Abstract. Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by persistent hyperglycemia and is associated with serious complications. The risk factors for T2DM include both genetic and lifestyle factors. Genome-wide association studies have indicated the association of genetic variations with many diseases, including T2DM. Glucokinase (GCK) plays a key role in the regulation of insulin release in the pancreas and catalyzes the first step in glycolysis in the liver. Genetic alterations in the GCK gene have been implicated in both hyperglycemia and hypoglycemia. Micro RNAs (miRNAs/miRs) are small non-coding RNA molecules that are involved in the important physiological processes including glucose metabolism. In the present study, the association of the single nucleotide polymorphisms (SNPs) in the GCK, MIR-196A-2 and MIR-423 genes with susceptibility to T2DM in patients from two regions of Saudi Arabia were examined, using the tetra-primer amplification refractory mutation system. The results showed that the AA genotype and the A allele of GCK rs1799884 were associated with T2DM (odds ratio (OR)=2.25, \( P=0.032\) and \( \text{OR}=1.55, \ P=0.021\), respectively. Likewise, the CT genotype and T allele of MIR-196A-2 rs11614913 were associated with an increased risk of T2DM (OR=2.36, \( P=0.0059\) and \( \text{OR}=1.74, \ P=0.023\), respectively). In addition, the CA genotype of MIR-423 rs6505162 C>A was found to be linked with T2DM (OR=2.12 and \( P=0.021\)). It was concluded in the present research study that gene variations in GCK, MIR-196A-2 and MIR-423 are potentially associated with an increased risk of T2DM. These results, in the future, may help in the identification and stratification of individuals susceptible to T2DM. Future longitudinal studies with larger sample sizes and in different ethnic populations are recommended to validate these findings.

Introduction

Diabetes mellitus (DM) is one of the major health issues worldwide and the Kingdom of Saudi Arabia (KSA) has a high prevalence of DM (1,2). In general, there are two types of DM: type 1 DM (T1DM) is caused by the destruction of pancreatic β cells that secrete insulin (3) and type 2 DM (T2DM) which develops by tissue resistance to insulin action and pancreatic β cell dysfunction (3). DM is associated with acute consequences including diabetic ketoacidosis, hyperosmolar hyperglycemic syndrome and chronic complications such as renal failure, blindness, cardiovascular disease and diabetic neuropathy (4). These complications unfortunately result in high rates of morbidity and mortality. Both T1DM and
T2DM are heterogenous and polygenic in nature with distinct characteristics (2,5).

Glucokinase (GCK) or hexokinase IV (EC 2.7.1.2) catalyzes the conversion of glucose to glucose-6-phosphate (step 1 in glycolysis) in the liver and pancreas; and in other cells, this reaction is catalyzed by hexokinase I (6). In hepatocytes, GCK enhances glucose uptake for glycogenesis and energy storage, whereas in the pancreas, GCK senses elevated blood sugar and stimulates the insulin release by pancreatic β cells (6,7). GCK activators enhance the pancreatic secretion of insulin and hence increase hepatic glycogenesis (7.8). The elevated liver glucose output is the main hepatic dysfunction associated with T2DM (8). Genetic variants of the GCK gene have been implicated in gestational diabetes mellitus (GDM) (9‑11), neonatal diabetes (12) and T2DM (13‑15). GCK SNP rs4607517 T>C has been reported to cause T2DM in American Indians (16), and rs1799884 G>A has been associated with T2DM in Dutch (17), French (18) and Moroccan (19) populations.

MicroRNAs (miRNAs/miRs), small non-coding RNA molecules, regulate gene expression and are involved in important physiologic processes (20). miRNA dysfunctions have been implicated in several diseases, such as cancer, cardiovascular disease and diabetes (21‑26). It has been reported that MIR‑196A‑2 is involved in the regulation of insulin signaling pathways (27) and that gene variation in MIR‑196A‑2 can induce T2DM through the regulation of body fat distribution (28). The miR‑423 blood levels are significantly decreased in cases with proliferative diabetic retinopathy (29). The inhibition of miR‑423‑5p decreases gluconeogenesis, reduces insulin resistance and decreases blood glucose (13). In contrast, overexpression of liver miR‑423‑5p increases gluconeogenesis, elevates blood glucose, and enhances the deposition of fat in mice (30).

In the present study, GCK, MIR‑196A‑2 and MIR‑423 genotyping was conducted using Tetra primer-amplification refractory mutation system-based polymerase chain reaction (T-ARMS-PCR) to evaluate the potential clinical association of GCK rs1799884 G>A, MIR‑196A‑2 rs11614913 C>T and MIR‑423 rs6505162 C>A with the development and progression of T2DM in individuals in the Asir and Tabuk regions of Saudi Arabia. This technique is based on the use of sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. It involves a single PCR followed by gel electrophoresis. Designing primers for the mutant [with single nucleotide polymorphisms (SNPs)] and normal (without SNP) alleles allows selective amplification which can be easily analyzed after electrophoresis. It utilizes four primers viz forward outer (FO), reverse outer (RO), forward inner (FI) and reverse inner (RI) primers. The FO/RO primer combination generates the outer fragment of the SNP locus and acts as an internal control for the PCR. The FI/RO and FO/RI primer combinations yield allele-specific amplicons depending on the genotype of the sample used. The inner primers are positioned unequally from the corresponding outer primer to generate amplicons with different sizes and hence easily resolvable in a gel and distinction is made accordingly. T-ARMS PCR is a flexible, rapid and economical SNP detection tool compared to contemporary genotyping tools such as allele-specific PCR (31).

Materials and methods

Study population. This population-based case-control, collaborative study was conducted on 110 T2DM patients and 110 healthy controls. Specimens were collected from Asir and Tabuk regions of Saudi Arabia in the following hospitals: Bisha: Diabetic Center, King Abdullah Hospital, Bisha; Abha: Asir General Hospital, Abha; Tabuk: King Fahd Specialty Hospital, Tabuk. The recruitment period of the patients and controls was from March 2021 to October, 2021. Informed consent was obtained prior to the collection of samples from all patients and control subjects.

