Association of CDKN2A/B gene polymorphisms (rs10811661 and rs2383208) with type 2 diabetes mellitus in a sample of Iraqi population

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Abstract
Background: Type 2 diabetes mellitus (T2DM) is chronic metabolic disorder manifested by increased blood glucose (hyperglycemia) due to pancreatic β-cell dysfunction and/or decreased sensitivity of peripheral tissue to insulin. T2DM is a multifactorial disease that may result from interaction of environmental and genetic factors.

Methods: A case–control study consisting of 400 T2DM patients in addition to 400 as control. Phenotyping as well as anthropometric data included body mass index BMI, fasting plasma glucose (FPG), serum total cholesterol, serum triglyceride, VLDL, LDL, HDL insulin levels and Homeostatic Model Assessment for Insulin Resistance HOMA-IR were estimated for the two groups. PCR–RFLP was used to carry out genotyping of CDKN2A/B gene (rs10811661 T>C and rs2383208 A>G) SNPs.

Results: For rs10811661 SNP the genotype and allele frequencies of CDKN2A/B gene for T2DM and control subjects showed that the co-dominant model in patients with the homozygous (TT) was found to be significantly higher than those in control group. In contrast, the heterozygous genotype (TC) did not reveal this significance ANOVA test for mean comparison of biochemical markers under the co-dominant model of rs10811661 SNP genotype in CDKN2A/B gene, revealed a significant difference for insulin (P < 0.0001) and HOMA-IR (P < 0.0001) in T2DM group as compared to control one; However (rs2383208) SNP did not show any significant association with T2DM and with the measured biochemical marker at any model.

Conclusions: CDKN2A/B gene rs10811661 SNP was implicated in T2DM pathogenesis in this sample of Iraqi population also it affects insulin level in those patients, whereas the rs2383208 SNP did not impact the disease.

Keywords: CDKN2A/B, T2DM, Polymorphism

Introduction
Type 2 diabetes mellitus T2DM is a chronic metabolic disorder manifested by increased blood glucose (hyperglycemia) due to pancreatic β-cell dysfunction and/or decreased sensitivity of peripheral tissue to insulin [9]. This condition occurs due to increased insulin resistance (IR) that is manifested especially in muscle, liver and fat cells [1]. Among the external risk factors are mainly obesity (BMI over 30), which demonstrate strong association with the development of insulin resistance [16]. Insulin resistance may be caused by several causes from the deficiency of the insulin receptor (its number and function) to glucose transporters damage [13], [2]. Genetically
insulin receptor defects represent the most severe form of insulin resistance [6].

T2DM is a multifactorial disease that may results from an interaction between environmental and genetic factors [9], [11]. Genome wide association studies (GWAS) of T2DM have identified several single nucleotide polymorphism SNPs in genes that predisposing to T2DM [5], of these genes are Cyclin-dependent kinase inhibitor 2A/2B genes CDKN2A/B which located on chromosome 9p21. CDKN2A/B locus human gene encodes proteins: p15 and p16 inhibitors for cyclin dependent kinase 4 (p15INK4b and p16INK4a) [10].

CDKN2B gene away about 30,000 bp from CDKN2A gene and the latter gene encode p16INK4a and p14ARF proteins while CDKN2B encodes p15INK4b protein. p16INK4a and p15INK4b considered as, tumor suppressor proteins which inhibit cyclin-dependent kinase 4 CDK4 in addition to CDK6 [12].

CDK4, act as a powerful regulator for replication of beta cells in pancreas [26]. The studies on murine model showed that any increased in expression of p15INK4b is associated with the development of hypoplasia in exocrine and endocrine glands [18]. Mice with inhibition of CDK4 may develop diabetes due to insulin deficiency because of a decrease in pancreatic β cells number [15, 21]. These findings collectively suggest that the polymorphisms of the CDKN2A/B genes may increases the likelihood of type 2 diabetes [12].

The objectives of this study were to evaluate the association of CDKN2A/B gene polymorphisms (rs10811661 and rs2383208) with type 2 diabetes mellitus in Iraqi population.

Materials and methods

Study subjects

The study design is a case–control, consisting of 400 T2DM patients in addition to 400 individuals as control. The specimen collections were done from Oct. 2019 till Jan. 2020. The analysis of phenotyping and genotyping were done in the postgraduate laboratory of department of biochemistry in faculty of medicine in Kufa University. The selection of sample size was done according to online software (osse.bii.a-star.edu.sg/calculation1.php).

