

## Variants in hepatocyte nuclear factor 4 $\alpha$ gene promoter region and type 2 diabetes risk in Chinese

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### Abstract

As a key regulator of insulin secretion and metabolism of glucose, cholesterol and fatty acid, hepatocyte nuclear factor 4 $\alpha$  (HNF4A) was suggested as a candidate gene for type 2 diabetes (T2D); however, no association study between HNF4A and T2D in the Chinese population has been conducted before. To address this issue, we evaluated the impact of the HNF4A variants (rs1884614 and rs2425637) on T2D and metabolic traits in 1912 unrelated patients and 2041 control subjects in the Chinese Han population. Our results suggested that no individual single nucleotide polymorphisms of HNF4A was significantly associated with T2D at either allele or genotype level. However, rs2425637 in the promoter region of HNF4A was found to have an effect on total cholesterol and high-density lipoprotein before multiple testing correction. To summarize, our investigation did not confirm the effects of HNF4A variants (rs1884614 and rs2425637) on T2D risk, but found that the risk HNF4A contributed to T2D might be population specific.

**Keywords:** type 2 diabetes, HNF4A, association, cholesterol, HDL, heterogeneity

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### Introduction

Hepatocyte nuclear factor 4 $\alpha$  (HNF4A) is a transcription factor in the steroid hormone receptor family widely expressed in liver, kidney, pancreas and other tissues.<sup>1,2</sup> To our knowledge, HNF4A interacts with approximately 12% of genes expressed in the liver and 11% in the pancreatic islets,<sup>3</sup> regulating glucose, cholesterol and fatty acid metabolism, as well as insulin secretion.<sup>4</sup> Thirteen exons and two promoters have been identified in the *HNF4A* gene. In addition, at least nine splicing isoforms driven by different promoters have also been identified. The P2 promoter, located about 46 kb upstream of the P1 promoter and the coding exons, is the major transcription start site in pancreatic  $\beta$ -cells while the P1 promoter is mainly active in hepatocytes, and partly accounts for the complex expression patterns.<sup>1,2,5</sup>

Loss of function mutations of HNF4A have been identified as causing type 1 maturity-onset diabetes of the young (MODY),<sup>6</sup> a monogenetic form of type 2 diabetes (T2D), characterized by early age of onset, autosomal-dominant inheritance and primary defects in glucose-dependent

insulin secretion.<sup>7</sup> Both MODY and T2D patients have reduced insulin sensitivity as a result of pancreatic islet  $\beta$ -cell dysfunction.

As an attractive candidate gene for linkage to T2D observed at 20q12–13 in Caucasians, Asians and Africans,<sup>8–14</sup> the *HNF4A* gene has been screened to investigate the possible association with T2D. Recent studies have shown that single nucleotide polymorphisms (SNPs) within a large haplotype block surrounding the P2 promoter have been associated with T2D in Finnish,<sup>15</sup> Ashkenazi Jewish,<sup>16</sup> Danish,<sup>17</sup> UK,<sup>18</sup> Norwegian<sup>19</sup> and Mexican American<sup>20</sup> subjects. However, no individual SNPs in that block have been associated with T2D in the Amish,<sup>21</sup> French,<sup>22</sup> Caucasian-American<sup>23</sup> and Thais,<sup>24</sup> another Norwegian<sup>25</sup> and another UK populations.<sup>26</sup> Association studies of the *HNF4A* gene in other regions, such as the P1 promoter, were also inconsistent.<sup>15,17,21,27</sup> It is unclear, therefore, whether HNF4A is a risk factor for T2D.

To explore the potential association between HNF4A and T2D, we investigated the most attractive tag SNPs, rs1884614, which is representative of the haplotype block

surrounding P2 promoter and rs2425637 in the P1 promoter of the HNF4A gene. In addition, we also analyzed the possible effect of the variants on clinical traits, to explore the role that HNF4A might play in metabolism.