Ethical approval. Ethical approval was obtained from the local RELOC Committee of the College of Medicine, University of Bisha (ref. no. UBCOM/H-06-BH-087(04/10), in accordance with the local guidelines which conformed in essence, to the principles of the Helsinki Declaration.

Inclusion criteria. All the study subjects were citizens of Saudi Arabia and included clinically confirmed cases with T2DM (both males and females). The selected patients included those with fasting plasma glucose levels >110 mg/dl and/or those clinically confirmed patients who were on oral hypoglycemic agents or insulin and had fasting glucose levels <110 mg/dl on the day of blood sampling. Patients with random blood glucose >200 mg/dl and/or those clinically confirmed patients who were on oral hypoglycemic agents or insulin and had random glucose levels <200 mg/dl on the day of blood sampling were also included.

Exclusion criteria. The T2DM patients with other significant chronic diseases, such as renal failure, liver cirrhosis and malignancies were excluded from the study. Type 1 diabetes patients were also excluded from the study.

Inclusion criteria for controls. The control subjects were healthy volunteers with no history of diabetes or any major clinical disorders (including dyslipidemia) and had normal fasting and random plasma glucose levels.

Data collection. This study included clinically confirmed cases of T2DM in Saudi Arabia who visited the hospitals in Abha, Bisha and Tabuk regions. This case-control study enrolled 110 subjects with T2DM and 110 normal control subjects for each SNP. T2DM was diagnosed according to the parameters of WHO criteria (who.int/diabetes/publications/Definition%20and%20diagnosis%20of%20diabetes_new.pdf). The various variables that were analyzed from the T2DM patients and controls included the case history, age and sex, duration of T2DM (only for patients), glycated hemoglobin (HbA1c), fasting and random blood glucose levels, total cholesterol, triacylglycerol (TG), high-density lipoprotein-cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) concentrations, total cholesterol/HDL-C ratios and serum creatinine. The biochemical parameters were assayed using standard protocols.

Sample collection from the T2DM patients. Approximately 3 ml of peripheral blood sample was collected in an EDTA
or lavender top tube for all T2DM patients. One aliquot of the blood specimens was immediately stored at -20 to -30°C until further molecular studies. Another aliquot of blood (~2 ml) was collected in a red top tube and immediately sent for biochemical analyses.

Sample collection from healthy controls. All healthy age-matched control specimens were timed around routine blood draws that were part of the routine workout and hence did not require additional phlebotomy. All participants provided a written informed consent form. Approximately 3 ml peripheral blood was collected in EDTA tubes. The blood specimens for molecular studies were immediately stored at -20 to -30°C until further analyses. Another aliquot of blood (~2 ml) was collected in a red top tube and immediately sent for biochemical analyses.

Genomic DNA extraction. Genomic DNA was extracted using DNeasy Blood K (Qiagen GmbH) as per the manufacturer’s instructions. The extracted DNA was dissolved in nuclease-free water and stored at 4°C until use. The quality and integrity of the DNA were checked by NanoDrop™ (Thermo Fisher Scientific, Inc.). All DNA samples from the patients and controls were screened for purity by measuring optical density (OD) at 260 nm (OD260) and 280 nm (OD280). The λ260/λ280 ratios ranged from 1.83-1.99 indicating good quality DNA.

Genotyping of GCK, MIR-196A-2 and MIR-423 genes by T-ARMS-PCR. The primers for GCK rs1799884 G>A and MIR-196A-2 rs11614913 C>T and MIR-423 rs6505162 C>A genes were designed by using primer3 software (version 0.4.0, Whitehead Institute of Biomedical Research). T-ARMS-PCR primers were optimized by gradient PCR. The ARMS-PCR primers for MIR-423 rs6505162 C>A were prepared by following previously used standard procedures (32,33). Reference sequence rs1799884 was used to design the primers for GCK. For MIR-196A-2 rs11614913 C>T and MIR-423 rs6505162 C>T ARMS primers were designed according to previously used procedures (32,33). The primers for all three SNPs are depicted in Table I.

Preparation of the PCR cocktail. T-ARMS-PCR was performed in a reaction volume of 25 µl containing template DNA (50 ng), 0.25 µl primer stock solution FO, RO, FI and RI, containing 5 pmol of each primer and 10 µl from GoTaq® Green Master Mix (cat no M7122; Promega Corp.). The final volume of 25 µl was adjusted by adding nuclease-free ddH2O. Finally, 2 µl of DNA was added from each subject.

Thermocycling conditions. The thermocycling conditions were used at 95°C for 10 min followed by 40 cycles of 95°C for 35 sec, annealing temperature GCK rs1799884 G→A (59°C), MIR-196A-2 rs11614913 C>T (61°C) and MIR-423

| Gene    | Amplicon size | Temperature |
|---------|---------------|-------------|
| GCK OF  | 390 bp        | 59°C        |
| GCK OR  |               |             |
| GCK IF-C| 181 bp        |             |
| GCK IR-A| 252 bp        |             |

| Gene    | Amplicon size | Temperature |
|---------|---------------|-------------|
| MIR-196A-2 OF | 297 bp     | 61°C        |
| MIR-196A-2 OR |           |             |
| MIR-196A-2 IF (T allele)  | 199 bp    |             |
| MIR-196A-2 IR (C allele) | 153 bp     |             |

| Gene    | Amplicon size | Temperature |
|---------|---------------|-------------|
| MIR-423 OF    | 336 bp       | 62°C        |
| MIR-423 OR    |               |             |
| MIR-423 IF (T allele): | 228 bp  |             |
| MIR-423 IR (C allele) | 160 bp     |             |

OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse. GCK, glucokinase.

| Gene    | Amplicon size | Temperature |
|---------|---------------|-------------|
| MIR-196A-2 OF | 297 bp     | 61°C        |
| MIR-196A-2 OR |           |             |
| MIR-196A-2 IF (T allele)  | 199 bp    |             |
| MIR-196A-2 IR (C allele) | 153 bp     |             |

| Gene    | Amplicon size | Temperature |
|---------|---------------|-------------|
| MIR-423 OF    | 336 bp       | 62°C        |
| MIR-423 OR    |               |             |
| MIR-423 IF (T allele): | 228 bp  |             |
| MIR-423 IR (C allele) | 160 bp     |             |

OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse. GCK, glucokinase.

or Lavender top tube for all T2DM patients. One aliquot of the blood specimens was immediately stored at -20 to -30°C until further molecular studies. Another aliquot of blood (~2 ml) was collected in a red top tube and immediately sent for biochemical analyses.

Sample collection from healthy controls. All healthy age-matched control specimens were timed around routine blood draws that were part of the routine workout and hence did not require additional phlebotomy. All participants provided a written informed consent form. Approximately 3 ml peripheral blood was collected in EDTA tubes. The blood specimens for molecular studies were immediately stored at -20 to -30°C until further analyses. Another aliquot of blood (~2 ml) was collected in a red top tube and immediately sent for biochemical analyses.

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Table I. Primer sequences of GCK rs1799884 G>A, MIR-196A-2 rs11614913 C>T and MIR-423 rs6505162 C>A genes.

A, ARMS primer sequences of GCK rs1799884 G>A

| Gene    | Sequence  | Temperature |
|---------|-----------|-------------|
| GCK OF  | 5'-GCTTTCTCCTCTGTTGTTTGGAG-3' | 59°C        |
| GCK OR  | 5'-GTCACAGTGTTGACAAAGGCA-3'   |             |
| GCK IF-C| 5'-CCTGGCCAGGGCTTACTGGGC-3'   | 61 bp       |
| GCK IR-A| 5'-GACAACCAAGGCCCTCTCAGTAA-3' | 62°C        |

B, ARMS primer sequences of MIR-196a-2 rs11614913 C>T

| Gene    | Sequence  | Temperature |
|---------|-----------|-------------|
| MIR-196A-2 OF | 5'-ACCCCTTCCCTTCTCCATCCAGATAGAT-3 | 61°C        |
| MIR-196A-2 OR | 5'-AAAAGCGGCTTCCAGACTTGTCTTG-3 |             |
| MIR-196A-2 IF (T allele)  | 5'-AGTTTTGAACTGGCAACAAAGACGTT-3 |             |
| MIR-196A-2 IR (C allele) | 5'-GACGAAAACCGACTGTATACTCCGG-3 |             |

C, ARMS primer sequences of MIR-423 rs6505162 C>A genes

| Gene    | Sequence  | Temperature |
|---------|-----------|-------------|
| MIR-423 OF    | 5'-TTTTCCCGGATGGAAGCAGCTTGGA-3' | 62°C        |
| MIR-423 OR    | 5'-TTTTGGCCGAACGTATACCCGTTTTCC-3' |             |
| MIR-423 IF (T allele): | 5'-TGAGGCCCCTCAGTCTTGTTCCCCAA-3' |             |
| MIR-423 IR (C allele) | 5'-CAAGCAGGAGGAACTCAAGCAGGAGG-3' |             |

OF, outer forward; OR, outer reverse; IF, inner forward; IR, inner reverse. GCK, glucokinase.
rs6505162 C>A genes (62°C), extension for 72°C for 45 msec and final extension at 72°C for 10 min.

**Gel electrophoresis for GCK amplification.** GCK rs1799884 G>A PCR products were separated on 2% agarose gel stained with 2 µl of SYBR Safe stain (Thermo Fisher Scientific, Inc.) and visualized on a UV transilluminator (Bio-Rad Laboratories, Inc.). Primers FO and RO flank the exon of the GCK rs1799884 G>A gene, resulting in a band of 390 bp to act as a control for DNA quality and quantity. Primers FO and RO amplify a wild-type allele (G allele), generating a band of 181 bp, and primers FO and reverse mutant) generate a band of 252 bp from the mutant allele (A allele). The results are depicted in Fig. 1.

**Gel electrophoresis for MIR-196A-2 amplification.** The amplification products for MIR-196A-2 rs11614913 C>T amplification were separated by electrophoresis through 2% agarose gel stained with 0.5 µg/ml ethidium bromide and visualized on a UV transilluminator. Primers FO and RO flank the exon of the MIR-196A-2 rs11614913 C>T gene, resulting in a band of 297 bp to act as a control for DNA quality and quantity. Primers FO and RO amplify a wild-type allele (C allele), generating a band of 153 bp, and primers FO and RI generate a band of 199 bp from the mutant allele (T allele). The electrophoresis gel image is shown in Fig. 2.

**Gel electrophoresis for MIR-423 amplification.** PCR products were separated on 2% agarose gel stained with 2 µl of SYBR
Safe stain and visualized on a UV transilluminator. Primers FO and RO flank the exon of the MIR-423 rs6505162 C>T gene, generating a band of 336 bp to act as a control for DNA quality and quantity. Primers FO and RO amplify a wild-type allele (C allele), generating a band of 160 bp, and primers FO and RO generate a band of 228 bp from the mutant allele (T allele).

