RESEARCH ARTICLE

Identification and functional study of GATA4 gene regulatory variants in type 2 diabetes mellitus

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Abstract

Background: Type 2 diabetes mellitus (T2D) is a common and complex disease. Dysfunction of pancreatic β cells, which cannot release sufficient insulin, plays a central role in T2D. Genetics plays a critical role in T2D etiology. Transcription factor GATA4 is required for the pancreatic development, and GATA4 gene mutations are implicated in neonatal or childhood-onset diabetes. In this study, we aimed to investigate whether regulatory variants in GATA4 gene may change GATA4 levels, conferring susceptibility to T2D development.

Methods: The promoter region of GATA4 gene was analyzed by targeted sequencing in T2D patients (n = 255) and ethnic-matched controls (n = 371). Dual luciferase activity assay was used for functional study, and EMSA (electrophoretic mobility shift assay) was performed for detecting transcription factor binding.

Results: Thirteen regulatory variants including 5 SNPs were identified. A novel heterozygous variant (32124C > T) and one SNP [31487C > G (rs1053351749)] were only identified in T2D. Both regulatory variants significantly affected GATA4 gene promoter activity in cultured HEK-293 and INS-1 cells. Furthermore, the variant (32124C > T) evidently enhanced the binding of unknown transcriptional activator.

Conclusions: Our data suggested that GATA4 gene regulatory variants may contribute to T2D development as a rare risk factor.

Keywords: Type 2 diabetes mellitus, Genetics, GATA4, Regulatory variants

Background

Type 2 diabetes mellitus (T2D) is a common and complex disease and caused by interactions between genetic and environmental factors [1]. Type 2 DM is mainly caused by inflammation due to overloading of adipose tissue. The resulting insulin resistance causes overstimulation of pancreatic beta-cells for compensation. This chronic overproduction, islet hyperplasia and inflammation of pancreatic islets eventually lead to beta-cell dysfunction and overt T2D. To date, genetic studies have associated hundreds of genetic loci with T2D susceptibility. A highly polygenic architecture of T2D has been established, which is dominated by common alleles with small and cumulative effects [2]. Since the identified genetic loci collectively accounts for a small portion of T2D cases, genetic etiology for T2D needs to be further investigated. Low-frequency and rare sequence variants have been implicated in T2D risk by modulating β-cell mass and function [3]. Therefore, genetic studies indicate that T2D is a highly heterogeneous and polygenic disease [4].
Transcription factor GATA4 plays essential roles in many cellular processes [5–8]. GATA4 gene is expressed in mesoderm and endoderm derived tissues, including pancreas in mice [5]. In human, GATA4 gene expressed is detected in heart, liver, pancreas, stomach, small intestines, gall bladder, ovary and testis [6]. Mice with GATA4 gene deletion die before birth, mainly due to severe defects in heart morphogenesis and ventral foregut closure [7, 8]. In mouse embryos, GATA4 regulates development of pancreatic progenitors, and morphogenesis of pancreas [9, 10]. Pancreatic-specific deletion of GATA4 gene results in mild pancreatic defects. Double deletion of GATA4 and GATA6 in pancreas causes severe agenesis [10]. In mice, GATA4 deficiency causes ectopic pancreas, which contains all three pancreatic lineage cells [11]. Therefore, GATA4 is required for pancreatic development.

Accumulating human studies indicate that defective pancreatic β-cells plays a central role in T2D pathogenesis [12]. A significant proportion of genetic variants for T2D risk impacts pancreatic islet cell function and insulin secretion [13]. Mutations in GATA4 gene have been implicated in neonatal or childhood-onset diabetes [14]. Human GATA4 is required for endoderm and pancreatic progenitors in a dosage-sensitive manner [15]. Thus, we postulated that regulatory variants of GATA4 gene may alter GATA4 level, conferring susceptibility to T2D development by affecting pancreatic formation and function. In this study, we aimed to investigate whether regulatory variants in GATA4 gene may change GATA4 levels, conferring susceptibility to T2D development. Therefore, the GATA4 gene promoter was genetically and functionally investigated with genomic samples from cohorts of T2D patients and ethnic-matched controls. Regulatory variants of GATA4 gene were identified and functionally analyzed.

