Genetic variants of nuclear factor erythroid-derived 2-like 2 associated with the complications in Han descents with type 2 diabetes mellitus of Northeast China

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Abstract

The transcription factor nuclear factor erythroid 2-like 2 (NFE2L2) is essential for preventing type 2 diabetes mellitus (T2DM)-induced complications in animal models. This case and control study assessed genetic variants of NFE2L2 for associations with T2DM and its complications in Han Chinese volunteers. T2DM patients with (n = 214) or without (n = 236) complications, or healthy controls (n = 359), were genotyped for six NFE2L2 single nucleotide polymorphisms (SNPs: rs2364723, rs13001694, rs10497511, rs1806649, rs1962142 and rs6726395) with TaqMan Pre-Designed SNP Genotyping and Sequence System. Serum levels of heme oxygenase-1 (HMOX1) were determined through enzyme-linked immunosorbent assay. Informative data were obtained for 341 cases and 266 controls. Between T2DM patients and controls, the genotypic and allelic frequencies of the SNPs were similar. However, there was a significant difference in genotypic and allelic frequencies of rs2364723, rs10497511, rs1962142 and rs6726395 between T2DM patients with and without complications, including peripheral neuropathy, nephropathy, retinopathy, foot ulcers and microangiopathy. Furthermore, HMOX1 levels were significantly higher in T2DM patients with complications than in controls. Multiple logistic regression analysis, however, showed that only rs2364723 significantly reduced levels of serum HMOX1 in T2DM patients for the GG genotype carriers compared with participants with CG+CC genotype. The data suggest that although NFE2L2 rs2364723, rs10497511, rs1962142 and rs6726395 were not associated with T2DM risk, they were significantly associated with complications of T2DM. In addition, only for rs2364723 higher serum HMOX1 levels were found in the T2DM patients with CG+CC than those with GG genotype.

Keywords: NFE2L2 gene mutation ● diabetes ● diabetic complications ● Chinese population ● Nrf2 polymorphism

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by insulin resistance, with or without defects in insulin production and secretion. Globally, up to 90% of all diabetes cases are T2DM, and the disease is a leading world heath challenge [1, 2]. In China, the prevalence of diabetes is rising at an alarming rate. In the year 2010, 11.6% of a representative sample of Chinese adults had T2DM, translating to as many as 113.9 million in the nation [1]. It was estimated that 382 million individuals suffered from diabetes in 2013, a number that could rise to 592 million by 2035 [2]. These findings indicate the importance of diabetes as a public health problem in the world and in China.

To date, there is no effective treatment for T2DM. Clinically, T2DM management consists of changes in lifestyle, lowering other cardiovascular risk factors and maintaining blood glucose levels in the normal range [3]. Routine medical treatment is challenging, especially the identification and management of complications associated with micro- and macrovascular damage in diabetes.

The development of T2DM and its complications are likely because of multiple factors, including the lifestyle and genetic changes [4-6]. The most common type of genetic variations is single nucleotide polymorphisms (SNPs), which have been shown to impact...
population susceptibility to diseases, and individual response to drug treatments. Many SNPs are silent (often called synonymous SNPs), that is, they have no direct effect on protein coding. However, some silent SNPs can be used as genetic markers of adjacent functional variants that contribute to disease by virtue of linkage disequilibrium. Other SNPs that affect the coding or regulatory regions of genes (usually promoter regions) may have direct functional consequences [4, 5, 7].

To date, there have been numerous studies regarding links between SNPs and the risk of T2DM. Through genetic association research, some SNPs of different genes have been found to increase the risk of developing T2DM in certain populations [4, 5]. In terms of the mechanism by which diabetes is induced and diabetic complications were developed, there are a few possibilities that have been proposed; however, a generally appreciated one is the oxidative stress caused by either overgeneration of reactive oxygen species or reduction in anti-oxidative mechanisms [3, 6, 8, 9].

The protein nuclear factor erythroid 2-like 2 (NFE2L2, also known as Nrf2; encoded by the NFE2L2 gene) is a basic leucine zipper [10] to play a master regulatory role in redox balance in the cytoprotective response [10, 11]. Many experimental studies have suggested that NFE2L2 plays a preventive role in the development of both T1DM and T2DM [12] as well as their complications such as diabetic cardiomyopathy, nephropathy [6, 13], neuropathy and retinopathy [14, 15]. However, it remains unknown whether NFE2L2 polymorphisms are associated with the risk of diabetes and diabetic complications.

During non-stressed conditions, NFE2L2 is sequestered in the cytoplasm by the repressor protein KEAP1 (kelch-like ECH [erythroid cell-derived protein with CNC homology]-associated protein 1) and targeted for proteasomal degradation [10, 11]. However, during oxidative stress NFE2L2 is translocated from the cytoplasm into the nucleus, where it activates transcription of a large battery of genes by binding to antioxidant response elements (AREs) in the upstream promoter regions of its downstream genes [6, 10, 11]. The activation of NFE2L2 leads to production of cytoprotective proteins including NADPH dehydrogenase, quinone 1 (NQO1), glutamate-cysteine ligase and heme oxygenase-1 (HMOX1) [10, 16]. The antioxidant response provided by the NFE2L2 and KEAP1-NFE2L2/ARE signalling pathways protect the pulmonary, hepatic, digestive, neural and cardiovascular systems [10, 11, 16].

In this study, therefore, we investigated NFE2L2 polymorphisms for associations with risk of T2DM and its complications in a cohort of Chinese volunteers of Han descent. We also investigated whether NFE2L2 SNPs are related to expression of its downstream gene, HMOX1.

