Hematopoietically expressed homeobox gene is associated with type 2 diabetes in KK Cg-A^{y}/J mice and a Taiwanese Han Chinese population

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Received November 8, 2016; Accepted November 29, 2017

DOI: 10.3892/etm.2018.6152

Abstract. Diabetes mellitus (DM) is a chronic disease. The KK Cg-A^{y}/J (KK-A^{y}) mouse is an animal model to study type 2 diabetes mellitus (T2D) disease. The present study assessed the expression of hematopoietically expressed homeobox (HHEX) protein in liver tissues of different age groups of mice (6, 16 and 42 weeks) by immunohistochemistry (IHC). The results demonstrated a significant decrease in the percentage of HHEX-positive cells in KK-A^{y} mice as compared with that in KK-α/α control mice. Furthermore, in Taiwan's Han Chinese population, genotypic and allelic frequency distributions of the rs61862780 single-nucleotide polymorphism (SNP) in the *HHEX* gene were investigated. The results demonstrated that in the rs61862780 SNP of the 3'-untranslated region (UTR) of HHEX, the frequency of the CC genotype was higher in patients (6.0%) than in controls (2.7%), while the TT genotype frequency was about equal. In the same SNP, the frequency of the C allele was higher in patients (21.0%) than in controls (17.3%), while the T allele frequency was about equal. These results may pave the road for exploring the KK-A^{y} mouse model and the HHEX SNP rs61862780, which was correlated with the susceptibility to T2D in a Chinese population. Based on these findings, an association of *HHEX* gene expression with pathological features of T2D was indicated.

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Key words: KK Cg-A^{y}/J mouse, Chinese population, type 2 diabetes mellitus, hematopoietically expressed homeobox, single-nucleotide polymorphism

Introduction

Diabetes mellitus (DM) is a chronic disease with high prevalence in Taiwan (1,2). DM is characterized by a relative or absolute lack of insulin, resulting in hyperglycaemia (3). Chronic hyperglycaemia leads to a variety of complications such as neuropathy, nephropathy and retinopathy and increased risk of cardiovascular disease (4-7). According to the World Health Organization Global report, 422 million individuals were diagnosed with DM in 2016 (8). In Taiwan, DM was reported to be the fifth leading cause of death in 2015 by the Ministry of Health and Welfare of Taiwan (9). DM has two subtypes, namely type 1 and type 2 DM (10,11). Type 1 DM (T1D) is an autoimmune disease that leads to the destruction of the insulin-producing pancreatic β cells in the islets of Langerhans (12). In children and young adults, T1D is the most commonly diagnosed type of DM. T1D is associated with low endogenous insulin production in affected patients, and insulin supplementation by subcutaneous injection is required (13). The blood glucose levels must be frequently monitored to manage the risk of hypoglycaemia (14). Genetic influences and environmental factors have an important role in disease development (15,16). However, T2D is the most common type of DM in Taiwan. It is most commonly diagnosed in middle-aged adults (17). T2D is associated with insulin resistance, which is a lack of appropriate compensation by the β cells, leading to a relative insulin deficiency (18,19). In the early stage, insulin resistance may be improved by weight reduction and exercise (20). A variety of drugs are available for treating T2D (21,22). Treatment with drugs such as sulphonylureas stimulates insulin production by the β cells (23,24), while that with drugs such as biguanides or metformin reduces hepatic glucose production (25,26), while α-glucosidase inhibitors delay carbohydrate uptake in the gut (27,28), thiazolidinediones improve insulin action (29,30)

and as glucagon-like peptide 1 (GLP-1) receptor agonists or dipeptidyl peptidase-4 inhibitors target the GLP-1 axis (31,32). DM represents is a complex disease involving various bodily systems. Thus, animal models should be carefully selected, depending on what aspect of the disease is being investigated (33).

One of the mouse models that has not been extensively examined by the National Institutes of Health-sponsored Animal Models of Diabetic Complications Consortium is the KK Cg-A^y/J (KK-A^y) strain (34,35). The KK-A^y mouse is a typical T2D model, and one of the inbred strains established from Japanese native mice (33,35). Yellow KK-A^y mice carry the yellow obesity gene A^y and develop marked adiposity and DM symptoms in comparison with control black KK-α/α mice (36). This model exhibits marked obesity, glucose intolerance and insulin resistance of peripheral tissue, hyperglycemia, dyslipidemia, hypertension and renal glomerular changes (33,35,36). Furthermore, the pancreatic islets in yellow KK-A^y mice are hypertrophic and de-granulated. This mouse strain also presents with signs of diabetic nephropathy (33,35,36).