## Methods

### Subject and DNA preparation

In the case-control investigation, 1912 unrelated type 2 diabetic patients (785 men, 1127 women; age  $63.9 \pm 9.5$  years) and 2041 healthy controls (635 men, 1406 women; age  $58.1 \pm 9.4$  years) with a fasting plasma glucose (FPG) concentration of  $<6.1$  mmol/L were recruited from Shanghai, China. T2D was defined in accordance with World Health Organization criteria (FPG  $\geq 7.0$  mmol/L and/or 2-h plasma glucose  $\geq 11.1$  mmol/L). All the participants were local residents who lived for years in two districts of Shanghai, China and all of them were Han population. No individual was diagnosed with severe diseases such as cancer, stroke, or with psychological disorder. This study was conducted with the permission of the Ethics Committee of Shanghai Institute for Biological Sciences and written informed consent was obtained from all participants before starting the investigation. At the site of investigation, a fasting blood sample was collected and blood pressure, height, weight, waist and hip circumferences were recorded from participants by trained medical professionals using a standardized protocol. Body mass index (BMI) was calculated as weight (kg)/[height (m)]<sup>2</sup>. Total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides, HbA1c and FPG were measured enzymatically according to standard methods on a Roche modular P800 autoanalyzer (Roche, Mannheim, Germany) with reagents (Roche Diagnostics GmbH, Mannheim, Germany). The clinical characteristics of the participants were summarized in Table 1 with all values expressed as mean  $\pm$  SD. It should be noted that 31 participants in this study did not complete their personal information table, so we could not get the information about their gender and age. High molecular

weight genomic DNA was prepared from venous blood using the QuickGene 610L Automatic DNA/RNA Extraction System (Fijifilm, Tokyo, Japan).

### Genotyping

Both of the SNPs (rs1884614, rs2425637) were genotyped using the TaqMan technology on an ABI7900 system (Applied Biosystems, Foster City, CA, USA). The probes for rs1884614 and rs2425637 were AGGGTGTAACTTACC CAGAGCTGCA[C/T]AGCTTGTCTTTGATGGCCAAGGTT A and TTGCCGAGTGCAGAGGGTGGTATGA[G/T]TGG CATTITAGGAGCTCAAGGAAGA, respectively. The standard 5  $\mu$ L polymerase chain reaction (PCR) reactions were carried out using TaqMan Universal PCR Master Mix reagent kits under the guidelines provided: 50°C for two minutes, followed by 95°C for 10 min and then 92°C for 15 seconds and 60°C for 1 min (these two steps together by 40 cycles). Genotype data were obtained in more than 95% of the DNA samples; replicate quality control samples (32 samples) were included and genotyped with 100% concordance.

### Statistical analysis

SHEsis was used to perform the Hardy-Weinberg equilibrium (HWE) test and to compare the differences of allele frequencies and genotype distributions between cases and controls.<sup>28</sup>

Logistic regression analysis was used to calculate odds ratios (ORs), 95% confidence intervals (CIs) and corresponding *P* values, after adjusting for sex, age and BMI. For regression modelling in the additive model, homozygotes for the major allele (1/1), and heterozygotes (1/0) and homozygotes for the minor allele (0/0) were coded to an ordered categorical variable for the genotype (0, 1 and 2). The dominant model was defined as 0/0 + 1/0 versus 1/1 and the recessive model as 0/0 versus 1/0 + 1/1.

Multiple linear regression was used to analyze the difference in the quantitative traits including BMI, waist-to-hip ratio (w/h), FPG, HbA1c, triglycerides (Trig), total cholesterol, LDL and HDL according to the genotypes in control subjects, with the clinical and biochemical characteristics above as the dependent variable and genotype, age and gender as the independent variable. The value of Trig was normalized by logarithmic transformation for analyses. Benjamin's method for multiple testing was used to control the false discovery rate.<sup>29</sup>

All data were analyzed using SPSS/Win programs (SPSS Inc, Chicago, IL, USA) and results are presented as means  $\pm$  SD. Power calculations were performed on the G\*Power program.<sup>30</sup> All *P* values reported were two-tailed. Statistical significance was defined at *P* < 0.05.

