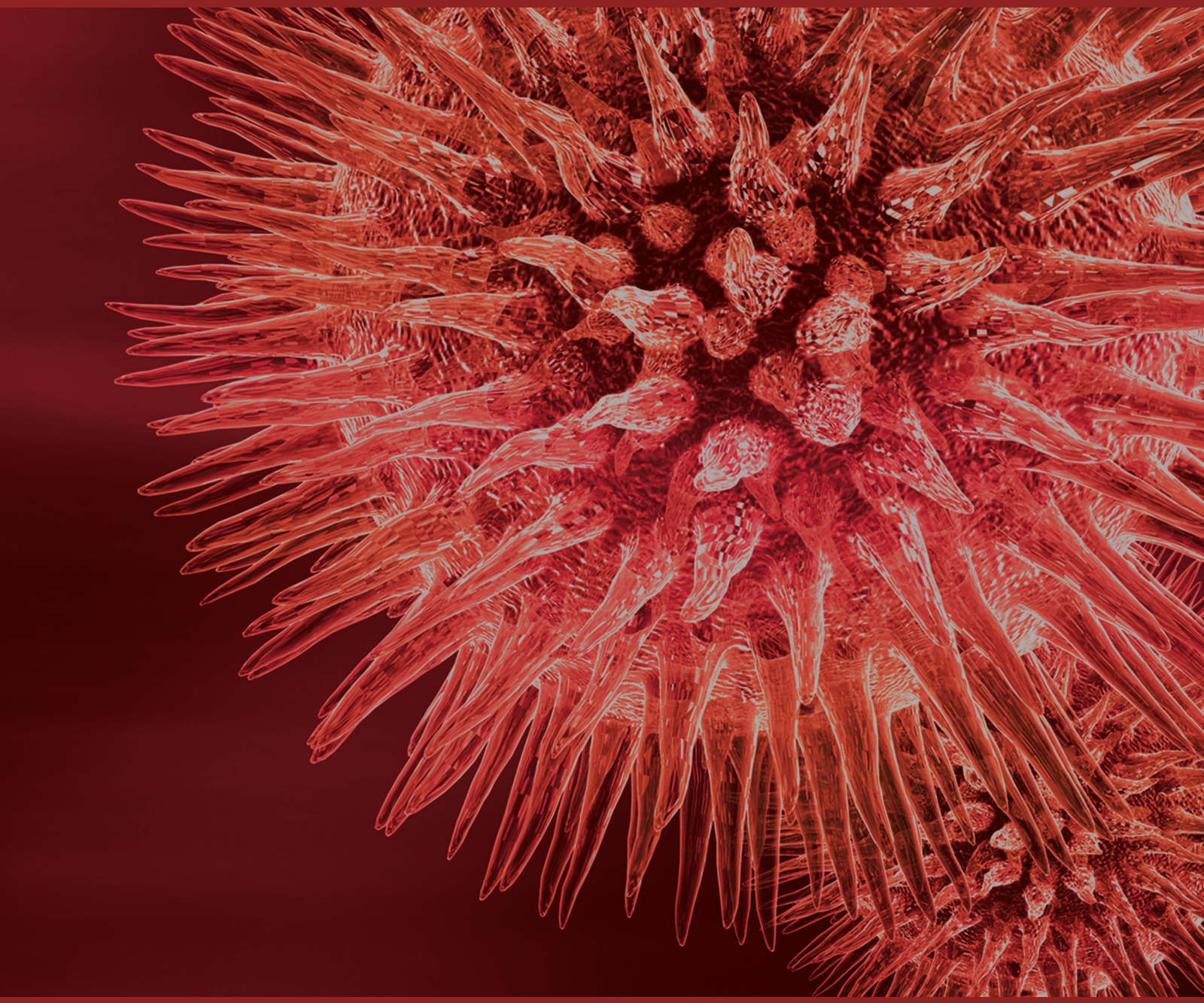
BioMed Research International

Biomarkers of Metabolic Disorders: Diagnostic and Prognostic Values, and Insights into the Pathogenesis

Guest Editors: Cheng Hu, Jiarui Wu, and Wei Jia



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Editorial

Biomarkers of Metabolic Disorders: Diagnostic and Prognostic Values, and Insights into the Pathogenesis

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In recent decades, overwhelming evidence has indicated that metabolic disorders, including obesity, diabetes, dyslipidemia, and nonalcohol fatty liver disease, are the leading cause of disability and death. However, the pathogenesis of metabolic disorder and its chronic complications involves multiple biological pathways and is largely unknown. Biological markers can be evaluated as indicators of pathogenic processes or pharmacological responses to therapeutic interventions. Taking type 2 diabetes, for example, though some clinical phenotypes such as obesity and impaired glucose tolerance are confirmed to be risk factors for the disease, the genetic information can be detected as earlier indicators for prevention and therapy. Due to the heterogeneity of the metabolic diseases in different ethnics, it is imperative to investigate the genetic characteristics in Chinese population. Therefore, the papers selected for this special issue mainly focused on exploring the genetic predisposition and pathogenesis for obesity and type 2 diabetes and their complications. The selected topics and papers are not exhaustive; they do represent the rich and many-faceted knowledge that we have the pleasure of sharing with the readers. We would like to thank the authors and reviewers for their excellent contributions in assisting us.

This special issue contains seven papers, where two papers discuss genetic susceptibility of type 2 diabetes and diabetic nephropathy and one paper identifies a novel mutation for severe obesity. In addition, one paper investigates the regulation of human fat mass and obesity associated gene (FTO) and one paper evaluates the advantages of different detection methods for ketones and establishes whether detection of the concentration of ketones in the breath is an effective and practical technique. Moreover, one paper performed functional studies to examine therapeutic effects of olmesartan on adipose tissue. Finally one review sheds light on the pathogenesis and its clinical applications of type 2 diabetes systematically.

In the paper entitled "*Association of genetic variants of BMP4 with type 2 diabetes mellitus and clinical traits in a Chinese Han population*," S. Tang et al. test the impacts of *BMP4* variants on type 2 diabetes in the Chinese including 3,410 diabetic patients and 3,412 normal glucose regulation individuals and finally present a minor effect of *BMP4* variants on glucose metabolism in Chinese population.

In the paper entitled "*Lack of association between TLR4 genetic polymorphisms and diabetic nephropathy in a Chinese population*," D. Peng et al. investigate the effects of *TLR4* genetic variants on diabetic nephropathy in 1,455 Chinese type 2 diabetic patients and detect no association with the disease.

In the paper entitled "*Genetics of type 2 diabetes: insights into the pathogenesis and its clinical application*," X. Sun et al. review the major genetic studies on the risk of T2D based on ethnicity and briefly discuss the potential mechanisms and clinical utility of the genetic information underlying T2D.

In the paper entitled "*A novel mutation in leptin gene is associated with severe obesity in Chinese individuals*," Y. Zhao et al. detect a novel mutation H118L in *leptin* gene in Chinese subjects. This novel mutation might be the casual variant leading to severe obesity; however, functional studies still needed to be confirmed.

In the paper entitled "*CCAAT/enhancer-binding protein α is a crucial regulator of human fat mass and obesity associated gene transcription and expression*," W. Ren et al. aim to study the possible mechanism of how the C/EBPα binding site regulates FTOgene expression. They suggest that C/EBPα may act as a positive regulator binding to FTO promoter and, consequently, activates the gene transcription.

In the paper entitled "*Breath ketone testing: a new biomarker for diagnosis and therapeutic monitoring of diabetic ketosis*," Y. Qiao et al. established breath ketone testing as a noninvasive, convenient method for the diagnosis and therapeutic monitoring of diabetic ketosis.

In the paper entitled "*Effects of the angiotensin receptor blocker olmesartan on adipocyte hypertrophy and function in mice with metabolic disorders*," A. Maeda et al. investigate the therapeutic effects of an AT1R-specific blocker, olmesartan, on genetically obese diabetic KKAy mice and analyze possible effects on adipose issue. They indicate that the blood pressure lowering effect of olmesartan in KKAy mice is associated with improvement in adipocyte dysfunction including suppression of adipocyte hypertrophy and inhibition of adipose IL-6-oxidative stress axis.

These studies will help readers to understand the current status and gain new insights into the genetic traits of metabolic diseases.

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Wei Jia

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Research Article

Effects of the Angiotensin Receptor Blocker Olmesartan on Adipocyte Hypertrophy and Function in Mice with Metabolic Disorders

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In the present study, we examined the therapeutic effects of olmesartan, an angiotensin II (Ang II) type 1 receptor (AT1R)-specific blocker, in genetically obese diabetic KKAy mice, a model of human metabolic disorders with visceral obesity, with a focus on an olmesartan effect on the adipose tissue. Olmesartan treatment (3 mg/kg per day) for 4 weeks significantly lowered systolic blood pressure but did not affect body weight during the study period in KKAy mice. However, there were three interesting findings possibly related to the pleiotropic effects of olmesartan on adipose tissue in KKAy mice: (1) an inhibitory effect on adipocyte hypertrophy, (2) a suppressive effect on IL-6 gene expression, and (3) an ameliorating effect on oxidative stress. On the other hand, olmesartan exerted no evident influence on the adipose tissue expression of AT1R-associated protein (ATRAP), which is a molecule interacting with AT1R so as to inhibit pathological AT1R activation and is suggested to be an emerging molecular target in metabolic disorders with visceral obesity. Collectively, these results suggest that the blood pressure lowering effect of olmesartan in KKAy mice is associated with an improvement in adipocyte, including suppression of adipocyte hypertrophy and inhibition of the adipose IL-6-oxidative stress axis. Further study is needed to clarify the functional role of adipose ATRAP in the pleiotropic effects of olmesartan.

1. Introduction

Recently, metabolic disorder with visceral obesity has come to be recognized as a major medical condition related to significantly increased risks of hypertension, type 2 diabetes, dyslipidemia, and ultimately life-threatening cardiovascular disease [1]. Accumulating evidence also indicates that adipose tissue functions as a distinct endocrine organ capable of producing adipokines, such as adiponectin and leptin [2]. Furthermore, dysregulation of adipose tissue function is suggested to be closely involved in the pathophysiology of metabolic disorders via the stimulated production of inflammatory cytokines and upregulation of oxidative stress [3–5].

The renin-angiotensin system (RAS) plays an important role in the maintenance of circulatory and water-electrolyte homeostasis based on the generation of angiotensin II (Ang II), a potent vasoactive peptide, and the pathological activation of RAS has been implicated as one of the major contributors to hypertension and cardiovascular disease. Recent evidence has also indicated an important role of adipose tissue RAS in the physiological regulation of adipose tissue

function, further suggesting a specific pathophysiological link between the dysregulated activation of adipose tissue RAS and the development of metabolic disorders and their complications [6, 7]. The physiological and pathophysiological actions of Ang II are principally mediated by the Ang II type 1 receptor (AT1R). In the present study, we examined the therapeutic effects of olmesartan, an AT1R-specific blocker, in genetically obese and diabetic KKAy mice, a model of human metabolic disorders with diabetes without any dietary loading [8], and focused our analysis on adipose tissue.

2. Materials and Methods

2.1. Animals and Treatment. C57BL/6 mice (male) and KKAy mice (male) were purchased from CLEA Japan, Inc. (Tokyo, Japan) for use as a nondiabetic normal control and a model of metabolic disorders with type 2 diabetes, respectively [9–11]. These mice were housed in a controlled environment with a 12 h light-dark cycle and were allowed free access to food and water. They were fed a standard diet (3.6 kcal/g; 13.3% energy as fat; Oriental MF, Oriental Yeast, Co., Ltd.). Male KKAy mice at 9 weeks of age were treated with the oral administration of olmesartan (3 mg/kg per day) in drinking water for 4 weeks, and body weight and food intake were measured. The KKAy mice treated with vehicle were previously described [12]. On the other hand, C57BL/6 control mice were treated with vehicle during the study period. Mice were sacrificed under anesthesia and the tissues were collected at the end of the experimental period. The protocol was reviewed and approved by the Animal Studies Committee of Yokohama City University and all experiments were performed in accordance with the National Institutes of Health guidelines for the use of experimental animals.

2.2. Blood Pressure Measurement by Tail-Cuff Method. Systolic blood pressure was measured noninvasively by the tail-cuff method (BP-monitor MK-2000; Muromachi Kikai Co.). The MK-2000 BP-monitor allowed determination of the blood pressure without any preheating of the animals, thus avoiding this very stressful condition [13–15]. At least eight readings were taken for each measurement.

2.3. Preparation of Tissue Sections and Histological Analysis. The epididymal white adipose tissue was isolated and fixed with 10% paraformaldehyde overnight and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin for cell size determination. The adipocyte diameter and area were quantified using Image-Pro Plus software.

2.4. Tissue RNA Isolation and Real-Time Quantitative Reverse Transcript-PCR (qRT-PCR) Analysis. Total RNA was extracted from epididymal adipose tissue with ISOGEN (Nippon Gene), and cDNA was synthesized using the SuperScript III First-Strand System (Invitrogen). Real-time qRT-PCR was performed with an ABI PRISM 7000 Sequence Detection System by incubating the reverse transcription product with TaqMan PCR Master Mix and a designed TaqMan probe (Applied Biosystems), essentially as described previously [15–18]. The mRNA levels were normalized to those of the 18S rRNA control.

2.5. Statistical Analysis. All data are shown as mean ± SEM. Differences were analyzed by ANOVA followed by the Newman-Keuls multiple-comparison test. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of Olmesartan on Blood Pressure, Body Weight, and Food Intake. The systolic blood pressure, heart rate, and body weight at baseline and after the study period in the control C57BL/6, vehicle-treated KKAy, and olmesartan-treated KKAy mice are shown in Figure 1. At baseline there were no significant differences in systolic blood pressure or heart rate between the groups before the treatment. Body weight at baseline was significantly greater in the KKAy mice of either treatment group than in the control C57BL/6 mice ($P < 0.01$ versus C57BL/6). With respect to the effects of treatment with olmesartan at a dose of 3 mg/kg per day for 4 weeks on heart rate and body weight, heart rate was not affected in the KKAy mice by the treatment with olmesartan for 4 weeks compared with baseline (baseline versus 4 weeks, 720 ± 11 versus 729 ± 8 bpm, NS), and there was a significant increase in body weight after the olmesartan treatment (baseline versus 4 weeks, 41.2 ± 0.4 versus 47.5 ± 0.8 g, $P < 0.01$). After 4 weeks, heart rate and body weight did not differ significantly between the vehicle-treated and olmesartan-treated KKAy mice (Figure 1).

On the other hand, systolic blood pressure in the KKAy mice was significantly decreased by the olmesartan treatment for 4 weeks compared with baseline (baseline versus 4 weeks; 108 ± 3 versus 95 ± 3 mmHg, $P < 0.01$), and systolic blood pressure was significantly lower in the KKAy mice treated with olmesartan than those treated with vehicle ($P < 0.01$ versus vehicle) (Figure 1). Furthermore, although the daily food intake after 4 weeks was significantly greater in the KKAy mice of either treatment group than the control C57BL/6 mice ($P < 0.01$ versus C57BL/6), the daily food intake was similar in the vehicle-treated and olmesartan-treated KKAy mice groups (Figure 2).

3.2. Effects of Olmesartan on Adipocyte Hypertrophy in KKAy Mice. We examined whether there was any phenotypic alteration in the adipose tissue of the KKAy mice treated with olmesartan. Although the KKAy mice treated with vehicle had significantly larger adipocytes than the control C57BL/6 mice ($P < 0.01$ versus C57BL/6) (Figures 3(a), 3(b), and 3(c)), adipocyte hypertrophy was significantly inhibited in the KKAy mice treated with olmesartan for 4 weeks (Figure 3(d); vehicle-treated KKAy mice versus olmesartan-treated KKAy mice; diameter: 113.7 ± 3.7 versus 91.2 ± 3.1 μm, $P < 0.01$; Figure 3(e), area: 11131 ± 765 versus 7264 ± 415 μm^2, $P < 0.01$).

3.3. Effects of Olmesartan on Adipokine and Adipose Tissue RAS Gene Expression. As shown in Figure 4, the KKAy mice treated with vehicle exhibited significantly suppressed

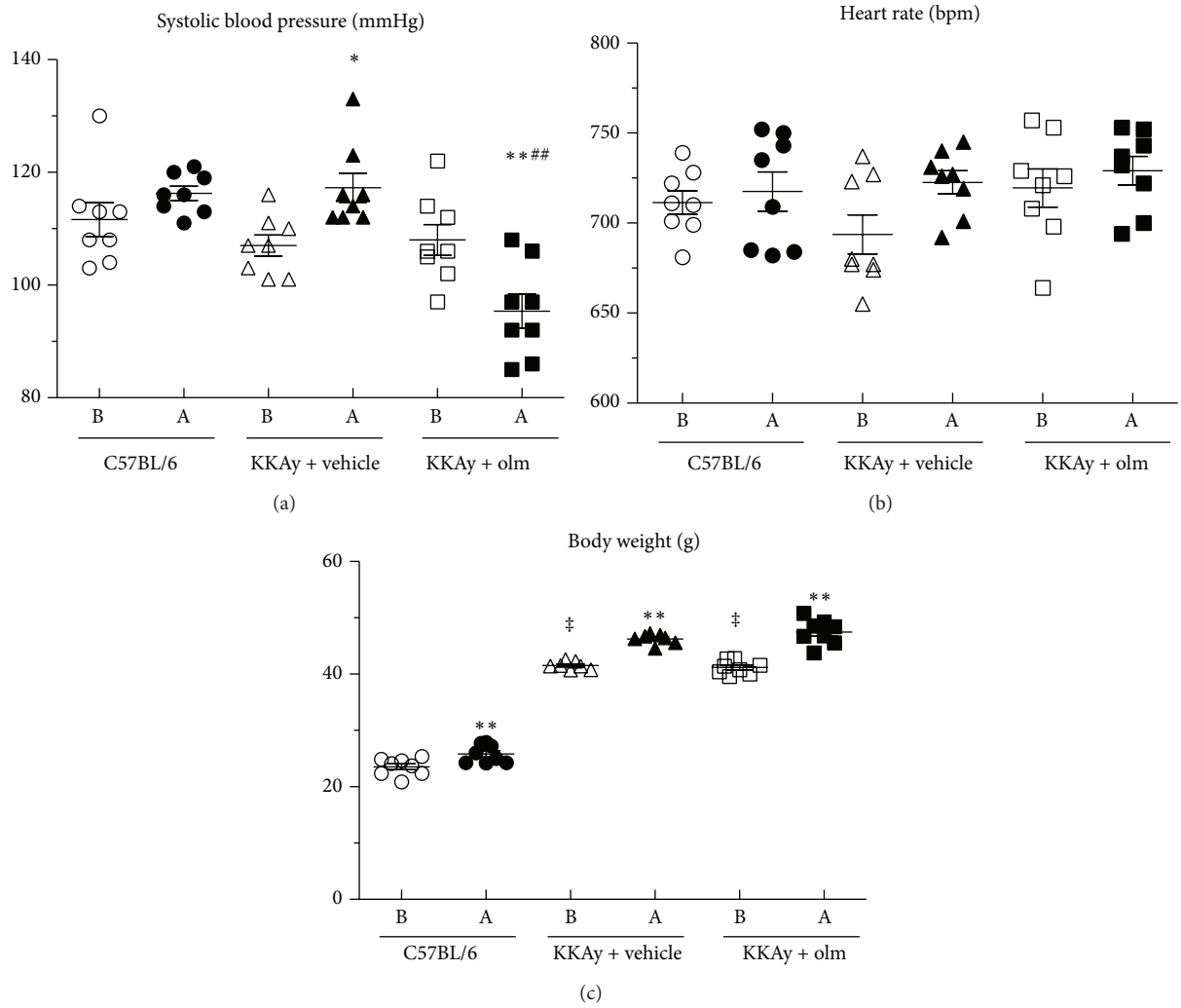

Figure 1: Effects of olmesartan (olm) on systolic blood pressure (a), heart rate (b), and body weight (c) in KKAy mice. Individual values are shown in the graphs and the values are also shown as the mean ± SEM ($n = 8$). B, before treatment; A, after treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ versus before treatment; $^{\#\#}P < 0.01$ versus KKAy + vehicle; $^{\ddagger}P < 0.01$ versus C57BL/6 (ANOVA).

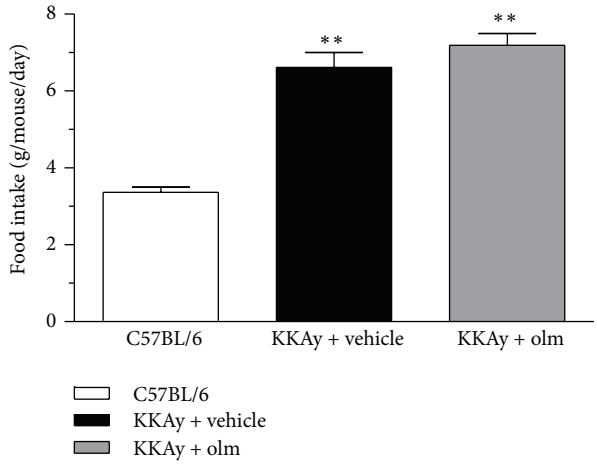

Figure 2: Effects of olmesartan (olm) on daily food intake in KKAy mice. The values are the mean ± SEM ($n = 8$). $^{**}P < 0.01$ versus C57BL/6 (ANOVA).

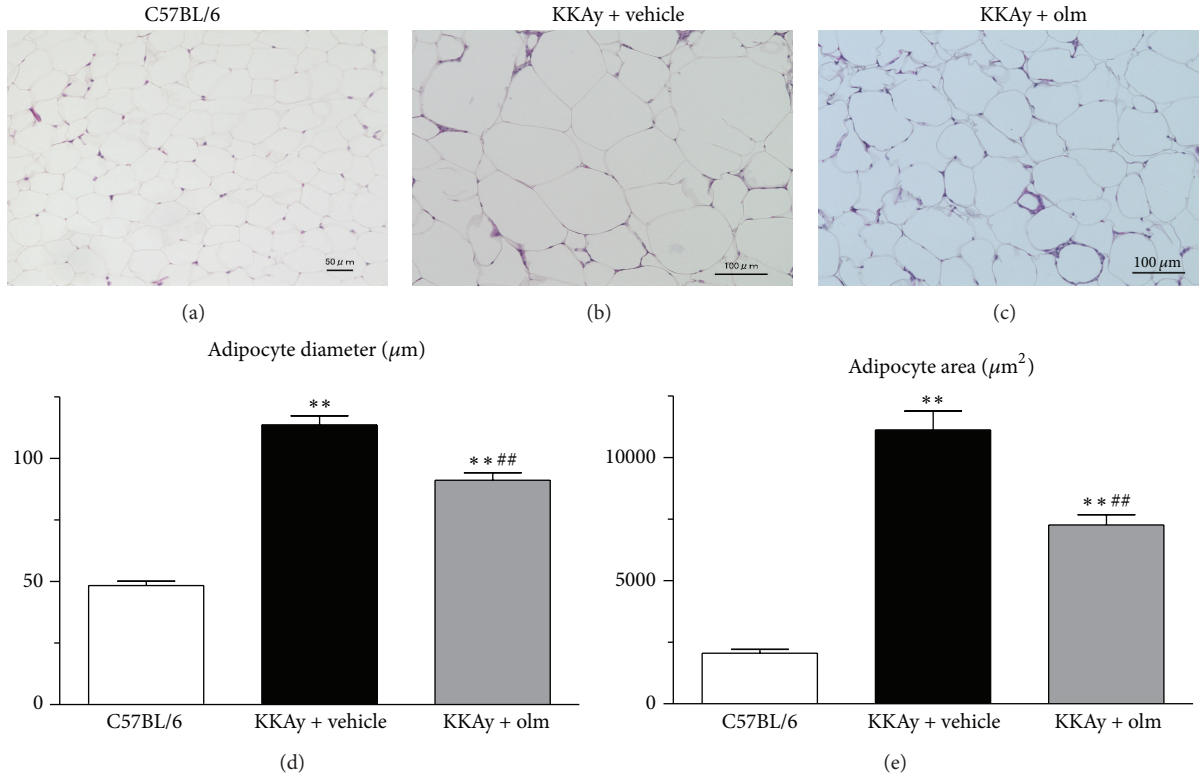

Figure 3: Effects of olmesartan (olm) on adipocyte hypertrophy in KKAy mice. Upper panel: histological analysis of epididymal adipose tissue sections ((a) C57BL/6; (b) KKAy + vehicle; (c) KKAy + olmesartan) stained with hematoxylin and eosin in each experimental group. Original magnification: ×200. Lower panel: adipocyte diameter (d) and area (e). The values are the mean ± SEM ($n = 8$). $^{**}P < 0.01$ versus C57BL/6; $^{\#\#}P < 0.01$ versus KKAy + vehicle (ANOVA). Olm indicates olmesartan.