T2D is a phenotypically and genetically diverse disease characterized by insulin resistance (22,28). Candidate gene mapping and positional cloning have suggested numerous putative susceptibility variants, but only a few genetic variants leading to T2D have been clearly identified, including transcription-factor-7-like 2 (TCF7L2) and hematopoietically expressed homeobox (HHEX) protein (37-39). The *HHEX* gene is located on chromosome 10q23.33 and encodes a 270 amino-acid protein (40). HHEX contains the insulin-degrading enzyme (IDE) and kinesin family member 11 (40,41). The *HHEX* gene encodes a transcription factor involved in hepatic and pancreatic development via the Wnt signal pathway, which is fundamental for cell growth and differentiation (42,43). Numerous studies have identified the *HHEX* gene polymorphisms of rs1111875 T>C and rs7923837 A>G in T2D patients (38,44,45). A genome-wide scan for association provided evidence that HHEX is an excellent candidate susceptibility gene for T2D, and indicated a significant association of rs1111875 and rs7923837 with T2D (38). Although the association between HHEX polymorphisms and T2D has been well studied in humans, it has remained elusive in KK-A^y mice. A preliminary study by our group clearly demonstrated that HHEX mRNA was downregulated in liver tissues of KK-A^y mice as compared with that in KK-α/α control mice by complementary DNA microarray analysis (unpublished data). The present study focused on investigating the association between HHEX and T2D in KK-A^y mice and in a Han Chinese Population in Taiwan.

Materials and methods

KK-α/α and KK-A^y mice. A total of 5 four-week-old male control KK-α/α mice and 5 four-week-old male KK-A^y mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA), the mice were divided into two groups (5 mice/group). The animals were housed in individual cages and provided lab chow (LabDiet 5k52; St. Louis, MO, USA) and water *ad libitum* in a room with a constant temperature (22-25˚C), relative humidity (50-70%) and photoperiod (12-h light/dark cycle). The study was approved by the Institutional Animal Care and Use Committee of China Medical University (IACUC permit no. 102-217) as previously described (46).

Immunohistochemical (IHC) analysis. IHC was performed on paraffin-embedded liver sections as previously described (46,47). IHC staining for HHEX was performed using a Leica Bond MAX automated immunostainer (Leica Microsystems Inc., Buffalo Grove, IL, USA). Tissue sections (5-μm-thick) were de-waxed, treated with Proteinase K enzyme (cat. no. P2308; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), followed by blocking with 3% hydrogen peroxide (cat. no. H1009; Sigma-Aldrich; Merck KGaA). The slides were incubated in anti-HHEX (cat. no. GTX84369; GeneTex, Hsinchu, Taiwan) antibodies (1:250 dilution) for 30 min at room temperature, followed by horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G secondary antibodies (cat. no. 61-6520; 1:1,000; Thermo Fisher Scientific, Inc., Waltham, MA, USA) for 15 min at 37˚C. Chromogen visualization was performed using 3,3'-diaminobenzidine tetrahydrochloride (DAB; cat. no. D12384, Sigma-Aldrich; Merck KGaA) for 5 min at room temperature. The sample was washed with 0.05% Tween 20 in Tris-buffered saline (DAKO, Carpinteria, CA, USA) between all steps.

Patients and sample collection for genotyping. A total of 570 patients diagnosed with T2D by endocrinologist at China Medical University Hospital (Taichung, Taiwan) were recruited between August 2014 and August 2015 for the present study from China Medical University Hospital. To compare the prevalence of polymorphisms in patients with that in a healthy population, the genotype frequency data of 1,700 healthy controls were downloaded from the Taiwan Biobank (https://taiwanview.twbiobank.org.tw/taiwanview/dl.do). The single nucleotide polymorphisms (SNPs) in the target gene were queried from the National Center of Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/snp). The SNPs of the genes of interest were obtained and compared between the disease and control groups. Chi-square tests were used to calculate odds ratios and P-values. The study was approved by the ethics committee/Institutional Review Board of China Medical University Hospital (no. CMUH103-REC2-071).

