Abstract. Diabetes mellitus (DM) is a complex disease caused by absolute or relative insulin deficiency. The C57BLKsJ-db/db mouse model is a useful animal model for studying type 2 DM (T2DM). The present study investigated the association between an antizyme inhibitor 1 (AZIN1) gene polymorphism (rs1062048) and T2DM susceptibility in 2,270 Taiwanese individuals (570 patients with T2DM and 1,700 controls). Additionally, the present study investigated AZIN1 gene and protein expression in the liver tissues of mice in three age groups (4, 16 and 32 weeks) through reverse transcription-quantitative PCR, western blotting and immunohistochemistry. The data indicated that the genotype frequency distribution of the rs1062048 single-nucleotide polymorphism differed significantly between the patients with T2DM and controls (P<0.05). Furthermore, gene and protein expression levels of AZIN1 were significantly lower in early stage and late stage T2DM mouse liver samples than in control samples. Overall, the data suggested that AZIN1 expression is involved in T2DM development.

Introduction

Globally, the number of adults affected by diabetes mellitus (DM) increased rapidly from ~108,000,000 in 1980 to 422,000,000 in 2014 (1). In the adult population, the global prevalence of DM has nearly doubled from 4.7 to 8.5% since 1980 (1). The prevalence of DM in adults reported by the International Diabetes Federation Western Pacific Region ranges between 2.6% in Cambodia and 16.9% in Malaysia (2). The major risk factors for type 2 DM (T2DM) are age, metabolic conditions (overweight and obesity) and lifestyle (unhealthy diet, physical inactivity and smoking); however, genetic factors, including ethnicity, family history of diabetes and previous gestational diabetes may serve crucial roles in increasing T2DM susceptibility (1). The mean height, weight, body mass index (BMI) and total visceral fat volume are lower in the East Asian population compared with in the African and Caucasian populations; however, non-obese East Asian individuals can also develop T2DM (3-6). A family history of T2DM is a crucial factor. If a parent has T2DM, their children are 40% as likely to develop T2DM. If both parents have T2DM, the probability of their children developing T2DM exceeds 70% (7,8). In addition, different ethnic groups exhibit different incidence rates; this also demonstrates the effects of genetic factors (9). In the majority of individuals, T2DM causes insulin resistance in its target tissues throughout the body, and eventually the b-cells of the pancreatic islets fail to secrete sufficient insulin to overcome the resistance. In addition to environmental factors, multiple genetic factors appear to cause predisposition to T2DM (10,11). Currently, >120 associated gene variants have been identified and have minor effects on diabetes risk (12). There is no complete information regarding genetic susceptibility of T2DM despite the availability of novel detection techniques; thus, additional pieces of the puzzle may help to explain the heritability of diabetes.

Polyamines are organic polycations essential for cell growth and differentiation, and impairment of the polyamine signaling
pathway can lead to numerous diseases and conditions, including cancer, inflammation, stroke, renal failure and diabetes (13). Ornithine decarboxylase (ODC) is involved in the commitment and rate-limiting step in polyamine biosynthesis, and polyamine homeostasis is maintained by the regulatory proteins antizyme isoform 1 (AZ1) and antizyme inhibitor 1 (AZIN1) (14). Translation of intracellular AZ1 occurs alongside upregulation of intracellular polyamine synthesis (15,16). AZ1 binds to ODC to form an ODC-AZ1 heterodimer that inhibits polyamine production (14). AZIN1 is obtained from rat liver extract, exhibits high similarity in sequence and structure to ODC, and has a greater affinity for AZ1 than ODC (17). AZIN1 inhibits the activity of ODC, promotes ubiquitin-dependent degradation of ODC and contributes to carcinogenesis (17). However, binding of AZ1 to ODC reduces the cellular polyamine level and affects the conversion of ASPC-1 cells into α-cells, which form islet-like structures and express the glucagon gene to regulate pancreatic endocrine cell function (18). Competition between AZIN1 and ODC for AZ1 binding effectively restores ODC activity and increases the cellular polyamine level, which may serve a role in diabetes (16). The present study demonstrated that AZIN1 genetic polymorphisms are potential candidate genes for diabetes in the Taiwanese population and revealed the downregulation of AZIN1 gene expression in T2DM.