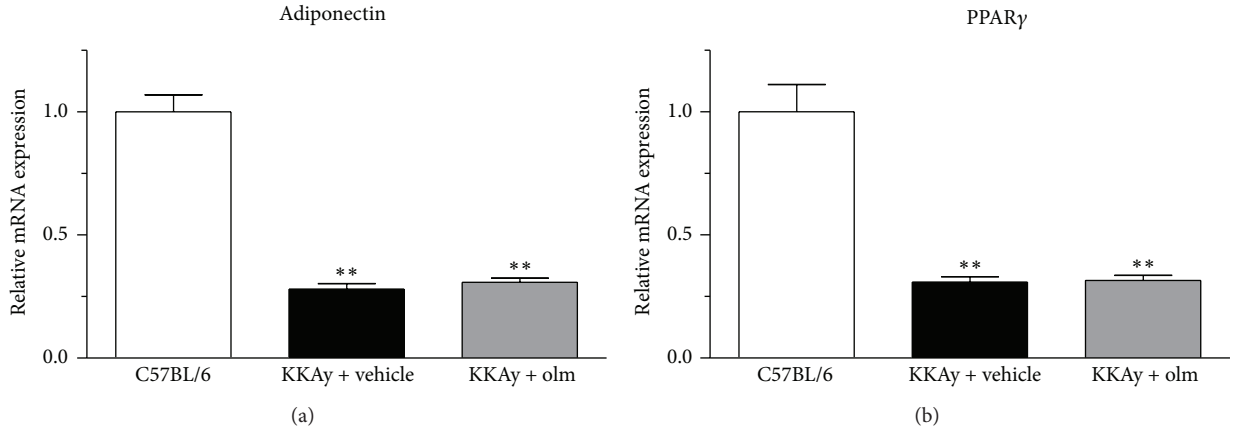

Figure 4: Effects of olmesartan (olm) on the adipose tissue mRNA expression of adiponectin (a) and PPARγ (b) in KKAy mice. The values are the mean ± SEM ($n = 8$). $^{**}P < 0.01$ versus C57BL/6 (ANOVA). Olm indicates olmesartan.

adipose tissue expression of adiponectin, an important adipokine, as well as peroxisome proliferator-activated receptor γ (PPARγ), compared with the control C57BL/6 mice (Figures 4(a) and 4(b)). With respect to a possible effect of olmesartan on adiponectin and PPARγ, the treatment with olmesartan did not affect adiponectin or PPARγ mRNA expression in the adipose tissue of KKAy mice (Figures 4(a) and 4(b)). We also examined the possible influence of olmesartan on adipose tissue expression of the RAS component genes (angiotensinogen, ATRAP, and AT1R) in KKAy mice. While the KKAy mice treated with vehicle exhibited a significantly lower expression of adipose angiotensinogen and ATRAP mRNA than the control C57BL/6 mice ($P < 0.01$ versus C57BL/6), adipose AT1R mRNA expression was not altered in vehicle-treated KKAy mice (Figures 5(a), 5(b), and 5(c)). In addition, treatment with olmesartan did not affect

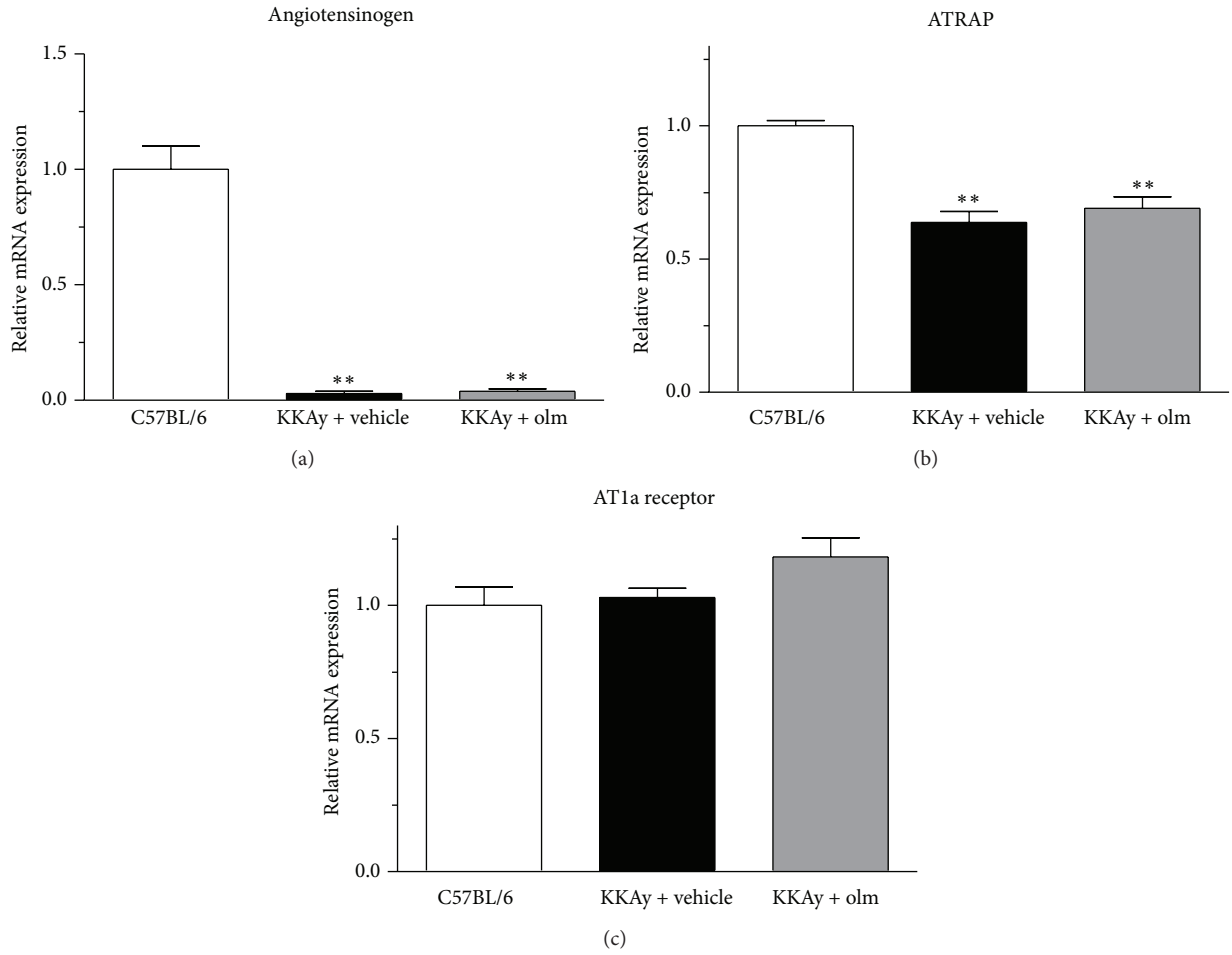

Figure 5: Effects of olmesartan (olm) on the adipose tissue mRNA expression of angiotensinogen (a), ATRAP (b), and AT1R (c) (AT1a receptor) in KKAy mice. The values are the mean ± SEM ($n = 8$). $^{**}P < 0.01$ versus C57BL/6 (ANOVA). Olm indicates olmesartan.

the expression of angiotensinogen, AT1R, or ATRAP mRNA in the adipose tissue of KKAy mice (Figures 5(a), 5(b), and 5(c)).

3.4. Effects of Olmesartan on Adipose Tissue Inflammatory Cytokines. Regarding the expression of tissue inflammatory cytokines (MCP-1, TNF-α, IL-6, and PAI-1) in adipose tissue (Figure 6), while the PAI-1 mRNA expression was not altered in the vehicle-treated KKAy mice (Figure 6(d)), the mRNA levels of MCP-1, TNF-α, and IL-6 were all significantly upregulated in the KKAy mice treated with vehicle compared with the control C57BL/6 mice (MCP-1 and TNF-α, $P < 0.01$; IL-6, $P < 0.05$ versus C57BL/6) (Figures 6(a), 6(b), and 6(c)). With respect to a possible effect of olmesartan on these inflammatory cytokine genes in the adipose tissue, the KKAy mice treated with olmesartan exhibited a blunted increase in adipose IL-6 mRNA expression (Figure 6(c)), in spite of the fact that there are no effects on the adipose MCP-1, TNF-α, and PAI-1 mRNA expression (Figures 6(a), 6(b), and 6(d)).

3.5. Effects of Olmesartan on Adipose Tissue Oxidative Stress. We finally examined the possible effects of olmesartan on the expression of the NADPH oxidase components (p22phox, gp91phox, p47phox, and p40phox) in the epididymal adipose tissue of KKAy mice. As shown in Figure 7, although the vehicle-treated KKAy mice exhibited a significantly elevated expression of these NADPH oxidase component mRNA levels in the adipose tissue compared with the control C57BL/6 mice (p22phox, gp91phox, p47phox, and p40phox, $P < 0.01$ versus C57BL/6), treatment with olmesartan for 4 weeks significantly suppressed the enhanced adipose tissue expression of p22phox, gp91phox, and p47phox mRNA in KKAy mice without affecting adipose p40phox mRNA expression mice (p22phox and gp91phox, $P < 0.01$; p47phox, $P < 0.05$ versus vehicle) (Figures 7(a), 7(b), 7(c), and 7(d)).

4. Discussion

Increasing evidence has indicated that adipose tissue is profoundly involved in the physiological and pathophysiological regulation of circulatory and endocrine systems *in vivo* via modulatory effects on the local production of inflammatory cytokines, adipokines, and vasoactive factors. In addition, it has been demonstrated that the genes of RAS components such as angiotensinogen and AT1R were substantially expressed in adipose tissue [6]. The local RAS

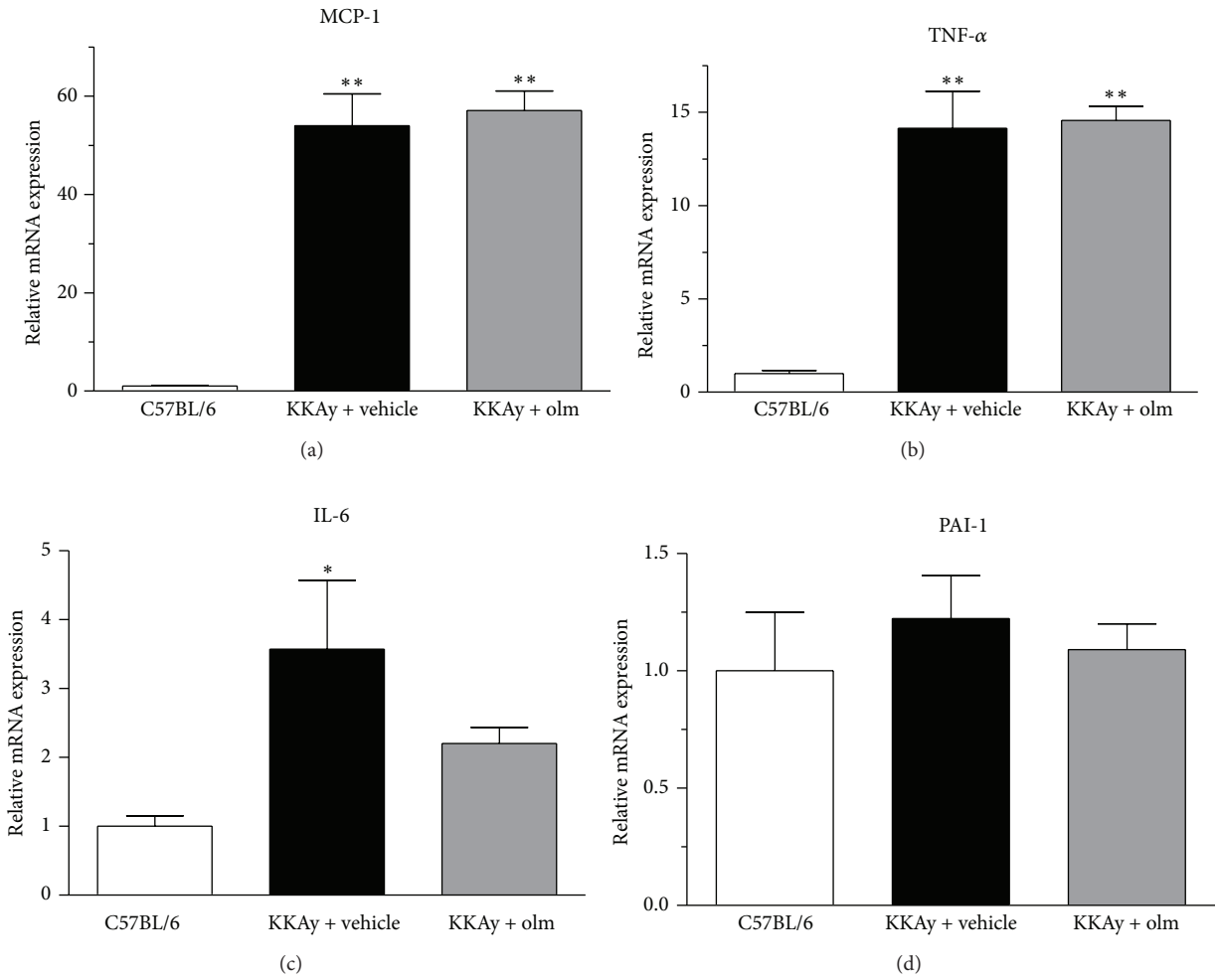

Figure 6: Effects of olmesartan (olm) on the adipose tissue mRNA expression of proinflammatory cytokines ((a) MCP-1; (b) TNF-α; (c) IL-6; and (d) PAI-1) in KKAy mice. The values are the mean ± SEM ($n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus C57BL/6 (ANOVA). Olm indicates olmesartan.

in adipose tissue is suggested to be critically involved in the modulation of the physiological function of adipocytes, and furthermore, the pathological activation of adipose tissue RAS reportedly plays a crucial role in the pathophysiology of metabolic disorders via the dysregulated production of oxidative stress, inflammatory cytokines, and adipokines in adipose tissue [7, 19]. Thus, it is certainly important to identify any beneficial effects of interventions on adipose tissue in order to develop a more efficient therapeutic strategy to treat metabolic disorders with obesity.

In the present study, 4-week olmesartan treatment significantly decreased blood pressure in KKAy mice, a human model of metabolic disorders, without any significant effects on dietary food intake or body weight gain. Thus, the hypotensive effect of olmesartan was exerted without any inhibitory effect on body weight gain in KKAy mice. However, from the point of view of possible pleiotropic effects of olmesartan on adipose tissue function, there are three interesting findings possibly related to adipose tissue in KKAy mice: (1) an inhibitory effect on adipocyte hypertrophy, (2) a suppressive effect on IL-6 gene expression, and (3) an ameliorating effect on oxidative stress.

Previous studies showed that the persistent low-grade activation of chronic inflammatory responses in adipose tissue plays an important role in the development of metabolic disorders with visceral obesity [20–26] and that chronic adipose tissue inflammation is provoked via the stimulated secretion of proinflammatory cytokines and factors derived from adipocytes [4, 27]. Although adiponectin is a well-established adipocyte-secreted endocrine factor involved in the pathophysiology of metabolic disorders and provides a functional link between adipose tissue and the immune system [5, 28], the circulating adiponectin level is reportedly decreased in metabolic disorders with visceral obesity [29]. In addition, PPARγ is reported to improve adipocytokine dysregulation in adipose tissue, including adiponectin, in metabolic disorders [30]. In the present study, while the treatment of KKAy mice with olmesartan did not affect the adipose expression of adiponectin or PPARγ, olmesartan inhibited the adipose tissue gene expression of IL-6, which

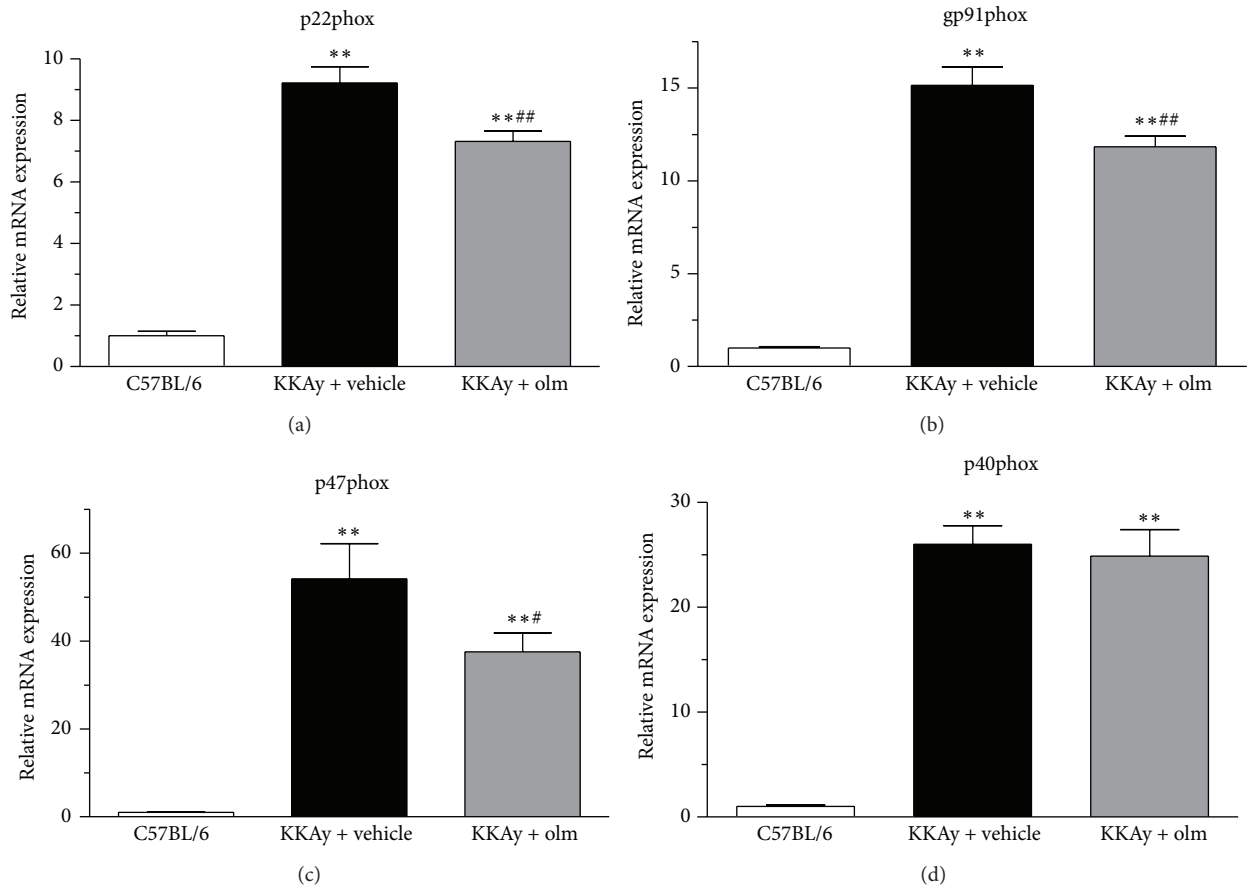

Figure 7: Effects of olmesartan (olm) on the adipose tissue mRNA expression of NADPH oxidase components ((a) p22phox; (b) gp91phox; (c) p47phox; and (d) p40phox) in KKAy mice. The values are the mean ± SEM ($n = 8$). $^{**}P < 0.01$ versus C57BL/6; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus KKAy + vehicle (ANOVA). Olm indicates olmesartan.

is also one of the key players in the inflammatory process in adipose tissue in metabolic disorders [22, 31].

Accumulated adipose tissue-induced dysregulated production of adipocytokines, including proinflammatory cytokines such as IL-6, is reported to activate NADPH oxidase components [32–35]. Adipose NADPH oxidase-derived reactive oxygen species (ROS) function as important intracellular second messengers to activate many downstream signaling molecules that modulate endothelial function, pathological growth and migration of vascular cells, expression of proinflammatory mediators and modification of extracellular matrix [36–39]. All of these processes play important roles in the development of insulin resistance and cardiovascular disease in metabolic disorders with visceral obesity. In the present study, olmesartan exerted a suppressive effect on adipocyte hypertrophy concomitant with an inhibitory effect on the IL-6-oxidative stress axis without any body weight reducing effect in KKAy mice, a human model of metabolic disorders.

We previously identified ATRAP as a novel molecule interacting with AT1R and showed that ATRAP suppressed the Ang II-induced pathological responses of cardiovascular cells and tissues by promoting AT1R internalization [17, 18, 40–42]. Thus, a tissue-specific regulatory balancing of ATRAP and AT1R expression may be involved in the modulation of AT1R signaling that specifically occurs in each tissue [43–46]. We showed that the upregulation of the cardiac ATRAP/AT1R ratio is one of the therapeutic benefits of olmesartan in inhibiting cardiac hypertrophy in hypertensive rats [47]. In addition, prepubertal transient blockade of AT1R signaling by olmesartan exerted a long-term therapeutic effect on salt-induced hypertension and renal injury in Dahl Iwai salt-sensitive rats, partly through a sustained enhancement of renal ATRAP expression [48].

Furthermore, a recent study employing mice with the gene-targeted systemic deletion of ATRAP has shown that the development of systemic insulin resistance related to ATRAP deficiency is attributable to the exaggerated adipose tissue inflammation that occurs via the secretion of proinflammatory cytokines and factors derived from enlarged adipocytes, thereby suggesting ATRAP to be a novel molecular target in metabolic disorders in visceral obesity [19]. However, in the present study, the treatment with olmesartan exerted no evident influence on adipose tissue ATRAP gene expression in KKAy mice. Therefore, further studies are necessary to examine whether the adipose ATRAP is involved in the olmesartan-induced beneficial suppressive effect on the IL-6-oxidative stress axis in KKAy mice.

