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Research Article

Single Nucleotide Polymorphism E23K of KCNJ11 Gene and Other Risk Factors Associated With Type-2 Diabetes Mellitus In Gaza

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Abstract: Type 2 diabetes mellitus (T2DM) is a multifactorial disease in which environmental triggers interact with genetic variants in predisposition to disease. The aim of this study was to evaluate (E23K) SNP in KCNJ11 gene as a possible contributor to T2DM among Gaza City patients. Also to consider relation between E23K SNP and other risk factors for T2DM. Two hundred male and female individuals were examined: 100 T2DM patients and 100 control individuals. The glucose level was determined, and the groups were genotyped for the SNP (E23K) by PCR/RFLP technique using the BanII restriction enzyme. A questionnaire was completed to evaluate the role of physical and environmental risk factors for T2DM. There was a strong statistically significant relation between the E23K polymorphism and T2DM (P=0.000). Forty three percent of cases were E/K compared to 29% of controls; and 15% of cases were K/K compared to 3% of controls. Obesity, persistent stress, absence of physical activity and low level of education were also significantly related to T2DM (P=0.000). The mean fasting blood sugar level was significantly higher among the cases than the control, and particularly among homozygous and heterozygous cases (P= 0.000). In conclusion, risk factors that are significantly related with T2DM patients in Gaza city include the E23K polymorphism, obesity, persistent stress, absence of physical activity, and low level of education. The inheritance of the K allele predisposes for T2DM, provided that other genetic or/and physical and environmental risk factors be present.

Keywords: E23K polymorphism, Diabetes, Gaza,

Introduction

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism due to defects in insulin secretion, insulin action, or both [1]. The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030 [2]. The prevalence of diabetes in Palestine ranges between 9 to 12% depending on the population studied and the same ratio exists in Gaza strip [3-5].

The two major types of diabetes are type 1 (insulin-dependent diabetes mellitus, IDDM) and type 2 (non-insulin-dependent diabetes mellitus, NIDDM or T2DM) [6]. Type 1 diabetes develops most frequently in children and adolescents and is characterized by failure of the pancreas to produce insulin [7]. Type 2 diabetes mellitus (T2DM) is a multifactorial disease, characterized by impaired insulin secretion and insulin action, in which environmental triggers interact with genetic variants in the predisposition to the disease [1].

Genes encoding for key components of insulin secretion and glucose metabolism pathways have been widely considered as targets for defects in T2DM and many associations have been reported [8]. ATP-sensitive potassium (K_{ATP}) channels in pancreatic β-cells comprise two sub-units: a pore-forming, K^+ inward rectifier (Kir6.2) encoded by the (KCNJ11) gene and a sulfonylurea receptor (SUR1) encoded by the (ABCC8) gene [9]. K_{ATP} channels regulate insulin secretion by coupling the metabolic state of the cell to membrane potential. Elevation of blood glucose level leads to an increase in ATP/ADP ratio and a decrease in K_{ATP} channel permeability that in turn leads to membrane depolarization, activation of voltage-dependent calcium channels, Ca^{2+} influx into the cell and finally insulin exocytosis [9].

Three common single nucleotide polymorphisms (SNPs): E23K, L270V, and I337V have been found in the KCNJ11 gene in Caucasians [10-13]. The E23K polymorphism in the K_{ATP} channel is consistently associated with disease risk, probably as a result of reduced insulin secretion [14]. E23K is caused by a switch of guanine to adenine, resulting in a glutamic acid (E) to lysine (K) substitution at codon 23. The E23K variant leads to over activity of the K_{ATP} channel, resulting in reduced insulin secretion [10, 11, 15, and 16]. Analysis of the E23K in various populations showed that K/K homozygosity has a stronger association with

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diabetes compared to heterozygous E/K or the wild-type E/E [12, 13].

Therefore the aim of the present study was to examine the role of (E23K) SNP as a possible genetic predisposing factor for T2DM among Gaza City patients. The genotype of (E23K) was determined in T2DM patients and matched normal control population and its relation to other risk factors for T2DM was evaluated.

Materials and Methods

Study population:

The present study is a case-control study comprised of two groups. The case group included 100 previously diagnosed T2DM patients according to criteria recommended by WHO (fasting blood glucose levels (FBG) of 126mg/dL (7.0mmol per L) or higher and/or two-hour postprandial plasma glucose (2hrPPG) readings of 200mg per dL (11.1mmol per L) or higher after a glucose load of 75g) [1]. The samples were collected from patients attending the diabetic clinic at Al Remal Clinic, Gaza City. The control group included 100 individuals with normal fasting blood glucose and negative family history of T2DM among first degree relatives, with matched age and sex.

Data collection and analysis:

A questionnaire was completed for each case and control. The collected data included personal data, life style, disease related data, family history and consanguinity. The questioner was completed by the researcher by interviewing each case and control to avoid misunderstanding. Patients and controls gave their oral consent for participation in the study and all information was kept confidential. The procedures of the study were approved by the local Helsinki committee according to the World Medical Association Declaration of Helsinki (17].