Materials and methods

Patients and sample collection for genotyping. In the present study, a total of 570 T2DM patients, aged 20 years and older (mean age, 63.6±11.5; 51.4% male individuals) were enrolled at China Medical University Hospital (Taichung, Taiwan) between August 2014 and July 2015. All patients met the diagnostic criteria (19) for T2DM with the exclusion criteria those without T2DM. To determine the prevalence of polymorphism in these patients, genotype frequency data of 1,700 healthy controls was downloaded from the Taiwan Biobank (https://taiwanview.twbiobank.org.tw/) (case no: TWBR10509-02; control no: TWBR10309-001) (9). In addition, to determine the association between polymorphism and clinical features in T2DM patients, the clinical features, including age, BMI, hemoglobin A1c, blood urea nitrogen, creatinine, uric acid, total calcium, phosphorus, parathyroid hormone (PTH), albumin, cholesterol, triglycerides, low-density lipoprotein-C and alanine aminotransferase (ALT) were also downloaded from the Taiwan Biobank. The present study obtained the rs1062048 single-nucleotide polymorphism (SNP) at chromosome region 8q22.3 in AZIN1 (Fig. 1) from the National Center for Biotechnology Information’s SNP database (http://www.ncbi.nlm.nih.gov/snp). The SNPs in the T2DM and control groups were then compared. Tag SNPs were selected using the Tagger function (http://software.broadinstitute.org/mpg/tagger/server.html) with the additional criteria: ii) A threshold minor allele frequency in the HapMap phase 3; and ii) Han Chinese in Beijing, China (CHB) + Japanese in Tokyo, Japan (JPT) population of 0.05 for ‘tag SNPs’. Chi-square tests were used to calculate odds ratios and P-values. All study protocols were approved by the Ethical Committee of China Medical University Hospital (approval no. CMUH103-REC2-071). Informed consent was obtained from all analyzed patients.

Animal model. A total 24 male background BKS.Cg-Dock 7m+/+ Leprdb/JNarl mice (age, 4 weeks old) were purchased from the National Laboratory Animal Center. Mice with the +Dock7m+/Dock7m genotype constituted the control group (n=12) and those with the +Leprdb/+Leprdb genotype constituted the T2DM group (n=12). The diabetic mice averaged 62±3 g in body weight at the time of the studies and their control littermates averaged 20±1 g. The animals were housed in individual cages and provided with lab chow ad libitum (Lab Diet 5k52; Purina) in a room at 22-25°C, with sufficient water, a relative humidity of 50-70%, and a 12-h light/dark cycle. The present study involved animal experiments and considered the 3R principles of ‘Replace’, ‘Reduce’ and ‘Refine’ to optimize experimental design (20). According to the information from Jackson Laboratory (JAX stock #000642) (21), the mouse model exhibited elevated blood sugar at 4-8 weeks and mortality by 10 months of age. The mice in the present study
were divided into six groups (four mice per group), namely three control groups of mice aged 4 (early stage), 16 (middle stage) and 32 (late stage) weeks, and three T2DM groups of mice aged 4 (early stage), 16 (middle stage) and 32 (late stage) weeks. Mice were sacrificed and the liver tissue was obtained at the scheduled time. Sample went through the methodology of flash freezing (22), which provided excellent specimen integrity and a wide array of options for tissue analysis, including extraction of RNA and proteins in the present research. The present study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University (IACUC permit no. 2106-221).