A total of 400 type 2 diabetes individuals were enrolled in present study (205 female and 195 male). Patients varied in age from 30 to 69 years old, the mean of age equal to 52.93 ± 7.00 years as SD. Specialist physicians have examined the patients. They were randomly selected from AL-Sader Teaching Hospital in Al Najaf Province. For the patients to be included in this study, the diagnosis of T2DM according to WHO criteria. Exclusion criteria were adopted to deport patients who have the following:

- Type 1 DM.
- Those who taking insulin.

A second group consist of 400 normal individuals were randomly selected from the general population (205 males and 195 female) as control group.

From all participants in this study a 5 ml of blood were taken by venipuncture and collected in two tubes, 3.0 ml of 5 placed in plain tube (without anticoagulant) and the remaining quantity (2 ml) has been placed in tube with EDTA. The first tube (plain tube) was centrifuged at 2000 × g for 10 min after being allowed to coagulate at room temperature for 10–15 min. The serum that obtained from previous step was stored at −20 °C until analysis for phenotyping estimation. Extraction of DNA was done from other tube that contains EDTA the samples of DNA were maintained frozen at −20 °C until gene polymorphisms were examined.

Informed consent has been taken from patients and control group before sampling, the protocol of this study was approved by the Kufa Medical Faculty Ethical Committee.

Measurements

Anthropometric data as BMI and biochemical parameters such as FPG, serum total cholesterol, serum triglyceride, VLDL, LDL, HDL insulin levels and HOMA-IR were measured for all individuals participating in the study. Serum total cholesterol, triglyceride, HDL in addition to fasting blood glucose estimated according to "standard enzymatic colorimetric assay" method in contrast LDL and VLDL were calculated by using mathematical formulas. Enzyme-linked immunosorbent assay ELISA was used to estimate insulin concentration whereas HOMA-IR was used for Insulin Resistance assessment.

Genotyping

DNA purification kit (G-spin) was used for DNA extraction from blood samples for all individuals participating in the study. Polymerase chain reaction–Restriction Fragment Length Polymorphism (PCR–RFLP) technique were used to determine CDKN2A/B rs10811661 T>C and rs2383208 A>G gene polymorphisms.

The primers of SNPs that used in current study were designed by Primer3plus software as the following:

**For rs10811661 SNP:**

Forward primer was 5′-CCGGCCCATTTTCTT TGTCA-3′
Reverse primer was 5′-CAAGCGCTGGATC ATAGG-3′
For rs2383208 SNP:

Forward primer was 5′-CTGTTACTGGCTGGG
GAAC-T-3′
Reverse primer was 5′-TGTCTGCAACCACCTGG
AGGTA-3′

The conditions of PCR–RFLP technique

A total volume of DNA amplification reaction was 25 μl which consist of GoTaq® Green Master Mix from Promega (12.5 μl), forward and reverse primer (1.5 μl) from both of them, genome DNA (5 μl) then Complete the final volume by nuclease-free water (4.5 μl). The conditions of thermo-cycle for rs10811661 SNP amplification was initial denaturation (5 min at 95 °C) for one cycle then for 30 cycles included denaturation step (1 min at 95 °C), annealing step (1 min at 62 °C) and extension step (1 min at 72 °C) then final extension step (10 min. at 72 °C). The product of PCR was 232 bp detected by 2% agarose gel electrophoresis. The same programed protocol of PCR reaction done for rs2383208 SNP, while the amplification protocol was as following: initial denaturation (5 min at 95 °C) then 35 cycles consisting of denaturation step (30 s at 95 °C), annealing step (30 s at 59.3 °C) and extension step (30 s at 72 °C) then the final extension step (5 min. at 72 °C). Also the size of PCR product also detected by 2% agarose gel electrophoresis which was 246 bp. PCR product for both SNPs were digested by specific restriction enzymes. For rs10811661 SNP the restriction enzymes was BspHI, the recognition sequence T^CATGA, the digestion temp. was 37 °C and the digestion time was 8 h. while for rs2383208 SNP the restriction enzymes was Hpy166II, the recognition sequence GTN^NAC.

The digestion temp. was 37 °C and the digestion time was 15 min. Digested fragments electrophoresed in 3% agarose gel agarose (75 V and 120 min) with diamond stain and visualized under UV light.

Statistical analysis

For statistical analysis, SPSS version 23 was used. Phenotypic data were expressed as mean±SD, T test used to determine the significance between the two groups. ANOVA test was used to comparing mean levels for continuous characteristics across genotypes Chi-square test was used to determine genotype frequencies among T2DM patients and healthy controls. The Hardy Weinberg Equilibrium equation was used to look at allelic frequencies. The associations between CDKN2A/B (rs10811661 and rs2383208) genotype with T2DM risk were estimated by calculating odds ratio with 95% CI using multinominal logistic regression analysis. P values of less than 0.05 were regarded significant.