In the present study, the body weight did not differ significantly between the vehicle-treated and olmesartan-treated KKAy mice after 4 weeks. A previous study showed that olmesartan treatment at a dose of 10 mg/kg per day for 6 weeks exerted an inhibitory effect on body weight gain with a trend of reduction in adiposity without any evident change in food intake in obese Otsuka Long-Evans Tokushima Fatty rats [49]. In contrast, another study reported that treatment with regular chow containing 0.0015% olmesartan for 2 weeks resulted in a significant reduction in blood pressure level without any effect on food intake or body weight gain in KKAy mice [50]. These results suggest that the inhibitory effects of olmesartan on body weight and adipose tissue mass may depend on the diabetic animal model used and the condition of olmesartan treatment (dose and duration). Furthermore, as a limitation of the present study, although olmesartan significantly suppressed the enhanced adipose tissue mRNA expression of NADPH oxidase, this does not constitute direct evidence of an effect. Further studies on the protein expression of NADPH oxidase and adipose tissue production of oxidative stress are needed to obtain a definitive result.

5. Conclusions

In summary, the results of the present study in a mouse model of human metabolic disorders showed a therapeutic effect of olmesartan on adipose tissue in addition to its blood pressure lowering effect. The results suggest that the olmesartan-mediated inhibitory effect on adipocyte hypertrophy in KKAy mice is associated with a beneficial suppression of the IL-6-oxidative stress axis in adipose tissue. Further studies are needed to demonstrate this beneficial effect of olmesartan on adipose tissue oxidative stress and to clarify whether there is a functional role of adipose ATRAP in the pathophysiology of metabolic disorders.

Disclosure

Kouichi Tamura has received honoraria, consulting fees, or funds from Novartis, Takeda, Daiichi-Sankyo, Dainippon-Sumitomo, Kyowa-Hakko Kirin, Chugai, Shionogi, Boehringer Ingelheim, Astellas, Mochida, Pfizer, Mitsubishi Tanabe, and Sanofi.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Breath Ketone Testing: A New Biomarker for Diagnosis and Therapeutic Monitoring of Diabetic Ketosis

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Background. Acetone, β-hydroxybutyric acid, and acetoacetic acid are three types of ketone body that may be found in the breath, blood, and urine. Detecting altered concentrations of ketones in the breath, blood, and urine is crucial for the diagnosis and treatment of diabetic ketosis. The aim of this study was to evaluate the advantages of different detection methods for ketones, and to establish whether detection of the concentration of ketones in the breath is an effective and practical technique. *Methods*. We measured the concentrations of acetone in the breath using gas chromatography-mass spectrometry and β-hydroxybutyrate in fingertip blood collected from 99 patients with diabetes assigned to groups 1 (−), 2 (±), 3 (+), 4 (++), or 5 (+++) according to urinary ketone concentrations. *Results*. There were strong relationships between fasting blood glucose, age, and diabetic ketosis. Exhaled acetone concentration significantly correlated with concentrations of fasting blood glucose, ketones in the blood and urine, LDL-C, creatinine, and blood urea nitrogen. *Conclusions*. Breath testing for ketones has a high sensitivity and specificity and appears to be a noninvasive, convenient, and repeatable method for the diagnosis and therapeutic monitoring of diabetic ketosis.

1. Introduction

Diabetic ketoacidosis (DKA) is a life-threatening condition that occurs predominantly in patients with newly diagnosed type 1 diabetes mellitus and is a consequence of a lack of insulin production by pancreatic islet cells, but it may also occur in patients with type 2 diabetes with poorly controlled blood glucose concentration or other diseases [1]. Diabetic ketosis and ketoacidosis are mainly caused by a lack of insulin or an inappropriate rise in blood glucagon concentration that leads to sugar, protein, fat, water, electrolyte, and acid-base imbalance. Identifying a testing method with high sensitivity and specificity would facilitate the early diagnosis and treatment of diabetic ketosis.

Ketone bodies are produced when the liver metabolizes fatty acids, including acetone, β-hydroxybutyrate, and acetoacetic acid: β-hydroxybutyrate can be converted into acetoacetic acid and accounts for 78% of all ketones in the body, followed by acetoacetic acid (20%) and acetone (2%). Clinically, when making the diagnosis of DKA, blood ketone concentration is generally inferred from the urinary ketone concentration. Commonly used detection methods for urinary ketones are more sensitive to acetoacetic acid than acetone but less sensitive to β-hydroxybutyrate, which appears earliest in DKA—explaining why patients with DKA may not have detectable concentrations of ketones in their urine. Urinary ketone excretion may also be impaired in patients with renal dysfunction. It can be argued that detecting urinary ketones is not a suitable means of diagnosing DKA.

A blood test that measures the concentration of serum β-hydroxybutyrate is available, but there has been a great deal of interest in developing means of measuring the concentration of ketones in the breath, as a convenient and noninvasive diagnostic tool that could also guide therapeutic interventions. The presence of acetone in the breath has

long been known to be correlated with ketone bodies in the plasma. Acetoacetate may be decarboxylated to produce volatile acetone, besides that the boiling point of acetoacetate and β-hydroxybutyric acid in exhaled breath is higher than acetone, with the content relatively small and difficult to detect, so we choose the acetone concentrations as a predictor of diabetic ketosis. We evaluated the advantages of various detection methods and explored the clinical value of acetone breath detection in the diagnosis and treatment of diabetic ketosis.

2. Materials and Methods

2.1. Participants. Ninety-nine patients with diabetes (49 males and 50 females; age range: 11–85 years) were recruited from the Department of Endocrinology of the Second Hospital of Jilin University in Changchun, China. According to the urinary ketone detecting package insert, color changes of –, ±, +, ++, and +++ correspond to concentrations of 0 mmol/L, 0.5 mmol/L, 1.5 mmol/L, 3.9 mmol/L, and 7.8 mmol/L, respectively. The patients were assigned into 5 groups on the basis of urinary ketone concentration: group 1 (−), urinary ketone recorded as negative, 9 males and 10 females ($n = 19$); group 2 (±), urinary ketone recorded as mild positive, 7 males and 9 females ($n = 16$); group 3 (+), urinary ketone recorded as positive, 14 males and 11 females ($n = 25$); group 4 (++), urinary ketone recorded as moderate positive, 9 males and 10 females ($n = 19$); and group 5 (+++), urinary ketone recorded as strong positive, 10 males and 10 females ($n = 20$). The study protocol was approved by the Ethics Committee of the Second Hospital of Jilin University, and written consent was obtained from all subjects before breath collection.

2.2. Inclusion Criteria. Type 2 diabetes mellitus was diagnosed according to the 1999 WHO diagnostic criteria [2]. Patients with gestational diabetes, diabetes mellitus complicating pregnancy, and secondary diabetes were excluded.

2.3. Measurement of Ketone Concentration. Fresh fingertip blood samples were obtained and the blood concentration of β-hydroxybutyrate was measured using an Optium Xceed (Abbott, USA) device: using the manufacturer-suggested cutoff of >0.5 mmol/L was considered to be positive. We used 3 L foil bags to collect exhaled breath from participants, which were analyzed within 5 days. Three samples of exhaled breath were obtained from each subject. The concentration of acetone was determined in the breath using gas chromatography-mass spectrometry (GC/MS). Operation was performed according to the instructions. Quality control of the exhaled breath has been described in our published paper [3]. A concentration ≥1.0 ppmv was considered positive. Urinary ketone concentrations were also measured, and the demographic and clinical characteristics of patients were recorded.

2.4. Statistical Analysis. All data were statistically processed using SPSS software (version 17; IBM, New York, NY, USA) and reported as mean ± standard deviation (SD). Intergroup comparisons were performed using t-tests for normally distributed data and nonparametric tests for data that were not normally distributed. Analysis of variance was used for multigroup comparisons. Categorical data were analyzed using chi-square tests and expressed as positive cases and constituent ratios (%). Correlation analysis was performed to examine the strength of relationships between variables. A receiver operating characteristic (ROC) curve was constructed to determine the optimal cut-off value of concentration of exhaled acetone and the urinary ketone, and sensitivity and specificity were calculated. Two-sided tests were used for all statistical analyses. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Demographic and Clinical Characteristics of Participants. Demographic and clinical data are shown in Table 1. Fasting blood glucose (FBG) concentration on admission was significantly higher in group 5 than groups 1, 2, 3, and 4 ($P < 0.001$, $P = 0.005$, $P = 0.029$, and $P = 0.008$, resp.), but there were no differences between groups 1 to 4. Patients in group 5 were significantly younger than those in groups 1 to 3 ($P = 0.005$, $P = 0.001$, and $P = 0.001$, resp.), and patients in group 4 were also younger than those in group 2 ($P = 0.037$), but there were no statistically significant differences in age between the other groups. Furthermore, there were no significant differences in sex, body mass index, blood hemoglobin A1c (HbA1c), total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Cr), and blood urea nitrogen (BUN) concentration between any of the five groups.

3.2. Comparison of Blood and Breath Concentrations of Ketones. Concentrations of blood β-hydroxybutyrate and exhaled acetone are shown in Table 2 and Figure 1. The blood concentration of β-hydroxybutyrate was significantly higher in groups 4 and 5 than groups 1 to 3 ($P = 0.003$, $P = 0.008$, and $P = 0.023$, resp., and $P < 0.001$, $P < 0.001$, and $P < 0.001$, resp.) and higher in group 5 than group 4 ($P < 0.001$), but there were no differences between groups 1 to 3. The breath concentration of acetone was higher in group 4 than groups 1 and 3 ($P = 0.028$ and $P = 0.035$, resp.) and higher in group 5 than groups 1 to 4 ($P < 0.001$, $P < 0.001$, $P < 0.001$, and $P = 0.002$, resp.), but there were no differences between the other groups. Blood β-hydroxybutyrate concentration was positive in 6.7%, 14.3%, 43.5%, 71.4%, and 89.5% of cases, respectively, in groups 1 to 5, and exhaled acetone concentration was positive in 18.8%, 20%, 60%, 80%, and 92.9% of cases, respectively, in groups 1 to 5 (Table 3).

3.3. Correlation of Urinary Ketone Concentration with Exhaled Breath Acetone. The exhaled acetone concentration was significantly correlated with the concentrations of FBG ($r = 0.428$, $P < 0.001$), blood β-hydroxybutyrate ($r = 0.817$,

Table 1: Demographic and clinical characteristics of study participants.

	1 Urine ketone (−)	2 Urine ketone (±)	3 Urine ketone (+)	4 Urine ketone (++)	5 Urine ketone (+++)	P
Age (yr)	45	48	45	37	30	0.004
Male (n)	9 (18.37%)	7 (14.29%)	14 (28.57%)	9 (18.37%)	10 (20.40%)	0.783
BMI (kg/m^2)	23.49	25.41	22.73	23.43	21.92	0.219
FBG (mmol/L)	13.27	14.36	16.29	15.08	20.36	0.006
HbA1c (%)	10.37	10.59	11.40	10.33	12.26	0.183
TC (mmol/L)	5.78	5.27	6.06	5.01	5.62	0.327
TG (mmol/L)	2.71	3.39	3.60	1.47	4.83	0.439
LDL-C (mmol/L)	3.08	3.04	3.02	2.86	3.02	0.99
HDL-C (mmol/L)	1.12	1.10	1.24	1.20	1.12	0.747
ALT (U/L)	31.35	31.45	23.47	22.69	23.31	0.833
AST (U/L)	23.65	24.18	25.04	25.36	18.94	0.830
BUN (mmol/L)	3.84	4.20	4.05	4.58	4.46	0.745
Cr (μmol/L)	58.06	64.16	61.66	62.13	70.32	0.584

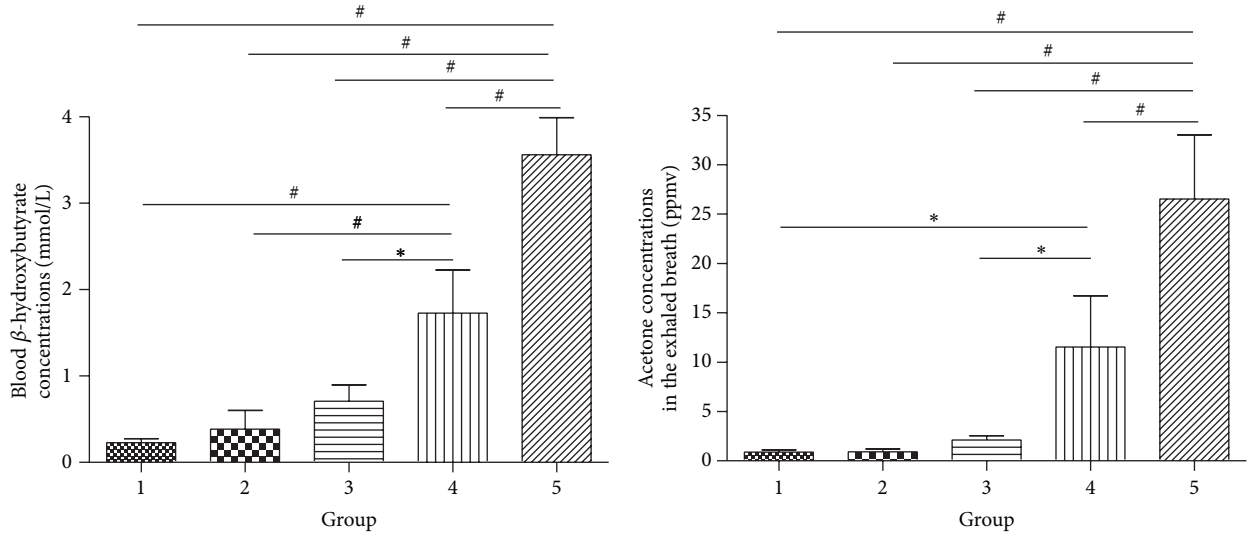
BMI: body mass index; FBG: fasting blood glucose; HbA1c: hemoglobin A1c; TC: total cholesterol; TG: triglyceride; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Cr: creatinine; BUN: blood urea nitrogen.

Table 2: Comparison of blood β-hydroxybutyrate concentrations and exhaled acetone concentrations between the groups.

	1 Urine ketone (−)	2 Urine ketone (±)	3 Urine ketone (+)	4 Urine ketone (++)	5 Urine ketone (+++)	P
Blood β-hydroxybutyrate (mmol/L)	0.23	0.39	0.71	1.73	3.56	<0.001
Acetone in the breath (ppmv)	0.89	0.93	2.04	13.82	33.12	<0.001

Table 3: Incidence of positive blood β-hydroxybutyrate and exhaled acetone detection in each group.

	1 Urine ketone (−)	2 Urine ketone (±)	3 Urine ketone (+)	4 Urine ketone (++)	5 Urine ketone (+++)	P
Blood β-hydroxybutyrate	6.7%	14.3%	43.5%	71.4%	89.5%	<0.001
Acetone in the breath	18.8%	20%	60%	80%	92.9%	<0.001



(a) Blood β-hydroxybutyrate concentrations

(b) Exhaled acetone concentrations $^{*}P < 0.05$, $^{\#}P < 0.01$

Figure 1: Blood β-hydroxybutyrate and exhaled acetone concentrations in patients with increasing concentrations of urinary ketones.

Table 4: Correlation between exhaled acetone concentration and other clinical variables.

	Correlation coefficient (r)	P
FBG	0.428	<0.001
Blood β-hydroxybutyrate	0.817	<0.001
Urine ketone	0.581	<0.001
LDL-C	0.255	0.047
Cr	0.385	0.002
BUN	0.362	0.003

FBG: fasting blood glucose; LDL-C: low-density lipoprotein cholesterol; Cr: creatinine; BUN: blood urea nitrogen.

$P < 0.001$), urinary ketone concentration ($r = 0.581$, $P < 0.001$), LDL-C ($r = 0.255$, $P = 0.047$), Cr ($r = 0.385$, $P = 0.002$), and BUN ($r = 0.362$, $P = 0.003$) (Table 4).

3.4. Exhaled Acetone Concentration as a Predictor of Diabetic Ketosis. Concentrations of blood β-hydroxybutyrate served as the standard to assess the sensitivity and specificity of exhaled acetone for detection of diabetic ketosis (Figure 2). The area under the curve (AUC) was 0.905 ($P < 0.001$), and the cut-off concentration of exhaled acetone for diagnosis of diabetic ketosis was 1.185 ppmv, with a sensitivity and specificity of 90.9% and 77.1%, respectively. Concentrations of blood β-hydroxybutyrate served as the standard to assess the sensitivity and specificity of urinary ketone for detection of diabetic ketosis (Figure 2). The area under the curve (AUC) was 0.815 ($P < 0.001$), and the cut-off concentration of urinary ketone for diagnosis of diabetic ketosis was 2.7 mmol/L, with a sensitivity and specificity of 63.6% and 85.7%, respectively.

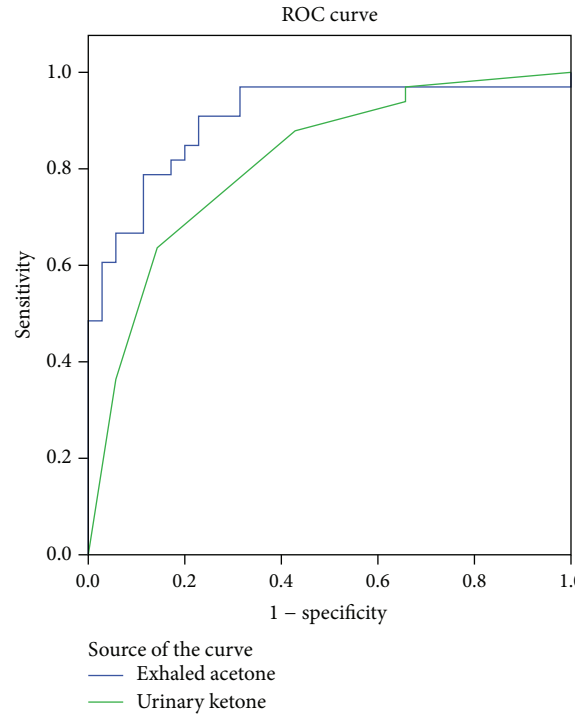


Figure 2: Receiver operating characteristic (ROC) curve for exhaled acetone and urinary ketone concentration for the diagnosis of diabetic ketosis.

4. Discussion

Ketoacidosis may occur in patients with diabetes of all ages [4]. A study of Austrian indicated that the incidence of DKA was negatively correlated with age [5]. Klingensmith and colleagues have reported that younger age, lack of private health insurance, and African American ancestral heritage are independent risk factors for DKA [6]. In our study, younger patients and higher FBG concentration tended to be strongly positive for urinary ketones, which is consistent with data reported.

Exhaled breath detection has been used to diagnose metabolic disease and monitor treatment for many years [7]. The techniques used to detect these compounds in exhaled breath are based on mass spectrometry, for example, proton transfer reaction mass spectrometry, selected ion flow tube mass spectrometry [8], and cavity ring down spectroscopy. The concentration of breath acetone is associated with glucose metabolism and lipolysis [7]. Previous studies have shown a close correlation between the concentrations of ketones released from the skin and blood levels [9]. Breath acetone concentration is also reported to be elevated in type 2 diabetes mellitus, and it can be used to diagnose the onset of diabetes [10]. We used the GC/MS method to detect exhaled acetone, which is capable of detecting over 200 constituents of exhaled breath and is highly sensitive to typical volatile organic compounds. In our study correlation analysis demonstrated that the concentration of exhaled acetone was significantly associated with urinary ketone concentration, blood FBG, LDL-C, Cr, and BUN concentrations. Prompt exhaled acetone maybe is a better index in reflecting the changes of blood glucose, and testing for exhaled acetone is a noninvasive, simple method, which is expected to be a promising indicator of blood glucose monitoring in the future.

When the concentration of blood β-hydroxybutyrate served as the standard in our study to assess the sensitivity and specificity of exhaled acetone and urine ketone, the sensitivity and specificity of exhaled acetone were 90.9% and 77.1%, respectively. However, the sensitivity and specificity of urine ketone were 63.6% and 85.7%, respectively. These results show that the specificity of exhaled acetone is similar to urine ketone, but its sensitivity is higher than urine ketones. In addition, the testing for blood β-hydroxybutyrate and the exhaled acetone is still positive in the urine ketone body negative group; the proportion is 6.7% and 18.8%, respectively. So the concentration of urine ketones may not be a timely predictor of early diabetic ketosis. Blood and exhaled testing for ketones helps to eliminate false negative results [11]. Another potential value for breath ketones testing is it being strongly influenced by physiological factors other than diet [3]. In the present method, the concentration of exhaled acetone higher than 1.185 ppmv was found in diabetic

ketosis patients; the detection just needs simple preparation and no organic solvent. Exhaled acetone analysis proves to be a noninvasive, convenient, sensitive, and solvent-free method and could be applied to diagnose and monitor the severity of diabetic ketosis. However, the technique is still preliminary and its wide clinical use requires further optimization.

Conflict of Interests

The authors declare that they have no financial and personal relationships with other people or organizations. They also declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

CCAAT/Enhancer-Binding Protein α Is a Crucial Regulator of Human Fat Mass and Obesity Associated Gene Transcription and Expression

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Several susceptibility loci have been reported associated with obesity and T2DM in GWAS. Fat mass and obesity associated gene (FTO) is the first gene associated with body mass index (BMI) and risk for diabetes in diverse patient populations. FTO is highly expressed in the brain and pancreas, and is involved in regulating dietary intake and energy expenditure. While much is known about the epigenetic mutations contributing to obesity and T2DM, less is certain with the expression regulation of FTO gene. In this study, a highly conserved canonical C/EBPα binding site was located around position −45~−54 bp relative to the human FTO gene transcriptional start site. Site-directed mutagenesis of the putative C/EBPα binding sites decreased FTO promoter activity. Overexpression and RNAi studies also indicated that C/EBPα was required for the expression of FTO. Chromatin immunoprecipitation (ChIP) experiment was carried out and the result shows direct binding of C/EBPα to the putative binding regions in the FTO promoter. Collectively, our data suggest that C/EBPα may act as a positive regulator binding to FTO promoter and consequently, activates the gene transcription.

1. Introduction

Human FTO consists of 505 amino acids, with the mature protein predicted to have a mass of approximately 58.3 kDa. The research of crystal structure confirmed FTO gene encodes a 2-oxoglutarate (2-OG) Fe^{2+}-dependent dioxygenase and is expressed widely in human tissues [1]. The functional domain contains several residues that are absolutely conserved among highly diverse species. Previous research on FTO mainly focused on the epigenetics. Several groups have revealed that single nucleotide polymorphisms (SNPs) within the first intron of FTO are strongly associated with adiposity and diabetes by genome-wide association studies (GWAS) [2]. FTO is highly expressed in the hypothalamus and pancreatic islets and widely expressed at a lower level in multiple tissues including adipose tissue, liver, and skeletal muscle. Berulava et al. showed that altered FTO levels affect the transcript of genes related to RNA processing and metabolism [3]. However, the molecular mechanisms responsible for transcriptional regulation of human FTO gene have not previously been completely elucidated.