**Methods**

**Study participants**

This was a prospective study. Enrolled T2D patients were newly diagnosed, and the clinical samples were collected from December 2017 to December 2018. The T2D patients (n = 255) were recruited from Yanzhou People’s Hospital, Affiliated Hospital of Jining Medical University, Jining Medical University (Jining, Shandong, China). Diagnosis criteria for T2D by WHO (World Health Organization) include fasting blood glucose > 7.0 mmol/L, or 2-h plasma glucose > 11.0 mmol/L, or HbA1C > 6.5% [16]. T2D patients included 138 males and 117 females. The age range were from 22 to 82 years. Ethnic-matched controls (n = 371) were from the subjects receiving routine check-up in the Physical Examination Center in the same hospital, including 197 males and 174 females. The age range were from 21 to 84 years. The controls with heart or kidney diseases were excluded. The patients with Type 1 diabetes (T1D) and the controls with familial history of T1D were excluded from this study. T1D was diagnosed according to the WHO 1999 screening criteria. In this study population, the primary hypertension was diagnosed with recorded systolic pressure > 140 mmHg or diastolic pressure > 90 mmHg or being actively treated for hypertension. The research protocol has been approved by the Ethics Committee of Jining Medical University. This study conforms to the provisions of the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013). Informed written consents were obtained from all participants.

**Targeted sequencing**

Peripheral leukocytes were isolated and genomic DNAs extracted using QIAamp DNA mini kit (Thermo Fisher Scientific, Waltham, MA, USA). Human GATA4 gene proximal promoter (1028 bp, −961 bp ~ +67 bp to the transcription start site) was directly sequenced. Two overlapped DNA fragments covering GATA4 gene proximal promoter, 510 bp (−961 bp ~ −451 bp) and 569 bp (−502 bp ~ +67 bp), were generated by PCR. The primers were designed using the human GATA4 genomic sequence (NCBI; NG_008177.2), which were previously reported [17]. PCR products were bi-directionally sequenced. Regulatory variants were identified by aligning the sequences with GATA4 gene promoter.

**Dual-luciferase reporter assay**

For functional analysis, wild type and variant GATA4 gene proximal promoters (971 bp, from -932 bp to +39 bp) were generated by PCR, and inserted upstream to reporter gene-luciferase (pGL3-basic). Human embryonic kidney cells (HEK-293) or rat insulinoma cells (INS-1) were cultured and transiently transfected with expression vectors, together with vector pRL-TK expressing renilla luciferase gene. The transfected cells were grown for 48 h. The cells were then collected and lysed. The dual-luciferases activities of the cell lysates were measured with Promega Glomax 20/20 luminometer. Ratios of luciferase activity over renilla luciferase activity was used to represent transcriptional activity. Wild type GATA4 gene promoter activity was set as 100%. Relative activity of GATA4 gene promoter was obtained. All experiments were repeated three times independently, in triplicate.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific) according to the procedure. HEK293 and INS-1 cell nuclear extracts were prepared with NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Protein
concentration was determined. Biotinylated probes were double-stranded oligonucleotides (30 bp) with or without regulatory variants. DNA-protein binding was carried out for 20 min (room temperature), and then separated on a 6% polyacrylamide gel. The DNA-protein complexes were subsequently transferred onto a nylon membrane (Thermo Fisher Scientific). Cross-link of oligonucleotides and membrane was conducted and signals detected by chemiluminescence.

Statistical analysis
SPSS v23.0 was used for statistical analysis in this study. Quantitative data was compared with standard student’s t-test. Frequencies of regulatory variants between two groups were compared with χ² test. P < 0.05 was considered as significant.