Results

The anthropometric and biochemical parameters result for the two groups were shown in Table 1. The results revealed significant differences for all parameters (except gender and age) between T2DM and control group.

RFLP analysis revealed that PCR product of rs10811661 SNP of CDKN2A/B gene was digested by BspHI restriction endonucleases enzyme. The products of digestion were electrophoresed in 3% agarose gel and demonstrate two (164, 68 bp) bands of wild type (TT), one (232 bp) band of homozygous (CC) and three (232, 164, 68 bp) bands of heterozygous (TC) genotypes as illustrated in Fig. 1.

The results of rs10811661 SNP genotype and allele frequencies of CDKN2A/B gene for control and T2DM subjects showed that the co-dominant model in patients with the homozygous (TT) was found to be significantly (OR 2.51, 95% CI 1.47–4.24, P = 0.004) higher than those in control group. In contrast, the heterozygous genotype (TC) did not reveal this significance (OR 1.14, 95% CI 0.77–2.62, P = 0.13) (Table 2).

The dominant, recessive, and additive style in T2DM persons displayed a significant (OR 1.99, 95% CI 1.11–3.25, P < 0.002), (OR 2.2, 95% CI 1.31–3.69, P < 0.0001) and (OR 2.2, 95% CI 1.31–3.69, P = 0.002) respectively, elevation in comparison to the subjects.

| Parameter | Control | T2DM subject | P value |
|-----------|---------|--------------|---------|
| No. (M/F) | 400 (196/204) | 400 (192/208) | – |
| Age (year) | 53.72±11.19 | 53.68±13.79 | 0.96 |
| BMI (kg/m²) | 25.33±2.10 | 29.53±2.98 | <0.0001 |
| FBS (mg/dl) | 104.32±7.88 | 228.65±35.92 | <0.0001 |
| Cholesterol (mg/dl) | 188.87±17.61 | 254.93±26.42 | <0.0001 |
| Triglyceride (mg/dl) | 135.73±18.39 | 236.77±24.20 | <0.0001 |
| VLDL-C (mg/dl) | 27.15±3.67 | 47.35±4.84 | <0.0001 |
| LDL-C (mg/dl) | 106.89±11.55 | 164.81±14.64 | <0.0001 |
| HDL-C (mg/dl) | 54.83±8.21 | 42.76±9.11 | <0.0001 |
| Insulin (μU/ml) | 12.93±2.61 | 32.85±3.83 | <0.0001 |
| HOMA-IR | 3.33±0.82 | 18.54±1.92 | <0.0001 |
of control group. For T2DM subjects the risk allele (T) frequency was significantly (OR 1.69, 95% CI 1.35–2.11, \( P < 0.0001 \)) elevated in T2DM (78%) with respect to the control individuals (68%) (Table 2). ANOVA test for mean comparison of biochemical markers under the co-dominant model of rs10811661 SNP genotype in CDKN2A/B gene, revealed a significant difference for insulin (\( P < 0.0001 \)) and HOMA-IR (\( P < 0.0001 \)), while BMI (\( P = 0.12 \)), FBG (\( P = 0.68 \)), cholesterol (\( P = 0.81 \)), TG (\( P = 0.51 \)), VLDL-C (\( P = 0.51 \)), HDL-C (\( P = 0.45 \)) and
LDL-C ($P=0.35$) failed to explore any significant difference (Table 4).

On the other hand, the other SNP (rs2383208) in CDKN2A/B gene did not show any significant association between T2DM and this SNP before and after the adjustment for BMI, age and gender (Table 3). In addition to ANOVA test for mean comparison of biochemical markers under the co-dominant model rs2383208 genotype in CDKN2A/B gene didn’t show any significance (table not included).