Materials and methods

Study population

For this study we recruited volunteers of Han Chinese descent: 450 patients (214 cases with complications and 236 cases without complications) and 359 healthy controls with the same age range (Table 1). The participants underwent genotyping analysis performed at the Research Center for Genomic Medicine of Jilin University (Changchun, China) between January 2010 and January 2012. A diagnosis of T2DM was determined in accordance with the diagnostic criteria set by the American Diabetes Association (ADA) in 1999 [17]. These patients were also checked with the updated ADA diagnostic criteria of T2DM and they are mostly consistent with the latest standards [18]. For the study participants, the inclusion criteria were as follows: no history of receiving pharmacologic treatment for diabetes, no clinically systemic diseases and no other acute or chronic inflammatory diseases, cancer and or acute respiratory infection when T2DM was diagnosed. This study was approved by the Second Hospital Ethics Committee of Jilin University [Protocol #: 2013L01477, (2014)018号] and written informed consent was obtained from all individuals. Diagnosed complications (after clinical examinations) in the group of T2DM patients with complications were developed after they were diagnosed diabetes, and included diabetic foot ulcer, nephropathy, retinopathy, microangiopathy, peripheral neuropathy or neurogenic bladder.

The T2DM patients were originally from Northeast China, and the age-matched healthy control participants (with neither T2DM nor family history of diabetes) were randomly selected from the health examination clinics of our hospital. During enrolment, each participant filled out a questionnaire regarding health, diagnosis of type of diabetes and T2DM duration, family history of diabetes and ethnic background (Table 1). Clinical exams included height and weight to determine body mass index (BMI; kg/m²).

SNP selection and TaqMan SNP genotyping

We used the website of the International HapMap (Haplotype Mapping) Project (http://www.hapmap.org) to download NFE2L2 SNP data from the Han Chinese in Beijing database. Data were processed with Haploview software 4.2.0.0 (Cambridge, MA, USA). Linkage disequilibrium blocks were constructed in accordance with a previous study [19]. The tag SNPs were assigned by the tagger function of Haploview. A minor allele frequency of ≥5% and pairwise tagging with a minimum $r^2$ of 0.80 were applied to capture the common variations within the blocks covering NFE2L2. Most of these sites were also checked in PubMed for its consistency with published studies [20, 21]. We therefore obtained six NFE2L2 SNPs (Table 3), including rs2364723 (SNP loci, 17812656); genotype, G/C), rs13001694 (17811990; A/G), rs10497511 (178119296; G/A), rs1806649 (178118125; C/T), rs1962142 (178113484; G/A) and rs6726395 (178103229; G/A).

To genotype volunteers for these SNPs, 5 ml of peripheral blood was collected from each into heparin-containing tubes. These blood samples were immediately stored at $−20^\circ C$ until further processed. Genomic DNA was extracted from the samples with a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) in accordance with the manufacturer’s protocol, and quantified by Nanodrop (Thermo-Fisher, Waltham, MA, USA).

The Taq SNPs were genotyped with TaqMan Pre-Designed SNP genotyping kits (Applied Biosystems, Foster City, CA, USA) and the ABI PRISM 7300 Sequence Detection System (Applied Biosystems). The polymerase chain reaction (PCR) amplification was performed in a 25 µl reaction mixture under the following conditions: 40 cycles of 95°C for 10 min., 92°C for 15 sec. and 60°C for 1 min. The primers to detect NFE2L2 SNPs were synthesized by Applied Biosystems. The ABI probe codes were C_351878_10 for rs2364723; C_31613510_10 for rs2364723.
The PCR products were randomly selected for DNA sequencing analysis by Shanghai Sangon Biological Engineering Technology & Services. For each SNP we choose three cases and the results were compared with the results of Taqman genotyping.

Measurement of serum HMOX1 levels

Serum HMOX1 levels were measured in 111 randomly selected T2DM patients (27 with complications and 84 without complications) and 53 controls with commercial ELISA kits (Enzo Life Sciences, Exeter, UK) in accordance with the manufacturer’s protocol.

Statistical analysis

The genotype frequency of NFE2L2 was assessed for Hardy–Weinberg equilibrium by the chi-squared goodness-of-fit test. A \( P \)-value >0.05 indicated Hardy–Weinberg equilibrium. Genotype frequency and distribution in patients and controls were analysed by the chi-squared test with statistical software Statistical Packages for Social Sciences (SPSS12.0, Chicago, IL, USA). The association between the combined effects of NFE2L2 SNPs and T2DM (with or without diabetic complications) was analysed by SHEsis software as in a previous study [22].

Results

Characteristics of the studied volunteers

The mean ages of the T2DM (55.9 ± 13.2 years) and control participants (55.7 ± 10.3 years) were similar, as were the gender distributions and BMI (25.7 ± 3.2 and 25.6 ± 2.7 kg/m\(^2\), respectively). The total cholesterol and total triglyceride in T2DM (4.62 ± 1.23 mmol/l and 1.81 ± 0.61 mmol/l, respectively) were higher than control participants (4.37 ± 1.16 mmol/l and 1.73 ± 1.16 mmol/l, \( P < 0.05 \), Table 1). There was no difference between T2DM patients and controls for high-density lipoprotein and low-density lipoprotein (Table 1).