## Results

Based on the linkage disequilibrium pattern and haplotype block structure defined in previous studies, we genotyped two SNPs (rs1884614 and rs2425637) in the HNF4A gene

**Table 1** Clinical characteristics of the subjects

|                                 | Type 2 diabetes    | Control            | <i>P</i> value       |
|---------------------------------|--------------------|--------------------|----------------------|
| BMI (kg/m <sup>2</sup> )        | 25.10 $\pm$ 3.45   | 24.59 $\pm$ 3.27   | 2.53e <sup>-6</sup>  |
| Waist-to-hip ratio              | 0.89 $\pm$ 0.06    | 0.86 $\pm$ 0.06    | 5.87e <sup>-56</sup> |
| Fasting blood glucose (mmol/L)  | 8.11 $\pm$ 2.91    | 4.83 $\pm$ 0.64    | 0                    |
| Triglycerides (mmol/L)          | 1.90 $\pm$ 1.58    | 1.60 $\pm$ 1.05    | 5.69e <sup>-12</sup> |
| Cholesterol (mmol/L)            | 4.48 $\pm$ 0.95    | 4.50 $\pm$ 0.87    | 0.432                |
| HDL (mmol/L)                    | 1.18 $\pm$ 0.31    | 1.27 $\pm$ 0.32    | 1.51e <sup>-18</sup> |
| LDL (mmol/L)                    | 2.75 $\pm$ 0.77    | 2.82 $\pm$ 0.73    | 0.002                |
| Systolic blood pressure (mmHg)  | 140.05 $\pm$ 20.35 | 131.96 $\pm$ 19.31 | 1.59e <sup>-36</sup> |
| Diastolic blood pressure (mmHg) | 81.87 $\pm$ 10.09  | 81.04 $\pm$ 10.07  | 0.009                |
| HbA1c (%)                       | 7.51 $\pm$ 1.61    | 5.78 $\pm$ 0.58    | 0                    |

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein

Data are shown as means  $\pm$  SD

*P* values are provided by *t*-test for equality of means

in 1912 unrelated type 2 diabetic patients and 2041 healthy controls. The allele and genotype frequencies for the two SNPs were summarized in Table 2, and all were in HWE in the control group. The association of SNPs with T2D was assessed by logistic regression after adjusting for sex, age and BMI in the additive model, the dominant model and the recessive model. We found that neither of the two SNPs revealed noticeable allelic or genotypic significance with T2D (Table 2).

Because of the important role that HNF4A plays in the metabolism, we specifically examined the relationship between the above two SNPs and BMI, w/h, FPG, HbA1c, Trig, total cholesterol, LDL and HDL in the control group using general linear model analysis (Table 3). We found that rs2425637 showed effects on total cholesterol ( $P = 0.004$ ) and HDL ( $P = 0.017$ ) after adjusting for age, sex and BMI, although rs1884614 had no effect on these characteristics. However, these associations seemed to be false positive by Benjamin's false discovery rate control.<sup>29</sup>

## Discussion

Rs1884614 and rs2425637 of HNF4A were originally reported to be associated with T2D by Love-Gregory *et al.*<sup>16</sup> and Silander *et al.*<sup>15</sup> For the Chinese population in our study, none of the individual SNPs was significantly associated with T2D. There are several possible explanations for this discrepancy such as sample size, genetic background and environmental factors.

This replication failure was unlikely to result from lack of power in our study. If an OR of 1.3 existed between the two SNPs and T2D in the Chinese population like that in the Ashkenazi Jewish population, we could not miss it with a power of 100.00%; for an OR of 1.2, we provided 99.99% power for both of the SNPs; even for an OR of 1.1, we have a 84.8% power for rs1884614 and 83.8% for rs2425637. However, if HNF4A contributed only a minor effect on T2D, it would have been hard to detect in our current sample size.

We followed strict inclusion and exclusion criteria on sample collection. As introduced in the method, we recruited only local residents in two districts in Shanghai and excluded those who had migrated here in recent years, and all the participants were of the Han population. This helped to reduce the risk of stratification within the population.

To further investigate the reason for discrepancy, we compared association results and allele frequencies of rs1884614 and rs2425637 in the Chinese population with those in other populations.

We first compared our association study with those conducted in the Japanese, who were considered to have a similar genetic background to the Chinese. As for rs1884614, no association was found between it and T2D in all studies performed in the Japanese.<sup>31–33</sup> The minor allele frequency was 0.48, 0.43 and 0.49 in the study by Tanahashi *et al.*<sup>33</sup> Takeuchi *et al.*<sup>32</sup> and Hara *et al.*,<sup>31</sup> respectively, while it was 0.44 in our study. The similar allele frequency may help to explain the consistent results in these two populations. Association results of rs2425637 were not compared because no association study between rs2425637 and T2D was performed in the Japanese population.