CCAAT/enhancer-binding proteins (or C/EBPs) are a family of transcription factors, composed of six members called C/EBPα to C/EBPζ. They promote the expression of certain genes through interaction with promoter region. Once bound to DNA, C/EBPs can open up chromatin structure or recruit basal transcription factors to regulate gene expression of many housekeeping and tissue-specific genes. C/EBPα is required for both adipogenesis and normal adipocytefunction [4]. For example, C/EBPα is not only

necessary but also sufficient to initiate the 3T3-L1 adipocyte differentiation program [5]. In mouse model, obese genes have been reported to be transcriptional activated by C/EBPα. Mice lacking C/EBPα show abnormal adipose tissue formation [6]. Moreover, ectopic expression of C/EBPα in various fibroblast cell lines promotes adipogenesis.

More recently, we have reported that transcription factor Foxa2 negatively regulates human FTO gene promoter, but the positive transcription factor has not been revealed. In the present study, the human FTO gene promoter structure and its transcriptional control elements have been identified. Mutational and functional analysis of the promoter revealed a functional C/EBPα binding sequence at positions -45~ -54 relative to the transcriptional initiation site in the FTO promoter. siRNA and cotransfection studies indicated that C/EBPα upregulates its transcription. C/EBPα associates with the binding sites of the FTO gene promoter, as demonstrated in ChIP assays *in vivo*. Thus, our study established a molecular basis for further understanding the mechanisms governing FTO gene expression, by which FTO may act as a regulator to enhance adipogenesis.

2. Materials and Methods

2.1. Cell Culture and Treatment. The human embryonic kidney 293 (HEK 293) and human cervical carcinoma (HeLa) cells were cell lines obtained from Shanghai Cell Biology Medical Research Institute, Chinese Academy of Sciences. These cells were maintained in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic agents.

2.2. Bioinformatics Transcriptional Elements Analyses. To identify transcriptional regulatory sequences and potential transcription factor binding sites on the putative promoter regions, the sequences of human, mouse, and rat were obtained from GenBank and aligned by Clustal X program. We analyzed the 5′-flanking regions at the transcription start site with TFsearch (http://mbs.cbrc.jp/research/db/TFSEARCH.html) and AliBaba 2.1 (http://www.gene-regulation.com).

2.3. Transient Transfections and Luciferase Assay. The cloning of the human FTO gene promoter region and the constructing of promoter luciferase report plasmids were performed as described previously [7]. For assaying luciferase expression, HEK 293 and Hela cells were seeded onto 24-well plates, cultured overnight, and cotransfected with pGL3 vector reporter construct and pRL-TK (Promega) as a transfection efficiency control. Cells were lysed and assayed for both firefly and Renilla luciferase using the Dual Luciferase Reporter Assay System Kit (Promega) at 24 h after transfection. Luminescence was determined in a Modulus luminometer (Turner Biosystems) after addition of substrate to allow adequate mixing. Relative firefly luciferase activities (RLU) were calculated by normalizing transfection efficiencies with the Renilla luciferase activity. All the data shown in this study were obtained from at least three independent experiments.

2.4. Overexpression of C/EBPα. In overexpression experiments, the expression plasmids pcDNA3.1-C/EBPα and pcDNA3.1 empty vector were purified and cotransfected by using Lipofectamine 2000 (Invitrogen). Total RNA was isolated 24 hours later and analyzed by RT-PCR. For western blotting experiments, lysates were obtained from cells cultured for 48 hours in 6-well plates.

2.5. Small Interfering RNA Transfection. In the RNA interference experiments, HEK293 cells were seeded in 6-well plates 24 h before transfection. Cells grown to 50% confluency were washed once with serum and antibiotic-free medium and transfected with 100 nM C/EBP siRNA using 2 μL Lipofectamine (Invitrogen) in serum-free medium. After 4 h incubation, complete medium without antibiotics was added and cells were incubated for 24 h. siRNAs specifically targeting C/EBPα (sense, 5′-GUCGGCCAGGAACUCGUCGTT-3′; and antisense, 5′-CGACGAGUUCCUGGCCGACTT-3′) were custom designed [8]. Scrambled siRNA (sense, 5′-GUAGUCCAUGGACCCGUAGTT-3′; and antisense, 5′-CUACGGGUCCAUGGACUACTT-3′) was used as a negative control.

2.6. Site-Directed Mutagenesis. Mutation of the putative C/EBPα sites at $-45/-54$ of human FTO promoter was performed using MutanBEST site-directed mutagenesis kit (Takara) with the pGL3-100 plasmid as the template. The mutagenesis primers designed for the mutations were as follows (the mutated sequences are underlined): mu-C/EBPα-Forward: 5′-CCTCCTG<u>AAC</u>AATGTAGTTCTC-3′, Reverse: 5′-CTACGGGAGCCTGCCATGTTTC-3′; mu-Sp1-Forward: 5′-GGGGTA<u>ATA</u>GACTACGCTCTT-3′, Reverse: 5′-CCGCCGACGACCGGGAACCTAC-3′. The mu-C/EBPα-Sp1 plasmid was created using mu-Sp1 as template. In the mutant expression clones, the sequences of the entire region mutated were amplified by PCR and the expected mutations were verified by DNA sequencing.

2.7. Real-Time PCR. The indicated plasmids or siRNAs were transfected into cells as described above. Total RNA was isolated according to the standard TRIZOL (Invitrogen) method. First-strand cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (Promega). Real-time PCR was performed with ABI system (ABI 7500). The Qiagen 2x SYBR Green master mix was used for PCR reaction. Negative control reactions contained sterilized double-distilled water instead of cDNA and were included in each run to ensure absence of contamination. Thermal denaturation (melt curve analysis) was used to confirm the specificity of desired PCR products. Quantitated mRNA levels of analyzed genes were normalized to GAPDH mRNA to generate a relative expression ratio. Primers utilized were as follows: FTO, forward 5′-ACTTGGCTCCCTTATCTGACC-3′ and reverse 5′-TGTGCAGTGTGAGAAAGGCTT-3′; GAPDH, forward 5′-AGGACTCATGTCCATGCCAT-3′ and reverse 5′-ACCCTGTTGCTGTAGCCAAA-3′.

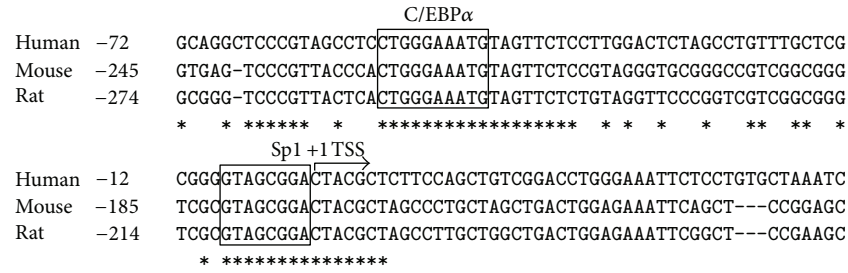

Figure 1: Putative C/EBPα binding site is located in FTO gene promoter. Human genomic sequence of FTO gene from −72 to +47 bp is shown. Asterisks indicate positions where the bases are absolutely conserved among the three species. The transcription start site (TSS) is shown as indicated in the figure. The boxed sequences represent the predicted C/EBPα (−45/−54) and Sp1 (−1/−8) binding sites based on the results of software.

2.8. Western Blot Analysis. After transfection and treatment, cells growing on 6-well cell-culture plates were washed in ice-cold PBS and lysed with 200 μL of lysis buffer containing a protease inhibitor cocktail (Complete; Roche). Protein concentrations were determined by the colorimetric BioRad Protein Assay (BioRad). Protein samples were prepared with 5x SDS sample buffer and loaded at 20 μg of protein per lane for SDS-PAGE. Western blot was performed with FTO (Abgent) and C/EBPα (Santa Cruz) antibodies, followed by goat anti-mouse IgG conjugated with HRP. GAPDH was detected as loading control. Chemoluminescence signals from three independent western analyses were quantified using an ECL imager and analyzed using Quantity One software (BioRad).

2.9. Chromatin Immunoprecipitation Assays. ChIP assays were performed according to the protocols provided by the manufacturer (Active Motif, Carlsbad, CA). Chromatin DNA was fragmented by sonication to an average length of 0.5 kb. Formaldehyde-fixed DNA/protein complex was immunoprecipitated with 5 μg of normal rabbit IgG, anti-C/EBPα antibody (Santa Cruz) and the DNA was purified using gel exclusion columns. The purified ChIP DNA fragment was subjected to semiquantitative PCR analysis (1 cycle of 95°C for 3 min, 35 cycles of 95°C for 20 s, 64°C for 20 s, and 72°C for 1 min). Specific forward (5′-CATTGCTATAGCGCCGACAGCG-3′) and reverse (5′-GAGAATTTCCCAGGTCCGACAG-3′) primers were designed to amplify the FTO promoter region between −133 and +36 bp relative to the transcription start site, which contains C/EBPα binding sites. The sample from total chromatin before immunoprecipitation (at 1:10 dilution) was used as positive control. The PCR products were analyzed on a 2% agarose gel and quantified by densitometry using Fluor's fluorimeter and Quantity One software (Bio-rad).

2.10. Statistical Analysis. Data are expressed as means ± SEM. Comparisons between means were performed by unpaired two-tailed Student's t-test using SPSS software. P-value less than 0.05 was considered significant.

3. Results

3.1. The FTO Promoter Contains a C/EBPα Binding Site. We have previously reported the preliminary analysis of the functional characteristics of human FTO promoter in HEK293 and Hela cells showed that the core promoter was located within the region of −100 bp relative to the transcription start site [7]. Further analysis of the region showed Foxa2 binding sequence (−26/−14) as a negative regulatory element to the expression of human FTO gene. To identify the positive regulatory transcription factors of FTO, the promoter sequences were subjected to search for transcription factor binding sites. A putative consensus C/EBPα binding site (TGGGAAAT at −45/−54 bp) and an Sp1 binding site (GTAGCGGA at −1/−8 bp) were predicted within the FTO core promoter region by TFSEARCH and Alibaba 2.1 program (Figure 1). The sequence TGGGAAAT was almost identical to the C/EBP consensus sequence TTNNGNAAT [9]. Sequence alignment by Clustal X reveals the putative nucleotide sequence for C/EBPα and Sp1 is well conserved between the mouse, the rattus, and the human FTO genes obtained from GenBank (Figure 1). The significant conservation across species suggesting C/EBPα and/or Sp1 transfactor may regulate FTO transcriptional expression.

3.2. C/EBPα Element Enhances the Basal Level of FTO Promoter Activity. To investigate further the function of the C/EBPα and Sp1 binding sites in the regulation of the human FTO gene, mutants were constructed by site-directed mutagenesis. The reporter gene plasmid pGL3-100 which contains human FTO gene core promoter (−100 to +34) was used as template. The luciferase activity of mutant constructs was measured and compared with that of the wild-type promoter (pGL3-100) using HEK 293 cell line.

The results showed the relative luciferase activities (RLU) of the C/EBPα site mutant (pGL3-mu-C/EBPα) decreased by 57.1% (Hela cell) or 55.6% (HEK 293 cell) compared with that of pGL3-100. In contrast, the change of luciferase activity is virtually absent in the reporter construct pGL3-mu-Sp1 containing the mutant Sp1 binding site. Furthermore, the two separate site-specific mutagenesis pGL3-mu-C/EBPα/Sp1 behaved similarly to the pGL3-mu-C/EBPα;

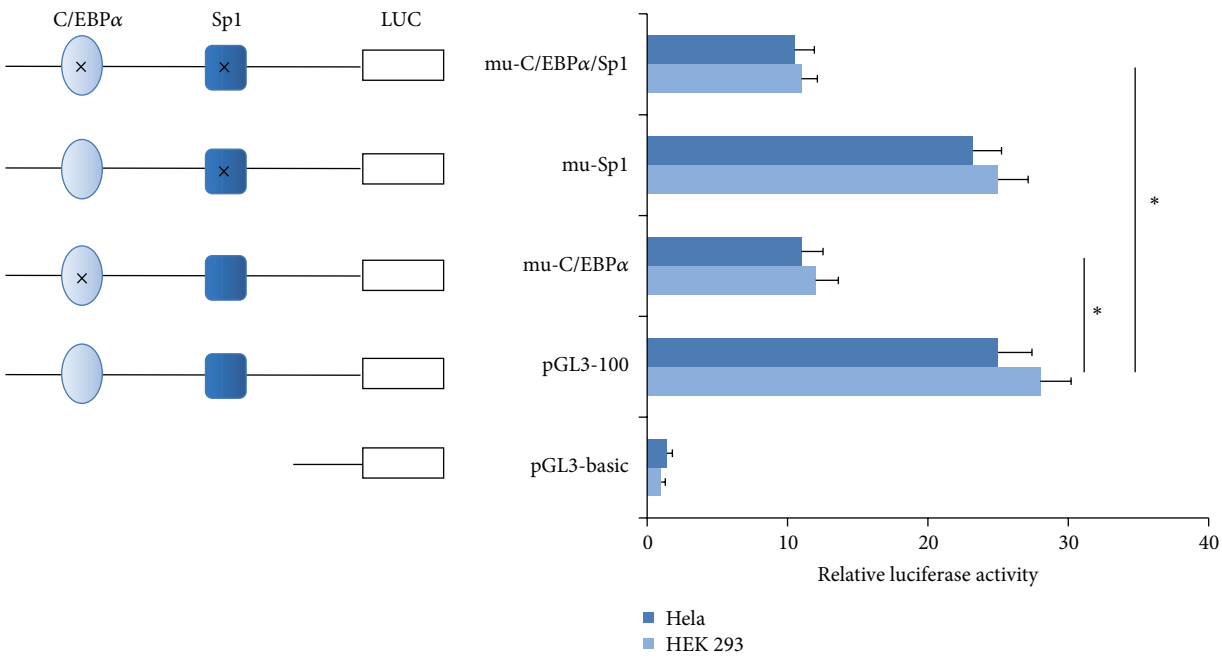

Figure 2: Luciferase reporter assay to determine the regulatory effect of C/EBPα on FTO promoter activity. Three mutant variants (mutated at C/EBP site, Sp1 site, and both of them, resp.) were constructed. Relative luciferase activities (RLU) were measured three independent times in Hela and HEK 293 cells. Results are presented as mean RLU ± SE of three independent experiments ($^*P < 0.05$).

luciferase activity was suppressed to 39.3% (Hela cell) or 41.7% (HEK 293 cell) (Figure 2).

3.3. C/EBPα is Involved in the Expression of FTO. The C/EBPα transfactor binding sites have been identified to play a role in regulating the activity of human FTO promoter. In the following experiments, we further examined the ability of C/EBPα to modulate the expression of FTO. C/EBPα expression vector pcDNA3.1-C/EBPα or C/EBPα siRNA was transfected into HEK 293 cells. FTO mRNA and protein levels were detected by RT-PCR or Western blotting, respectively. As illustrated in Figure 3, more robust overexpression of C/EBPα increased the FTO transcript level 2.1-fold (Figure 3(a)) and FTO protein level by 78.2% above control (Figure 3(b)). Conversely, RNAi-mediated reduction of C/EBPα significantly inhibited the FTO expression at both the mRNA level (66.3%) and protein level (56.2%).

3.4. C/EBPα Binds to the FTO Promoter In Vivo. ChIP analysis was performed to test if C/EBPα binds to the FTO promoter. Nuclear lysates prepared from HEK 293 were subjected to sonication to shear DNA to lengths between 200 and 1000 bp on ice followed by phenol/chloroform extraction to recover protein/DNA complexes. C/EBPα antibody was used to pull down the complexes as instructed. The resultant precipitates were then used as templates for PCR amplification of FTO promoter sequence containing the C/EBPα binding motif. As shown in Figure 4, precipitates resulting from C/EBPα antibody yielded a corresponding band as that of amplification with input (1 : 10), an aliquot of chromatin that was not incubated with an antibody, while no band was showed in the negative control (IgG). These results clearly demonstrate an *in vivo* recruitment of C/EBPα binding element on the human FTO promoter. Taken together, our results suggest that C/EBPα specifically binds to the predicted motifs to regulate FTO transcription and expression directly.

4. Discussion

The association of the genetic variants of FTO gene with obesity and diabetes was recently identified by several independent GWA studies. Subsequent studies have revealed the influence of FTO variants on measures of appetite, food intake, or energy expenditure. FTO is highly expressed in the brain (hypothalamic) and pancreas. The putative influence of FTO in hypothalamic-pituitary-thyroid axis has been determined [10, 11]. A number of studies have been observed the expression of Fto in hypothalamic correlates with changes in the nutritional status of animals, and furthermore, the abundant hypothalamic expression of FTO also supports a potential role in the control of satiety or appetite [12]. Although these studies reveal the connection between FTO expression and energy metabolism, the molecular mechanism for regulating FTO gene expression remains unclear.

The human FTO gene transcription has been studied previously by our group. We had reported that the region (−100/+34) seems to be crucial because deletion of this fragment would not remain as the basal promoter activity. Transfactor Foxa2 was defined to regulate the transcription and expression of FTO gene negatively. There are no data about transcription factors positively involved in FTO gene expression. Here, we improved the regulatory mechanism of

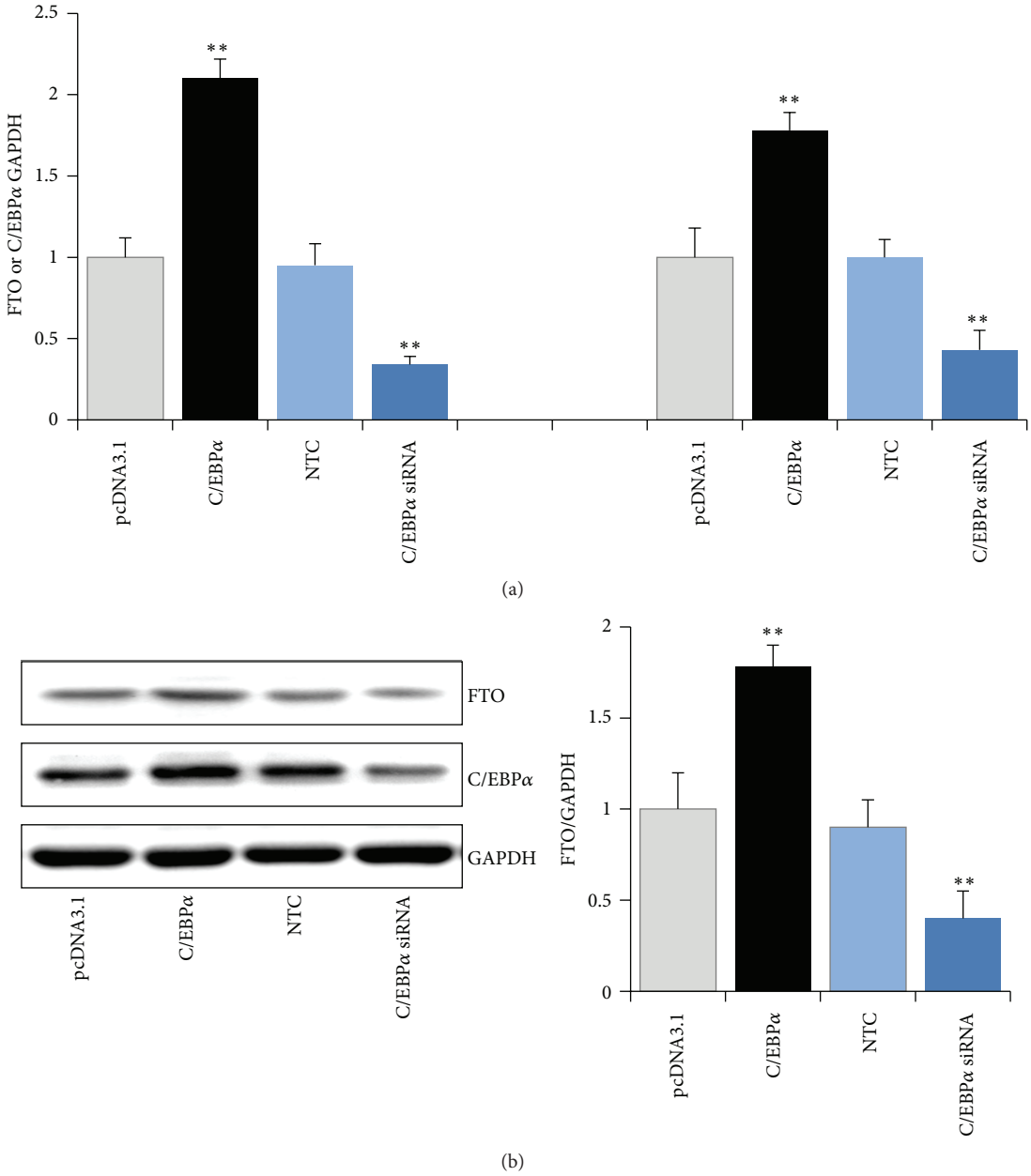

Figure 3: Effect of C/EBPα on FTO expression. HEK 293 cells were transfected with pcDNA3.1-C/EBPα or C/EBPα siRNA. FTO and C/EBPα mRNA and protein levels were normalized by their respective GAPDH values. Bars are the mean of three independent experiments ± S.D. ($^{**}P < 0.01$).

human FTO gene expression. A conserved C/EBPα binding sequence was identified in the core promoter region of the FTO gene, suggesting that the binding sites may play important roles in regulating the expression of FTO gene. Promoter-reporter gene constructs that contain the proximal C/EBP binding site showed that C/EBPα strongly activates reporter gene expression. Moreover, mutation of the C/EBPα binding site within the promoter completely blocked transactivation by C/EBPα. We then performed gene transfection-mediated overexpression and RNA interference- (RNAi-) mediated gene silencing of C/EBPα on HEK 293 cells and the results suggested that C/EBPα promoted the expression of FTO. Consistent with this, ChIP experiments showed that C/EBPα binds to the predicted binding element in the FTO promoter. Taken together, these findings provide compelling evidence that C/EBPα to be a positive transcriptional factor contributes to the transcription and expression regulation of human FTO gene.