Reverse transcription-quantitative PCR (RT-qPCR). The RNeasy Mini kit (Qiagen, Inc.) was used for total RNA isolation from the liver tissues of the control and T2DM mice, and the Superscript First-Strand Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used for complementary DNA transcription, as previously described (23). Briefly, the cDNA synthesis reaction protocol including reverse transcription step, 30 min at 42˚C and RT inactivation step, 1 min at 95˚C. To study gene expression, qPCR was performed using the ABI ViiA™ 7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TaqMan™ MGB (minor groove binder)-NFQ (nonfluorescent quencher) Universal ProbeLibrary (Roche Diagnostics) probes, as previously described (24). The primer sequences were as follows: Mus musculus AZIN1 (NM_018745.5) forward, 5'-TGCTAAGAA AGTTGTTGAAAATGTATAA-3'; murine AZIN1 reverse, 5'-CTGCTCACACTCCTCCTTT-3' with UPL probe #6 (Roche, cat. no. 04685032001); mouse GAPDH (M32599.1) forward, 5'-GAGCCAACGGGTCATCA-3'; and murine GAPDH reverse, 5'-CATATTCTCGTGTTCCACACC-3' with UPL probe #29 (Roche, cat. no. 04687612001). PCR amplification conditions were: Initial denaturation at 95˚C for 5 min, followed by 25 cycles of 95˚C for 10 sec, 56˚C for 10 sec and 72˚C for 20 sec, with a final extension at 72˚C for 5 min. The target gene expression levels were normalized to mouse GAPDH expression. The relative quantification gene expression of AZIN1 was determined using the 2^-ΔΔCq method (25). The assay was run in triplicate for each group to allow for assessment of technical variability. To account for PCR amplification of contaminating genomic DNA, a control without reverse transcription was included. To improve the accuracy of RT-qPCR for quantification, amplifications were performed in triplicate for each RNA sample.

Western blot (WB) analysis. In the present study, an anti-AZIN1 (cat. no. orb154904; Biorbyt, Ltd.) polyclonal anti-rabbit antibodies were used to detect AZIN1 in the WB analysis. Samples were separated by SDS-PAGE on a 12.5% gel. WB was performed as described previously (26). Frozen liver tissue samples were homogenized with three volumes of 10 mM ice cold phosphate buffer (pH 7.0), containing 1 mM ethylenediaminetetraacetic acid, 0.25 M sucrose, 1 mM sodium azide and 0.1 mM phenylmethylsulfonyl fluoride. Samples were centrifuged at 20,000 x g for 30 min at 4˚C. The protein concentration was measured using a bichoronic acid assay (Pierce; Thermo Fisher Scientific, Inc.). The tissue lysate was obtained through electrophoresis by SDS-PAGE on a 12.5% with 60 mg protein loaded per lane, electrotransferred to a polyvinylidine difluoride membrane, the blots were incubated with blocking buffer (1X PBS and 5% nonfat dry milk) for 1 h at room temperature and then probed with primary anti-AZIN1 antibodies (1:1,000; cat. no. orb154904; Biorbyt, Ltd.) overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. GTX213110; GeneTex) for 1 h at RT. To control equal loading of total protein in all lanes, blots were stained with mouse anti-β-actin antibody (1:5,000; cat. no. ab8226; Abcam). The bands were visualized using an enhanced chemiluminescence kit (GE Healthcare) according to the manufacturer's protocol. Finally, the blots were visualized using an ImageQuant™ LAS 4000 system (GE Healthcare) (27).