Results
Clinical and biochemical characteristics
Clinical data and biochemical characteristics were shown in Table 1. In this population, percentages of hypertension and smoking in T2D group were significantly higher than those in control group (P < 0.05). No significant difference in age, body mass index (BMI), SBP (systolic blood pressure), DBP (diastolic blood pressure), triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL) and low density lipoprotein cholesterol (LDL) existed between two groups (P > 0.05).

| Table 1 Clinical and biochemical characteristics of T2D patients and controls |
|---------------------------------------------------------------|
| Controls(n = 371) | T2D(n = 255) | P value |
|-------------------|--------------|---------|
| Age (years, mean ± SD) | 51.12 ± 12.52 | 53.00 ± 11.88 | 0.062 |
| Male (n, %) | 197 (53.10%) | 138 (54.12%) | 0.807 |
| Smoking (n, %) | 42 (11.32%) | 80 (31.37%) | 0.000 |
| BMI (Kg/M²) | 25.11 ± 3.58 | 24.86 ± 3.56 | 0.438 |
| Hypertension (n, %) | 94 (25.34%) | 97 (38.04%) | 0.001 |
| SBP (mmHg) | 129.08 ± 21.12 | 132.28 ± 19.71 | 0.067 |
| DBP (mmHg) | 78.26 ± 13.42 | 80.19 ± 11.89 | 0.075 |
| TG (mmol/L) | 1.57 ± 1.10 | 1.66 ± 1.07 | 0.091 |
| TC (mmol/L) | 5.02 ± 0.95 | 4.88 ± 1.09 | 0.227 |
| HDL (mmol/L) | 1.27 ± 0.30 | 1.21 ± 0.53 | 0.359 |
| LDL (mmol/L) | 2.91 ± 0.83 | 2.81 ± 0.86 | 0.127 |

BMI body mass index, DBP diastolic blood pressure, HDL high density lipoprotein cholesterol, LDL low density lipoprotein cholesterol, SBP systolic blood pressure, TG triglyceride, TC total cholesterol. Quantitative data including age, BMI, TG, TC, HDL and LDL was expressed as mean ± SD.

Functional analysis of regulatory variants
GATA4 gene regulatory variants were functionally analyzed in cultured cells. The results were shown in Fig. 2. In HEK-293 cells, variant (32124C > T) significantly increased GATA4 gene promoter activity (P < 0.01). SNP [31487C > G (rs1053351749)] significantly decreased GATA4 gene promoter activity (P < 0.01). In contrast, variants (31,403 G > T, 31566G > C and 31567A > G) found in controls did not significantly changed GATA4 gene promoter activity (P > 0.05) (Fig. 2a).

To determine tissue-specific effects of the regulatory variants, we examined the GATA4 gene promoter activity in INS-1 cells (Fig. 2b). Variant (32124C > T) significantly increased GATA4 gene promoter activity (P < 0.01). SNP [31487C > G (rs1053351749)] significantly decreased GATA4 gene promoter activity (P < 0.01). As expected, variants (31,403 G > T, 31566G > C and 31567A > G) did not significantly changed GATA4 gene promoter activity (P > 0.05). Therefore, these regulatory variants identified in T2D patients affected GATA4 gene promoter activity in both HEK-293 and INS-1 cells, suggesting that their effects was non-tissue specific.

Regulatory variants-affected transcription factor binding
EMSA was performed with nuclear extracts of HEK-293 and INS-1 cells. DNA sequences of the oligonucleotides (30 bp)
Fig. 1 Identified regulatory variants of GATA4 gene. a. Locations of the regulatory variants. The transcription start site is at the position of 32,284 (+1) in the first exon of the human GATA4 gene (NG_008177.2). b. Sequencing chromatograms of the regulatory variants identified in T2D patients. Sequence orientations are all forward. Top panels are wild type and bottom variant sequences. Arrows indicate heterozygous variant.

Table 2 Regulatory variants in the GATA4 gene promoters in T2D patients and controls