Then, we compared our association study with those conducted in Caucasians. The allele frequencies were distinct between Chinese and Caucasians.<sup>15,17–19,21,27,34</sup> For rs1884614, the minor allele frequency ranged from 0.14 to 0.20 in Caucasians, while it was 0.44 in Chinese. For rs2425637, the T allele frequency is 0.48; however, the T allele turned out to be a major allele in several studies conducted in Caucasians.<sup>15,17,22,23</sup> This really indicates that Chinese and Caucasians might have a different genetic background. It was noted that the association results diverged in the Caucasians, in that although they shared similar allele frequencies, a positive association was only confirmed in the Ashkenazi population while negative results were just substantiated in the people of the United Kingdom.<sup>16,18,26</sup> As for other populations in Europe, it was not clear whether HNF4A was a risk factor for T2D because of inconsistent results. A recent study performed

**Table 2** Association study between the two SNPs and T2D in Chinese Han population

| SNP       | Risk allele | Genotype distribution |                | Risk-allele frequencies |                 | Unadjusted OR<br>OR (95% CI)                        | Adjusted OR                                        |                                                    |                                                    |
|-----------|-------------|-----------------------|----------------|-------------------------|-----------------|-----------------------------------------------------|----------------------------------------------------|----------------------------------------------------|----------------------------------------------------|
|           |             | Control<br>n (%)      | T2D<br>n (%)   | Control<br>n (%)        | T2D<br>n (%)    |                                                     | OR (95% CI)*                                       | OR (95% CI)†                                       | OR (95% CI)‡                                       |
| rs1884614 | T           | TT 384<br>(0.190)     | 385<br>(0.205) | 1776<br>(0.439)         | 1707<br>(0.454) | 1.065<br>(0.974–1.164)<br>$P_{\text{freq}} = 0.168$ | 1.064<br>(0.968–1.170)<br>$P_{\text{add}} = 0.197$ | 1.091<br>(0.945–1.260)<br>$P_{\text{dom}} = 0.235$ | 1.079<br>(0.914–1.275)<br>$P_{\text{rec}} = 0.370$ |
|           |             | TC 1008<br>(0.498)    | 937<br>(0.499) |                         |                 |                                                     |                                                    |                                                    |                                                    |
|           |             | CC 632<br>(0.312)     | 557<br>(0.296) |                         |                 |                                                     |                                                    |                                                    |                                                    |
| rs2425637 | T           | TT 489<br>(0.244)     | 437<br>(0.239) | 1935<br>(0.484)         | 1772<br>(0.484) | 1.003<br>(0.917–1.097)<br>$P_{\text{freq}} = 0.953$ | 0.993<br>(0.904–1.090)<br>$P_{\text{add}} = 0.876$ | 1.015<br>(0.874–1.180)<br>$P_{\text{dom}} = 0.845$ | 0.964<br>(0.825–1.127)<br>$P_{\text{rec}} = 0.643$ |
|           |             | TG 957<br>(0.478)     | 898<br>(0.491) |                         |                 |                                                     |                                                    |                                                    |                                                    |
|           |             | GG 554<br>(0.277)     | 494<br>(0.270) |                         |                 |                                                     |                                                    |                                                    |                                                    |