Among 450 T2DM patients, 214 T2DM have various complications that were associated with diabetes, including peripheral neuropathy (\( n = 115 \)), nephropathy (\( n = 85 \)), retinopathy (\( n = 33 \)), foot ulcers (\( n = 8 \)), microangiopathy (\( n = 9 \)) and neurogenic bladder

| Table 1 Demographics and clinical characteristics of T2DM patients and healthy control participants |
|---------------------------------|----------------|----------------|
| Diagnosis, \( n \) (%)          | Control        | T2DM           | \( P \)-value |
| Diagnosis, \( n \) (%)          | 359 (44.4)     | 450 (55.6)     | 0.876         |
| Age, years                      | 55.7 ± 10.7    | 55.9 ± 13.2    | 0.746         |
| Gender, male/female             | 220/139        | 250/200        | 0.115         |
| BMI, kg/m\(^2\)                 | 25.6 ± 2.7     | 25.7 ± 3.2     | 0.724         |
| Total cholesterol, mmol/l       | 4.37 ± 1.16    | 4.62 ± 1.23    | 0.003         |
| Total triglyceride, mmol/l      | 1.73 ± 1.16    | 1.81 ± 0.61    | 0.033         |
| High-density lipoprotein, mmol/l| 1.03 ± 0.23    | 1.02 ± 0.23    | 0.810         |
| Low-density lipoprotein, mmol/l | 3.39 ± 0.39    | 3.44 ± 0.62    | 0.144         |
| Diabetic foot, \( n \)           | 0              | 8              | –             |
| Diabetic nephropathy, \( n \)   | 0              | 85             | –             |
| Diabetic microangiopathy, \( n \)| 0              | 9              | –             |
| Diabetic retinopathy, \( n \)   | 0              | 33             | –             |
| Diabetic peripheral neuropathy, \( n \)| 0 | 115 | – |
| Diabetic neurogenic bladder, \( n \) | 0 | 1 | – |
| Hyperlipidaemia, \( n \)        | 0              | 158            | –             |
| Non-alcoholic fatty liver disease, \( n \) | 0 | 53 | – |
There was no difference for age, gender, BMI, total cholesterol and high-density lipoprotein (Table 2). However, total triglyceride (1.93 ± 0.60 mmol/l) and low-density lipoprotein (3.60 ± 0.40 mmol/l) in the group without complications were significantly higher and low, respectively, than that of patients with complications (1.68 ± 0.60 mmol/l and 3.29 ± 0.73 mmol/l, \( P < 0.05 \), Table 2).

**Genotyping distribution of NFE2L2 SNPs**

The six NFE2L2 SNPs genotyped in this study were highly polymorphic and their genotypic distributions were in Hardy–Weinberg equilibrium (\( P > 0.05 \)), suggesting the suitability of this sample pool for genetic analysis.

After genotyping the patients and control participants, we only analysed the data for 341 patients and 266 control, respectively (Table 3); for the remaining cases, the serum samples or PCR readings were abandoned if one of the six sites in the sample was failed to be genotyped. Our data showed no significant difference between the T2DM patients and controls with regard to genotypic or allelic frequencies of the six NFE2L2 SNPs (\( P > 0.05 \)). However, there were significant differences both in genotypic frequencies and in allelic frequency of rs2364723, rs10497511, rs1962142 and rs6726395 between the T2DM patients without and with complications (\( P = 0.000–0.046 \), Table 4). There were also significant differences for both Dominants and Recessives of rs10497511, rs1962142 and rs6726395 between the patients with and without complications too (\( P = 0.001–0.019 \), Table 4).

Haplotypes of NFE2L2 were constructed for the patients and controls, and six major haplotypes with frequencies >3% were identified (Table 5). The data showed no statistically significant difference in prevalence of haplotypes (H1, H2, H3, H4, H5, H6 and H7) between the T2DM patients and controls (\( P > 0.05 \)).

**Serum HMOX1 levels and association with NFE2L2 SNPs**

We randomly selected a subset of T2DM patients and control participants to evaluate serum HMOX1 levels with regard to its association with these NFE2L2 SNPs (Tables 6 and 7). The mean serum HMOX1 level of T2DM patients trends higher than that of the controls and was significantly high in patients with complications compared with those without complications (Table 6).

Multiple logistic regression analysis was performed to examine the correlations of HMOX1 level with NFE2L2 SNPs or other variables including gender, diabetic complication and age (Table 7). We found that only for rs2364723 there was a significant decrease in the serum HMOX1 levels in T2DM patients for the GG genotype carriers.