| Regulatory variants | Genotypes | Location\(^a\) | Controls \((n = 371)\) | T2D \((n = 255)\) | \(P\) value |
|---------------------|-----------|----------------|----------------------|------------------|---------|
| 31,360 T > C (rs372004083) | TC        | -924 bp       | 1                    | 0                | –       |
| 31403 G > T        | GT        | -881 bp       | 1                    | 0                | –       |
| 31437 C > A (rs769262495) | CA        | -847 bp       | 1                    | 0                | –       |
| 31487 C > G (rs1053351749) | CG        | -797 bp       | 0                    | 1                | –       |
| 31,492 T > A       | TA        | -792 bp       | 1                    | 0                | –       |
| 31566 G > C        | GC        | -718 bp       | 1                    | 0                | –       |
| 31567 A > G        | AG        | -717 bp       | 1                    | 0                | –       |
| 31715 C > A        | CA        | -569 bp       | 1                    | 0                | –       |
| 31730 A > G (rs56306152) | AG        | -554 bp       | 1                    | 0                | –       |
| 31979_80InsG       | −/G       | -304 bp       | 7                    | 3                | 0.747   |
| 32124 C > T       | CT        | -160 bp       | 0                    | 1                | –       |
| 32171 A > G (rs944611351) | AG        | -113 bp       | 1                    | 0                | –       |
| 32190 C > T       | CT        | -84 bp        | 1                    | 0                | –       |

\(^a\)Variants are located upstream \((-)\) to the transcription start site of GATA4 gene at 32284 of NG_008177.2
were “GACACATTCCCCTC(C/G)CCCATACCCTGGAA
G” for SNP [31487C > G (rs1053351749)], and “CCCCA-
GAGCCTGGA(C/T)TTTGCCTGCTGGGGG” for variant
(32124C > T). The results showed that in both line cells, the
variant (32124C > T) evidently enhanced the binding ability
of a transcription factor (Fig. 3). Since variant (32124C > T)
increased GATA4 gene promoter activity, this transcription
factor probably functions as an activator. The effect of SNP
[31487C > G (rs1053351749)] on transcription factor binding
was not detected, likely due to low level of the transcription
factors or EMSA sensitivity limit.

Discussion
Manipulation of GATA4 gene expression by targeting its
promoter with genetic or therapeutic approaches might
provide a potential way for clinical purposes. GATA4
gene mutations have been associated with several human
diseases, including congenital heart disease, coronary ar-
tery disease, hypertension, type 1 diabetes and various
cancers [14, 19]. A GATA4 mutation has been found in
a child with atrial septal defect and neonatal diabetes
caused by pancreatic agenesis [20]. In human ectopic
pancreatic tissues, GATA4 gene expression is
downregulated [11]. In previous studies, we have found
several GATA4 gene regulatory variants in patients with
congenital heart disease [17]. In this study, we identified
two functional regulatory variants of GATA4 gene in
T2D patients. Considering the complex genetic hetero-
geneity in T2D etiology, GATA4 gene regulatory vari-
ants may probably contribute to the T2D development
as a rare risk factor.

The human GATA4 gene is mapped to chromosome
8p23.1-p22, and contains seven exons [21]. In normal
human tissues, GATA4 is highly detected in heart, liver,
pancreas, stomach, small intestines, ovaries and testes
[6]. There are conserved GC-box, E-box, AP-1 and
GATA motif in the GATA4 gene promoter region [22].
FOXA2 regulates GATA4 gene expression through an
intronic enhancer [23]. A distant enhancer of GATA4
gene is bound by pancreatic duodenal homeobox 1,
directing its expression in the mouse endoderm [24]. In
this study, conserved binding motifs for transcription
factors in GATA4 gene promoter were not disrupted by
identified regulatory variants. Therefore, the identified
regulatory variants may change GATA4 gene expression
levels by altering the binding of transcription factors.
Recent studies on genomic occupancy of GATA4 indicate that GATA4 exhibits cell-type-specific binding [25]. To date, few GATA4-interacting proteins and downstream targets have been identified in the pancreatic formation. GATA4 and GATA6 coordinate to regulate embryonic pancreatic regulators [9, 10]. GATA4 and GATA6 inhibit hedgehog signaling to regulate pancreatic endoderm specification [26]. GATA4 co-occupies genomic regions with TCF7L2, and represses TCF7L2/β-catenin complex in adult heart [27]. Interestingly, TCF7L2 gene polymorphisms are linked to type 1 diabetes and T2D [28]. In addition, GATA4 contributes to the regulation of pancreas development by initiating pancreatic gene regulation, and is involved in regulating pancreatic glucagon gene expression [29]. In mouse neuroendocrine tumor derived cells, GATA4 increases insulinotropic polypeptide gene expression [30]. Collectively, GATA4 plays essential roles for beta cell activity and pancreatic development. As GATA4 is a dosage-sensitive regulator, deficient or excessive GATA4 may disrupt pancreatic β-cell function.