T2D, type 2 diabetes; OR, odds ratio; CI, confidence interval

\*Additive model

†Dominant model

‡Recessive model, all adjusted for sex, age and BMI

**Table 3** Genotype distributions of rs1884614 and rs2425637 on clinical and biochemical parameters in control subjects

| Variant   | Allele | Trait                      | Genotype*    |              |              | P value†<br>Additive |
|-----------|--------|----------------------------|--------------|--------------|--------------|----------------------|
|           |        |                            | 1/1          | 1/0          | 0/0          |                      |
| Rs1884614 | C > T  | BMI (kg/m <sup>2</sup> )   | 24.47 ± 3.23 | 24.54 ± 3.28 | 24.59 ± 3.14 | 0.479                |
|           |        | FPG (mmol/L)               | 4.80 ± 0.64  | 4.84 ± 0.64  | 4.84 ± 0.62  | 0.227                |
|           |        | Triglycerides (mmol/L)     | 1.62 ± 1.05  | 1.58 ± 1.05  | 1.61 ± 1.05  | 0.814                |
|           |        | HbA1c (%)                  | 5.79 ± 0.54  | 5.77 ± 0.59  | 5.74 ± 0.62  | 0.192                |
|           |        | Waist-to-hip ratio         | 0.86 ± 0.06  | 0.86 ± 0.06  | 0.86 ± 0.06  | 0.398                |
|           |        | Total cholesterol (mmol/L) | 4.53 ± 0.85  | 4.49 ± 0.90  | 4.51 ± 0.85  | 0.696                |
|           |        | LDL (mmol/L)               | 2.84 ± 0.70  | 2.81 ± 0.75  | 2.84 ± 0.74  | 0.910                |
|           |        | HDL (mmol/L)               | 1.27 ± 0.33  | 1.28 ± 0.32  | 1.24 ± 0.32  | 0.477                |
|           |        |                            |              |              |              |                      |
| Rs2425637 | G > T  | BMI (kg/m <sup>2</sup> )   | 24.45 ± 3.26 | 24.51 ± 3.20 | 24.71 ± 3.27 | 0.249                |
|           |        | FPG (mmol/L)               | 4.79 ± 0.63  | 4.84 ± 0.64  | 4.86 ± 0.65  | 0.122                |
|           |        | Triglycerides (mmol/L)     | 1.57 ± 1.05  | 1.62 ± 1.10  | 1.60 ± 0.96  | 0.483                |
|           |        | HbA1c (%)                  | 5.77 ± 0.57  | 5.76 ± 0.58  | 5.82 ± 0.61  | 0.281                |
|           |        | Waist-to-hip ratio         | 0.86 ± 0.06  | 0.86 ± 0.06  | 0.86 ± 0.06  | 0.064                |
|           |        | Total cholesterol (mmol/L) | 4.60 ± 0.89  | 4.48 ± 0.88  | 4.45 ± 0.82  | 0.004                |
|           |        | LDL (mmol/L)               | 2.87 ± 0.74  | 2.80 ± 0.74  | 2.80 ± 0.70  | 0.114                |
|           |        | HDL (mmol/L)               | 1.31 ± 0.35  | 1.25 ± 0.31  | 1.25 ± 0.31  | 0.017                |
|           |        |                            |              |              |              |                      |

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; FPG, fasting plasma glucose

Data are given as means ± SD

\*1/1, subjects with homozygous for common allele; 1/0, heterozygous for rare allele; 0/0, homozygous for rare allele

†P values were derived using the additive model; it was adjusted for age and sex on BMI and waist-to-hip ratio and adjusted for age, sex and BMI on other clinical traits

by Barroso *et al.*<sup>26</sup> suggested that the risk conferred by HNF4A P2 promoter was population specific. Similarly, Bento *et al.*'s<sup>35</sup> study found heterogeneity in gene loci associated with T2D on human chromosome 20q13.1, which harbors the *HNF4A* gene region.

As discussed above, it is possible that the variation in results among the studies is related to the potential genetic background differences among the populations. Besides, other differences, such as sample inclusion criteria and environmental factors may also contribute to variations in estimated risk.

Besides the association study between rs1884614, rs2425637 and T2D, we also investigated the possible effects of the variants on several clinical and biochemical traits. We found that rs2425637 was associated with HDL and total cholesterol level in healthy people before multiple correction. However, these associations disappeared after false discovery rate by Benjamin's method.<sup>29</sup> This suggested that the former associations between rs2425637 and HDL and cholesterol might be due to false-positive error and so rs2425637 may not have a real effect on them. It is also possible that rs2425637 does have a weak effect on HDL and total cholesterol but the power is not enough to detect such a tiny effect by our sample size. It should be noted that this study was only conducted in healthy controls but not in T2D patients, as medicines taken by patients may have affected cholesterol metabolism.<sup>36,37</sup>

According to our study, it is almost impossible that rs1884614 and rs2425637 in the *HNF4A* gene play a major role in the pathogenesis of T2D in the Chinese population. However, we could not absolutely deny the possible association between the *HNF4A* gene and T2D, and more association studies covering more tag SNPs in the whole gene region are still needed to verify the potential effect of *HNF4A* on T2D. Moreover, replication studies in different populations will help us to understand the genetic heterogeneity of T2D more deeply.

**Author contributions:** All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; ZC, YL, DZZ and TZ conducted the experiments, ZC and DZ analyzed the data, ZC wrote the manuscript while YFY, LH and HX reviewed the manuscript. ZC and DZ contributed equally to this work.

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