Immunohistochemistry (IHC) analysis. AZIN1 protein expression was determined using IHC analysis of paraffin-embedded liver sections. Anti-AZIN1 IHC staining was conducted using the LAB-SA Detection System (cat. no. 85-8943; Invitrogen; Thermo Fisher Scientific, Inc.). IHC was performed as described previously (28). Paraffin tissue sections (5 µm thick) were dewaxed, treated with proteinase K enzyme and incubated in 3% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase activity. After being washed in PBS (pH 7.6) for 5 min, the sections were incubated in 0.1% Triton X in PBS with primary anti-AZIN1 antibody (cat. no. orb154904; 1:200 dilution; Biorbyt, Ltd.) at 4˚C for overnight followed by staining with secondary rabbit anti-rat antibody conjugated with horseradish peroxidase (1X reagent B; cat. no. 1454284A; Invitrogen; Thermo Fisher Scientific, Inc.) at room temperature for 20 min. The immunocomplexes were visualized after treatment with 3,3’-diaminobenzidine (cat. no. 00-2014; Invitrogen; Thermo Fisher Scientific, Inc.) solution for 5 min at room temperature. The sections were then washed with PBS (pH 7.6) for further processing (29-31). The AZIN1 protein expression was defined using light microscopy (Leica DM 1000 LED Lab; cat. no. 10052-384; Leica Microsystems, Inc.) at a magnification of x100 or x400.

Statistical analysis. Statistically significant differences in allele/genotype frequencies of AZIN1 SNP (rs1062048) between the T2DM and control groups were determined using the χ² test. Odds ratios were calculated from the genotypic frequency and allelic frequency at 95% CI for the AZIN1 SNP (rs1062048). Statistical analysis was performed using SPSS software (version 11; SPSS, Inc.). Data from three independent experiments are expressed as the mean ± SE. Statistical comparisons between the T2DM and control groups were performed using Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Genotypic and allelic frequencies of AZIN1 gene polymorphisms in patients with T2DM and controls. Table I presents the allelic and genotypic frequencies of the rs1062048 AZIN1 gene polymorphism distribution in patients with T2DM and controls. It was observed that the T allele in the rs1062048 polymorphism was higher in the patients with T2DM (87.6%; 999/1,140) than in the controls (85.2%; 2,897/3,400). The
frequencies of the TT genotype at rs1062048 differed between the control group (72.3%; 1229/1700) and patients with T2DM (77.4%; 441/570). Genotype frequencies were significantly different between T2DM and control groups (P=0.039). The data suggested that the T allele and TT genotype at rs1062048 SNP are risk factors for T2DM.

**Downregulation of AZIN1 gene expression in liver tissues of T2DM mice.** The control (+Dock7+/+Dock7+) and T2DM (+Leprdb/+Leprdb) mice were sacrificed at 4, 16 and 32 weeks of age, and liver tissue RNA was extracted for RT-qPCR. The qPCR data in Fig. 2 shows AZIN1 gene expression in the liver tissues of mice aged 4, 16 and 32 weeks. AZIN1 gene expression in the liver tissues of mice in the T2DM group was significantly lower than that in the control group (P<0.05; Fig. 2). These results suggested that downregulation of AZIN1 gene expression occurred in the T2DM mice.

**Downregulation of AZIN1 protein expression in liver tissues of T2DM mice.** Liver tissues were homogenized and 60 µg protein was used in WB analysis using an anti-AZIN1 antibody and β-actin as a control. Representative blots are shown in Fig. 3A. Samples from the control mice are in lanes 1, 3 and 5 and those from the T2DM mice are in lanes 2, 4 and 6 at 4, 16 and 32 weeks, respectively. Compared with T2DM mice, no matter which stage, the AZIN1 protein expression level was always higher in the control group. In addition, the protein expression levels of AZIN1 were significantly lower in early stage (4th week) and late stage (32nd week) T2DM mouse liver samples when compared with that in control samples (Fig. 3B). These results suggested that downregulation of AZIN1 gene expression occurred in the T2DM mice.