**Statistical analysis.** Group differences were compared using the Student's two-sample t-test or one-way analysis of variance (ANOVA) for continuous variables and the Chi-squared test for categorical variables. Differences in the GCK rs1799884 G>A, MIR-196A-2 rs11614913 C>T and MIR-423 rs6505162 C>A allele and genotype frequencies between groups were evaluated using the Chi-square test. The associations between GCK, MIR-196A-2 and MIR-423 genotypes with the risk of T2DM were estimated by computing the odds ratios (ORs), risk ratios (RRs) and risk differences (RDs) with 95% confidence intervals (CIs). OR was calculated by dividing the odds of the first group by the odds in the second group. The interpretation of the OR depends on whether the predictor is categorical or continuous. ORs that are >1 indicate that the event is more likely to occur as the predictor increases. Odds ratios that are <1 indicate that the event is less likely to occur as the predictor increases. OR >1.0 indicates that the odds of exposure among patients are greater than the odds of exposure among controls. For example, an OR of 1.2 is above 1.0, but is not a strong association while as an OR of 10 suggests a stronger association. Deviation from Hardy-Weinberg disequilibrium (HWD) was calculated by Chi-square (χ²) ‘goodness of fit test’. Allele frequencies among patients and controls were evaluated by using the Chi-square Hardy-Weinberg equilibrium test. A P-value <0.05 was considered as indicative of a statistically significant difference. The univariate and multivariate analyses were calculated by using MedCalc software, version 20.027 (medcalc.org/calc/odds_ratio.php )/SPSS 16.0 (SPSS, Inc.).

**Results**

**Demographic features and baseline characteristics.** The demographic features and the baseline characteristics of the 110 consecutive T2DM patients are summarized in Table II. Of the 110 consecutive patients, 61 were males and 49 were females, 20 patients were ≤40 years of age and 23 were >40 years of age. The age range of the patients was 24-77 years with a mean age of 51.46 years. Among the 110 T2DM patients, 64 had triglycerides (TG) >150 mg/dl and 46 had triglycerides ≤150 mg/dl. The HDL-cholesterol was ≤55 mg/dl in 82 while it was >55 mg/dl in 28 patients, respectively. The LDL-cholesterol was ≤100 mg/dl in 30 while it was >100 mg/dl in 75 patients, respectively. Differences in the mean of the serum lipid profile for HDL-C, LDL-C, total cholesterol and TG were significant between the patient and controls (P=0.0001). A total of 80 T2DM patients had HbA1c >6% and 30 had HbA1c ≤6%.

| Subject characteristics | T2DM group | Control group |
|-------------------------|------------|---------------|
|                        | n   | %   | n   | %   |
| **Sex distribution**    |      |      |      |      |
| Males                   | 61  | 55.45 | 65  | 59.09 |
| Females                 | 49  | 44.55 | 45  | 40.91 |
| **Age distribution (years)** |  |      |      |      |
| Age ≤40                 | 20  | 18.18 | 23  | 20.91 |
| Age >40                 | 23  | 81.82 | 87  | 79.09 |
| **Fasting blood glucose (mg/dl)** |  |      |      |      |
| Glucose ≤110            | 28  | 25.45 | 96  | 87.27 |
| Glucose >110            | 82  | 74.55 | 14* | 12.73 |
| **Association with RBG (mg/dl)** |  |      |      |      |
| RBG ≤200                | 56  | 59.01 | 110 | 100  |
| RBG >200                | 54  | 49.09 | 0   | 0    |
| **Total cholesterol (mg/dl)** |  |      |      |      |
| Cholesterol ≤200        | 60  | 54.55 | 104 | 94.55 |
| Cholesterol >200        | 50  | 45.46 | 6†  | 0.45 |
| **HDL-C (mg/dl)**       |      |      |      |      |
| HDL-C ≤55               | 82  | 74.55 | 110 | 100  |
| HDL-C >55               | 28  | 25.45 | 0   | 0    |
| **LDL-C (mg/dl)**       |      |      |      |      |
| LDL ≤100                | 30* | 28.57 | 107 | 97.27 |
| LDL >100                | 75* | 71.43 | 3†  | 2.73 |
| **TG (mg/dl)**          |      |      |      |      |
| TG ≤150                 | 46  | 41.82 | 110 | 100  |
| TG >150                 | 64  | 58.18 | 0   | 0    |
| **HbA1c**               |      |      |      |      |
| HbA1c ≤6%               | 30  | 27.27 | 110 | 0    |
| HbA1c >6%               | 80  | 72.73 | 0   | 0    |
| **Creatinine (mg/dl)**  |      |      |      |      |
| Creatinine ≤1.35        | 83  | 75.45 | 110 | 100  |
| Creatinine >1.35        | 27  | 24.55 | 0   | 0    |

*14 subjects in the control group had fasting glucose in the range of 112-115 mg/dl. *6 subjects in the control group had total cholesterol in the range of 204-226. *The LDL-cholesterol values were available in 105 patients only. *3 controls had LDL-cholesterol in the range of 102-109. T2DM, type 2 diabetes mellitus; RBG, random blood glucose; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triacylglycerol; HbA1c, glycated hemoglobin.

The serum creatinine values were ≤1.35 mg/dl in 83 and >1.35 in 27 patients, respectively.

**Statistical comparisons of GCK (rs1799884 G>A) genotypes in the T2DM patients and controls.** At the time of analysis, all of the 110 T2DM patients displayed results in gel electrophoresis whereas only 107 healthy controls displayed sharp bands in the
Table III. Statistical comparisons of GCK (rs1799884 G>A) genotypes in the T2DM patients and controls.

| Subjects       | N   | GG (%) | GA (%) | AA (%) | χ²  | Df | G   | A     | P-value |
|----------------|-----|--------|--------|--------|-----|----|-----|-------|---------|
| T2DM patients  | 110 | 26 (23.7) | 43 (39) | 41 (37.3) | 8.4 | 2  | 0.43 | 0.57 | 0.0150* |
| Controls       | 107 | 30 (28)  | 56 (52.33) | 21 (19.62) |     |    | 0.54 | 0.46 |         |

*P<0.05 (statistically significant). T2DM, type 2 diabetes mellitus; GCK, glucokinase.