Statistical analysis. The Chi-square test was used to determine statistically significant differences in allele/genotype frequencies of HHEX SNP rs61862780 between the case and control groups. Differences were considered statistically significant when $P<0.05$. The odds ratios (ORs) with 95% confidence intervals (95% CIs) were calculated for the genotypic and allelic frequencies of the HHEX SNP rs61862780. The statistical analysis was performed using SPSS version 11 (SPSS, Inc., Chicago, IL, USA).

Results

Diabetes-associated features of yellow KK-A^y mouse model. The diabetes-associated features of yellow KK-A^y mice according to the supplier's information and previous studies are listed in Table I (33,34). In the late stage (42 weeks), KK-A^y mice presented with a variety of T2D-like characteristics,

Table I. General characteristics of diabetic features of yellow KK Cg-A^y/J mice according to the supplier.

Parameter	6 weeks	16 weeks	42 weeks
Abnormal lipid homeostasis	-	+	++
Increase in blood glucose levels	+	++	+++
Marked hyperglycemia (400-500 mg/dl)	-	-	+
Impaired glucose tolerance	-	+	++
Increased circulating insulin level	+	++	+++
Increased urine glucose level	+	++	+++
Insulin resistance	-	+	++
Adipose tissue weight increases	-	+	++
Increased susceptibility to weight gain	+	++	+++
Abnormal pancreatic islet morphology	-	+	++
Degranulated pancreatic beta cells	+	++	+++
Abnormal renal glomerulus morphology	+	++	+++
Abnormal renal tubule morphology	+	++	+++
Dilated renal tubules	+	++	+++
Expanded mesangial matrix	+	++	+++
Increased renal glomerulus basement membrane thickness	+	++	+++
Hyaline cast is present in tubules	+	++	+++

including abnormal lipid homeostasis, increase in blood glucose levels, hyperglycemia (>400 mg/dl), glucose intolerance, increased circulating insulin and urine glucose levels, insulin resistance, adipose tissue weight increases, increased susceptibility to weight gain, abnormal morphology of pancreatic islets, renal glomerulus and renal tubules, de-granulated pancreatic β cells, dilated renal tubules, expanded mesangial matrix, increased renal glomerulus basement membrane thickness and hyaline cast present in tubules (2,21,31,33).

HHEX protein expression in liver tissues of KK-A^y T2D model mice. KK-α/α mice (control group) and KK-A^y mice (T2D group) were individually sacrificed at 6, 16 and 42 weeks of age, and liver tissues were excised, fixed, embedded and sectioned for IHC staining. As presented in Fig. 1, IHC analysis indicated that the HHEX protein expression in liver tissue was decreased with time. Based on these results, HHEX protein expression in liver tissues of KK-A^y mice (T2D group) was significantly lower than that in KK-α/α mice (control group). In addition, it was demonstrated that DM mice (KK-A^y) grew in the period that relative HHEX protein expression were decreased (Fig. 2). Although KK-α/α mice (control group) grew in the same period HHEX protein expression did not change.

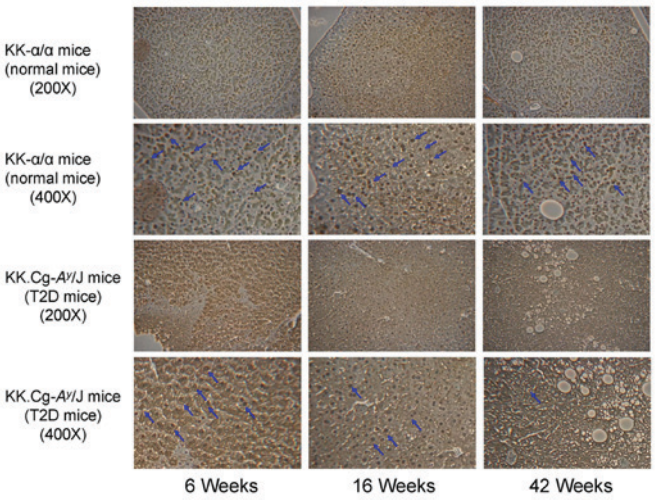

Figure 1. Representative immunohistochemical images for detecting HHEX expression in the livers of KK Cg-A^y/J mice with T2D compared with that in normal KK-α/α mice (arrows indicate HHEX expression identified by brown staining; magnification, x200 or x400). HHEX, hematopoietically expressed homeobox; T2D, type 2 diabetes.