| Diagnosis, n (%) | T2DM with complications | T2DM without complications | \( P \)-value |
|------------------|-------------------------|----------------------------|---------------|
| Diagnosis, n (%) | 214 (47.6)              | 236 (52.4)                 | –             |
| Age, years       | 56.9 ± 11.6             | 55.1 ± 14.5                | 0.144         |
| Gender, male/female | 121/93                 | 129/107                    | 0.688         |
| BMI, kg/m\(^2\)  | 25.7 ± 3.3              | 25.7 ± 3.2                 | 0.803         |
| Total cholesterol, mmol/l | 4.57 ± 1.43          | 4.68 ± 1.09                | 0.356         |
| Total triglyceride, mmol/l | 1.68 ± 0.60          | 1.93 ± 0.60                | 0.000         |
| High-density lipoprotein, mmol/l | 1.03 ± 0.23        | 1.03 ± 0.22                | 0.835         |
| Low-density lipoprotein, mmol/l | 3.60 ± 0.40         | 3.29 ± 0.73                | 0.000         |
| Diabetic foot, n | 8                       | 0                          | –             |
| Diabetic nephropathy, n | 85                     | 0                          | –             |
| Diabetic microangiopathy, n | 9                      | 0                          | –             |
| Diabetic retinopathy, n | 33                     | 0                          | –             |
| Diabetic peripheral neuropathy, n | 115                   | 0                          | –             |
| Diabetic neurogenic bladder, n | 1                      | 0                          | –             |
| Hyperlipidaemia | 78                      | 80                         | 0.737         |
| Non-alcoholic fatty liver disease | 28                     | 25                         | 0.495         |
| Genotype | T2DM, n (%) | Control, n (%) | OR (95% CI) | P |
|----------|-------------|---------------|-------------|---|
| rs2364723 |             |               |             |   |
| GG       | 99 (0.29)   | 73 (0.27)     | 1*          | – |
| GC       | 174 (0.51)  | 135 (0.50)    | 1.16 (0.73–1.84) | 0.537 |
| CC       | 68 (0.20)   | 58 (0.22)     | 1.10 (0.73–1.67) | 0.656 |
| HWE P-value | 0.592   | 0.766        |             |   |
| G        | 372 (0.54)  | 281 (0.53)    | 1*          | – |
| C        | 310 (0.46)  | 251 (0.47)    | 1.07 (0.85–1.35) | 0.55 |
| rs13001694 |            |               |             |   |
| AA       | 259 (0.76)  | 200 (0.75)    | 1*          | – |
| AG       | 77 (0.23)   | 64 (0.24)     | 1.93 (0.37–10.05) | 0.435 |
| GG       | 5 (0.015)   | 2 (0.08)      | 0.93 (0.64–1.36) | 0.704 |
| HWE P-value | 0.789   | 0.197        |             |   |
| A        | 595 (0.87)  | 464 (0.87)    | 1*          | – |
| G        | 87 (0.13)   | 68 (0.13)     | 1.00 (0.71–1.41) | 0.990 |
| rs10497511 |            |               |             |   |
| AA       | 172 (0.50)  | 141 (0.53)    | 1*          | – |
| AG       | 142 (0.42)  | 105 (0.40)    | 0.90 (0.49–1.68) | 0.748 |
| GG       | 27 (0.08)   | 20 (0.07)     | 1.00 (0.53–1.88) | 0.996 |
| HWE P-value | 0.758   | 0.941        |             |   |
| A        | 486 (0.71)  | 387 (0.73)    | 1*          | – |
| G        | 196 (0.29)  | 145 (0.27)    | 1.03 (0.80–1.33) | 0.824 |
| rs1806649 |            |               |             |   |
| CC       | 279 (0.82)  | 213 (0.80)    | 1*          | – |
| CT       | 59 (0.17)   | 52 (0.19)     | 0.44 (0.05–4.23) | 0.474 |
| TT       | 3 (0.009)   | 1 (0.004)     | 0.38 (0.04–3.75) | 0.406 |
| HWE P-value | 0.951   | 0.242        |             |   |
| C        | 617 (0.90)  | 478 (0.90)    | 1*          | – |
| T        | 65 (0.10)   | 54 (0.10)     | 1.07 (0.73–1.57) | 0.719 |
| rs1962142 |            |               |             |   |
| GG       | 185 (0.54)  | 146 (0.55)    | 1*          | – |
| GA       | 134 (0.39)  | 101 (0.38)    | 1.09 (0.57–2.10) | 0.786 |
| AA       | 22 (0.07)   | 19 (0.07)     | 1.15 (0.59–2.23) | 0.689 |
compared with participants with CG-CC genotype \([0.592 (0.230–0.152), \ P = 0.038]\), suggesting that NFE2L2 polymorphism of rs2364723 may cause an increase in serum HMOX1 level in T2DM patients.

### Discussion

Nuclear factor erythroid 2-like 2 is a transcription factor and functions to up-regulate expression of antioxidant genes in response to oxidative stress \([6, 13–15, 22, 23]\). We conducted this study to investigate NFE2L2 SNPs for possible associations with either T2DM or diabetic complications (including diabetic foot, nephropathy, retinopathy, microangiopathy and peripheral neuropathy) in a cohort of Chinese volunteers of Han descent. We did not find significant difference in genotype frequencies of these six SNPs between T2DM patients and healthy controls. However, T2DM patients with complications had a higher frequency of mutant rs2364723 C allele, rs10497511 G allele, rs1962142 A allele and rs6726395 G allele in the T2DM patients without complications. Increased serum HMOX1 levels were also associated with mutant genotypes of rs2364723 in T2DM patients. Thus, this study suggests that NFE2L2 SNPs are associated with T2DM patients with complications and serum levels of HMOX1.

The first issue is that we did not find the significant difference between NFE2L2 SNPs and T2DM in this study. This may be related to the diversity of risks responsible for the development of T2DM, including diet, age, environmental alterations and genetic factors. Although these risks may exist with the presence of oxidative stress, the latter is not the pivotal step for the development of T2DM. In a published study, the association between different NFE2L2 SNPs and human cancers was established \([24]\); however, there were also several studies that did not find the association between NFE2L2 polymorphisms and the risk of Alzheimer’s \([25]\), Parkinson’s diseases \([26]\) or oxidative stress biomarkers in patients with amyotrophic lateral sclerosis \([27]\). Consistent with these previous studies, we found no association of NFE2L2 polymorphisms with the risk of T2DM. These results suggest that there was no significant association of NFE2L2 polymorphisms with these chronic diseases that were not predominantly caused by oxidative stress.

Although diabetic complications were developed based on diabetes, the susceptibilities of diabetic individuals to diabetes-caused secondary complications are different. For instance, generally speaking the risk for heart disease is six times higher for women with diabetes, but only two- to threefold in men with diabetes, compared with those without diabetes \([1–3]\), suggesting that except for diabetes there are other factors to also determine the risk of complication development in these diabetic individuals.