The C/EBP transcription factors consist of six members (α, β, δ, γ, ε, and ζ), containing conserved DNA binding domain at C-terminus and an activation domain at their N-terminus. Data presented that different C/EBP number occurs at different point-in-time in adipogenesis, indicative of their own distinct roles during the progress [13]. C/EBPα

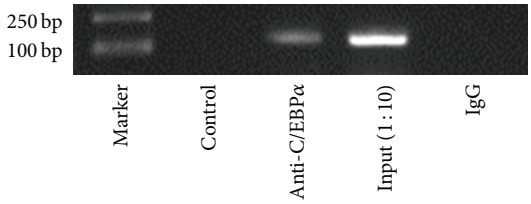

Figure 4: Identification of the sequence motif responsible for activation of the FTO promoter by C/EBPα. ChIP assay in HEK 293 cells showing that C/EBPα can bind to the human FTO promoter site. Amounts of coprecipitated DNA (Anti-C/EBPα) and the corresponding amounts in the input chromatin samples (input 1 : 10) were measured by PCR.

is expressed in liver, adipose tissue, and muscle and is required for both adipogenesis and normal adipocyte function. Ectopic expression of C/EBPα in various fibroblast cell lines promotes adipogenesis. Moreover, it was reported that C/EBPα promotes adipogenesis by inducing the expression of peroxisome proliferator activated receptor γ (PPARγ). It was reported that PPARγ was able to promote adipogenesis even in the absence of C/EBPα, whereas C/EBPα could not promote adipogenesis in the absence of PPARγ [14, 15]. Previous research has also shown that in the early phase of 3T3-L1 cell differentiation, FTO expression was transiently increased; however, partial reduction of FTO did not impact PPARγ expression and adipocyte differentiation [16]. Thus, one can speculate that C/EBPα mediates different signaling pathways involved in adipogenesis. While some researchers have found that FTO expression was not modulated during differentiation of preadipocytes into mature adipocytes, some have even demonstrated that FTO expression is decreased during differentiation of primary preadipocytes isolated from human subcutaneous adipose tissue or preadipocytes derived from Simpson-Golabi-Behmel syndrome (SGBS) [17, 18]. These results somewhat contrast with each other suggesting that FTO expression and functions are not entirely clear in the different cell types and tissues.

FTO has also shown a positive association with the expression of adiponectin, an anti-inflammatory adipokine in adipose tissues. NF-κB and C/EBP family can be activated by inflammatory cytokines such as interleukin-1 (IL-1) and IL-6, respectively, whereas IL-6 suppresses the transcription of adiponectin [19–21]. Much more rigorous research needs to be conducted to understand the regulation mechanism as in the inflammatory reaction of adipose tissue.

In summary, we have confirmed so far that C/EBPα and Foxa2, respectively, positively and negatively regulated the expression of human FTO gene. Given the complex interacting network of transcription factors involved in time-space characteristics of gene expression, it can often be difficult to determine which factor plays a crucial role in the transcriptional regulation process. Data derived from further experiments would unravel the details of FTO functions and regulatory networks.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Genetics of Type 2 Diabetes: Insights into the Pathogenesis and Its Clinical Application

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With rapidly increasing prevalence, diabetes has become one of the major causes of mortality worldwide. According to the latest studies, genetic information makes substantial contributions towards the prediction of diabetes risk and individualized antidiabetic treatment. To date, approximately 70 susceptibility genes have been identified as being associated with type 2 diabetes (T2D) at a genome-wide significant level ($P < 5 \times 10^{-8}$). However, all the genetic loci identified so far account for only about 10% of the overall heritability of T2D. In addition, how these novel susceptibility loci correlate with the pathophysiology of the disease remains largely unknown. This review covers the major genetic studies on the risk of T2D based on ethnicity and briefly discusses the potential mechanisms and clinical utility of the genetic information underlying T2D.

1. Introduction

The prevalence of type 2 diabetes (T2D) is rising rapidly owing to increased economic growth and lifestyle changes in both developed and developing countries. According to a recent report, the number of diabetics is estimated to reach 439 million by 2030 worldwide [1]. Therefore, strategies to prevent and treat diabetes are urgently needed in order to stem this global pandemic. It is well known that T2D is caused by β-cell dysfunction and/or insulin resistance, which is promoted by multifactorial genetic or environmental factors. Over the years, linkage analysis, candidate gene approach, large-scale association studies, and genome-wide association studies (GWAS) have successfully identified multiple genes that contribute to T2D susceptibility. Combined analyses of these loci, such as construction of genetic risk scores, have contributed significantly to the prediction of T2D diabetes and thus facilitated the adoption of early diagnosis and preventative strategies to reduce this growing disease burden [2–5].

Pharmacogenomics is an emerging discipline that highlights the role of inherited and acquired genetic variations in drug response and which is beneficial for appropriate selection of antidiabetic drugs [6]. So far, pharmacogenomics has proven to be valuable in guiding therapeutic choices in maturity onset diabetes in the young (MODY) and in neonatal diabetes; however, its extension to T2D still needs detailed studies [7]. The present review summarizes recent genetic research on T2D in both ethnic and chronologic contexts and briefly discusses the potential mechanisms and clinical utilities of genetic information in T2D.

2. Advances in Type 2 Diabetes Genetic Research

Linkage analysis, candidate gene approach, large-scale association studies, and GWAS have identified approximately 70 loci conferring susceptibility to T2D. Among them, 45 loci were identified in European populations (Table 1), and the other 29 loci were identified in Asian populations, especially in East and South Asians (Tables 2 and 3). The immediate benefit derived from these findings was the better understanding of the pathophysiology of T2D.

Table 1: European-derived susceptibility loci for type 2 diabetes.

	Locus	SNP	Chr.	Position	Allele (risk/other)	RAF*	OR	Probable mechanism	
2000	*PPARγ* [8]	rs1801282	3	12368125	C/G	0.92	1.14	Insulin action	Candidate and large-scale association study
2003	*KCNJ11* [9]	rs5219	11	17366148	T/C	0.5	1.14	β-Cell function	Candidate and large-scale association study
2006	*TCF7L2* [10]	rs7903146	10	114748339	T/C	0.25	1.37	β-Cell function	Candidate and large-scale association study
2007	*WFS1* [11]	rs10010131	4	6343816	G/A	0.6	1.11	β-Cell function	Candidate and large-scale association study
2007	*HNF1B* [12]	rs4430796	17	rs4430796	A/G	0.47	1.1	β-Cell function	Candidate and large-scale association study
2007	*IGF2BP2* [13–15]	rs4402960	3	186994381	T/G	0.29	1.14	β-Cell function	GWAS
2007	*CDKN2A-CDKN2B* [13–15]	rs10811661	9	rs10811661	T/C	0.79	1.2	β-Cell function	GWAS
2007	*CDKAL1* [13–16]	rs10946398	6	20769013	C/A	0.31	1.12	β-Cell function	GWAS
2007	*SLC30A8* [17]	rs13266634	8	118253964	C/T	0.75	1.12	β-Cell function	GWAS
2007	*HHEX/IDE* [17]	rs1111875	10	94452862	C/T	0.56	1.13	β-Cell function	GWAS
2007	*FTO* [13, 15, 18]	rs8050136	16	rs8050136	A/C	0.45	1.17	Obesity	GWAS
2008	*NOTCH2* [19]	rs10923931	1	120230001	T/G	0.106	1.13	Unknown	GWAS
2008	*ADAMTS9* [19]	rs4607103	3	64686944	C/T	0.761	1.09	Insulin action	GWAS
2008	*THADA* [19]	rs7578597	2	43644474	T/C	0.902	1.15	β-Cell function	GWAS
2008	*TSPAN8/LGR5* [19]	rs7961581	12	69949369	C/T	0.269	1.09	β-Cell function	GWAS
2008	*CDC123/CAMK1D* [19]	rs12779790	10	12368016	G/A	0.183	1.11	β-Cell function	GWAS
2008	*JAZF1* [19]	rs864745	7	28147081	T/C	0.501	1.1	β-Cell function	GWAS
2009	*MTNR1B* [20]	rs1387153	11	92313476	T/C	0.283	1.15	β-Cell function	GWAS
2009	*IRS1* [21]	rs2943641	2	226801989	C/T	0.633	1.19	Insulin action	GWAS
2010	*DGKB/TMEM195* [22]	rs2191349	7	15030834	T/G	0.333	1.06	β-Cell function	GWAS
2010	*GCKR* [22]	rs780094	2	27594741	C/T	0.394	1.06	Insulin action	GWAS
2010	*GCK* [22]	rs4607517	7	44202193	A/G	0.195	1.07	β-Cell function	GWAS
2010	*PROX1* [22]	rs340874	1	212225879	C/T	0.492	1.07	β-Cell function	GWAS
2010	*ADCY5* [22]	rs11708067	3	124548468	A/G	0.226	1.12	β-Cell function	GWAS
2010	*RBMS1/ITGB6* [23]	rs7593730	2	160879700	C/T	0.23	0.9	Insulin action	GWAS
2010	*KCNQ1* [24]	rs231362	11	2648047	G/A	0.52	1.08	β-Cell function	GWAS
2010	*DUSP9* [24]	rs5945326	X	152553116	A/G	0.79	1.27	Insulin action	GWAS
2010	*PRC1* [24]	rs8042680	15	89322341	A/C	0.22	1.07	Unknown	GWAS
2010	*ZFAND6* [24]	rs11634397	15	78219277	G/A	0.6	1.06	Unknown	GWAS
2010	*HNF1A* [24]	rs7957197	12	119945069	T/A	0.85	1.07	Unknown	GWAS
2010	*HMGA2* [24]	rs1531343	12	64461161	C/G	0.1	1.1	Insulin action	GWAS
2010	*CENTD2* [24]	rs1552224	11	72110746	A/C	0.88	1.14	β-Cell function	GWAS
2010	*CHCHD9* [24]	rs13292136	9	81141948	C/T	0.93	1.11	Unknown	GWAS
2010	*TP53INP1* [24]	rs896854	8	96029687	T/C	0.48	1.06	Unknown	GWAS
2010	*KLF14* [24]	rs972283	7	130117394	G/A	0.55	1.07	Insulin action	GWAS
2010	*ZBED3* [24]	rs4457053	5	76460705	G/A	0.26	1.08	Unknown	GWAS
2010	*BCL11A* [24]	rs243021	2	60438323	A/G	0.46	1.08	Unknown	GWAS

Table 1: Continued.

	Locus	SNP	Chr.	Position	Allele (risk/other)	RAF*	OR	Probable mechanism	
2012	*HMG20A* [25]	rs7177055	15	75,619,817	A/G	0.68	1.08	Unknown	GWAS
2012	*GRB14* [25]	rs13389219	2	165,237,122	C/T	0.6	1.07	Insulin action	GWAS
2012	*ZMIZ1* [25]	rs12571751	10	80,612,637	A/G	0.52	1.08	Unknown	GWAS
2012	*ANK1* [25]	rs516946	8	41,638,405	C/T	0.76	1.09	β-cell function	GWAS
2012	*KLHDC5* [25]	rs10842994	12	27,856,417	C/T	0.8	1.1	Unknown	GWAS
2012	*TLE1* [25]	rs2796441	9	83,498,768	G/A	0.57	1.07	Unknown	GWAS
2012	*ANKRD55* [25]	rs459193	5	55,842,508	G/A	0.7	1.08	Insulin action	GWAS
2012	*CILP2* [25]	rs10401969	19	19,268,718	C/T	0.08	1.13	Unknown	GWAS
2012	*MC4R* [25]	rs12970134	18	56,035,730	A/G	0.27	1.08	Unknown	GWAS
2012	*BCAR1* [25]	rs7202877	16	73,804,746	T/G	0.89	1.12	β-Cell function	GWAS

*Data were derived from HapMap East Asian or original studies. Position is given for NCBI Build 36.
SNP: single nucleotide polymorphism; Chr.: chromosome; RAF: risk allele frequency; OR: odds ratio.

Table 2: Type 2 diabetes susceptibility loci identified in East Asians.

	Locus	SNP	Chr.	Position	Allele (risk/other)	RAF*	OR	Probable mechanism	
2009	*KCNQ1* [26]	rs2237892	11	2796327	C/T	0.683	1.43	β-Cell function	GWAS
2010	*UBE2E2* [27]	rs7612463	3	23311454	A/C	0.134	1.19	Unknown	GWAS
2010	*C2CD4A-C2CD4B* [27]	rs7172432	15	60183681	A/G	0.42	1.13	Unknown	GWAS
2010	*SPRY2* [28]	rs1359790	13	79615157	G/A	0.273	1.15	Unknown	GWAS
2010	*CDC123/CAM K1D* [28]	rs10906115	10	12355003	A/G	0.561	1.13	Unknown	GWAS
2010	*SRR* [29]	rs391300	17	2163008	G/A	0.367	1.28	β-Cell function	GWAS
2010	*PTPRD* [29]	rs17584499	9	8869118	T/C	0.226	1.57	Insulin action	GWAS
2011	*MAEA* [30]	rs6815464	4	1299901	C/G	0.640	1.13	Unknown	GWAS
2011	*PSMD6* [30]	rs831571	3	64023337	C/T	0.688	1.09	Unknown	GWAS
2011	*ZFAND3* [30]	rs9470794	6	38214822	C/T	0.203	1.12	Unknown	GWAS
2011	*GCC1-PAX4* [30]	rs6467136	7	126952194	G/A	0.182	1.11	Unknown	GWAS
2011	*KCNK16* [30]	rs1535500	6	39392028	T/G	0.398	1.08	β-Cell function	GWAS
2011	*PEPD* [30]	rs3786897	19	38584848	A/G	0.547	1.1	Unknown	GWAS
2011	*FITM2-R3HD* [30]	rs6017317	20	42380380	G/T	0.545	1.09	β-Cell function	GWAS
2011	*GLIS3* [30]	rs7041847	9	4277466	A/G	0.529	1.1	β-Cell function	GWAS
2012	*ANK1* [31]	rs515071	8	41,638,405	C/T	0.8	1.18	Unknown	GWAS
2013	*GRK5* [32]	rs10886471	10	121139393	C/T	0.756	1.12	Insulin action	GWAS
2013	*RASGRP1* [32]	rs7403531	15	36610197	T/C	0.317	1.1	β-Cell function	GWAS
2013	*PAX4* [33]	rs10229583	7	127034139	G/A	0.829	1.18	Unknown	GWAS
2013	*MIR129-LEP* [34]	rs791595	7	127650038	A/G	0.08	1.17	Unknown	GWAS
2013	*SLC16A13* [34]	rs312457	17	6881117	G/A	0.078	1.2	Unknown	GWAS
2013	*GPSM1* [34]	rs11787792	9	138371969	A/G	0.874	1.15	Unknown	GWAS

*Data were derived from HapMap East Asian or original studies. Position is given for NCBI Build 36.
SNP: single nucleotide polymorphism; Chr.: chromosome; RAF: risk allele frequency; OR: odds ratio.

2.1. Genetics of Type 2 Diabetes in European Populations

2.1.1. Linkage Analysis, Candidate Gene Approach, and Large-Scale Association Studies. Linkage analysis has proved to be valuable in the exploration of genetic factors of monogenic diseases, such as MODY, neonatal mitochondrial diabetes, insulin resistance, and Wolfram syndromes [38–40]. However, it has not been particularly useful in identifying the genetic factors for common forms of T2D. Over the years, linkage studies have reported many predisposing associations with chromosomal regions for T2D, including segments in chromosomes 5 and 10, and have identified putative, causative

Table 3: Type 2 diabetes susceptibility loci identified in South Asians.

	Locus	SNP	Chr.	Position	Allele (risk/other)	RAF*	OR	Probable mechanism	
2011	*ST6GAL1* [35]	rs16861329	3	188149155	G/A	0.86	1.09	β-Cell function	GWAS
2011	*HNF4A* [35]	rs4812829	20	42422681	A/G	0.29	1.09	β-Cell function	GWAS
2011	*VPS26A* [35]	rs1802295	10	70601480	A/G	0.26	1.08	Unknown	GWAS
2011	*AP3S2* [35]	rs2028299	15	88175261	C/A	0.31	1.1	Unknown	GWAS
2011	*HMG20A* [35]	rs7178572	15	75534245	G/A	0.52	1.09	Unknown	GWAS
2011	*GRB14* [35]	rs3923113	2	165210095	A/C	0.74	1.09	Insulin action	GWAS
2013	*TMEM163* [36]	rs998451	2	135145758	G/A	1	1.56	β-Cell function	GWAS
2013	*SGCG* [37]	rs9552911	13	22762657	A/G	0.07	0.67	Unknown	GWAS

*Data were derived from HapMap East Asian or original studies. Position is given for NCBI Build 36.
SNP: single nucleotide polymorphism; Chr.: chromosome; RAF: risk allele frequency; OR: odds ratio.

genetic variants in *CAPN10* [41], *ENPP1* [42], *HNF4A* [43, 44], and *ACDC* (also called *ADIPOQ*) [45], but most of the findings from these reports could not be replicated.

During the past several decades, only a few loci conferring risk of T2D were identified through candidate gene approach with *PPARγ* Pro12Ala polymorphism being the first reported locus [8]. PPARγ is a transcription factor that plays a pivotal role in adipocyte differentiation. It was reported that *PPARγ* Pro12Ala variant was associated with increased insulin sensitivity in the general population and thus may protect an individual from T2D [46]. The *KCNJ11* (potassium inwardly rectifying channel subfamily J, member 11) encodes potassium inwardly rectifier 6.2 subunit (Kir6.2) of the ATP-sensitive potassium (K_{ATP}) channel, which has an impact on glucose-dependent insulin secretion in pancreatic β-cells [9]. The E23K variant in this gene demonstrated a robust association with T2D using the candidate gene approach [9]. *WFS1* and *HNF1B* were also uncovered as established genes associated with T2D [11, 12]. *WFS1* encodes wolframin, a membrane glycoprotein that maintains calcium homeostasis of the endoplasmic reticulum. Rare mutations in *WFS1* cause Wolfram syndrome, which is characterized by a significant β-cell loss as a result of enhanced endoplasmic reticulum stress [47–49]. *HNF1B* encodes hepatocyte nuclear factor 1 homeobox B, which is a liver-specific factor of the homeobox-containing basic helix-turn-helix family. Mutation of this gene was demonstrated to cause MODY5 [38].

In 2006, a large-scale association study identified *TCF7L2* as an important genetic factor for T2D in Icelandic individuals [10]. This discovery was a significant breakthrough as this association was then widely confirmed in populations of European origin and other ethnic groups, such as Japanese and American individuals [50–57]. Therefore, *TCF7L2* was regarded as the most significant T2D susceptibility gene identified to date.

2.1.2. Genome-Wide Association Study (GWAS). With the advent of GWAS, exploration of the genetic basis for T2D susceptibility has made significant breakthroughs. In 2007, the results of five genome-wide association studies were published. These studies increased the number of confirmed T2D susceptibility loci to nine (*PPARγ*, *KCNJ11*, *TCF7L2*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *HHEX/IDE*, *FTO,* and *SLC30A8*) [13–18]. Except for *PPARγ* and *FTO*, which mainly affect insulin sensitivity, all the other genes may affect β-cell function, although the exact mechanisms remain largely unknown [16]. *HHEX*, which is located on chromosome 10q, is a member of the homeobox family and encodes a transcription factor that maybe involved in Wnt signaling [58]. Nevertheless, these studies established the utility of GWAS approach in elucidating complex genetic traits.

In 2008, to increase the power of identifying variants with modest effects, a meta-analysis of three GWAS, including Diabetes Genetics Initiative (DGI), Finland-United States Investigation of NIDDM Genetics (FUSION), and Wellcome Trust Case Control Consortium (WTCCC), were conducted. This study detected at least six previously unknown loci that reached genome-wide significance for association with T2D ($P < 5 \times 10^{-8}$), with the loci being *JAZF1*, *CDC123-CAMK1D*, *TSPAN8-LGR5*, *THADA*, *ADAMTS9*, and *NOTCH2* [19]. Genetic variants in *JAZF1*, *CDC123-CAMK1D*, *TSPAN8-LGR5, and THADA* have been reported to affect pancreatic β-cell functions [59, 60].

In 2009, a novel genetic variant rs2943641, which is located adjacent to the insulin receptor substrate 1 gene (*IRS1*), was shown to have a significant association with insulin resistance and hyperinsulinemia and further studies also showed that this variant is implicated in reduced basal *IRS1* protein level and decreased *IRS1*-associated phosphatidylinositol-3-OH kinase activity in human skeletal muscle biopsies [21]. In the same year, a variant near *MTNR1B* was found to be associated with increased fasting plasma glucose level and higher risk of T2D (odds ratio = 1.15, 95% CI = 1.08–1.22, $P = 6.3 \times 10^{-5}$) [20]. Ten GWAS involving a total of 36,610 individuals of European descent and a meta-analysis of 13 case-control studies replicated this result and found that risk alleles in this gene are associated with reduced β-cell function as measured by homeostasis model assessment (HOMA-β, $P = 1.1 \times 10^{-15}$) [61].

In 2010, a meta-analysis of 21 genome-wide association studies performed by Dupuis and colleagues identified *ADCY5*, *PROX1*, *GCK*, *GCKR*, and *DGKB/TMEM195* as new genetic loci for T2D susceptibility [22]. Among these loci, *DGKB/TMEM195*, *GCK*, *PROX1,* and *ADCY5* mainly affect

β-cell functions, whereas the locus mapped in *GCKR* shows a primary effect on insulin action [22]. In the same year, another genome-wide association study by Qi and colleagues discovered new variants near *RBMS1* and *ITGB6* genes at 2q24, and these variants were found to affect glucose metabolism and insulin resistance [23]. In addition, an expanded meta-analysis of existing GWAS by Voight and colleagues identified 12 new signals with a combined $P < 5 \times 10^{-8}$, including *BCL11A*, *ZBED3*, *KLF14*, *TP53INP1*, *TLE4*, *CENTD2*, *HMGA2*, *HNF1A*, *PRC1*, *ZFAND6*, *DUSP9,* and *KCNQ1* [24]. *HNF1A* was previously recognized as the causal gene of MODY3 [62] and also harbored the common variant (G319S) that contributes to early-onset T2D [63, 64]. *DUSP9*, mapped on chromosome X, encodes a member of the family of mitogen-activated protein kinase phosphatase 4, MKP4, which is important in cell cycle regulation and plays pivotal roles in regulating insulin action [65–67].

In 2012, a meta-analysis conducted by Morris and colleagues identified additional ten previously unreported T2D susceptible loci, including *BCAR1*, *MC4R*, *CILP2*, *ANKRD55*, *TLE1*, *KLHDC5*, *MGC21675*, *ANK1*, *ZMIZ1*, and *GRB14* [25]. To assess the potential function of these loci, OGTT was employed to test insulin release and insulin sensitivity. *ANK1* was found to be associated with insulinogenic and disposition indices, indicating that this gene probably had an effect on insulin secretion [68]. In this study, *GRB14* and *AKNRD55* were associated with decreased Matsuda index, an index of insulin sensitivity [68].

As described above, genetic studies of T2D in European populations have made significant progress in our understanding of T2D susceptibility. However, existing data can only provide partial explanation for the heritability of T2D. It is well known that discrepancies exist in allelic frequencies and effect sizes in different ethnic groups. It is, therefore, important to understand whether these variants are also applicable to other ethnic populations.

2.2. Genetics of T2D in East Asians. Epidemiological studies have documented consistent increases in the prevalence of diabetes in Asia, especially in China, with diabetes prevalence having increased from 2.6% in 2000 to 9.7% in 2010 [69]. However, our understanding of the genetic basis of T2D in East Asia remains limited. It is therefore imperative to identify specific genes associated with this disease in East Asians.

In 2008, two papers provided the first reports of GWAS for T2D in East Asian populations and ascertained *KCNQ1* as a new susceptibility locus [70, 71]. *KCNQ1* encodes the pore-forming α-subunit of the voltage-gated K^+ channel (KvLQT1), which is expressed mainly in the heart and pancreas. Its association with T2D was further replicated in Korean [72], Chinese [26], and Singaporean [73] populations, as well as individuals of European descent [70]. Therefore, *KCNQ1* is regarded as the most significant locus for T2D in East Asians. This genetic variant is implicated in insulin secretion, which may be the explanation for its association with T2D [73, 74].