Genotypic and allelic frequency distributions of the rs61862780 SNP in the HHEX gene among Taiwan's Han Chinese population. The genotypic and allelic frequency distributions of the rs61862780 SNP in the HHEX gene located within Chromosome 10q23.3 region (92,708,886 bp) are summarized in Table II. The Hardy-Weinberg model was used to describe and predict genotype and allele frequencies in the study cohort. It was observed that for the rs61862780 (C/T) SNP in the 3'-UTR of HHEX, the frequency of the CC genotype was higher in patients (6.0%) than in controls (2.7%). In addition, the frequency of the CT genotype was higher in patients (30.0%) than in controls (29.2%). In comparison with the TT genotype, the OR of the CC genotype was 2.4 (95% CI=1.51-3.8; P<0.001). The allelic frequency of the C allele was higher in patients (21.0%) than in controls (17.3%). In comparison with the T allele, the OR for the C allele was 1.27 (95% CI=1.08-1.5; P<0.005). It was therefore indicated that the HHEX SNP rs61862780 is associated with the susceptibility to T2D.

Discussion

Numerous animal models of T2D are obese to reflect the human condition where obesity is closely linked to T2D

Table II. Genotypic and allelic frequencies in the rs61862780 single nucleotide polymorphism of the 3' untranslated region of the HHEX gene in patients with T2D (n=570) and controls (n=1,698).

Parameter	Patients with T2D, n (%)	Control, n (%)	OR (95% CI)	P-value
Genotype				
CC	34 (6.0)	45 (2.7)	2.4 (1.51-3.8)[a]	<0.001
CT	171 (30.0)	496 (29.2)	1.09 (0.89-1.35)	
TT	365 (64.0)	1,157 (68.1)		
Allele frequency				
C	239 (21)	586 (17.3)	1.27 (1.08-1.5)[b]	<0.005
T	901 (79)	2,810 (82.7)		

[a]Comparison with the TT genotype; [b]Comparison with the T-allele frequency. CI, confidence interval; OR, odds ratio; T2D, type 2 diabetes.

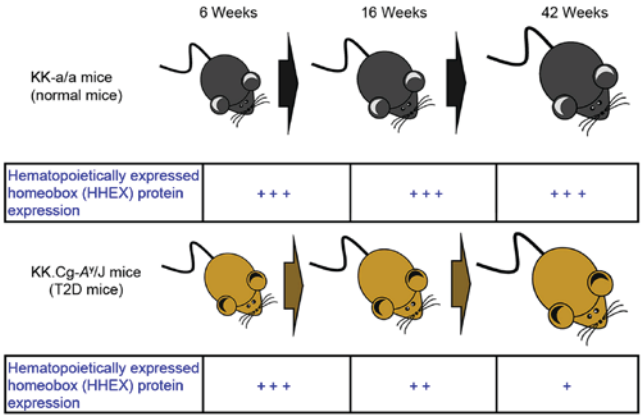

Figure 2. Cartoon representation of HHEX protein expression in KK-α/α mice (normal mice; black color) and KK Cg-A^{y}/J mice (T2D mice; yellow color) depending on the age of the mice. The amount of HEXX expression is indicated by + symbols. HHEX, hematopoietically expressed homeobox; T2D, type 2 diabetes.