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) version 13 for Windows. For normally distributed data, the means and standard deviations were calculated. Chi squire and student T test with confidence intervals of (95%) were applied where appropriate. Statistical significance was at the 5% level.

Sample collection:

Blood samples were collected from patients and controls in the morning after 10–12hours fasting. 5 milliliters of venous blood were drawn and divided into 3ml in plane tubes and 2ml in EDTA containing tubes. The plane tube samples were processed within 2hours from collection to prevent reduction in glucose concentration. Serum was separated by centrifugation at 3000 rpm for 10 minutes. DNA was extracted from EDTA tube samples within 5 hours from collection.

Glucose determination:

The glucose level was determined by using DiaSys kit according to the kit instruction (DiaSys Diagnostic Systems- Germany).

DNA extraction from whole blood:

DNA was extracted from 300µl whole blood samples using the Wizard Genomic DNA Purification kit (Promega, USA), according to the kit instructions. The quantity and quality of DNA was determined by measuring the optical density at 260, 280 and 230 nm.

E23K polymorphism determination by PCR/RFLP:

Primers for amplification of the E23K polymorphism were modified from previously published sequences and were flanking the RFLP site [18]. The forward primer: 5`-GAATACGTGCTGACACGCCT-3`and the reverse primer: 5`-GCCAGCTGCACAGGAAGGACAT-3' were purchased from Operon, Germany.

PCR reactions were carried out in 25µl final volume including 0.025 U/µl Taq DNA polymerase in the provided buffer (both from Promega, USA); and in the presence of 1.5 mM $MgCl_2$, 200µM each dNTP and 0.8µM of the forward and reverse primers. The cycling conditions were: initial denaturation at 95˚C for 5 min followed by 35 cycles of 95˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min. A final extension step was allowed at 72˚C for10 min. 5µl of PCR products were analyzed on 2% agarose gel stained with ethidium bromide and visualized by UV. A positive PCR gave a 222bp fragment.

The amplified fragments were digested using Ban*II* restriction endonuclease (Promega, USA) for two hours at 37°C. The digestion reactions were carried out in 20µl volumes using 2.5 U of Ban *II* in the provided buffer (Promega, USA) and in the presence of 2µg acetylated BSA and 16µl PCR products. Three fragments were obtained in case of wild type (37bp, 28bp and 157bp), two fragments were produced in case of mutant (65 bp, and 157 bp) and four fragments were produced in case of heterozygous (28bp, 37bp, 65bp and 157bp) samples.

Results

E23K polymorphism:

DNA samples from all cases and controls were successfully amplified by PCR and gave the

desired 222 bp amplification fragment. All of the amplified fragments were also successfully digested with *Ban* II restriction enzyme and gave the wild type, the heterozygous or the homozygous pattern of digestion.

The majority of the K/K and E/K combinations was found among cases (83.3% and 59.7% respectively), while the majority of the E/E genotype (61.8%) was found among the controls (Table.1). This strong association between the K allele and T2DM was statistically significant (X^2= 16.87, *P*= 0.000).

Table.1: Distribution of cases and controls by risk factors for T2DM

		Case	Control	*P*-value
Genotype	E/E	42	68	0
	E/K	43	29	
	K/K	15	3	
Social Status	Married	87	97	0.029
	Divorced	1	0	
	Widow	7	0	
Employment	Yes	22	63	0
	No	78	37	
Living in risky area	Yes	20	4	0
	No	80	96	
Hospitalization for surgery	Yes	40	5	0
	No	60	95	
Exposure to problems	Yes	62	62	1
	No	38	38	
Ability to solve Problems	Yes	46	62	0
	No	16	0	
Loss of a close person	Yes	70	76	0.339
	No	30	24	
Other diseases	Yes	56	12	0
	No	44	88	
Parents' consanguinity	Yes	53	40	0.060
	No	47	60	
Degree of parents consanguinity	First	33	23	0.642
	Second	20	17	
Practice of physical activity	Yes	55	99	0
	No	45	1	

Stress:

A number of factors that may contribute to living a stressful life were investigated and found positively related to T2DM (Table.1). These include being divorced or widow (X^2=9.04, P= 0.029); unemployment (X^2=34.39, P=0.000); living in risky areas (P=0.000) and hospitalization for surgery (P=0.000). An equal number of cases and controls (62%) reported that they have been exposed to stressful problems. However, significantly more control subjects were able to solve their problems (100%) than case subjects (74%) (X^2=18.37, P=0.000) as they reported. Significantly lower mean family income was also reported in the case (1025 ± 282 NIS) than in the control group (1980 ± 1067 NIS) (t= -8.65, P=0.000). A reported loss of one close relative did not appear to be significantly related to T2DM as approximately equal numbers of the cases and the controls lost at least one close relative (Table.1).

The body mass index:

A statistically significant relationship between T2DM and the mean BMI was found (t=7.28, *P* = 0.000). The mean BMI of case 32.9 ± 8.5kg/m^2 was higher than of control 25.9 ± 4.4 kg/m^2).