In 2010, another GWAS conducted in a Japanese group identified two new loci in *UBE2E2* and *C2CD4A-C2CD4B*. Genetic variants in *C2CD4A-C2CD4B* were then validated in European populations [27]. When the GWAS reports sprung up in East Asians, Chinese investigators performed their first GWAS in the Han Chinese residing in Taiwan and identified two new susceptible loci for T2D in *PTPRD* (protein tyrosine phosphatase receptor type D) and *SRR* (serine racemase) [29]. PTPRD is a protein tyrosine phosphatase and may play a role in the pathogenesis of T2D through increased insulin resistance [75]. *SRR* encodes a serine racemase that synthesizes D-serine from L-serine and which confers risk for T2D via the glutamate signaling pathway [76, 77]. In the same year, a fast-track, multiple-stage study conducted in Han Chinese population by Shu and colleagues discovered a novel genetic susceptibility locus rs1359790, at *13q31.1* for T2D, and this variant was also validated in European Americans, Koreans, and Singapore Chinese [28].

In 2011, in order to identify additional genes in East Asians, Cho and colleagues carried out a meta-analysis of three-stage GWAS in populations of East Asian descent. Compelling evidence for association with T2D of eight novel loci was demonstrated by this study. All of these loci are mapped in or near *GLIS3*, *PEPD*, *FITM2-R3HDML-HNF4A*, *KCNK16*, *MAEA*, *GCC1-PAX4*, *PSMD6, and ZFAND* [30].

In 2012, another GWAS in Japanese populations revealed that rs515071 in *ANK1* was associated with T2D at the genome-wide significance level [31]. *ANK1*, which encodes a member of the ankyrin family, is also reported to be associated with impaired insulin secretion and abnormal level of HbA_{1c} [68, 78]. In addition, GWAS in Beijing and Shanghai populations added two new loci to the list, *GRK5* and *RASGRP1*, and the association signal for *GRK5* seems to be specific to East Asians [32]. GRK5 is regarded as a positive regulator of insulin sensitivity and this protein is a potential therapeutic target for the treatment of insulin resistance [79].

In 2013, a novel variant rs10229583 at 7q32 near *PAX4* was identified in a meta-analysis of three GWAS from Southern Han Chinese descents [33]. As a member of the paired box family of transcription factors, *PAX4* plays a critical role in pancreatic β-cell development and β-cell functions [80]. Further three new predisposing loci, *MIR129-LEP*, *GPSM1*, and *SLC16A13*, with genome-wide significance for T2D were identified [34]. Rs791595 is located between *MIR129-1* and *LEP*. The coding product of *LEP*, leptin, is closely related to body weight regulation and its deficiency in mice and human causes morbid obesity and diabetes, while the role of *MIR129* in diabetes remains unknown [81].

Besides these newly identified loci, some susceptible genes identified in Caucasians were also replicated in East Asians, such as *PPARγ*, *KCNJ11*, TCF2, TCF7L2, *CDKAL1*, *CDKN2A-CDKN2B*, *IDE-KIF11-HHEX*, *IGF2BP2*, MTNR1B, *SLC30A8*, *KCNQ1*, *CDC123*, *GLIS3*, *HNF1B*, *and DUSP9* [32, 82–93].

Together, all these T2D risk loci, initially identified or replicated in East Asians, provide new perspectives on the etiology of T2D and uncover the need for further studies to explore additional loci with strong effects on T2D.

2.3. Genetics of T2D in South Asians. South Asia, with more than a quarter of the world's population, harbors the highest number of patients suffering from T2D [94]. Currently, the number of diabetic patients is reaching 62.4 million, and the number of prediabetic individuals is reaching 77.2 million [95]. Compared to European populations, South Asians are at a fourfold higher risk of T2D [96, 97]. Therefore, significant efforts should be made to identify common genetic variants underlying the T2D risk in individuals of South Asian ancestry.

In 2011, a GWAS in South Asians identified six novel loci harboring disease-predisposing variants, including *GRB14*, *ST6GAL1*, *VPS26A*, *HMG20A*, *AP3S2,* and *HNF4A*. Single nucleotide polymorphisms (SNPs) at *GRB14* were associated with insulin sensitivity and SNPs at *ST6GAL1* and *HNF4A* were associated with pancreatic β-cell function [35].

In 2013, a GWAS performed in Indians identified *TMEM163* on chromosome 2q21 as a new signal for T2D. *TMEM163* encodes a putative vesicular transporter in nerve terminals and shows a plausible effect on T2D by impairing insulin secretion [36]. Concurrently, a novel locus at 13q12 in the *SGCG* gene was identified to confer T2D susceptibility in Punjabi Sikhs from Northern India. This association demonstrated excellent consistency across the three Sikh samples, but no significant association was observed in a large East Asian replication study, indicating that the detected locus is specific to the Indian Punjabi Sikh population [37].

In consideration of India's complex demographic history, cultural diversity, differences in risk allele frequency, and pattern of linkage disequilibrium existing between European and South Asian populations, large replication studies were conducted to evaluate the contribution of European-derived loci in South Asian populations. SNPs in or near *PPARG, KCNJ11, TCF7L2, SLC30A8, HHEX, CDKN2A/B, IGF2BP2, CDKAL1, FTO, KCNQ1, JAZF1, IRS1, KLF14, CHCHD9,* and *DUSP9* displayed significant associations with T2D in Pakistani populations, with similar effect sizes as those seen in European populations [98–102].

2.4. Genetics of Type 2 Diabetes in Other Populations. The discovery of new susceptibility loci for T2D by GWAS in different ethnic groups emphasizes the need to conduct more GWAS based on ethnic background. In addition to European and Asian populations, researchers also conducted studies in Pima Indians and Mexican Americans aimed at identifying new risk loci.

In Pima Indians, a few genes have been reported to confer risk of T2D. In 2007, researchers found that variants within *ARHGEF11* nominally increased the risk of T2D, possibly as a result of increased insulin resistance [103]. In 2008, variation within *PCLO* was confirmed to have a modest effect on early-onset T2D, possibly by reduction of insulin action [104]. In 2010, *ACAD10* variation was found to increase T2D risk by impairing insulin sensitivity via abnormal lipid oxidation [105]. Soon afterwards, an *ASK1* variant was identified to confer susceptibility to T2D by decreasing insulin sensitivity owing to reduced ASK1 expression in skeletal muscle [106]. However, a replication study, which genotyped SNPs mapped in *CDKAL*, *SLC30A8*, *HHEX*, *EXT2*, *IGF2BP2*, *LOC387761*, and *FTO* previously associated with T2D in Caucasians, did not provide any evidence for association with T2D or obesity among full-heritage Pima Indians. Instead, they found that *CDKAL1, HHEX,* and *EXT2* were evidently associated with either insulin secretion or insulin action in Pima Indians with normal glucose tolerance [107].

Similarly, analysis of T2D risk genes in Mexican American populations had identified several novel candidate loci for T2D, such as rs979752 and rs10500641 near *UBQLNL* and *OR52H1* on chromosome 11, rs2773080 and rs3922812 in or near *RALGPS2* on chromosome 1, and rs1509957 near *EGR2* on chromosome 10 [108]. In 2011, the largest GWAS and meta-analysis of T2D in Mexican populations identified 49 SNPs in eight gene regions (*PER3, PARD3B, EPHA4, TOMM7, PTPRD, HNT, LOC729993, and IL34*) and six intergenic regions with an unadjusted P value $< 1 \times 10^{-5}$ [109]. In consideration of the fact that all the above loci did not reach genome-wide significance ($P < 5 \times 10^{-8}$), Williams and colleagues analyzed 9.2 million SNPs in 8,214 Mexicans and other Latin Americans and identified a novel locus associated with T2D spanning the solute carriers *SLC16A11* ($P = 3.9 \times 10^{-13}$; odds ratio (OR) = 1.29). They observed that SLC16A11 mainly localizes with the endoplasmic reticulum membrane protein, calnexin, in liver, salivary gland, and thyroid. Importantly, overexpression of SLC16A11 in HeLa cells resulted in substantial increases in triacylglycerol, suggesting that SLC16A11 may have a role in hepatic lipid metabolism [16, 110]. Nevertheless, the role of all these risk loci in the pathogenesis of diabetes remains unclear and needs further investigations.

3. Correlation of the Susceptibility Loci with the Pathogenesis of T2D

With the large number of aforementioned genetic loci susceptible to T2D, the question pertains to how they participate in the pathogenesis of T2D. A great number of studies have suggested that genetic variants in or near *KCNJ11, TCF7L2, WFS1, HNF1B, IGF2BP2, CDKN2A-CDKN2B, CDKAL1, SLC30A8, HHEX/IDE, KCNQ1, THADA, TSPAN8/LGR5, CDC123/CAMK1D, JAZF1, MTNR1B, DGKB/TMEM195, GCK, PROX1, ADCY5, SRR, CENTD2, ST6GAL1, HNF4A, KCNK16, FITM2-R3HDML-HNF4A, GLIS3, GRB14, ANK1, BCAR1, RASGRP1,* and *TMEM163* may confer T2D risk through impaired β-cell function [16, 24, 44, 68, 111–114], whereas *PPARγ, ADAMTS9, IRS1, GCKR, RBMS1/ITGB6, PTPRD, DUSP9, HMGA2, KLF14, GRB14, ANKRD55,* and *GRK5* have an impact on insulin action [21, 24, 115, 116] (Tables 1, 2, and 3). *FTO* and *MC4R*, previously identified genes associated with obesity, appear to confer T2D risk through their primary effects on BMI, but recent GWAS have shown that their effects on T2D were independent of BMI, though *FTO* may have a small but detectable influence on T2D risk through insulin action [117, 118].

3.1. Impact of TCF7L2 on the Risk of T2D. *TCF7L2* is the most intensively studied locus for T2D risk so far. The risk

alleles of *TCF7L2* were associated with enhanced expression of this gene in human islets as well as impaired insulin secretion both *in vitro* and *in vivo*. The authors also observed an impaired incretin effect in subjects carrying risk alleles of *TCF7L2* and proposed the engagement of the enteroinsular axis in T2D [119]. Dennis and colleagues then verified this result and indicated that *TCF7L2* variant rs7903146 affected risk of T2D, at least in part, through modifying the effect of incretins on insulin secretion. This was not due to reduced secretion of glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), which exhibit an important physiological role in boosting insulin secretion following meals, but rather due to the effect of *TCF7L2* on the sensitivity of β-cells to incretins [120]. TCF7L2 has also been linked to altered pancreatic islet morphology as exemplified by increased individual islet size and altered alpha and beta cell ratio/distribution within human islets [121]. This phenomenon is also observed in other *in vivo* or *in vitro* studies [122–124]. This further strengthened the evidence for the role of TCF7L2-associated alteration of cell types in islets in the pathogenesis of T2D.

TCF7L2 encodes the transcription factor TCF4 which is related to Wnt signaling pathway and which plays a critical role in the pathogenesis of T2D. The major effector of the canonical Wnt signaling pathway is known as β-catenin/TCF. This bipartite transcription factor is formed by free β-catenin (β-cat) and a member of the TCF protein family, including TCF7L2 (previously known as TCF-4) [125]. GWAS have revealed the involvement of a Wnt ligand (Wnt-5b), Wnt coreceptor (LRP-5), and the Wnt pathway effector TCF7L2 in the development of diabetes [126]. Several previous studies also provide evidence that the β-catenin/TCF axis participates in pancreatic cell proliferation and differentiation [127–131]. Treatment of β-cells with purified Wnt protein or activated β-catenin augmented the proliferation of these cells [132]. Intriguingly, deletion of β-catenin within the pancreatic epithelium resulted in an almost complete lack of acinar cells, whereas deletion of β-catenin specifically in differentiated acinar cells had no such effect [128], suggesting that the *TCF7L2*-related Wnt signaling mainly perturbs pancreatic growth but not pancreatic function. However, deletion of islet *TCF7L2* expression from β-cells did not show any demonstrable effects on glucose-stimulated insulin secretion (GSIS) in adult mice, whereas manipulating *TCF7L2* levels in the liver caused hypoglycemia and reduced hepatic glucose production [133]. In concordance with these results, risk alleles in *TCF7L2* were associated with hepatic but not peripheral insulin resistance and enhanced rate of hepatic glucose production in human [119]. Therefore, TCF7L2-related disruption of β-cell function is probably the indirect consequence of primary events in liver or other organs/systems.

3.2. Impact of SCL30A8 on the Risk of T2D. Besides *TCF7L2*, solute carrier family 30 member 8 gene (*SCL30A8*) has also been explored in depth. *SCL30A8* encodes the islet-specific zinc transporter ZnT-8, which delivers zinc ions from cytoplasm into intracellular insulin-containing granules, and is implicated in insulin maturation and/or storage processes in β-cells [134]. Expression level of ZnT-8 was remarkably downregulated in the pancreas of db/db and Akita mice in the early stage of diabetes [135]. Global *SCL30A8* knockout mice demonstrated reduced plasma insulin, impaired GSIS, and markedly reduced islet zinc content [136]. Remarkably, both ZnT-8 knockout mice and human individuals carrying risk alleles of *SLC30A8* exhibited increased hepatic insulin clearance, with significantly increased c-peptide/insulin ratios [137]. Contrary to the previous findings, overexpression of ZnT-8 in INS-1 cells stimulated zinc accumulation and enhanced GSIS of these cells [138]. Importantly, a recent study discovered that *SCL30A8* gene transcription was regulated by Pdx-1, a β-cell-enriched transcription factor, and involved in the development of islets, through an intrinsic enhancer. Restriction of Pdx-1 in pancreatic islet β-cells correlated with the induction of *SCL30A8* gene and ZnT-8 protein expression [139]. Therefore, the specific pathways by which *SL30A8* correlates with the pathogenesis of T2D still need further exploration.

It should be noted that a great number of low frequency variants might not be identified by GWAS owing to the required genome-wide significance level. According to the existing studies, many important loci are also obscured as a result of borderline associations. The known variants account for only a small amount of the overall estimated genetic heritability; therefore, there is still a long way to go in terms of understanding the pathogenesis of type 2 diabetes.

4. Clinical Utility of Genetic Information: Prediction of Type 2 Diabetes

One of most important clinical utilities of genetic information is to predict the risk of developing T2D among nondiabetic individuals. This will facilitate the early interventional strategies to prevent or delay the onset of the disease. A vast number of recent studies have constructed genetic risk score models by summing up numerous independently inherited susceptible variants for T2D to evaluate the predictive ability from the current genetic information. For example, the area under the receiver operating characteristic (ROC) curves (AUCs) is used to assess discriminative accuracy of this approach. The AUC value can range from 0.5 to 1.0, where the AUC of 0.5 stands for the lack of discrimination and AUC of 1 stands for perfect discrimination. An AUC value of greater than 0.75 is considered to be clinically useful [140]. Imamura and colleagues created a genetic risk score model using 49 susceptibility alleles (GRS-49) for T2D in a Japanese population and discovered an increased level of AUC with combined GRS-49 and clinical factors (including age, sex, and BMI) compared with each individually. But the AUC value is only 0.773, which shows a clinically modest but statistically significant effect on T2D [141]. This phenomenon is also observed in many other studies from different ethnic groups [142, 143]. Controversially, it was proposed that phenotype-based risk models are superior to models based on 20 common independently inherited diabetes risk alleles in discrimination for T2D, with the observation of only

minimal improvement in accuracy of risk estimation when adding genotypes to phenotype-based risk models [144]. The discrepancy may result from the fact that prediction for T2D using genetic information is largely affected by age. For example, the Framingham Offspring Study conducted with 3,471 subjects followed over 34 years found out that common genetic variations appropriately reclassified younger people for T2D risk beyond clinical risk factors, but it failed in older people [145]. In addition, along with the rapid economic growth and lifestyle changes, we may underscore the role of environmental factors in the pathogenesis of T2D. A recent study suggested that the potential deleterious effect of several T2D loci may be abolished or at least attenuated by higher physical activity levels or healthy lifestyle, whereas they may be augmented by low physical activity and dietary factors that are similar to a Western dietary pattern [146]. Therefore, these inconsistencies will need further investigations.

5. Pharmacogenomics of Type 2 Diabetes

With the advent of GWAS, studies on the roles of inherited and acquired genetic variations in drug response have undergone an evolution from pharmacogenetics into pharmacogenomics, with a shift from the focus on individual candidate genes to GWAS [147]. Clinically, it is often observed that even patients who receive similar antidiabetic regimens demonstrate large variability in drug disposition, glycemic response, tolerability, and incidence of adverse effects [148]. This interindividual variability can be attributed to specific gene polymorphisms involved in the metabolism, transportation, and therapeutic mechanisms of oral antidiabetic drugs. Pharmacogenomics is on the agenda to explore feasible genetic testing to predict treatment outcome, so that appropriate steps could be taken to treat type 2 diabetes more efficiently.

In general, the oral antidiabetic drug (OAD) is the first line treatment for T2D after failure of lifestyle intervention. The most commonly prescribed OADs include sulfonylureas (SU), biguanides, thiazolidinediones (TZDs), glinides, and α-glucosidase inhibitors. To date, numerous pharmacogenetic studies comparing these drugs have been conducted in populations with different ethnic backgrounds. With respect to sulfonylureas, genetic variants at multiple loci such as *KCNJ11*, *ABCC8*, *IRIS1*, *TCF7L2*, *NOS1AP*, *KCNQ1*, *CDKAL1*, and *CAPN10* affect pharmacokinetics and/or pharmacodynamics of these drugs [149–157]. Among them, *KCNJ11* encodes a major subunit of the ATP-sensitive K^+ channel, and *ABCC8* encodes a modulator of ATP-sensitive potassium channels (SUR1). They both play pivotal roles in insulin secretion and are both shown in pharmacogenomic studies to impact sulfonylureas efficacy [151, 158]. The Arg (972) IRS-1 variant is associated with increased risk for secondary failure to sulfonylurea and it is noteworthy that the genotype frequency of this variant is twice as high in patients with secondary failure to sulfonylurea compared to the diabetic patients whose blood glucose levels were well controlled with oral therapy [157]. In diabetic patients carrying risk alleles in *NOS1AP* gene, glibenclamide is less effective in reducing glucose levels. The increased mortality in users of sulfonylurea was also shown in this paper, reminding us of the fact that genetic variation could alter responses to T2D therapy [155]. Consistent with this notion, studies have shown that genetic variants in *SLC22A1, SLC22A2, SLC47A1, SLC47A2,* and *ATM* [159–167] were found to affect metformin efficacy. *SLC22A1* encodes organic cation transporter 1 (OCT1), which participates in the transportation of metformin into hepatocytes. *SLC47A1* encodes the multidrug and toxin extrusion 1 protein (MATE1), which facilitates metformin excretion from hepatocytes into bile. *ATM*, a gene known to be involved in DNA repair and cell cycle control, plays a role in metformin efficacy upstream of AMPK, and variation in this gene alters glycemic responses to metformin [167].

Gene polymorphisms associated with glinide (repaglinide and nateglinide) responses were mapped in *CYP2C8, SLCO1B1, TCF7L2, CYP3A4, IGF2BP2, SLC30A8, KCNQ1, KCNJ11, NAMPT, UCP2, MDR1, NeuroD1,* and *PAX4* [168–174]. Among them, *SLCO1B1* is mainly expressed in the basolateral membrane of hepatocytes and can facilitate hepatic uptake of repaglinide [175]; polymorphisms of this gene have significant influence on the pharmacokinetics of repaglinide with reduced pharmacokinetic exposure after a single oral dose administration of 2 mg repaglinide [176]. Thiazolidinediones, also known as glitazones, act as agonists for their molecular target, peroxisome proliferator-activated receptor-γ (PPAR-γ). The direct antioxidant action of glitazones may contribute to its effect on insulin resistance [177]. Recent studies have also reported several loci involved in the pharmacogenetics of thiazolidinediones, including *PGC-1α*, resistin, adiponectin, leptin, TNF-alpha, and *CYP2C8* [178–183].

Pharmacogenetic research provides a means to better understand and improve pharmacotherapy. Despite all these advances in the field of pharmacogenetics, adequately designed and rigorously conducted clinical trials are still needed for guiding therapeutic decisions in T2D treatment.

6. Conclusion

To date, approximately 70 loci associated with T2D have been identified. Despite this excellent progress, the current knowledge from these genetic data is still not sufficient to support the clinical utility for the prediction, early identification, and prevention of diabetes. As an emerging field, pharmacogenomics aims at exploring possible molecular mechanisms of drugs and specific genetic variants associated with drug efficacy and thus can make contributions for decisions regarding drug selection, dose titration, treatment duration, and avoidance of adverse drug reactions. However, the loci identified so far explain only a small amount of the estimated heritability of type 2 diabetes and the clinical utility of genetic information is still in its preliminary stage. There is no doubt that intensive studies should be conducted to further identify T2D inheritability factors and promote the translation of novel findings from GWAS to clinical application.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Xue Sun and Weihui Yu contributed equally to this paper.

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Research Article

Lack of Association between *TLR4* Genetic Polymorphisms and Diabetic Nephropathy in a Chinese Population

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Objective. Toll-like receptor 4 (TLR4) plays a central role in innate immunity. Activation of innate immune response and subsequent chronic low-grade inflammation are thought to be involved in the pathogenesis of diabetic nephropathy. In this study, we aimed to investigate whether *TLR4* variants are associated with diabetic nephropathy in the Chinese population. *Methods*. Seven tagging single nucleotide polymorphisms (SNPs) of *TLR4* based on HapMap Chinese data were genotyped in 1,455 Chinese type 2 diabetic patients. Of these patients, 622 were diagnosed with diabetic nephropathy and 833 were patients with diabetes for over 5 years but without diabetic nephropathy. *Results*. None of the SNPs and haplotypes showed significant association to diabetic nephropathy in our study. No association between the SNPs and quantitative traits was observed either. *Conclusion*. We concluded that common variants within *TLR4* genes were not associated with diabetic nephropathy in the Chinese type 2 diabetes patients.

1. Introduction

Type 2 diabetes has become an epidemic all around the world, resulted in large loss in economy, and threatened the human health. Diabetic microvascular complications are the major causes of morbidity and early mortality in diabetes [1, 2]. As one of the most important long-term complications of diabetes, diabetic nephropathy is the leading cause of chronic kidney failure and end-stage renal disease [3], and patients with diabetic nephropathy have an increased risk of cardiovascular mortality [4, 5]. At present, it is widely accepted that diabetic nephropathy is a heterogeneous disorder caused by the interaction between environmental factors and genetic factors. Diabetes duration and glycemic control are the strongest environmental risk factors [6], and family history of kidney disease appears to be the strongest risk factor for initiation of diabetic nephropathy [7, 8]. Recently, several studies have suggested that the innate immunity changes may be associated with type 2 diabetes and diabetic complications [9–11]. Thus, genes encoding the innate immune system components might be good candidates for studying diabetic nephropathy.