In addition, to our knowledge, T2DM can increase oxidative stress that is a major reason to induce T2DM complications \([6, 13, 14]\); however, T2DM itself is caused by various factors among which oxidative stress may not be an essential one \([1–3, 8]\). In a line with our finding, a few previous studies have shown that NFE2L2 polymorphisms were related to progression of Alzheimer’s disease, although it was not associated with the risk of Alzheimer’s disease \([25]\). Similarly, although NFE2L2 polymorphisms were not associated with a susceptibility to childhood-onset systemic lupus erythematosus, it could confer a risk in developing kidney malfunction in patients with the disease \([28]\). These studies suggest that Alzheimer’s diseases, childhood-onset systemic lupus erythematosus and T2DM may be developed predominantly independent on the oxidative stress; however, their secondary effects on the organs such as various complications are related to the oxidative stress.

It has been well-reported that the oxidative stress was increased in the patients with diabetes and diabetic animals, reflected by enhanced oxidative stress biomarkers in the bloods and tissues, such as malondialdehyde, 3-nitrotyrosine, 4-hydroxynonenal and reactive oxygen species \([29, 30]\). The oxidative stress

### Table 3. Continued

|              | T2DM, n (%) | Control, n (%) | OR (95% CI)       | \(P\)   |
|--------------|-------------|----------------|-------------------|--------|
| HWE P-value  | 0.409       | 0.789          |                   |        |
| G            | 504 (0.74)  | 393 (0.74)     | 1*                |        |
| A            | 178 (0.26)  | 139 (0.26)     | 1.10 (0.85–1.43)  | 0.476  |
| rs6726395    |             |                |                   |        |
| AA           | 67 (0.20)   | 47 (0.18)      | 1*                |        |
| AG           | 175 (0.51)  | 134 (0.50)     | 1.22 (0.76–1.96)  | 0.402  |
| GG           | 99 (0.29)   | 85 (0.32)      | 1.12 (0.78–1.62)  | 0.541  |
| HWE P-value  | 0.512       | 0.642          |                   |        |
| A            | 309 (0.45)  | 228 (0.43)     | 1*                |        |
| G            | 373 (0.55)  | 304 (0.57)     | 1.07 (0.85–1.34)  | 0.580  |

*Reference category (odds ratio, 1.0); HWE: Hardy–Weinberg equilibrium.
| Genotype and allele frequencies of NFE2L2 SNPs stratified by T2DM complications | T2DM ± complications | Co-dominants (11 versus 12) | Dominants (11 versus 12 + 22) | Recessives (22 versus 11 + 12) |
|---|---|---|---|---|
| rs2364723 (G>C) | |  | | |
| GG | 41 (0.25) | 58 (0.33) | 1 | 0.67 (0.42–1.08) | 0.100 |
| GC | 85 (0.52) | 89 (0.51) | 0.53 (0.28–0.98) | 0.044* |
| CC | 39 (0.24) | 29 (0.16) | 0.71 (0.40–1.25) | 0.235 |
| G  | 167 (0.51) | 205 (0.58) | 1 |  |  |
| C  | 163 (0.49) | 147 (0.42) | 0.74 (0.54–0.99) | 0.046* |
| rs13001694 (G>A) | |  | | |
| AA | 124 (0.75) | 135 (0.77) | 1 | 0.26 (0.03–2.37) | 0.233 |
| AG | 40 (0.24) | 37 (0.21) | 0.27 (0.03–2.47) | 0.247 |
| GG | 1 (0.006) | 4 (0.023) | 1.18 (0.71–1.96) | 0.531 |
| A  | 288 (0.87) | 307 (0.87) | 1 |  |  |
| G  | 42 (0.13) | 45 (0.13) | 1.01 (0.64–1.58) | 0.982 |
| rs10497511 (A>G) | |  | | |
| AA | 95 (0.58) | 77 (0.44) | 1 | 1.75 (1.14–2.68) | 0.011* |
| AG | 63 (0.38) | 79 (0.45) | 3.53 (1.42–8.77) | 0.007* |
| GG | 7 (0.04) | 20 (0.11) | 2.28 (0.91–5.73) | 0.080 |
| A  | 253 (0.77) | 233 (0.66) | 1 |  |  |
| G  | 77 (0.23) | 119 (0.34) | 1.68 (1.20–2.35) | 0.003* |
| rs1806649 (C>T)  | |  | | |
| CC | 132 (0.80) | 147 (0.84) | 1 | 1.27 (0.73–2.20) | 0.400 |
| CT | 32 (0.19) | 27 (0.15) | 1.80 (0.16–20.03) | 0.634 |
| TT | 1 (0.006) | 2 (0.01) | 2.37 (0.20–27.59) | 0.491 |
| C  | 296 (0.90) | 321 (0.91) | 1 |  |  |
| T  | 34 (0.10) | 31 (0.09) | 1.19 (0.71–1.98) | 0.506 |
| rs1962142 (G>A) | |  | | |
| GG | 105 (0.64) | 80 (0.46) | 1 | 0.48 (0.31–0.74) | 0.001* |
| GA | 56 (0.34) | 78 (0.44) | 5.91 (1.92–18.13) | 0.002* |
| AA | 4 (0.02) | 18 (0.10) | 3.23 (1.04–10.07) | 0.043* |
| G  | 266 (0.81) | 238 (0.68) | 1 |  |  |
| A  | 64 (0.19) | 114 (0.32) | 1.26 (1.12–1.42) | 0.000† |

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in the patients with T2DM may play a crucial role in the development of diabetic complications [23, 31]. To support this concept, we demonstrated here that multiple NFE2L2 SNPs are associated with the development of various complications in T2DM patients caused by lack of the prevention of oxidative stress that has been appreciated as a key responsible factor for the development of diabetic complications [6, 8, 9, 12, 31].