**Table 2** Distribution of genotypes and allele frequency in CDKN2A/B gene polymorphism (rs10811661) for T2DM and control subjects

| rs10811661 (T/C) | T2DM n = 400 | Control n = 400 | Unadjusted OR (95% CI) | P value | Adjusted OR (95% CI) | P value |
|------------------|--------------|-----------------|------------------------|---------|----------------------|---------|
| **Co-dominant**  |              |                 |                        |         |                      |         |
| CC (reference)   | 23 (6%)      | 44 (11%)        |                        |         |                      |         |
| TT               | 249 (62%)    | 188 (47%)       | 2.53 (1.47–4.34)       | 0.0007  | 2.51 (1.47–4.24)     | 0.004   |
| TC               | 128 (32%)    | 168 (42%)       | 1.45 (0.83–2.53)       | 0.18    | 1.14 (0.77–2.62)     | 0.132   |
| **Dominant**     |              |                 |                        |         |                      |         |
| CC (reference)   | 23           | 44              |                        |         |                      |         |
| TT + TC          | 377          | 356             | 2.02 (1.19–3.42)       | 0.008   | 1.99 (1.11–3.25)     | 0.002   |
| **Recessive**    |              |                 |                        |         |                      |         |
| TT + CC (reference) | 151         | 212             | 1.86 (1.40–2.46)       | <0.0001 | 1.918 (1.48–2.55)    | <0.0001 |
| **Additive**     |              |                 |                        |         |                      |         |
| CC (reference)   | 23           | 44              |                        |         |                      |         |
| 2(TT)+TC         | 626          | 544             | 2.2 (1.31–3.69)        | 0.002   |                      |         |
| **Risk allele frequency (T)** | | | | | | |
| C                | 174 (22%)    | 256 (32%)       | 1.69 (1.35–2.11)       | <0.0001 |                      |         |
| T                | 626 (78%)    | 544 (68%)       |                        |         |                      |         |

**Table 3** Distribution of genotypes and allele frequency in CDKN2A/B gene polymorphism (rs2383208) for T2DM and control subjects

| rs2383208 (A/G) | T2DM n = 400 | Control n = 400 | Unadjusted OR (95% CI) | P value | Adjusted OR (95% CI) | P value |
|------------------|--------------|-----------------|------------------------|---------|----------------------|---------|
| **Co-dominant**  |              |                 |                        |         |                      |         |
| GG (reference)   | 26 (6%)      | 35 (9%)         |                        |         |                      |         |
| AA               | 223 (56%)    | 203 (51%)       | 1.47 (0.86–2.54)       | 0.15    | 1.51 (0.92–2.94)     | 0.11    |
| AG               | 151 (38%)    | 162 (40%)       | 1.25 (0.72—2.18)       | 0.42    | 1.14 (0.68–2.02)     | 0.632   |
| **Dominant**     |              |                 |                        |         |                      |         |
| GG (reference)   | 26           | 35              |                        |         |                      |         |
| AA + AG          | 374          | 365             | 1.37 (0.81–2.33)       | 0.23    | 1.19 (0.72–1.95)     | 0.455   |
| **Recessive**    |              |                 |                        |         |                      |         |
| GG + AG (reference) | 177         | 197             |                        |         |                      |         |
| AA               | 223          | 203             | 1.22 (0.92—1.61)       | 0.15    | 0.998 (0.78—1.26)    | 0.661   |
| **Additive**     |              |                 |                        |         |                      |         |
| GG               | 26           | 35              |                        |         |                      |         |
| 2(AA) + AG       | 597          | 568             | 1.41 (0.84–2.38)       | 0.19    |                      |         |
| **Risk allele frequency (A)** | | | | | | |
| G                | 203 (25%)    | 232 (29%)       | 1.2 (0.96–1.49)        | 0.1     |                      |         |
| A                | 597 (75%)    | 568 (71%)       |                        |         |                      |         |

**Discussion**

The causes and implications of T2DM in humans have been extensively researched in various global populations in attempt to reduce the disease’s burden on the healthcare system. Several SNPs in genes that predispose to T2DM have been discovered in GWAS of T2DM. These genetic variations were found in many researches including various populations, confirming several correlations [14].

In this study, we examined the association of the CDKN2A/B gene SNPs rs10811661 and rs2383208 with...
the T2DM development in an Iraqi population sample (Table 4).

The genetic power in the current study was verified for each studied SNP to assess the results’ reliability, since 80 percent genetic power is necessary to consider a real association between genotyping findings and illness or phenotypic characteristics. Only one SNP (rs10811661) was found to have adequate genetic capabilities (89.1 percent). While the other SNP (rs2383208) it has just 24.6 percent of genetic power.

The results of the highlighted two SNPs showed consistency with Hardy Weinberg equilibrium (HWE) in healthy control persons, implying that the examined genotypes had consistent frequencies throughout generations. Based on this, changes in allele frequencies in the diabetic group might be substantially linked to T2DM.