Toll-like receptors (TLRs) are the family of type I transmembrane receptors involved in innate immunity and pathogen recognition [12]. While toll-like receptor 4 (TLR4) is predominantly expressed on dendritic cells and macrophages and plays an important role in the activation of the innate immune response and subsequent proinflammatory reactions, it not only recognizes the lipopolysaccharide (LPS) of Gram-negative bacteria, but also interacts with some endogenous ligands, such as heat-shock proteins, fibronectin, oxidized low-density lipoprotein cholesterol, Fetuin-A, and high-mobility group box 1 (HMGB1) [12–17]. Several studies have indicated that there may be a link between TLR4 pathway and diabetic nephropathy [18–20]. Therefore, in this study, we aim to investigate whether *TLR4* genetic polymorphisms are associated with diabetic nephropathy and its related quantitative traits in the Chinese population.

Table 1: Clinical characteristics of the study samples.

	Cases	Controls
Samples (*n*)	621	832
Male/female (*n*)	348/274	352/480
Age (years)	62.42 ± 12.67	63.31 ± 10.56
BMI (years)	24.95 ± 3.86	24.00 ± 3.35
Age at diagnosis of diabetes (years)	53.56 ± 12.27	42.38 ± 10.22
Duration of diabetes (years)	9.00 (3.00, 14.00)	10.00 (7.00, 14.00)
Hemoglobin A_{1C} (%)	9.34 ± 2.29	8.68 ± 2.10
Systolic blood pressure (mmHg)	140.04 ± 19.27	134.52 ± 18.27
Diastolic blood pressure (mmHg)	82.53 ± 9.90	79.57 ± 9.05
AERs (mg/24 H)	93.09 (47.47, 296.27)	9.23 (6.40, 14.58)
eGFR*	109.81 (81.62, 136.87)	121.57 (103.40, 144.84)

Data are *n*, mean ± SD, or median (interquartile range). BMI: body mass index. AERs: albumin excretion rates. eGFR: estimated glomerular filtration rate. eGFR* was calculated by using a formula developed by the Modification of Diet in Renal Disease study group with adjustment for Chinese ethnicity.

2. Methods

2.1. Participants. This study involved 1,455 patients with type 2 diabetes recruited from the Shanghai Diabetes Institute Inpatient Database of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. All participants were unrelated type 2 diabetic patients meeting the 1999 WHO criteria. Of these patients, 622 were diagnosed with diabetic nephropathy and 833 were patients with diabetes for over 5 years but without diabetic nephropathy, considered as cases and controls for diabetic nephropathy, respectively. This study was approved by the institutional review board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital, with written informed consent obtained from each participant.

2.2. Clinical Measurement. The 24 h albumin excretion rates (AERs) and estimated glomerular filtration rate (eGFR) were applied to asses nephropathy. AERs were measured in 3 consecutive days, and the mean value was recorded for each patient. Patients with AER < 30 mg/24 h, 30 mg/24 h ≤ AER < 300 mg/24 h, or AER ≥ 300 mg/24 h were classified as having normoalbuminuria, microalbuminuria, or proteinuria, respectively. Patients having microalbuminuria or proteinuria were diagnosed with diabetic nephropathy. eGFR was calculated using a formula developed by the Modification of Diet in Renal Disease study group with adjustment for Chinese ethnicity: $186 \times (\text{serum creatinine in mmol/L} \times 0.011)^{-1.154} \times (\text{age in years})^{-0.203} \times (0.742 \text{ if female}) \times (1.233 \text{ if Chinese})$ [21]. Glycemic control was evaluated by measuring glycated HbA1c levels. Data of blood pressures and lipid profiles were also collected for each participant.

2.3. Single Nucleotide Polymorphisms (SNPs) Selection, Genotyping, and Quality Control. We selected tagging SNPs according to HapMap phase III (release 28) Han Chinese database with a threshold of $r^2 > 0.8$ by using Haploview (v 4.2). The seven tagging SNPs selected could cover 100% of common SNPs (14 out of 14 SNPs in the HapMap Chinese Han samples) with a minor allele frequency (MAF) > 0.05. All genotyping was done using the primer extension of multiplex products with detecting by matrix-assisted laser desorption ionization time of flight mass spectroscopy using a MassARRAYCompact Analyzer (Sequenom, San Diego, CA, USA). The genotyping data underwent a series of quality control checks as described previously [22] and cleared data were used in further statistical analysis. Overall, 2 individuals were excluded from the sample call rate checks and all the seven SNPs passed the SNP call rate check with an average call rate of 98%.

2.4. Statistical Analysis. The Hardy-Weinberg equilibrium test was performed before the association analysis (a two-tailed P value < 0.05 was considered statistically significant). The allelic frequencies between the patients with or without diabetic nephropathy were compared by χ^2 test, and odds ratios with 95% confidence intervals (CIs) were presented. Linear regression was applied to test the effect of genotype on quantitative traits with adjustment of confounding factors under an additive model. Skewly distributed quantitative traits (eGFR and AER) were logarithmically transformed (log 10) to approximate normality before linear regression analysis. All these analyses were performed using SAS 9.3 (SAS institute, Cary, NC, USA) unless specified otherwise. A two-tailed P value < 0.05 was considered statistically significant.

3. Results

All the seven SNPs were in accordance with Hardy-Weinberg equilibrium. The clinical characteristics of the samples passed genotype quality control were shown in Table 1. The linkage disequilibrium pattern of these SNPs was shown in Figure 1. Two haplotype blocks were constructed in this region.

The single SNP association analysis showed that no SNP was significantly associated with diabetic nephropathy in our samples. The minimum P value was 0.417 for rs7044464 (Table 2). Then we compared the frequencies of haplotypes between type 2 diabetic patients with or without diabetic nephropathy and observed that there was no nominal difference between two groups either (Table 3). Further, we

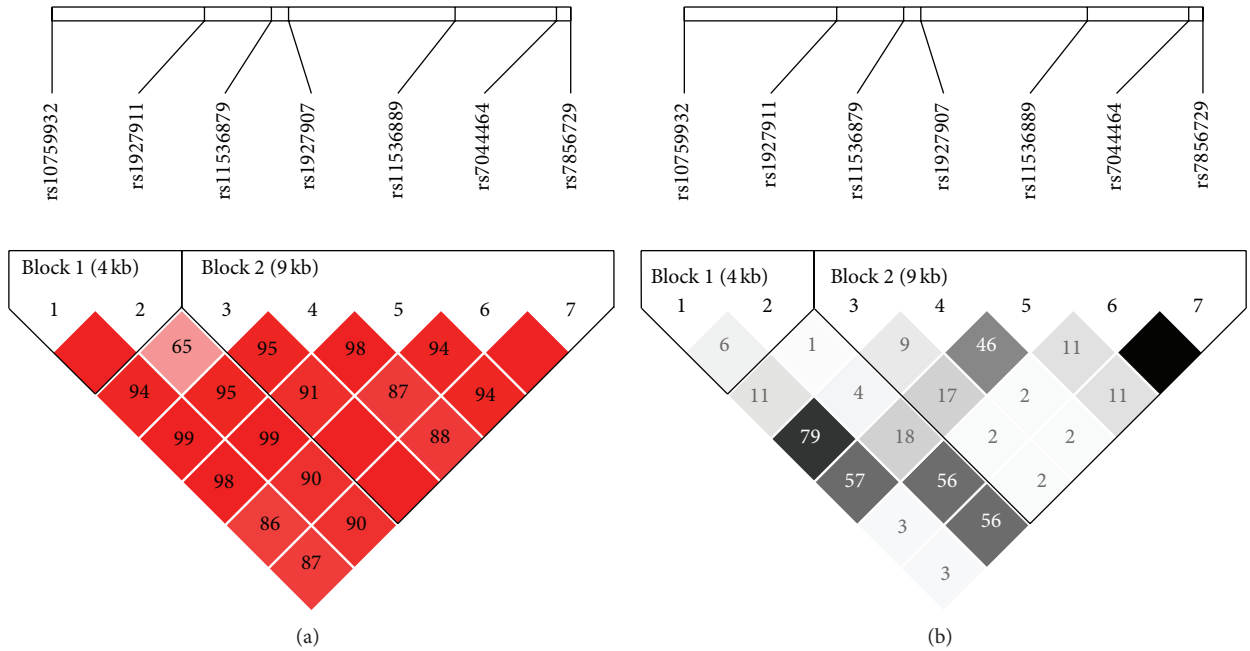

Figure 1: Linkage disequilibrium maps for SNPs genotyped in *TLR4* region. (a) Shades of red demonstrate the strength of the pairwise linkage disequilibrium based on D′ and numbers represent the value of D′ expressed as a percentage. (b) Shades of grey show the strength of the pairwise linkage disequilibrium based on r^2 and numbers indicate the value of r^2 expressed as a percentage.

analyzed the effect of these SNPs on nephropathy related quantitative traits. With adjustment of age, sex, body mass index, duration of diabetes, HbA1C, diastolic pressure, and systolic pressure, we found that no SNP was nominally associated with AER and eGFR. The minimum P value was 0.087 for the association between rs10759932 and AER (Table 4).

4. Discussion

It has been shown that TLR4 and its signal pathway participated in the pathogenesis of diabetes and diabetic nephropathy. Dasu et al. [23] reported that *TLR4* expression and its ligand, signaling, and functional activation were increased in recently diagnosed type 2 diabetes subjects and contributed to the proinflammatory state. Furthermore, knockout of *tlr4* attenuated the proinflammatory state of diabetes in animal models [24]. Exposure of isolated islets of Langerhans to LPS reduced insulin gene expression; insulin secretion was inhibited as well. However, those effects were not observed in islets from TLR4-deficient mice [25]. In vitro, TLR4 expression and activity were increased under hyperglycemia in mesangial cells and could contribute to the progression of diabetic nephropathy [18]. Liu et al. [20] observed that renal TLR4 expression was significantly higher in diabetic nephropathy in animal model, as well as kidney/body weight ratio, serum creatinine, CRP, and TNF-α level. TLR4 has also been proved to accelerate the progression of diabetic nephropathy induced by hyperlipidemia [26]. The research of Lin et al. [17] also showed that TLR4 pathway promoted tubular inflammation in diabetic nephropathy. And their further study investigated that TLR4 antagonist CRX-526 could reduce albuminuria and blood urea nitrogen without altering blood glucose and systolic blood pressure in diabetic mice, thus protecting diabetic mice from advanced nephropathy [19]. Above all, it is convincible that TLR4 and its signal pathway play an important role in diabetic nephropathy.

The data of *TLR4* genetic polymorphisms and diabetic complications was limited. It was reported in a German population that Asp299Gly and Thr399Ile genotypes of the *TLR4* were associated with diabetic neuropathy in type 2 diabetes, but not with diabetic nephropathy [27]. Asp299Gly was associated with early onset of diabetic retinopathy in the type 2 patients, reported in a Poland population [28]. However, these two polymorphisms were not detected in the Chinese population [29–32]. In the present study, we aimed to investigate the association between *TLR4* polymorphisms and diabetic nephropathy. However, we failed to find any evidence of association between SNPs from this locus and traits related to diabetic nephropathy in our samples. One possible explanation might be that the statistical power of our samples was not enough to detect the effects of this locus in the Chinese population. With our samples, we had over 75% power to detect an effect SNP (OR = 1.4) with minor allele frequency of 0.1 at a 0.05 level. We could not exclude the possibility that associated SNPs with lower minor allele frequency or lower effect in the Chinese existed. Secondly, although the tagging SNPs we selected covered all the common SNPs of *TLR4*, it is still possible that rare variants of *TLR4* associated with diabetic nephropathy existed. Thirdly, in our study, the diagnosis of diabetic nephropathy was not determined by the histological analysis of tissue samples obtained from renal

Table 2: Associations of *TLR4* SNPs with type 2 diabetic nephropathy.

SNP	Chr. position (Build 37.3)	Major/minor allele	Risk allele	Cases ($n = 621$)		Controls ($n = 832$)		OR for minor allele (95% CI)	P value
				Minor allele frequencies	Genotype count 11/12/22[#]	Minor allele frequencies	Genotype count 11/12/22[#]		
rs10759932	9:120465144	T/C	T	0.310	290/236/65	0.309	392/336/83	1.001 (0.851–1.177)	0.993
rs1927911	9:120470054	C/T	T	0.435	201/289/121	0.427	267/403/148	1.030 (0.887–1.196)	0.698
rs11536879	9:120472211	A/G	G	0.125	471/129/12	0.120	630/169/13	1.047 (0.835–1.312)	0.691
rs1927907	9:120472764	G/A	A	0.272	320/218/51	0.260	449/294/62	1.064 (0.894–1.260)	0.477
rs11536889	9:120478131	G/C	G	0.221	371/224/25	0.216	511/275/41	1.031 (0.862–1.231)	0.741
rs7044464	9:120481397	T/A	A	0.093	503/110/2	0.084	694/127/6	1.113 (0.859–1.443)	0.417
rs7856729	9:120481856	G/T	T	0.093	506/111/2	0.086	691/131/6	1.084 (0.838–1.402)	0.541

[#]11, major allele homozygotes; 12, heterozygotes; 22, minor allele homozygotes.
The OR with 95% CI shown is for the minor allele.

Table 3: Associations of two haplotypes in *TLR4* region with diabetic nephropathy.

Haplotype	Haplotype frequencies		*P* value
	Cases	Controls	
Block 1 (rs10759932-rs1927911)			
TA	0.566	0.570	0.827
CA	0.309	0.309	0.973
TG	0.125	0.120	0.705
Block 2 (rs11536879-rs1927907-rs11536889-rs7044464-rs7856729)			
GGCTG	0.342	0.361	0.288
GATTG	0.269	0.253	0.341
CGCTG	0.215	0.205	0.520
GGTAT	0.088	0.080	0.404
GGTTG	0.071	0.081	0.320

Table 4: Associations of *TLR4* SNPs with clinical features related to diabetic nephropathy in type 2 diabetic patients without nephropathy.

SNP	AERs			eGFR		
	Beta	SE	*P* value#	Beta	SE	*P* value#
rs10759932	−0.022	0.013	0.087	0.003	0.006	0.683
rs1927911	−0.011	0.012	0.354	0.002	0.006	0.781
rs11536879	0.019	0.019	0.312	−0.001	0.009	0.894
rs1927907	−0.019	0.013	0.165	0.001	0.006	0.839
rs11536889	0.000	0.015	0.974	−0.003	0.007	0.712
rs7044464	0.022	0.022	0.314	0.002	0.011	0.865
rs7856729	0.024	0.022	0.271	0.006	0.011	0.581

The additive model was used in the analysis.
P value# was adjusted for age, sex, BMI, diabetes duration, HbA1C, and blood pressure.

biopsies, which is the golden standard [33]. So we cannot exclude the possibility that patients diagnosed with diabetic nephropathy also may include nondiabetic renal disease and a superimposed nondiabetic condition on underlying diabetic nephropathy. However, we excluded patients with history of renal diseases in the enrollment of study subjects, and the blood pressures of patients with or without diabetic nephropathy were similar, and thus the influence of the diagnostics of diabetic nephropathy on our study was limited.

5. Conclusions

In conclusion, although many functional researches have implied that TLR4 played an important role in diabetic nephropathy, our study suggested that common variants within *TLR4* gene were not associated with diabetic nephropathy in the Chinese type 2 diabetes patients. However, due to the limitation of the current study, the effects of SNPs from this locus on diabetic nephropathy needed to be tested in further studies with larger samples with accurate diagnosis for diabetic nephropathy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Danfeng Peng, Jie Wang, and Jiemin Pan contribute equally to this work.

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Research Article

A Novel Mutation in *Leptin* Gene Is Associated with Severe Obesity in Chinese Individuals

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Obesity is a clinical syndrome which is driven by interactions between multiple genetic and environmental factors. Monogenic obesity is a rare type of obesity which is caused by a mutation in a single gene. Patients with monogenic obesity may develop early onset of obesity and severe metabolic abnormalities. In this study, we screened mutations of *LEP* in a total of 135 Chinese individuals including 35 obese patients whose BMI $\geq 32\,kg/m^2$ and 100 controls with BMI $<25\,kg/m^2$. Moreover, detailed information and clinical measurements of the participants were also collected. Finally, we identified a novel nonsynonymous mutation H118L in exon 3 of *LEP* in one patient with BMI $46.0\,kg/m^2$. This mutation was not identified in the controls. We speculated that the mutation H118L in *LEP* might be associated with severe obesity in Chinese subjects. However, the substantial mechanism should be further investigated.

1. Background

Nowadays, obesity has become an important public health issue, for its prevalence is increasing through the years [1, 2]. Besides, it can also induce severe metabolic abnormalities including type 2 diabetes, hypertension, dyslipidaemia and cardiovascular diseases. It has been confirmed that obesity is influenced by both genetic and environmental factors. Monogenic obesity is a special and rare type of obesity which is caused by a mutation in a single gene and is not affected by the environmental factors. So far, several genes, such as *proopiomelanocortin (POMC)*, *leptin receptor (LEPR)*, *leptin (LEP)*, *proconvertase 1 (PC1)*, and *melanocortin 4 receptor (MC4R)*, have been confirmed as the casual genes to the onset of monogenic obesity [3–10]. *LEP* in humans, for instance, was cloned and identified and in 1995. Human *LEP* locates on chromosome 7q31.3, and its translational product is leptin, which plays a decisive role in the regulation of human appetite and results in severe metabolic disorders [11, 12]. Several mutations in *LEP* have been confirmed to be associated with monogenic obesity [3, 4]. In this study, we aimed to screen the potential mutations in *LEP* in obese patients with BMI $\geq 32\,kg/m^2$ to explore the mechanism of severe obesity in these patients.

2. Methods

2.1. Ethics Statement. This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Written informed consent was obtained from each participant.

Table 1: The clinical characteristics of the subjects.

	Cases	Controls
Male/female (*n*)	16/19	47/53
Age (years)	24 (19, 33)	31 (23, 35)
BMI (kg/m^2)	40.64 ± 7.70	21.58 ± 1.94

Data are expressed as mean ± SD or median (interquartile range).

2.2. Subjects. 35 Chinese Han obese participants with BMI ≥32 kg/m^2 and 100 Chinese Han control subjects with BMI <25 kg/m^2 in Shanghai were enrolled in this study. All the participants underwent a detailed clinical investigation. Anthropometric parameters such as body height, body weight, blood pressure, body mass index (BMI), and waist and hip circumference were measured. The clinical characteristics of the two groups are listed in Table 1.

2.3. Sequences Analysis. Genomic DNA was extracted from the leukocytes in the peripheral blood samples. The coding region of *LEP* consisted of two fragments and amplified by PCR using the primers designed with the software "Primer Premier 5." Detailed information of the primers and the products were shown in Table 2. The encoding regions of *LEP* were amplified by a thermal cycler (Veriti, ABI, USA). Then the PCR products were depurated and sequenced directly using the 3130 genetic analyzer (Applied Biosystems, USA). Moreover, we use PolyPhen2 (Polymorphism Phenotyping v2) and SIFT Human Protein to predict the function of the mutation. It divides mutation into several categories such as benign, possibly damaging, or probably damaging on the basis of structure information and functional annotation. PolyPhen2 and SIFT Human Protein are available at http://genetics.bwh.harvard.edu/pph2 and http://sift.jcvi.org/www/SIFT_enst_submit.html, respectively.

3. Results

After direct sequencing, one novel missense mutation in exon 3 of *LEP* which changed the amino acid from hydrophilic His to hydrophobic Leu (H118L) was detected in one obese patient with BMI 46.0 kg/m^2 (Figure 1(a)). In addition, his physical examination also showed a typical abdominal obesity appearance, with the waist circumference 145 cm, as well as hip circumference 135 cm. Besides extreme obesity, the carrier also has complications of hypertension, metabolic syndrome, fatty liver syndrome, sleep apnea syndrome, gastric ulcer, and chronic superficial gastritis. However, such mutation was not detected in other obese subjects nor the normal controls (Figure 1(b)). PolyPhen2 showed that the mutation was possibly damaged with a score of 0.041 (sensitivity: 0.94; specificity: 0.83), which is predicted to be benign. In addition, SIFT human protein indicated the mutation to be "DAMAGING." The two prediction outcomes are also in accordance with the clinical phenotypes of that patient.

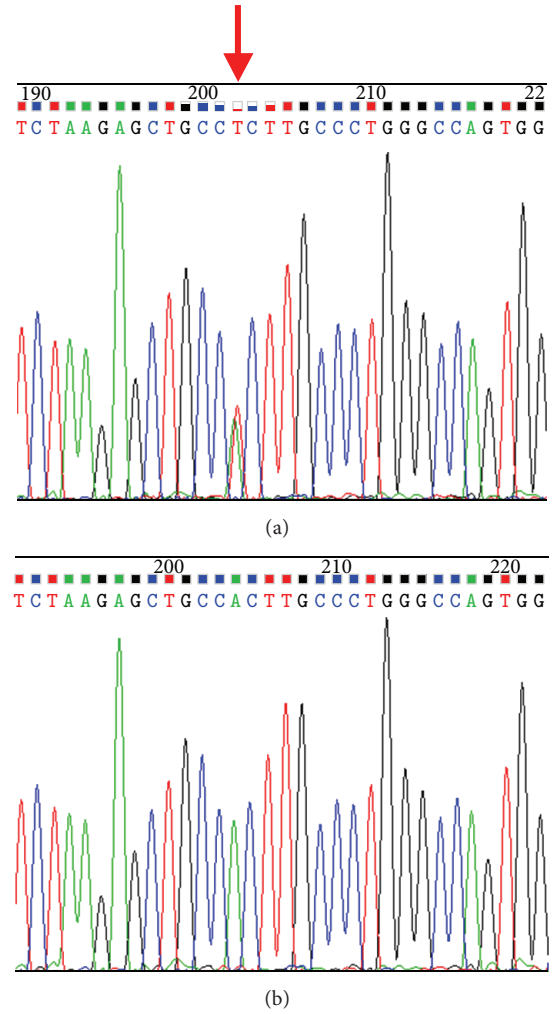

Figure 1: Heterozygous sequence of H118L mutation (a) and normal sequence of *LEP* (b). The red arrow indicated the heterozygous mutation H118L of *LEP*.

4. Discussion

LEP was expressed in adipose tissue and its product leptin was a classic adipokine, which participated in the food intake and energy expenditure. Therefore, the mutations in *LEP* can possibly damage the function of leptin and disturb the metabolic balance in humans, which is directly responsible for severe obesity and other metabolic disorders. Up to now, several mutations like R105W, N103K, and L72S in *LEP* have been reported to be related with a phenotype of extreme obesity around the world.

In our present study, we screened the whole exons of *LEP* in the obese samples as well as the normal controls in the Chinese and detected a novel mutation H118L in one obese individual but not the normal controls. This novel mutation was not reported in the 1000 Genomes Project neither [13]. Moreover, the missense mutation led to the amino acid residue substitution from His to Leu, which is predicted to be possibly damaged with a score of 0.041 (sensitivity: 0.94; specificity: 0.83) by Polyphen2 and "DAMAGING" by

Table 2: Primer Sequences for the amplification.