At the cellular level, activation of NFE2L2 results in the up-regulated expression of many cytoprotective proteins, including HMOX1. The latter is an essential enzyme in heme catabolism and has anti-inflammatory properties via up-regulation of IL-10 and IL-1R antagonist expression [32]. It was reported that oxidative stress was increased and associated with the endothelial cell injury in HMOX1-deficiency patients [33]. Liu et al. [34] demonstrated that the absence

Table 4. Continued

| rs6726395 (A>G) | T2DM ± complications | Co-dominants (11 versus 12 versus 22)* | Dominants (11 versus 12 + 22)* | Recessives (22 versus 11 + 12)* |
|-----------------|-----------------------|---------------------------------------|-------------------------------|--------------------------------|
|                 | +, n (%)†              | −, n (%)†                              | OR (95% CI) P                  | OR (95% CI) P                  | OR (95% CI) P                  | P                      |
| rs6726395 (A>G) | AA 22 (0.13)           | 45 (0.26)                              | 1§                            | 2.23 (1.27–3.92) 0.005§        |
|                 | AG 85 (0.52)           | 90 (0.51)                              | 0.35 (0.18–0.66) 0.001†        |
|                 | GG 58 (0.35)           | 41 (0.23)                              | 0.67 (0.41–1.10) 0.112         |
|                 | A 129 (0.39)           | 180 (0.51)                             | 1§                            | 0.67 (0.41–1.10) 0.112         |
|                 | G 201 (0.61)           | 172 (0.49)                             | 1.18 (1.06–1.30) 0.002§        |

*11: homozygotes for the major allele, 12: heterozygotes and 22: homozygotes for the minor allele. †n = 165. ‡n = 176. §Reference category (odds ratio, 1.0). ‖Logistic regression analyses were used for calculating statistic value.

Table 5 Frequency of haplotypes of NFE2L2 of T2DM patients and controls

| Haplotypes | T2DM patients | Controls | P | OR (95% CI) |
|------------|---------------|----------|---|--------------|
| H1-C A A C A A | 17.95 (0.026) | 18.16 (0.034) | 0.47 | 0.78 (0.40–1.52) |
| e            | 273.60 (0.401) | 231.04 (0.434) | 0.91 (0.72–1.14) |
| H3-G A A C G G | 89.04 (0.131) | 64.89 (0.122) | 0.55 | 1.11 (0.79–1.57) |
| H4-G A G C A A | 152.64 (0.224) | 112.61 (0.212) | 0.47 | 1.11 (0.84–1.46) |
| H5-G A G C G A | 35.42 (0.052) | 25.35 (0.048) | 0.66 | 1.12 (0.67–1.90) |
| H6-G A G C G A | 21.65 (0.032) | 15.03 (0.028) | 0.67 | 1.16 (0.59–2.26) |
| H7-G G A T G A | 60.70 (0.089) | 52.97 (0.100) | 0.62 | 0.91 (0.62–1.34) |

Order of polymorphisms: rs2364723, rs13001694, rs10497511, rs1806649, rs1962142, rs6726395. Global $\chi^2 = 2.194, df = 6, P = 0.901$. Haplotypes were omitted if the estimated haplotype probability was <3%.

Table 6 Serum HMOX1 levels of the participants

|                          | Participants, n | HMOX1 (ng/ml) | P-value |
|--------------------------|-----------------|---------------|---------|
| Controls                 | 53              | 0.29 ± 0.16   | Reference |
| T2DM                     | 111             | 0.36 ± 0.21   | 0.097   |
| Without complications    | 84              | 0.35 ± 0.22   | 0.14    |
| With complications       | 27              | 0.38 ± 0.17   | 0.048*  |

The Kruskal-Wallis test was used to analyse differences in serum HMOX1 levels, and then the post hoc Mann-Whitney U-test. *P < 0.05, between T2DM patients with and without complications.
of HMOX1 exacerbated myocardial ischaemia/reperfusion injury in diabetic mice, and expression of HMOX1 could ameliorate T1DM in mice [35]. The relationship and associated pathways of NFE2L2 with downstream biomarkers have been reviewed [36]. Normally, NFE2L2 locates in cytoplasm and binds to Keap1 remaining inactive. Under oxidative stress conditions, NFE2L2 is phosphorylated, released from Keap1 and transferred into the nuclei. Then the phosphorylated NFE2L2 binds to the ARE in the upstream promoter region of many anti-oxidative genes such as HMOX1, and initiates their transcription [36]. Thus, we speculate that serum HMOX1 levels might be negatively associated with the development of T2DM and/or its complications. Unexpectedly, however, our finding showed that the serum level of HMOX1 in the group of T2DM patients with complications was the highest (0.38 ± 0.17 ng/ml). In fact in patients with T2DM accompanied by kidney disease, the severity of renal failure was found to positively correlate with serum HMOX1 levels [37]. In a line with this finding, a few other studies also demonstrated the association of the elevated plasma HMOX1 contents with the patients with impaired glucose regulation [38] and T2DM [39] in a Chinese population. In addition, HMOX1 protein levels in peripheral blood mononuclear cells were higher in patients with T2DM and tuberculosis than in patients with tuberculosis only [41]. Plasma levels of HMOX1 were higher in patients with T2DM and tuberculosis and in patients with tuberculosis only [41]. This positive association of serum HMOX1 with the several diseases was considered as the body’s stress response with a potential to protect the oxidative stress induced by diseases such diabetes [37]. This notion was supported by our experimental studies with animal models where we demonstrated the increased expression and late decreases in cardiac expression of NFE2L2 and HMOX1, along with the late development of diabetic cardiomyopathy in the T1DM mouse model [42]. The correlation analysis of serum HMOX1 and T2DM factors illuminated that only mutant rs2364723 G carriers could significantly decrease the serum HMOX1 levels in T2DM patients, which supports the hypothesis we described above.