Table IV. Statistical comparisons between T2DM patients and controls for GCK (rs1799884 G>A) genotypes using multivariate analysis.

| Mode of inheritance | Controls (N=107) | Patients (N=110) | OR (95% CI) | RR (95% CI) | P-value |
|---------------------|------------------|------------------|-------------|-------------|---------|
| Co-dominant         |                  |                  |             |             |         |
| GCK-GG              | 30               | 26               | (ref.)      | (ref.)      |         |
| GCK-GA              | 56               | 43               | 0.86 (0.46 to 1.71) | 0.94 (0.71 to 1.27) | 0.7100 |
| GCK-AA              | 21               | 41               | 2.25 (1.07 to 4.74) | 1.58 (1.03 to 2.42) | 0.0320* |
| Dominant            |                  |                  |             |             |         |
| GCK-(GA+AA)         | 77               | 84               | (ref.)      | (ref.)      |         |
| Recessive           |                  |                  |             |             |         |
| GCK (GG+GA)         | 86               | 69               | (ref.)      | (ref.)      |         |
| GCK-AA              | 21               | 41               | 2.43 (1.32 to 4.49) | 1.63 (1.13 to 2.38) | 0.0045* |
| Allele              |                  |                  |             |             |         |
| GCK-G               | 116              | 95               | (ref.)      | (ref.)      |         |
| GCK-A               | 98               | 125              | 1.55 (1.07 to 2.27) | 1.25 (1.03 to 1.52) | 0.0210* |

*P<0.05 (statistically significant). Only 107 control samples displayed sharp bands in the gel electrophoresis. T2DM, type 2 diabetes mellitus; GCK, glucokinase; OR, odds ratio; RR, risk ratio; CI, confidence interval.

In gel. As such only 107 results were included for the analyses. The results indicated that there were significant differences in genotype distribution of the GCK rs1799884 G>A genotypes between T2DM patients and controls (P<0.015) (Table III). The frequency of the genotypes (GG, GA and AA) between the patients and controls was 23.7%, 39 and 37.3 and 28, 52.3 and 19.7%, respectively. A higher frequency of the A allele (0.57) was reported in T2D patients in comparison to the healthy controls (0.46).

Multivariate analysis to estimate the association between GCK genotypes and risk to T2DM. The presented study, significantly, yielded the following results. a) The AA genotype was associated with T2DM with OR=2.25 (1.071 to 4.737), RR=1.58 (1.034 to 2.418), P<0.0320 (Table IV). b) The A allele of the rs1799884 G>A was associated with T2D with OR=1.55 (1.066 to 2.274), 1.25 (1.032 to 1.515), P<0.0210 (Table IV). c) There was a significant difference (P<0.05) in genotype distribution of rs1799884 G>A between males and females (Table V). d) There were significant differences in rs1799884 G>A genotype distribution between patients with normal and elevated fasting and random glucose and HbA1c (P<0.05) (Table V). e) Finally, there were significant differences in the rs1799884 G>A genotype distribution between patients with normal and abnormal lipid profiles (Table V).

Association of MIR-196A-2 rs11614913 C>T genotypes with T2DM. At the time of analysis, out of 110, only 100 T2DM patient samples gave sharp bands in gel electrophoresis for MIR-196A-2 rs11614913 C>T genotyping. Similarly, for controls only 100 displayed sharp bands. The results in this analysis indicated there was a significant difference (P<0.0190) in the MIR-196A-2 rs11614913 C>T genotype between patients and controls (Table VI).

Multivariate analysis to estimate the association between MIR-196A-2 rs11614913 C>T genotypes and T2DM risk. Results showed that MIR-196A-2 rs11614913 CT genotype was associated with T2DM with OR=2.36 (1.2816 to 4.348), RR=1.57 (1.1124 to 2.225), P=0.0059 (Table VII). The T allele of the MIR-196A-2 rs11614913 was associated with T2DM with OR=1.74 (1.0787 to 2.807), RR=1.35 (1.0217 to 1.787), P=0.023 (Table VII).

Statistical correlation of MIR-196A-2 rs11614913 C>T genotypes with patient characteristics. The results indicated that
there were significant differences in the MIR-196A-2 rs11614913 genotype distribution between patients with normal and those with elevated random blood glucose (RBG) and HbA1c (P=0.0050 and =0.0380, respectively) (Table VIII).

Association of MIR-423 rs6505162 C>A gene variation with T2DM. At the time of analysis, out of 110, only 100 T2DM patient samples gave sharp bands in gel electrophoresis. Similarly, for the controls only 107 displayed sharp bands. As such only 107
Table VII. Statistical comparisons between T2DM patients and controls for MIR-196A-2 rs11614913 C>T genotypes using multivariate analysis.a.

| Genotypes          | Healthy controls | T2DM cases | OR (95% CI) | RR     | P-value |
|--------------------|------------------|------------|-------------|--------|---------|
| Codominant         | (N=100)          | (N=100)    |             |        |         |
| MIR-196A-2-CC      | 70               | 51         | 1 (ref.)    | 1 (ref.) |        |
| MIR-196A-2-CT      | 25               | 43         | 2.36 (1.28 to 4.35) | 1.57 (1.11 to 2.23) | 0.0059c |
| MIR-196A-2-TT      | 05               | 06         | 1.64 (0.48 to 5.69) | 1.27 (0.65 to 2.47) | 0.4300  |
| Dominant           |                  |            |             |        |         |
| MIR-196A-2-CC      | 70               | 51         | 1 (ref.)    | 1 (ref.) |        |
| miR-196-CT+TT      | 30               | 49         | 2.24 (1.25 to 4.01) | 1.52 (1.11 to 2.09) | 0.0060c |
| Recessive          |                  |            |             |        |         |
| MIR-196A-2-(CC+CT) | 95               | 98         | 1 (ref.)    | 1 (ref.) |        |
| MIR-196A-2-CT      | 05               | 06         | 1.16 (0.34 to 3.94) | 1.08 (0.56 to 2.10) | 0.8000  |
| Allele             |                  |            |             |        |         |
| MIR-196A-2-C allele| 165              | 149        | 1 (ref.)    | 1 (ref.) |        |
| MIR-196A-2-T allele| 35               | 55         | 1.74 (1.08 to 2.81) | 1.35 (1.02 to 1.78) | 0.0230  |
| Over-dominant      |                  |            |             |        |         |
| MIR-196A-2-CC+TT   | 75               | 57         | 1 (ref.)    | 1 (ref.) |        |
| MIR-196A-2-CT      | 25               | 43         | 2.26 (1.24 to 4.13) | 1.54 (1.09 to 2.18) | 0.0070c |