Name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Product length (bp)	Annealing temperature (°C)
Primer 1	GCCAGAGCAGAAAGCAAA	TCAGGAGGCGTTCAATAA	**397**	**59**
Primer 2	GAGCACTTGTTCTCCCTCTT	TTCCCTTAACGTAGTCCTTG	**435**	**61**

SIFT human protein. Since this mutation carrier exhibited a phenotype of severe obesity with BMI 46.0 kg/m^2 and complicated with other metabolic disorders including hypertension, metabolic syndrome, fatty liver syndrome, and sleep apnea syndrome, we considered that this mutation might be associated with those abnormal phenotypes.

There are several limitations of our study. Firstly, since the pedigrees and phenotypes of the mutation carrier were not available, whether the mutations were cosegregated with obesity individuals in China or not still remains unknown. In addition, more clinical data such as history of overweight, visceral and subcutaneous fat will be a great help to the certain conclusion. Finally, the functional study of this novel mutation H118L was not carried out to elucidate the mechanism of the disease.

We initially identified a mutation in *LEP* which might be associated with severe obesity in Chinese individuals in our study. However, functional investigations are still needed to confirm our findings and elucidate the mechanism underlying such association between the variants and obesity.

5. Conclusion

A novel mutation H118L of *LEP* was detected in the severe obese patient but not the normal controls in the Chinese. We speculated this mutation to be a casual mutation to monogenic obesity in the Chinese. However, further functional studies should be performed to elucidate the substantial mechanism.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yue Zhao, Nanchao Hong, Xiao Liu, and Beibei Wu contributed equally to this paper. Yue Zhao, Nanchao Hong, Xiao Liu, and Beibei Wu performed the experiments. Shanshan Tang and Jianjun Yang contributed to samples collection. Cheng Hu and Weiping Jia designed the study.

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Research Article

Association of Genetic Variants of *BMP4* with Type 2 Diabetes Mellitus and Clinical Traits in a Chinese Han Population

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BMP4 is one of the transforming growth factor-β superfamily, which can participate in adipogenesis. Gene encoding *BMP4* is acknowledged as a convincing candidate that may contribute to both glucose and lipid metabolism. In this paper, we aimed to test the impacts of *BMP4* variants on type 2 diabetes in a large sample of Chinese population. We genotyped 10 tagging single nucleotide polymorphisms within the *BMP4* region in 6822 participants and acquired detailed clinical investigations and biochemistry measurements. We found that *BMP4* rs8014363 showed nominal association towards type 2 diabetes, with the T allele conferring a high risk of type 2 diabetes (OR = 1.108, 95%CI 0.999–1.229, $P = 0.051$ for allele; OR = 1.110, 95%CI 1.000–1.231, $P = 0.050$ for genotype), but it was no longer statistically significant after adjusting for multiple testing (empirical $P = 0.3689$ for allele based on 10,000 permutations). Moreover, we observed a significant association of rs8014363 with triglyceride level and a trend towards association with high-density lipoprotein cholesterol after adjusting for age, gender, and BMI ($P = 0.035$ and 0.068, resp.). Our data suggested that the genetic variants of *BMP4* may not play a dominant role in glucose metabolism in Chinese Han population, but a minor effect cannot be ignored.

1. Background

According to the IDF Diabetes Atlas 2012, there are more than 371 million diabetic patients worldwide and 4.8 million patients died due to diabetes [1]. With the staggering increase of diabetes pandemic creating an overwhelming array of serious complications and high mortality rate, exploring the etiology behind diabetes is of great essentiality. Although environmental factors contribute significantly to diabetes, it is generally considered that the importance of genetic factors cannot be ignored as well [2, 3]. Recently along with the powerful genome-wide association study, the candidate gene approach can also guide a better understanding of the pathophysiology of complex diseases. Up to now, more than 60 loci have been confirmed to confer susceptibility to type 2 diabetes [4, 5] however, they are still not enough to interpret the genetic mechanism of the disease. As a consequence, it is worthy for further research.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β superfamily, which is now acknowledged to be involved in regulating embryonic development and differentiation as well as cellular function [6–8]. Among them, *BMP4* has been suggested to play an important role in adipogenesis, especially the white adipocyte differentiation through interaction with BMP receptor (BMPR) and subsequently activating the Smad signaling pathways [9–13]. White adipocyte tissues (WAT) are originally recognized as the primary site of triglycerides storage; however, accumulating evidences indicate that WAT is an endocrine organ that participates in the whole body energy metabolism and is highly associated with the risk of developing metabolic syndrome [14, 15]. Thus, *BMP4*, which is considered as a convincing candidate gene that may contribute substantially to both glucose and lipid metabolism, should arise more attention. Nevertheless, up to now, there is no report focusing on the genetic association studies of *BMP4* with type 2 diabetes and related clinical traits in East Asian population. In view of this, the aim of this current study was to test for the possible correlation between them in a Chinese Han population.

Table 1: Clinical characteristics of the study sample.

	Cases	Controls
Samples (*n*)	3410	3412
Male/female (*n*)	1812/1597	1364/2048
Age (years)	60.33 ± 12.49	51.41 ± 14.39
BMI (kg/m^2)	24.20 (22.00, 26.60)	23.23 (21.27, 27.68)
Fasting plasma glucose (mmol/L)	12.78 (9.00, 16.00)	5.02 (4.70, 5.40)
2 h plasma glucose (mmol/L)	17.00 (13.00, 22.00)	5.42 (4.60, 6.30)
Total cholesterol (mmol/L)	4.70 (4.00, 5.50)	4.70 (4.04, 5.35)
Triglyceride (mmol/L)	1.49 (0.99, 2.18)	1.25 (0.87, 1.82)
HDL-C (mmol/L)	1.11 (0.94, 1.33)	1.33 (1.13, 1.51)
LDL-C (mmol/L)	2.97 (2.42, 3.57)	3.04 (2.49, 3.61)

Data are shown as *n* or median (interquartile range).
BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

2. Methods

2.1. Ethics Statement. The study was approved by the institution review board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital in accordance with the principle of the Helsinki Declaration II. Written informed consent was obtained from each participant.

2.2. Subjects. A total of 6822 participants of Han ancestry residing in Shanghai were recruited, including 3410 cases with type 2 diabetes and 3412 controls. Detailed information concerning this study population has been described elsewhere [16, 17]. In brief, all cases were unrelated type 2 diabetes patients defined according to 1999 WHO criteria and were recruited from the clinical inpatient database of Shanghai Diabetes Institute [18]. The controls were enrolled from community-based random sample epidemiological studies of diabetes and related metabolic diseases. All of them were unrelated subjects with normal glucose tolerance as assessed by 75 g oral glucose tolerant tests (OGTTs) and with negative family history of diabetes. The clinical characteristics of all participants were shown in Table 1.

2.3. Clinical Measurements. All participants underwent a detailed clinical investigation as described previously [19]. Briefly, anthropometric parameters such as height, weight, blood pressure, and waist and hip circumference were measured. Body mass index (BMI) was calculated as weight in kilometers divided by height in meters squared. For the controls, OGTTs which were assessed by standard 75 g glucose in the morning after an overnight fast were performed. And blood samples were obtained at the fasting and 2 h during OGTTs. Plasma glucose, serum insulin and lipid profile were measured. Homeostasis model assessment (HOMA), which was calculated by fasting plasma glucose and insulin, was used for estimating insulin resistance index and β cell function [20]. In addition, Insulin sensitivity from the OGTT was also estimated according to the insulin sensitivity index (ISI) proposed by Gutt et al. [21]. ISI = [75000 + (fasting plasma glucose − 2 h plasma glucose) × 0.19 × weight]/120/mean plasma glucose/$\log_{10}$ mean insulin.

2.4. Single Nucleotide Polymorphism (SNP) Selection and Genotyping. In the present study, 10 tagging SNPs were selected according to the HapMap Phase III (release 27) Han Chinese database using the threshold of $r^2 \geq 0.8$, which stretched 9 kb in the upstream to 9 kb in the downstream of the *BMP4* gene region. The 10 tagging SNPs could tag 73% SNPs (14 SNPs out of 19 SNPs in the HapMap Chinese Han sample) with a minor allele frequency (MAF) of >0.05. All the SNPs were genotyped using the primer extension of multiplex products with detection by matrix-assisted laser desorption ionization-time of flight mass spectroscopy using a MassARRAY Compact Analyzer (Sequenom, San Diego, CA, USA) and the overall call rate was 98.7%.

2.5. Statistical Analysis. The Hardy-Weinberg equilibrium test was performed in the cases and controls separately for each variant before association analysis. SNPs that failed this test ($P < 0.01$ in the controls) should be excluded. Pairwise linkage disequilibrium including $|D'|$ and r^2 was estimated using Haploview (version 4.2). Allele and genotype distributions between the patients and control subjects were compared with χ^2 test or logistic regression [22], and odds ratios (ORs) with 95% confidence intervals (CIs) were presented. All skewly distributed quantitative traits (including fasting plasma glucose, 2 h plasma glucose, fasting insulin, 2 h insulin, triglycerides, total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), estimated ISI, HOMA for β-cell function, and insulin resistance) were logarithmically transformed to approximate univariate normality. Quantitative traits were analyzed in the control group by linear regression under an additive genetic model adjusted for age, gender, and BMI as confounding factors. Correction of multiple testing on allele association was performed using Haploview (version 4.2) through 10,000 permutations that randomly permutated the case/control status independently of genotypes. The statistical analyses were performed using SAS for Windows (version 8.0; SAS Institute, Cary, NC, USA). A two-tailed P value of 0.05 was considered statistically significant.

The statistic power was calculated under an additive model based on the allele frequency observed in our samples. Upon the assumption that the population risk was 9.6% and two-side α of 0.05, for SNPs with the minor allele frequency over 0.2, our case-control sample size has over 80% power to detect the minimum OR of 1.15.

3. Results

The genotype distributions of all SNPs were in Hardy-Weinberg equilibrium. Pairwise linkage disequilibrium indicated that these 10 SNPs were in modest linkage disequilibrium and formed 3 haplotype blocks in this region (Figure 1).

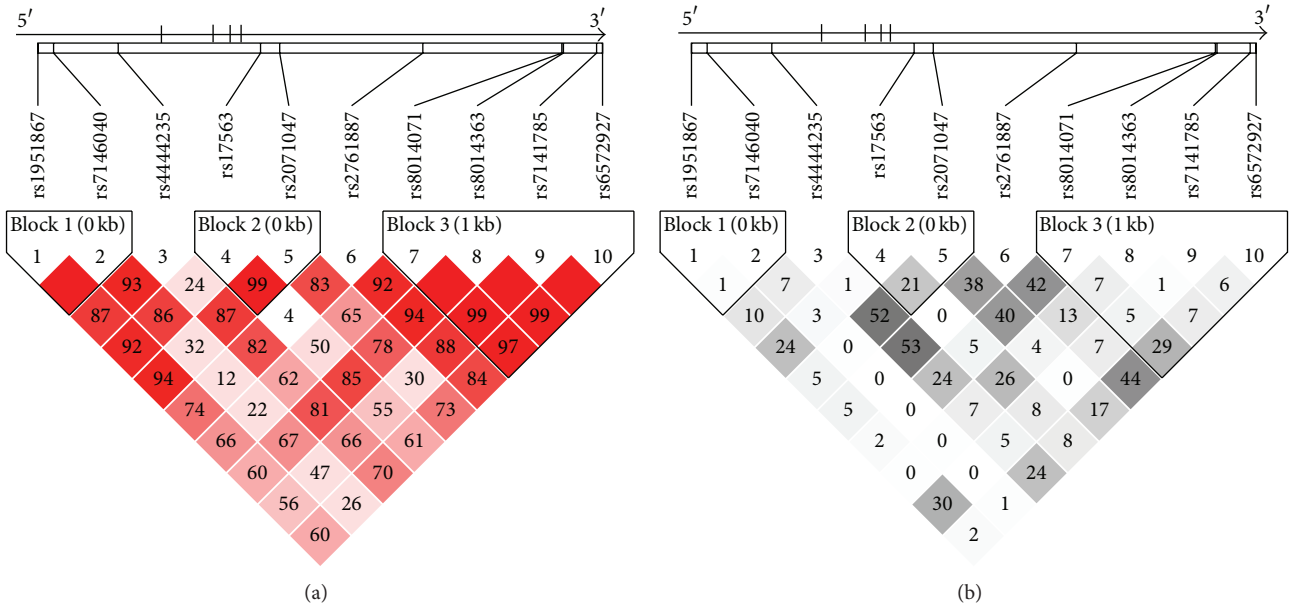

FIGURE 1: Linkage disequilibrium maps for SNPs genotyped in *BMP4* region. (a) Shades of red demonstrate the strength of the pairwise linkage disequilibrium based on D' and numbers represent the value of D' expressed as a percentage. (b) Shades of grey show the strength of the pairwise linkage disequilibrium based on r^2 and numbers indicate the value of r^2 expressed as a percentage.

We firstly examined the associations of these 10 SNPs with type 2 diabetes. Nominal evidence of the association was observed only for rs8014363, with the T allele conferring a high risk of type 2 diabetes (OR = 1.108, 95%CI 0.999 – 1.229, $P = 0.051$ for allele; OR = 1.110, 95%CI 1.000 – 1.231, $P = 0.050$ for genotype, Table 2). However, the result was no longer significant after correction for multiple testing by 10,000 permutations (empirical $P = 0.3689$). For the haplotype analysis, by comparing the frequencies between the cases and control subjects, we found that haplotype ACCT in block 3 comprised by rs8014071-rs8014363-rs7141785-rs6572927 indicated a marginal association with type 2 diabetes ($P = 0.056$, Table 3). After adjusting for multiple testing, the statistical significance was remarkably attenuated (empirical $P = 0.4263$).

In addition, we further analyzed the effect of the SNP rs8014363 on clinical characteristics in the control group under an additive model. As shown in Table 4, rs8014363 was significantly associated with triglyceride level, with carriers of more type 2 diabetes risk alleles (T) exhibiting higher values of triglyceride than CC homozygotes after adjusting for age, gender and BMI as confounding variants ($P = 0.035$). Moreover, we also observed a trend towards association between rs8014363 and HDL-C, with carriers of the risk alleles (T) demonstrating lower values ($P = 0.068$). However, no conspicuous associations were detected in other lipid profile indexes.

4. Discussion

In the current study, we tested the effects of 10 tagging SNPs in *BMP4* region on type 2 diabetes in a Chinese population. We identified modest effects of *BMP4* variants on the risk of type 2 diabetes.

Over the past years, it has become increasingly obvious that obesity is a major independent risk factor for developing type 2 diabetes [23, 24]. In accompany with obesity, there is always increase of adipose tissues results from both adipocyte hypertrophy and hyperplasia [25]. *BMP4* is considered generally to induce commitment of pluripotent stem cells to the white adipocyte lineage [9–11]. In our present analysis, we observed that the triglyceride level significantly increased with the increasing number of risk allele in rs8014363. Nonetheless, an opposite situation between HDL-C levels and the number of risk allele in rs8014363 could also be seen. They are consistent with that WAT is a depot which is not only specialized in storing energy in the form of triglyceride but also a crucial target organ for insulin action. While HDL-C is mainly responsible for reverse-transporting cholesterol to the liver, it serves as a beneficial effect toward atherosclerosis, which is in contrast with triglyceride. That is to say, *BMP4* pose an influence on the glucose and lipid metabolism, which may through participating in WAT differentiation.

Furthermore, One functional analysis using multiple genetic approaches showed that *BMP4* and its affinity BMPR1A were both expressed in β cells; besides, results not only in vitro but also in vivo all provided consistent evidences that *BMP4* in β cells was required for insulin secretion and advantageous to ameliorate glucose tolerance through significantly stimulating glucose-stimulated insulin secretion (GSIS) [26]. However, in our analysis, we failed to find any association with β cell function for variants of *BMP4*. Accordingly, the exact mechanisms behind these results should be warranted for further investigation.

Some limitations should be considered in the present study. Firstly, as the genetic effect of *BMP4* variants on type 2 diabetes was mild, our samples may not have enough power

Table 2: Associations of *BMP4* SNPs with type 2 diabetes.

SNP	Chr. position (Build 37.3)	Major/minor allele	Risk allele	Cases (n = 3410)		Controls (n = 3412)		OR for risk allele (95% CI)	P value for risk allele	OR for genotype (95% CI)	P value for genotype (empirical P value)
				Risk allele frequencies	Genotype count 11/12/22[#]	Risk allele frequencies	Genotype count 11/12/22[#]				
rs1951867	54407192	G/C	G	0.903	2748/586/35	0.896	2673/617/36	1.073 (0.959, 1.201)	0.220	1.071 (0.957, 1.198)	0.233
rs7146040	54407920	A/G	G	0.097	2755/586/36	0.096	2768/572/38	1.017 (0.908, 1.140)	0.767	0.983 (0.878, 1.101)	0.769
rs4444235	54410919	T/C	C	0.459	996/167/717	0.452	1034/1593/716	1.026 (0.959, 1.098)	0.461	0.975 (0.912, 1.043)	0.465
rs17563	54417522	T/C	T	0.720	1800/1319/273	0.717	1733/1375/269	1.042 (0.967, 1.124)	0.280	1.042 (0.967, 1.123)	0.283
rs2071047	54418411	C/T	T	0.368	1380/1522/485	0.355	1428/1527/444	1.056 (0.985, 1.133)	0.126	0.948 (0.885, 1.016)	0.131
rs2761887	54425052	C/A	A	0.519	793/1675/920	0.511	795/1674/866	1.033 (0.965, 1.105)	0.348	0.968 (0.905, 1.036)	0.348
rs8014071	54431500	A/G	G	0.350	1426/1572/404	0.341	1462/1539/386	1.039 (0.968, 1.115)	0.290	0.962 (0.896, 1.033)	0.287
rs8014363	54431575	T/C	T	0.884	2617/690/45	0.873	2574/774/45	1.108 (0.999, 1.229)	**0.051**	1.110 (1.000, 1.231)	**0.050** (0.3689)
rs7141785	54433114	C/T	C	0.904	2740/572/36	0.902	2727/594/32	1.028 (0.916, 1.154)	0.639	1.022 (0.912, 1.146)	0.705
rs6572927	54433390	T/A	T	0.633	1345/1534/456	0.632	1327/1533/455	1.008 (0.940, 1.082)	0.816	1.008 (0.939, 1.081)	0.834

P values <0.1 were shown in bold.

The additive model was used in the association analyses between genotype and type 2 diabetes.

[#]11: major allele homozygotes; 12: heterozygotes; 22: minor allele homozygotes. Empirical P values are for the alleles based on 10,000 permutations.

Table 3: Associations of three haplotypes in *BMP4* region with type 2 diabetes.

Haplotype	Haplotype frequencies		*P* value (empirical *P* value)
	Cases	Controls	
Block1 (rs1951867-rs7146040)			
GA	0.805	0.801	0.522
CA	0.097	0.103	0.248
GG	0.097	0.096	0.755
Block2 (rs17563-rs2071047)			
TT	0.368	0.355	0.116
TC	0.358	0.361	0.688
CC	0.274	0.284	0.210
Block3 (rs8014071-rs8014363-rs7141785-rs6572927)			
ATCA	0.365	0.366	0.960
GTCT	0.348	0.338	0.223
ACCT	0.117	0.127	**0.056** (0.4263)
ATTT	0.096	0.098	0.705
ATCT	0.073	0.068	0.247

P values <0.1 were shown in bold.
Empirical *P* values are for the haplotypes based on 10,000 permutations.

Table 4: Association analyses of the rs8014363 genotype with clinical characteristics in the normal glucose tolerant group.

	CC (n = 45)	CT (n = 774)	TT (n = 2574)	β	SE	*P*	*P**
Age (years)	48.33 ± 14.93	51.02 ± 14.44	51.58 ± 14.39	0.7892	0.5317	0.138	/
BMI (kg/m^2)	22.98 (21.48, 24.65)	23.26 (21.12, 25.53)	23.23 (21.30, 25.51)	0.0003	0.0022	0.884	/
Fasting plasma glucose (mmol/L)	5.06 (4.60, 5.40)	5.00 (4.63, 5.39)	5.03 (4.70, 5.40)	0.0015	0.0017	0.371	0.462
2 h plasma glucose (mmol/L)	5.09 (4.50, 6.14)	5.44 (4.70, 6.30)	5.43 (4.60, 6.34)	0.0017	0.0035	0.637	0.888
Fasting insulin (mU/L)	5.41 (3.39, 6.52)	6.00 (4.23, 8.34)	6.23 (4.39, 8.72)	0.0181	0.0115	0.114	0.127
2 h insulin (mU/L)	29.36 (12.43, 51.29)	28.34 (14.07, 45.17)	27.81 (16.10, 47.10)	0.0182	0.0158	0.249	0.365
Total cholesterol (mmol/L)	4.75 (4.04, 5.25)	4.64 (4.01, 5.32)	4.70 (4.05, 5.35)	0.0034	0.0036	0.341	0.643
Triglyceride (mmol/L)	1.11 (0.74, 1.71)	1.24 (0.84, 1.82)	1.26 (0.88, 1.83)	0.0188	0.0087	**0.031**	**0.035**
HDL-C (mmol/L)	1.38 (1.19, 1.58)	1.34 (1.14, 1.52)	1.33 (1.12, 1.51)	−0.0054	0.0036	0.134	**0.068**
LDL-C (mmol/L)	3.00 (2.45, 3.62)	3.03 (2.45, 3.60)	3.04 (2.50, 3.62)	0.0031	0.0049	0.527	0.846
HOMA-IR	1.13 (0.79, 1.56)	1.27 (0.91, 1.89)	1.34 (0.93, 1.91)	0.0201	0.0119	**0.092**	0.104
HOMA-B	79.81 (47.82, 128.65)	89.25 (61.45, 137.90)	90.30 (62.92, 135.68)	0.0115	0.0128	0.369	0.382
Gutt-ISI	104.36 (83.45, 138.38)	100.24 (81.62, 131.43)	99.56 (80.97, 127.80)	−0.0095	0.0068	0.160	0.220

Data are shown as mean ± SD or median (interquartile range).
BMI: body mass index; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; HOMA-IR: homeostasis assessment model of insulin resistance; HOMA-B: homeostasis assessment model of β-cell function; Gutt-ISI: insulin sensitivity index proposed by Gutt.
P values <0.1 were shown in bold.
*Adjusted for age, gender, and BMI.

to detect the association. Secondly, because the patients we enrolled were not newly diagnosed and they were treated with antidiabetic drugs and/or insulin. The effect of *BMP4* variants on type 2 diabetes which is definitely through impact on impaired insulin secretion or insulin sensitivity is rather ambiguous. Thirdly, the status of taking lipid lowering medication in the control subjects was not clear. Nonetheless, the condition is very rare. Finally, although we found modest correlation between *BMP4* variant and type 2 diabetes and related clinical characteristics, we did not perform a replicated research in another independent sample to confirm these results. Thus, it is imperative to further replicate the influence of the variant of *BMP4* on type 2 diabetes and metabolic traits in other Chinese samples.

5. Conclusion

Our data suggested that the genetic variants of *BMP4* may not play a dominant role in glucose metabolism in Chinese Han population, but a minor effect cannot be ignored. Further investigations are of great necessity to confirm our observations and elucidate the unequivocal mechanisms underlying such association.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Shanshan Tang and Rong Zhang contributed equally to this paper.

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