Limitations of this study include the high number of non-informative samples, which was because of inadequate samples or PCR readings. Moreover, our study population was limited to the geographic area of Northeast China, which contains certain specific factors such as temperature (cold at winter and cool at summer compared with Southern populations of China), dietary and environmental differences, and even genetic background. All these may cause certain impacts on the serum antioxidant or oxidative stress marker patterns. In addition, we also found a significantly higher plasma level of HMOX1 in those carrying the rs2364723 GG or CC allele than in those carrying the rs2364723 GG allele. As a result of relatively small sample size specifically for this observation in this study, we could not have exact explanation now. Thus, further studies with a larger sample size including diverse populations from different geographic areas are needed to verify differences relative to controls.

In summary, this study investigated the association of NFE2L2 polymorphisms with T2DM and its complications. We found that multiple mutations of NFE2L2 rs2364723, though not associated with T2DM, were significantly associated with the prevalence of complications in T2DM, indicating that this gene locus may predispose towards diabetic complications. The mutation of NFE2L2 rs2364723 G allele was significantly associated with increased serum HMOX1 levels in T2DM patients.

**Table 7 Multivariate logistic regression analysis of association between clinical factors or NFE2L2 SNPs and serum HMOX1 levels in T2DM patients**

|                                      | Estimate | Wald | P-value | Point estimate OR (95% CI) |
|--------------------------------------|----------|------|---------|---------------------------|
| rs10497511 (AA versus AG+GG)         | 0.13     | 0.058| 0.43    | 0.871 (0.242-2.668)       |
| rs1962142 (GG versus GA+AA)          | 0.43     | 0.55 | 0.12    | 1.546 (0.963-4.876)       |
| rs6726395 (AG versus GG+AA)          | −0.068   | 0.021| 0.88    | 1.107 (0.426-2.689)       |
| rs2364723 (GG versus CG+CC)          | 0.52     | 1.17 | 0.30    | 2.583 (0.417–16.001)      |
| rs13001694 (AA versus AG+GG)         | 0.94     | 1.03 | 0.30    | 2.583 (0.417–16.001)      |
| rs1806649 (CC versus CT+TT)          | −0.67    | 0.41 | 0.52    | 0.534 (0.079-3.116)       |
| Gender (male versus female)          | 0.52     | 1.27 | 0.25    | 1.681 (0.682-4.145)       |
| T2DM complications (yes versus no)   | 0.27     | 0.33 | 0.86    | 1.315 (0.520-3.330)       |
| Age (<60 versus ≥60)                 | −0.30    | 0.41 | 0.52    | 0.734 (0.287–1.881)       |

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Conflict of interest

The authors have no conflicts of interest to declare for this work.
Author contribution

Lu Cai and Lining Miao initiated and designed the study. Xiaohong Xu, Jing Sun, Xiaomin Chang, Ji Wang and Manyu Luo participated in recruiting samples and/or performed laboratory studies. Lu Cai, Lining Miao and Kupper A. Wintergerst periodically discussed the progression of project, data interpretation and wrote the draft of manuscript. Lu Cai and Kupper A. Wintergerst revised and formed the final manuscript. All authors contributed to review of the draft and revised manuscript with certain suggestions.