*aMultivariate analyses was calculated by using MedCalc's software/SPSS 16.0 https://www.medcalc.org/calc/odds_ratio.php. bOnly 100 T2DM patient and control samples gave sharp bands in the gel electrophoresis for MIR-196A-2 rs11614913 C>T genotyping. cP<0.05 (statistically significant). T2DM, type 2 diabetes mellitus; OR, odds ratio; RR, risk ratio; CI, confidence interval.

results were included for the analyses. The results indicated that there was a significant difference in genotype distribution of the MIR-423 rs6505162 C>A genotypes between T2DM patients and controls (P<0.0240) (Table IX). The frequency of CC, CA and AA genotypes was 23, 67 and 10% for patients, and 35, 48 and 17% for the controls, respectively. A higher frequency of C allele (0.62) was reported in T2DM patients than among the healthy controls (0.59) (Table IX).

Multivariate analysis to estimate the association between MIR-423 rs6505162 C>A gene genotypes and risk to T2DM. The results showed that the CA genotype was associated with T2DM with OR=2.12 (1.1160 to 4.0426), RR=1.44 (1.0708 to 1.952), P<0.0210 in the codominant model (Table X). The results also indicated that there were no significant differences in the MIR-423 rs6505162 C>A genotypes in dominant, recessive and over-dominant alleles (Table X).

Association of MIR-423 rs6505162 C>A with T2DM patient characteristics. The statistical comparisons (P-values) of MIR-423 rs6505162 C>A genotypes with comorbid conditions and T2DM severity was conducted by using multivariate analysis based on logistic regression such as odds ratio (OD) and risk ratio (RR) with 95% confidence intervals (CI) (Table XI). A significant correlation was reported between the MIR-423 rs6505162 C>A genotypes and the age of the subjects (P<0.0001). A significant correlation was reported between the MIR-423 rs6505162 C>A genotypes with biochemical parameters such as fasting glucose, RBG, total serum cholesterol, LDL-C, TG and HbA1c.

Discussion

Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by hyperglycemia resulting from impaired insulin action caused by insulin resistance in the liver, muscles and adipose tissues (3,34). Insulin resistance leads to hyperinsulinemia and pancreatic β cell dysfunction (34). Glucokinase is very important for glucose homeostasis, since it is essential for insulin secretion, energy storage as glycogen, and gluconeogenesis (35). The rs1799884 SNP is found in the specific promoter region of the glucokinase (GCK) gene (14). The results revealed that there was a significant difference in rs1799884 G>A genotype distribution between the T2DM patients and the controls. The a allele of the rs1799884 G>a was also associated with T2DM (Table V). This result is consistent with previous studies that indicated the association of rs1799884 with an increased fasting blood glucose concentration and susceptibility to T2DM (13‑16). Simultaneously, the result is also in agreement with previous studies as well which reported that i) rs1799884 influences glucokinase activity and that the reduced glucokinase activity is associated with T2DM (14,36‑39), and ii) rs1799884 SNP is associated with dyslipidemia and coronary artery disease (CAD) in Han Chinese and Austrian populations (40,41).

The present results also revealed that rs1799884 GA and AA genotypes were associated with hyperlipidemia. Hyperlipidemia and cardiovascular diseases are among the traditional complications of diabetes mellitus (38‑40). This result is substantiated by a recent study by Ormazabal et al, who reported that the systemic metabolism of lipids is altered in the insulin resistance that leads to the so-called lipid triad; hypertriglyceridemia, reduced HDL and the development of small dense LDL (41).
analysis by ethnicity, significant associations have been found in
Caucasians for the polymorphism in all genetic models; while
no associations were detected among Asians (13,19). There are
several possible reasons for such differences. First, the distribu-
tion of the a allele varies extensively between different races,
ethnicities, with a prevalence of ~23% among Asians and ~17%
among Caucasians. The frequency of three genotypes GG, Ga,
aa between the T2dM patients and controls was found to be
23, 39 and 37.3% and 28, 52.3 and 19.7% respectively. A higher
frequency of a allele (0.57) was reported in our T2dM cases
when compared with the healthy controls (0.46). Therefore,
additional studies are warranted to further validate the ethnic
difference in the effect of this polymorphism on T2dM risk.

The results on MIRNA SNPs showed that the CT genotype
and the T allele of MIR-196A-2 rs11614913 were associated
with T2DM. This result is in agreement with a recent study that
reported MIR-196A-2 rs11614913 to be associated with T2DM
in a Pakistani population (42). The MIR-196A-2 rs11614913 C>T
SNP has been reported to influence the expression of mature
mRNAs by binding with target mRNAs (25,43), and the T allele
is associated with reduced mature mir-196a-2 levels (44). It has
been reported that mir-196a-2 directly targets and inhibits the
expression of Scm like with four Mbt domains 1 (SFMBT1)
and homeobox c8 (HOXC8) genes (43,44). The HOXC8
gene was reported to increase white fat cells and the susceptibility
to obesity (44), while SFMBT1 was demonstrated to be
among the adiponectin level-regulating loci (45). The blood
levels of adiponectin are genetically determined and correlate
negatively with the susceptibility to T2dM and cardiovascular
diseases (45). It has also been suggested that a reduction
in the mature mir-196a-2 by rs11614913 T allele increases
the expression of HOXC8 and SFMBT1 leading to obesity