development (33). Two genetic mouse models of T2D exist, namely monogenic models (including Lep$^{ob/ob}$ and Lepr$^{db/db}$ mice) and polygenic models (including KK Cg-A^{y}/J mice) (33). A variety of different polygenic mouse models of obesity, glucose intolerance and diabetes are available, and the variety of genotypes, for example KK Cg-A^{y}/J mice, which are one of the spontaneous animal models of T2D, might allow for more better modeling of T2D in humans compared with other obesity models (33,34,48). In the present study, KK-A^{y} mice were used to study the protein expression of HHEX in the liver. The KK-A^{y} mouse strain is glucose-intolerant, insulin-resistant, dyslipidemic and hypertensive (2,21,31). The complex pathogenesis of T2D in obese and non-obese patients involves genetic and environmental factors (49,50). Genome-wide association studies (GWAS) have indicated an association of T2D with several newly identified genes (51,52). Associations of T2D with common variants in HHEX, hepatocyte nuclear factor 4α, potassium voltage-gated channel subfamily J member 11 (KCNJ11), peroxisome proliferator-activated receptor γ, cyclin dependent kinase inhibitor 2A (CDKN2A)/2B, solute carrier family 30 member 8, cell division cycle 123 (CDC123)/calcium/calmodulin dependent protein kinase ID (CAMK1D), TCF7L2, ATP binding cassette subfamily A member 1 and solute-carrier-family-16-member 11 (SLC16A11) genes have been reported in European, Asian and Latin American populations (53-59). Kong *et al* (53,60,61) demonstrated associations of SNPs in wolframin ER transmembrane glycoprotein, CDK5 regulatory subunit associated protein 1 like 1, CDKN2A/2B, CDC123/CAMK1D, HHEX, TCF7L2, KCNQ1 and melatonin receptor 1B with T2D in a Chinese population. Sladek *et al* (62) reported variants of the *TCF7L2*, *SLC30A8* and *HHEX* genes as novel loci associated with T2D by GWAS analysis in a French population. The present study proved that the frequency of the CC genotype of the rs61862780 (C/T) SNP in the 3'UTR of HHEX was higher in T2D patients than in controls compared with that of the TT genotype. In addition, the allelic frequency of the C allele was higher in patients than in controls compared with that of the T allele, which is in agreement with the previous studies (53,60,61). The *HHEX* gene regulates cell proliferation and tissue differentiation underlying vascular, hepatic differentiation and forebrain neuro-development (63-65). HHEX is essential for heart (66,67), thyroid (68), hepatic and pancreatic development and is a target of the Wnt signaling pathway (43,69). Decreased levels in HHEX-deficient islets cause disrupted paracrine inhibition of insulin release from β cells (70). HHEX activity has been described in the mouse lung, thyroid and liver. Numerous studies reported associations between HHEX/insulin-degrading enzyme variants (rs1111875) and the insulin secretion response following a glucose load, suggesting that the *HHEX* gene may influence the T2D risk primarily through an effect on β-cell function (47,71,72). However, to the best of our knowledge, HHEX protein expression has not been previously reported in KK-A^{y} T2D mice. The present study was the first to demonstrate the role of HHEX in KK-A^{y} mice. The IHC results were corroborated by results of a complementary DNA microarray analysis of the liver tissues (data not shown), which indicated a significant decrease in the expression of HHEX in KK-A^{y} mice at 16 and 42 weeks. High insulin levels (hyperinsulinemia) are a major feature of T2D in the early stage, and the pancreas fails to produce sufficient insulin to overcome the insulin resistance; however, failure of β-cell function in the late stages of T2D causes a reduction of insulin secretion (73,74). The present results suggested that downregulation of HHEX expression in the liver may contribute to disrupted paracrine

control of insulin secretion in KK-A^y T2D mice at 16 and 42 weeks.

Diabetes is a risk factor of various types of cancer, including colorectal cancer (CRC), as well as breast, bladder, liver and pancreatic cancer (75-77). Ma *et al* (54) demonstrated that T2D-associated variants of HHEX (rs7923837), TCF7L2 (rs290481) and CDKAL1 (rs7756992) increased the risk of cancer in patients with diabetes. Sun *et al* (78) also demonstrated that two variants on the T2D susceptibility gene HHEX are associated with the risk of CRC in a Chinese population. Therefore, the factors associated with an increased risk for CRC in T2D patients and the underlying mechanisms still require further study. The time-dependent changes in the protein levels of HHEX in KK-A^y T2D mice are summarized in Fig. 2. The present results demonstrated a downregulation of HHEX protein in the liver of KK-A^y T2D mice and an association of the HHEX SNP rs61862780 with T2D in Taiwanese populations, indicating that loss/mutation of HHEX may have an important role in the pathogenesis of T2D.

Acknowledgements

Not applicable.

Funding

This study was supported by the Biosignature project, Academia Sinica, Taiwan and the China Medical University Hospital (Taichung, Taiwan; grant no. DMR-107-123).

Availability of data and materials

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

Authors' contributions

CCLu, JSY and FJT conceived and designed the experiments. JWT and YNJ performed the experiments. YTC, SYC, YMH and CCLi analyzed the data. CCLu, JSY and FJT wrote and modified the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of China Medical University (IACUC permit no. 102-217). The human experiments were approved by the ethics committee/Institutional Review Board of China Medical University Hospital (no. CMUH103-REC2-071).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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