**Association between AZIN1 rs1062048 SNP and clinical features in patients with type 2 diabetic nephropathy (T2DN).** There were 246 patients with T2DM developing into T2DN in the cohort of the present study. A comparison between the clinical features of the patients with T2DN with and without the TT genotype at rs1062048 SNP is detailed in Table II. The two patient groups did not differ significantly in most of terms. However, the levels of hemoglobin A1c, blood urea nitrogen, creatinine, total calcium and triglycerides in the patients with T2DN with and without the TT genotype at rs1062048 SNP were higher than the normal values. In addition, the laboratory tests did not reveal differences between the TT genotype and non-TT genotype groups at rs1062048 SNP in the AZIN1 gene. In addition, a significantly higher level of parathyroid hormone (PTH) was observed in the TT group (202.7±176.62) than in the non-TT group (41.0±8.91; P=0.009; Table II).
Discussion

According to a literature review, the present study was the first to reveal human AZIN1 gene polymorphisms in patients with T2DM. Furthermore, to the best of our knowledge, it was the first to use a time serial T2DM animal model from the early to the late stage obese mice to determine the association between AZIN1 gene expression and protein levels by IHC staining of liver sections. The animal model exhibited the characteristics of T2DM and obesity, namely significant increases in body weight and blood glucose (32,33).

Notably, the qPCR, WB and IHC assay data demonstrated downregulation of AZIN1 gene and protein expression in the T2DM group in liver tissue samples collected from mice of

### Table II. Association between AZIN1 rs1062048 SNP and clinical features in patients with type 2 diabetic nephropathy.

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>TT (n=189), mean ± SD</th>
<th>Non-TT (n=57), mean ± SD</th>
<th>P-value</th>
<th>Normal value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>68.12±12.61</td>
<td>68.28±13.0</td>
<td>0.933</td>
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<tr>
<td>BMI, kg/m²</td>
<td>25.95±5.26</td>
<td>25.38±4.84</td>
<td>0.480</td>
<td>20-25</td>
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<td>Hemoglobin A1c, %</td>
<td>7.22±1.64</td>
<td>6.95±1.34</td>
<td>0.269</td>
<td>3.8-6.0</td>
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<tr>
<td>Blood urea nitrogen, mg/dl</td>
<td>38.59±26.24</td>
<td>32.34±18.07</td>
<td>0.066</td>
<td>7-20</td>
</tr>
<tr>
<td>Creatinine, mg/dl</td>
<td>2.87±3.21</td>
<td>2.56±2.33</td>
<td>0.490</td>
<td>0.6-1.3</td>
</tr>
<tr>
<td>Uric acid, mg/dl</td>
<td>7.16±4.31</td>
<td>6.27±1.59</td>
<td>0.171</td>
<td>2.3-7.6</td>
</tr>
<tr>
<td>Total calcium, mg/dl</td>
<td>10.34±12.36</td>
<td>9.20±0.65</td>
<td>0.558</td>
<td>2.15-2.5</td>
</tr>
<tr>
<td>Phosphorus, mg/dl</td>
<td>4.90±4.10</td>
<td>4.25±0.93</td>
<td>0.316</td>
<td>2.5-5.0</td>
</tr>
<tr>
<td>Parathyroid hormone, pg/ml</td>
<td>202.76±176.62</td>
<td>41.00±8.91</td>
<td>0.009*</td>
<td>12-65</td>
</tr>
<tr>
<td>Albumin, g/dl</td>
<td>4.04±0.50</td>
<td>4.18±0.40</td>
<td>0.077</td>
<td>3.5-5.7</td>
</tr>
<tr>
<td>Cholesterol, mg/dl</td>
<td>172.13±41.61</td>
<td>168.49±41.65</td>
<td>0.609</td>
<td>&lt;200</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>181.54±162.91</td>
<td>173.51±127.95</td>
<td>0.762</td>
<td>&lt;150</td>
</tr>
<tr>
<td>Low density lipoprotein-C, mg/dl</td>
<td>99.32±36.84</td>
<td>88.77±32.39</td>
<td>0.136</td>
<td>&lt;130</td>
</tr>
<tr>
<td>Alanine aminotransferase, IU/l</td>
<td>24.64±16.09</td>
<td>21.74±12.71</td>
<td>0.308</td>
<td>0-41</td>
</tr>
</tbody>
</table>

*Statistically significant. AZIN1, antizyme inhibitor 1; SNP, single nucleotide polymorphism; BMI, body mass index.