Table VIII. Association of MIR-196A-2 rs11614913 C>T SNP genotypes with the T2DM patient characteristics.

| Subject characteristics | N=100 | CC | CT | TT | \(\chi^2\) | df | P-value |
|-------------------------|-------|----|----|----|----------|----|---------|
| Association with sex    |       |    |    |    |          |    |         |
| Males                   | 61    | 30 | 27 | 04 | 0.24     | 2  | 0.8800  |
| Females                 | 39    | 21 | 16 | 02 |          |    |         |
| Association with age (years) |       |    |    |    |          |    |         |
| Age ≤40                 | 18    | 8  | 8  | 2  | 2.4      | 2  | 0.3000  |
| Age >40                 | 82    | 40 | 36 | 6  |          |    |         |
| Fasting glucose (mg/dl) |       |    |    |    |          |    |         |
| Glucose ≤110            | 21    | 13 | 6  | 02 | 2.6      | 2  | 0.2900  |
| Glucose >110            | 79    | 38 | 37 | 04 |          |    |         |
| Association with RBG (mg/dl) |     |    |    |    |          |    |         |
| RBG ≤200                | 52    | 22 | 23 | 07 | 10.55    | 2  | 0.0050a |
| RBG >200                | 48    | 26 | 15 | 07 |          |    |         |
| Association with total cholesterol (mg/dl) |     |    |    |    |          |    |         |
| Cholesterol ≤200        | 57    | 19 | 34 | 4  | 19.89    | 2  | 0.0002a |
| Cholesterol >200        | 43    | 32 | 09 | 02 |          |    |         |
| Association with HDL-C (mg/dl) |     |    |    |    |          |    |         |
| HDL-C ≤55               | 71    | 16 | 13 | 02 | 03      | 2  | 0.9800  |
| HDL-C >55               | 29    | 35 | 30 | 04 |          |    |         |
| Association with LDL-C (mg/dl) |     |    |    |    |          |    |         |
| LDL ≤100                | 28    | 21 | 05 | 02 | 10.11    | 2  | 0.0060a |
| LDL >100                | 72    | 30 | 38 | 04 |          |    |         |
| Association with TG (mg/dl) |     |    |    |    |          |    |         |
| TG ≤150                 | 37    | 24 | 10 | 03 | 6.13     | 2  | 0.0470a |
| TG >150                 | 63    | 27 | 33 | 03 |          |    |         |
| Association with HBA1c % |       |    |    |    |          |    |         |
| HBA1c ≤6                | 24    | 10 | 10 | 4  | 6.54     | 2  | 0.0380  |
| HBA1c >6                | 76    | 41 | 33 | 2  |          |    |         |
| Association with creatinine (mg/dl) |     |    |    |    |          |    |         |
| Creatinine ≤1.35        | 76    | 29 | 33 | 14 | 4.11     | 2  | 0.1200  |
| Creatinine >1.35        | 24    | 22 | 10 | 02 |          |    |         |

*P<0.05 (statistically significant). T2DM, type 2 diabetes mellitus; RBG, random blood glucose; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triacylglycerol; Hba1c, glycated hemoglobin.
probably by the promotion of white fat cells (44). Obesity is a well-established risk factor for insulin resistance and the development of T2DM (46). The results indicated that there were significant differences (P<0.05) in MIR‑196A‑2 rs11614913 c>T SNP genotype distribution between the subjects with normal and abnormal lipid profiles.

Human miR‑196 (mir‑196a‑1, mir‑196a‑2, and mir‑196b) is transcribed from three different genes located on chromosomes 17q21, 12q13, and 7p15, respectively. The nucleotide sequences of mir‑196a‑1 and mir‑196a‑2 are identical, while the sequence of mir‑196b differs from that of mir‑196a by only one nucleotide in the non‑seed region. Previous research has shown that the expression level of mature mir‑196a‑3p is higher in cc carriers with lung cancer compared to cT and TT individuals (47). Hoffman et al reported elevated expression of mature miR‑196a‑2 forms in MCF‑7 cells transfected with a pre‑miR‑196a‑c vector when compared with cells transfected with a pre‑miR‑196a‑T vector (43). It is plausible to believe that rs11614913 C>T SNP may affect the binding efficiency of mir‑196a‑2 to its target mRNA or it might affect the processing of the pre‑miRNA into its mature form, thereby predisposing the individuals to T2DM (48).

It was observed that the cases with a c T genotype had low serum cholesterol and TG values. Since this is a cross sectional study, it is possible that these cases have received cholesterol‑lowering medications and their normal lipid profiles were already maintained prior to the sample collection. This result is rather expected as the MIR‑196A‑2 rs11614913 C>T SNP has been associated with cardiovascular disease (cVD) in previous studies in different populations (49‑52).

Table IX. Association of MIR‑423 rs6505162 C>A gene variation in T2DM patients and controls.

| Subjects  | N  | CC (%) | CA (%) | AA (%) | Df | χ²  | C   | A   | P‑value |
|-----------|----|--------|--------|--------|----|-----|-----|-----|--------|
| Patients  | 100| 23 (23)| 67 (67)| 10 (10)| 2  | 7.44| 0.62| 0.38| 0.0240 |
| Controls  | 100| 35 (35)| 48 (48)| 17 (17)|   | 0.59| 0.59| 0.41|        |

*P<0.05 (statistically significant). T2DM, type 2 diabetes mellitus.

