no effect on GDM in an Asian Indian population as well. Therefore, the role of the VPS26A gene in the development of diabetes mellitus requires further clarification.

There were some limitations to the present study. First, functional studies were not carried out to further confirm the binding between predicted miRNA and targeted mRNA. Second, only common variants in 3’UTR of type 2 diabetes mellitus candidate loci were examined, thus some rare SNPs with large effects were probably omitted. Third, Bonferroni adjustments were not carried out so as to avoid missing possible SNPs predisposed to GDM. In addition, because no replication was carried out, it is possible that some of the findings might be false positives.

In conclusion, the present study extensively investigated the influence of miR-binding SNP of type 2 diabetes mellitus candidate loci on GDM susceptibility. The minor alleles of three genes (PAX4 rs712699, KCN1 rs1051295, MFN2 rs1042842) were identified to increase the risk of GDM. In addition, four miR-binding SNPs (rs1051295, rs1042842, LPIN1 rs1050800, VPS26A rs1802295) were significantly associated with glucose metabolism traits. The present findings facilitated a better understanding of the genetic contribution to the risk of GDM, and further underscored the similar genetic architecture underlying GDM and type 2 diabetes mellitus. In future, functional studies are required to elucidate the biological mechanisms underlying these significant associations.

ACKNOWLEDGMENTS

We thank all the participants in this study. The study was funded by research grants from the National Natural Science Foundation of China (Grant No. 81270879) and National Key Program of Clinical Science (WBYZ2011873).

DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1** | Sanger sequencing trace of six micro-ribonucleic acid-binding single-nucleotide polymorphisms.
**Table S1** | Reference of 28 selected genes.
**Table S2** | Clinical characteristics of the participants.
**Table S3** | Genotype distribution and gestational diabetes mellitus susceptibility in the recessive and dominant model.
**Table S4** | Association between minor allele and 1-h oral glucose tolerance test and 2-h oral glucose tolerance test plasma glucose.