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Figure 3. Hepatic AZIN1 protein expression determined by western blot analysis. (A) AZIN1 protein expression levels in the liver tissues of control (lanes 1, 3 and 5) and T2DM (lanes 2, 4 and 6) mice at 4, 16 and 32 weeks, respectively. (B) Densitometric analysis of western blotting of AZIN1 from T2DM mice at 4, 16 and 32 weeks. *P<0.05; **P<0.01. AZIN1, antizyme inhibitor 1; con, control; db/db, mice in the T2DM group.
varying ages (4, 16 and 32 weeks). These findings improved the understanding of the role of AZIN1 gene expression in the pathological features of T2DM. Oka et al (18) reported that AZI binds to ODC, which reduces the cellular polyamine level. ASPC-1 cells are converted into α-cells, which regulate the glucagon gene during pancreatic endocrine cell function and form an islet-like structure (18). AZIN1 effectively competes with ODC and has a higher AZ1 affinity, which restores the ODC activity of polyamine accumulation; in addition, the cellular polyamine level may serve a role in preventing diabetes (34). The animal model in the present study revealed the downregulation of AZIN1 gene and protein expression in T2DM mice. This may indirectly imply that polyamine homeostasis is impaired by a regulatory protein, such as AZ1 or AZIN1, and AZ1 can bind to ODC to reduce polyamine biosynthesis (14). Furthermore, these findings suggest that AZIN1 may serve a crucial role in the T2DM mechanism.

The present study had several limitations. First, the data indicated that the patients with T2DN exhibited a high TT genotype distribution at rs1062048 SNP in the AZIN1. However, to the best of our knowledge, no methods detecting AZIN1 activity to demonstrate the functional associations between rs1062048 SNP and the enzyme activity of AZIN1 are currently available. A second limitation was that although a time serial of the T2DM animal model was used to determine gene transcription and protein expression levels of AZIN1 by WB and IHC staining. Considering the disease/phenotype information from Jackson Laboratory (JAX stock #000642), the phenotype findings in the present study demonstrated that compared with the control, the T2DM mice exhibited more severe fatty change during the time course of their life as they gain weight. Therefore, it is possible that liver dysfunction interfered with AZIN1 expression or its clearance. However, the level of ALT did not differ significantly between the two groups. Therefore, the liver function in all animals appeared consistent. In summary, another dedicated experimental design is required to explore the associations among AZ1/AZIN1/ODC and polyamines in human and animal models.

The data indicated that the increase of the PTH in the patients with T2DN with the TT (risk) genotype was significantly higher than that in the non-TT genotype patients. Recently, Mary et al (35) reported that higher PTH prevalence is associated with increased below-the-knee arterial calcification in patients with T2DM. Therefore, the present study suggested that the patients with T2DN with the TT (risk) genotype were at risk of increased calcification of the inferior knee artery due to a high prevalence of PTH.
In conclusion, the present study explored the downregulation of AZIN1 gene and protein expression in T2DM mice, and demonstrated the association between AZIN1 and T2DM. To the best of our knowledge, the present study was the first to use a time serial animal model to investigate the association between rodent AZIN1 gene expression and the progression of T2DM disease from the gene expression to the phenotype level. Further research is required to understand the mechanisms of AZIN1 at the gene and protein levels, and its association with pancreatic endocrine cells in patients with T2DM.

Acknowledgements

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Funding

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Availability of data and materials

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

Authors' contributions

SYC, CHC, YHW and FJT conceived and designed the study. SYC, CHC, YHW and FJT analyzed and interpreted the data. SYC, CHC, YHW and FJT participated in the drafting of the manuscript. SYC, CHC, YHW, FJT, SFT and TMY revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of China Medical University (IACUC permit no. 2016-221). The human experiments were approved by the Ethics Committee/Institutional Review Board of China Medical University Hospital (approval no. CMUH103-REC2-071). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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