The results of rs10811661 SNP in CDKN2A/B showed a significant association for allele T with the existence of T2DM. The carriers of the TT (homozygous variant) have a 2 folds’ risk relative to the non-carriers to develop T2DM. In addition, the adjustment for gender, age and BMI had no effect on the results. The TC (heterozygous variant) variants do not show significant changes neither before nor after the adjustment for age, gender, and BMI. On the other hand, the dominant, recessive, and additive models have approximately two-fold risk for the disease development. However, the allele frequency (T) increases the risk of the disease by 1.69 folds. Several studies have indicated that the CDKN2A/B gene is linked to diabetes in the Arab ethnic population, but the present study is the first in Iraq. Most of these studies used a case–control design such as in KSA [17], Palestine [7] and Oman [24]. A study in Tunisian people showed a female gender-specific association of rs10811661 SNP with T2DM (Amira [3]). A study in India, demonstrated a significant association with the disease [4] and also in Thai population [19]. On the contrary, other studies revealed non-significant association of this SNP with T2DM in Qatar [23] and in Lebanon [22] populations.

Our study demonstrated significant effect of this SNP on insulin levels and HOMA-IR this finding could explain how CDKN2B and CDKN2A genes that encoding p15\textsuperscript{INK4b} and p16\textsuperscript{INK4a}, respectively, implicated in pancreatic islet regenerative capacity. As studies shown that transgenic mice overproducing p16\textsuperscript{INK4a} showed decreased islet proliferation with ageing, and aged mice lacking p16\textsuperscript{INK4a} demonstrated enhanced islet proliferation [12].

Results of rs2383208 SNP in CDKN2A/B gene polymorphism showed non-significant association with T2DM in the analyzed individuals. Results seemed to be insignificant whatever the type of the inheritance model was used. The findings of the present study are consistent with those of others [20]. In contrast, a study on Japanese population showed a significant association of this SNP with the incidence of T2DM [8]. It has been concluded that CDKN2A/B loci (rs10811661 and rs2383208) may impact T2DM risk by modulating gene expression of islet cells and proliferation of β-cell [25]. The variation of the results is worthy to point out that ethnicity may play a fundamental role in various diabetogenic gene influences on T2DM development.

**Conclusion**

CDKN2A/B gene polymorphism rs10811661 was implicated in T2DM pathogenesis in this sample of Iraqi population also its significantly affect insulin levels in those patients, whereas the rs2383208 SNP do not impact the disease nor the measured biochemical markers.

| Clinical characteristic | Genotype M ± SD | P |
|-------------------------|----------------|---|
|                         | TT (n = 249)   | TC (n = 128) | CC (n = 23) |
| BMI (kg/m²)             | 29.83 ± 2.90  | 29.31 ± 2.96 | 28.89 ± 2.90 | 0.12 |
| FBS (mg/dl)             | 229.83 ± 35.88 | 227.11 ± 36.39 | 224.72 ± 36.86 | 0.68 |
| Cholesterol (mg/dl)    | 254.98 ± 26.03 | 254.62 ± 27.78 | 251.24 ± 23.63 | 0.81 |
| Triglycerides (mg/dl)  | 237.62 ± 22.23 | 237.25 ± 22.11 | 232.02 ± 18.09 | 0.51 |
| VLDL-C (mg/dl)         | 47.52 ± 4.45  | 47.45 ± 4.42  | 46.40 ± 3.62  | 0.51 |
| LDL-C (mg/dl)          | 165.22 ± 15.86 | 164.26 ± 14.2  | 160.60 ± 13.62 | 0.35 |
| HDL-C (mg/dl)          | 42.23 ± 8.77  | 43.11 ± 9.32  | 44.23 ± 8.77  | 0.45 |
| Insulin (μU/ml)        | 33.14 ± 3.44  | 32.78 ± 3.87  | 30.76 ± 3.97  | 0.01 |
| HOMA-IR                | 18.80 ± 1.86  | 18.38 ± 1.74  | 17.06 ± 1.93  | <0.0001 |
Abbreviations
BMI: Body mass index; CDKAL1: CDK5 Regulatory Subunit Associated Protein 1 Like 1; CI: Confidence interval; EDTA: Ethylenediaminetetraacetic acid; FPG: Fasting plasma glucose; GWAS: Genome-wide association study; IDF: International Diabetic Federation; MAF: Minor allele frequency; OR: Odds ratio; PCR-RFLP: Polymerase Chain Reaction–Restriction Fragment Length Polymorphism; T2DM: Type 2 diabetes mellitus; SNP: Single-nucleotide polymorphism; WHO: World Health Organization.

Authors’ contributions
All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Abbreviations
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