References

1. Xu Y, Wang L, He J, et al. Prevalence and control of diabetes in Chinese adults. JAMA 2013; 310: 948–59.
2. Guariguata L, Whiting DR, Hambleton I, et al. Global estimates of diabetes prevalence for 2013 and projections for 2030. Diabetes Res Clin Pract 2014; 103: 137–49.
3. Ripsin CM, Kang H, Urban RJ. Management of blood glucose in type 2 diabetes mellitus. Am Fam Physician. 2009; 79: 29–36.
4. Zhang C, Bao W, Rong Y, et al. Genetic variants and the risk of gestational diabetes mellitus: a systematic review. Hum Reprod Update. 2013; 19: 376–90.
5. Sousa AG, Selvatici L, Krieger JE, et al. Association between genetics of diabetes, coronary artery disease, and macrovascular complications: exploring a common ground hypothesis. Rev Diabet Stud. 2011; 8: 230–44.
6. Li B, Liu S, Miao L, et al. Prevention of diabetic complications by activation of Nrf2: diabetic cardiomyopathy and nephropathy. Exp Diabetes Res. 2012: 2012: 216512.
7. Hunt R, Sauna ZE, Ambudkar SV, et al. SNPs: should we care about them? Methods Mol Biol. 2009; 578: 23–39.
8. Yang H, Jin X, Kei Lam CW, et al. Oxidative stress and diabetes mellitus. Clin Chem Lab Med. 2011; 49: 1773–82.
9. Rizvi S, Raza ST, Mahdi F. Association of genetic variants with diabetic nephropathy. World J Diabetes. 2014; 5: 809–16.
10. Moi P, Chan K, Asunis I, et al. Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. Proc Natl Acad Sci USA. 1994; 91: 9296–300.
11. Kaspar JW, Niture SK, Jaiswal AK. Nrf 2: Nrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med. 2009; 47: 1304–9.
12. Yu ZW, Li D, Ling WH, et al. Role of nuclear factor (erythroid-derived 2)-like 2 in metabolic homeostasis and insulin action: a novel opportunity for diabetes treatment? World J Diabetes. 2012; 3: 19–28.
13. He X, Kan H, Cai L, et al. Nrf2 is critical in defense against high glucose-induced oxidative damage in cardiomyocytes. J Mol Cell Cardiol. 2009; 46: 47–58.
14. Negi K, Kumar A, Sharma SS. Nrf2 and NF-kappaB modulation by sulforaphane counteracts multiple manifestations of diabetic neuropathy in rats and high glucose-induced changes. Curr Neurovasc Res. 2011; 8: 294–307.
15. Uno K, Prow TW, Bhutto IA, et al. Role of Nrf2 in retinal vascular development and the vaso-obliterative phase of oxygen-induced retinopathy. Exp Eye Res. 2010; 90: 493–500.
16. Numazawa S, Yoshida T. Nrf2-dependent gene expressions: a molecular toxicological aspect. J Toxicol Sci. 2004; 29: 81–93.
17. American Diabetes Association. Standards of medical care for patients with diabetes mellitus. Diabetes Care. 2000; 23 (Suppl.1): S32–42.
18. American Diabetes Association. Classification and diagnosis of diabetes. Sec. 2. In Standards of Medical Care in Diabetes 2015. Diabetes Care. 2015; 38 (Suppl.1): S8–16.
19. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. Cell Res. 2005; 15: 97–8.
20. Sasaki H, Suzuki A, Shitara M, et al. Polymorphisms of Nrf2 gene correlated with decreased FEV1 in lung cancers of smokers. Biomed Rep. 2013; 1: 484–8.
21. Synowiec E, Siwiński T, Danisz K, et al. Association between polymorphism of the NQO1, NOS3 and NFE2L2 genes and AMD. Front Biol. 2013; 18: 80–90.
22. Li Z, Zhang Z, He Z, et al. A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis. Cell Res. 2009; 19: 519–23. Available at http://analysis.bio-x.cn.
23. Urano A, Yagiishiha Y, Yamamoto M. The Keap1-Nrf2 system and diabetes mellitus. Arch Biochem Biophys. 2015; 566: 76–84.
24. Arisawa T, Tahara T, Shibata T, et al. Nrf2 gene promoter polymorphism and gastric carcinogenesis. Hepatogastroenterology. 2008; 55: 750–4.
25. von Otter M, Landgren S, Nilsson S, et al. Nrf2-encoding NFE2L2 haplotypes influence disease progression but not risk in Alzheimer’s disease and age-related cataract. Mech Ageing Dev. 2010; 131: 105–10.
26. Chen YC, Wu YR, Wu YC, et al. Genetic analysis of NFE2L2 promoter variation in Taiwanese Parkinson’s disease. Parkinsonism Relat Disord. 2013; 19: 247–50.
27. LoGerfo A, Chico L, Borgia L, et al. Lack of association between nuclear factor erythroid-derived 2-like 2 promoter gene polymorphisms and oxidative stress biomarkers in amyotrophic lateral sclerosis patients. Oxid Med Cell Longev. 2014; 2014: 432626.
28. Cordova EJ, Velazquez-Cruz R, Centeno F, et al. The NRF2 gene variant, -653G/A, is associated with nephritis in childhood-onset systemic lupus erythematosus. Lupus. 2010; 19: 1237–42.
29. Karimi P, Farhangi MA, Sarmadi B, et al. The therapeutic potential of resistant starch in modulation of insulin resistance, endotoxemia, oxidative stress and antioxidant biomarkers in women with type 2 diabetes: a randomized controlled clinical trial. Ann Nutr Metab. 2016; 68: 85–93.
30. Palem SP, Abraham P. A study on the level of oxidative stress and inflammatory markers in type 2 diabetes mellitus patients with different treatment modalities. J Clin Diagn Res. 2015; 9: BC04–7.
31. Robertson RP. Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. J Biol Chem. 2004; 279: 42351–4.
32. Plantadosi CA, Willers CM, Bartz RR, et al. Heme oxygenase-1 couples activation of mitochondrial biogenesis to anti-inflammatory cytokine expression. J Biol Chem. 2011; 286: 16374–85.
33. Yachie A, Nuida Y, Wada T, et al. Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficient. J Clin Invest. 1999; 103: 129–35.
34. Liu X, Wei J, Peng DH, et al. Absence of heme oxygenase-1 exacerbates myocardial ischemia/reperfusion injury in diabetic mice. Diabetes. 2005; 54: 778–84.
35. Hu CM, Lin HH, Chiang MT, et al. Systemic expression of heme oxygenase-1 ameliorates...
type 1 diabetes in NOD mice. *Diabetes.* 2007; 56: 1240–7.

36. Zhang Z, Zhou S, Jiang X, et al. The role of the Nrf2/Keap1 pathway in obesity and metabolic syndrome. *Rev Endocr Metab Disord.* 2015; 16: 35–45.

37. Calabrese V, Mancuso C, Sapienza M, et al. Oxidative stress and cellular stress response in diabetic nephropathy. *Cell Stress Chaperones.* 2007; 12: 299–306.

38. Bao W, Rong S, Zhang M, et al. Plasma heme oxygenase-1 concentration in relation to impaired glucose regulation in a non-diabetic Chinese population. *PLoS ONE.* 2012; 7: e32223.

39. Bao W, Song F, Li X, et al. Plasma heme oxygenase-1 concentration is elevated in individuals with type 2 diabetes mellitus. *PLoS ONE.* 2010; 5: e12371.

40. Xin G, Du J, Wang YT, et al. Effect of oxidative stress on heme oxygenase-1 expression in patients with gestational diabetes mellitus. *Exp Ther Med.* 2014; 7: 478–82.

41. Andrade BB, Pavan Kumar N, Sridhar R, et al. Heightened plasma levels of heme oxygenase-1 and tissue inhibitor of metallo-proteinase-4 as well as elevated peripheral neutrophil counts are associated with TB-diabetes comorbidity. *Chest.* 2014; 145: 1244–54.

42. Bai Y, Cui W, Xin Y, et al. Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. *J Mol Cell Cardiol.* 2013; 57: 82–95.