Table X. Multivariate analysis to estimate the association between MIR‑423 rs6505162 C>A gene genotypes and risk to T2DM.

| Genotypes | Healthy controls (N=100) | T2DM patients (N=100) | OR (95% CI) | RR (95% CI) | P‑value |
|-----------|--------------------------|-----------------------|-------------|-------------|--------|
| Codominant |                          |                       |             |             |        |
| MIR‑423‑CC | 35                       | 23                    | 1 (ref.)    | 1 (ref.)    |        |
| MIR‑423‑CA | 48                       | 67                    | 2.12 (1.12 to 4.04) | 1.44 (1.07 to 1.95) | 0.0210 |
| MIR‑423‑AA | 17                       | 10                    | 0.89 (0.35 to 2.29) | 0.95 (0.67 to 1.37) | 0.8100 |
| Dominant   |                          |                       |             |             |        |
| MIR‑423‑CC | 35                       | 23                    | 1 (ref.)    | 1 (ref.)    |        |
| MIR‑423‑(CA+AA) | 65                   | 77                    | 1.80 (0.97 to 3.35) | 1.31 (1.01 to 1.74) | 0.6300 |
| Recessive  |                          |                       |             |             |        |
| MIR‑423‑(CC+CA) | 83                  | 90                    | 1 (ref.)    | 1 (ref.)    |        |
| MIR‑423‑AA | 17                       | 10                    | 0.54 (0.24 to 1.25) | 0.76 (0.55 to 1.06) | 0.1500 |
| Allele     |                          |                       |             |             |        |
| MIR‑423‑C  | 118                      | 113                   | 1 (ref.)    | 1 (ref.)    |        |
| MIR‑423‑A  | 82                       | 87                    | 1.10 (0.75 to 1.65) | 1.05 (0.86 to 1.29) | 0.6100 |
| Over‑dominant |                     |                       |             |             |        |
| MIR‑423‑CC+AA | 52                   | 33                    | 1 (ref.)    | 1 (ref.)    |        |
| MIR‑423‑A  | 17                       | 10                    | 0.92 (0.38 to 2.27) | 0.97 (0.69 to 1.36) | 0.8600 |

*P<0.05 (statistically significant). T2DM, type 2 diabetes mellitus; OR, Odds ratio; RR, risk ratio; CI, confidence interval.
MIR-196A-2 rs11614913 affects the maturation of mir-196a-2 and its interaction with its target mRNAs (55,56). The rs6505162 C>A is located in the pre-miRNA sequence of MIR-423 that expresses two microRNAs, MIR-423-3P and MIR-423-5P (52). The current results showed that there was a significant difference in MIR-423 rs6505162 C>A genotype distribution between T2 DM patients and controls, and that the ca genotype of the MIR-423 rs6505162 C>A was associated with T2dM. The a allele of rs6505162 has been reported to increase the expression of the mature mi r-423 (53,54). The result of this study is quite consistent with the study by Yang et al who reported that in obese diabetic mice suppression of liver mir-423-5p inhibits gluconeogenesis and ameliorates insulin resistance, and promotes blood sugar and fatty liver (30). They further reported that the overexpression of mir-423-5p enhanced gluconeogenesis, increased blood glucose levels and obesity in healthy mice through the suppression of the hepatic FAM3A/ATP/Akt pathway (30). However, the result is in disagreement with a study that reported no association of rs6505162 with the induction of T2DM in the Pakistani population (42). This dissimilarity of findings is probably due to different subject ethnicity and sample size and requires further validation. The present results showed that there were significant differences in rs6505162 genotype distribution in cases with normal and abnormal lipid profiles. This result is rather expected as the rs6505162 SnP has been associated with cardiovascular disease (57,58). The result of this study is also consistent with studies that demonstrated that the over-expression of miR-423-5p enhanced fat deposition and that miR-423-5p is specifically increased in the blood of heart failure subjects (30,58).

In the present study Tetra primer-amplification refractory mutation system-based polymerase chain reaction (T-ARMS-PCR) was successfully used, although
the genotyping methods including high-resolution melting (HRM), pyrosequencing, TaqMan assay, Mass ARRAY are highly accurate and acknowledged as gold standard for detecting SNPs but require expensive equipment and kits. The other alternative methods that could have been used include quantitative PCR, PCR-RFLP and direct sequencing but T-ARMS-PCR has been reported to be cost-effective, reliable and simple (31). The results of T-ARMS-PCR have been reported to be consistent with DNA sequencing results by Jin et al (58) that reiterates our belief that T-ARMS-PCR can offer a viable, simple and reliable alternative for the detection of SNPs.

To conclude, the SNPs of GCK rs1799884 G>A, MIR-196A-2 rs11614913 C>T, MIR-423 rs6505162 were examined for their association with T2DM in a section of the Saudi population by using T-ARMS-PCR. The results indicated that the AA genotype and the A allele of the GCK rs1799884 G>A were strongly associated with T2DM susceptibility in the patient population. The results also indicated that the MIR-196A-2 rs11614913 CT genotype and T allele and MIR-423 rs6505162 CA genotype were also associated with T2DM. Since this is the first study of its kind in Saudi Arabia, the results will help in uncovering more loci that are associated with T2DM in different ethnic populations and the stratification of individual susceptible to T2DM. The limitations of this study include the small sample size and no strict age matching between patients and healthy controls. More longitudinal studies with larger sample sizes and in different ethnic populations are recommended to further validate these observations.

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Availability of data and materials

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

Authors’ contributions

All the authors were involved in the conception and planning of the study. MMM, RM, MAAA, MJ, VM and MHA designed the study. MAAA, JJW, ZUS, MA and AMA were involved in the recruitment of patients. MMM, RM, MJ and IE performed the experiments. RM and MMM confirm the authenticity of all the raw data. MMM, RM and IE wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the local RELOC Committee of the College of Medicine, University of Bisha (ref. no. UBCOM/H-06-BH-087/04/10), in accordance with the local guidelines which conformed in essence, to the principles of the Helsinki Declaration. Informed consent was obtained prior to the collection of samples from all patients and control subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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