In conclusion, two functional regulatory variants in GATA4 gene were identified in T2D patients. Our study suggested that GATA4 gene regulatory variants may probably contribute to the T2D development as a rare risk factor by influencing beta cell development and activity.

Since GATA4 is involved in the pancreatic development and acts in a dose-sensitive manner, we first investigated the regulatory variants of GATA4 gene in T2D patients, which was the strength of the study. As this was a primary study, the sample size was relatively small, which was the limitation of the study. Further larger studies are needed to confirm the finding from this study.

Abbreviations
GATA4: Transcription factor GATA4; GATA6: Transcription factor GATA6; HEK-293: Human embryonic kidney cells; HNF4A: Hepatocyte nuclear factor 4 alpha; HNF4G: Hepatocyte nuclear factor 4 gamma; INS-1: Rat insulinoma cells; KLF5: Kruppel like factor 5; THAP1: THAP domain containing 1; T2D: Type 2 diabetes; ZNF148: Zinc finger protein 148

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Authors’ contributions
LD, MC, LC and HY performed the experiments and analyzed the results. SL and SP collected and prepared the clinical samples. SP validated the data and analysis. LD and BY wrote and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The novel variants of GATA4 gene promoter database has been deposited in the NCBI SNP promoter database. The NCBI Sub_SNP numbers are following, 31403G > T (ss213754185), 31492 T > C (ss236862200), 31566G > C (ss236862200), 31567A > G (ss236862210), 31715C > A (ss236862211), 32124C > T (ss236862213), 32190C > T (ss236862214) and 31979_80insG (ss236862212).

Declarations

Ethics approval and consent to participate
The research protocol was approved by the Ethics Committee of Jining Medical University. This study conforms to the provisions of the Declaration of Helsinki (as revised in Fortaleza, Brazil, October 2013). Informed consents were obtained from all participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Kahn SE, Cooper ME, Del Prato S. Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future. Lancet. 2014; 383(9922):1068–83. https://doi.org/10.1016/S0140-6736(13)6254-6.
2. Langenberg C, Lotta LA. Genomic insights into the causes of type 2 diabetes: perspectives on the past, present, and future. Lancet. 2014;383(9922):1068–74. https://doi.org/10.1016/j.jad.2014.01.024.
3. Steinthorsdottir V, Thorleifsson G, Sulem P, Helgason H, Grarup N, Pedersen NL, et al. Loss of GATA4 causes ectopic pancreas in the stomach. J Pathol. 2020;250(4):362–73. https://doi.org/10.1002/path.5378.
4. Molkentin JD. The zinc finger-containing transcription factors GATA-4, −6, and −11.8.1048.
5. Kuo CT, Morrisey EE, Anandappa R, Sigrist K, Lu MM, Parmacek MS, et al. GATA4 transcription factor is required for ventral morphogenesis and heart tube formation. Genes Dev. 1997;11(8):1048–60. https://doi.org/10.1101/gad.11.8.1048.
6. Molkentin JD, Lin Q, Duncan SA, Olson EN. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Dev. 1997;11(8):1061–72. https://doi.org/10.1101/gad.11.8.1061.
7. Carrasco M, Delgado I, Soria B, Martin F, Rojas A. GATA4 and GATA6 control mouse pancreas organogenesis. J Clin Invest. 2012;122(10):3504–15. https://doi.org/10.1172/JCI63524.
8. Xuan S, Boroi DJ, Decker KL, Battle MA, Duncan SA, Hale MA, et al. Pancreas-specific deletion of mouse Gata4 and Gata6 causes pancreatic agenesis. J Clin Invest. 2012;122(10):3516–28. https://doi.org/10.1172/JCI63524.
9. Rodríguez-Seguel E, Villamayor L, Arroyo N, De Andrés MP, Real FX, Martín F, et al. Loss of GATA4 causes ectopic pancreas in the stomach. J Pathol. 2020; 250(4):362–73. https://doi.org/10.1002/path.5378.
10. Xuan S, Boroi DJ, Decker KL, Battle MA, Duncan SA, Hale MA, et al. Pancreas-