Education:

The number of years of education was found significantly related to T2DM (t=-13.58, P= 0.000). The mean number of cases years of education was (7.2 ± 4.91 years) compared to (14.7 ± 2.6 years) of controls.

Physical activity:

Most of the controls reported they were practicing different levels of physical activity (99%) compared to (55%) of the cases (Table.1). The relationship between T2DM and physical activity was statistically significant (X^2=54.66, P=0.000).

Health conditions:

Most of the controls were not complaining of any diseases (88%). On the other hand more than half of the cases were complaining of other diseases (Table.1). This distribution was statistically significant (X2= 55.143, P=0.000). It is noteworthy to mention that all of these medical complications occurred after the onset of T2DM as reported by the patients.

Family history of T2DM and single nucleotide polymorphism:

The parents of (53%) of the cases were consanguineous compared to (40%) of the controls (Table 1). The relationship between consanguinity of parents and T2DM was statistically significant (X^2= 3.4, P= 0.06). On the other hand the degree of consanguinity was not statistically related to T2DM (X^2= 0.216, P= 0.642).

There was statistically significant correlation between E23K genotype and parents' consanguinity. E/K and K/K genotypes were more represented among cases or controls with consanguineous parents compared to those with non-consanguineous parents (Table.2). The correlation between parents' consanguinity and E23K polymorphism was statistically significant among controls (X^2= 33.849, P= 0.000) but not among the cases (X^2= 4.290, P= 0.117).

Table.2: Distribution of E23K genotype among cases and controls with consanguineous parents.

	Consanguinity	E/E	E/K	K/K	*P*-value
Cases	Yes	27	18	8	0.117
	No	15	25	7	
Controls	Yes	14	23	3	0
	No	54	6	0	

The results showed that 80% of cases had a family history of T2DM, where the highest percentage of cases with family history of T2DM was among those of E/K (88.4%) compared to 73.3% of K/K, and 37.8% in E/E (Table.3). This distribution was not statically significant (P = 0.191). The mean age of T2DM onset among cases was 44.3 ± 11.36 years. The mean age of onset of T2DM among cases with the E/E alleles was (43.1 ± 12.8 years), whereas that of the cases with the K/K (47.6 ± 10.4 years). This distribution however was not statistically significant.

Table.3: Distribution of cases genotype by family history of T2DM

		E/E	E/K	K/K	*P*-value
Family history	Yes	31	38	11	0.191
	No	11	5	4	

Relationship between risk factors and mean fasting blood glucose:

The mean fasting blood glucose (FBG) of the cases was (182.9 ± 66.8) mg/dl compared to (94.5 ± 4.2) mg/dl of the controls (P= 0.000). The mean FBS was significantly correlated with E23K genotype, category of obesity, level of education and exercise, exposure to problem, ability to solve these problem, employment, family income, marital status, living in risky area, exposure to sorry events and hospitalization for surgery (Table. 4).

Discussion

The results of the present study show that the three possible genotypes of E23K SNP were significantly distributed among the cases and the controls (X^2=16.87, *P*=0.000). The results suggest a possible role of the homozygous K/K genotype, and to a lesser extent the heterozygous genotype, as a genetic risk factor for T2DM among our population. Similar results were obtained by several case-control studies in different populations which indicated the involvement of homo- and heterozygous E23K polymorphism as risk factor for T2DM [12, 18-21]. Despite a high incidence of T2DM, the role of E23K variant of KCNJ11 among Arabs has not been well established, except for a case-control study of Saudi Arab that showed a positive association of the E23K variant with T2DM [22].

Table.4: Mean FBG level of cases and controls categorized by the identified risk factors for T2DM.

		No.	Mean FBG (mg\dl)	SD	*P*-value
Genotype	E/E	110	128.6	66.1	0.024
	E/K	72	146.8	61.1	
	K/K	18	167.8	60.6	
Obesity (BMI)	Under weight	1	554	-	0
	Normal weight	71	105.4	32	
	Over weight	50	141	59	
	Obese	63	155.2	55	
	Severe obese	15	191.4	82.6	
Practice of physical activity	Yes	154	127.7	63.1	0
	No	46	175.2	59.6	
Employment	Yes	85	112.3	36.5	0
	No	115	158.2	73.7	
Exposure to problems	Yes	124	141.5	63.2	0.434
	No	76	134.1	67.3	
	Total	200	138.7	64.8	
Ability to solve problem	Yes	108	135.1	61.1	0.012
	No	16	184.4	62.3	
	No problem	76	134.1	67.3	
	Total	200	138.6	64.8	
Loss of a close person	Yes	79	163	77.3	0
	No	121	122.8	49.3	
	Total	200	138.7	64.8	
Live in risky area	Yes	24	183.2	69.2	0
	No	176	132.6	61.9	
	Total	200	138.7	64.8	
Hospitalization	Yes	45	168.8	50.4	0
	No	155	129.9	65.9	
	Total	200	138.7	64.8	
Social status	Single	8	214.2	158.9	0
	Married	184	132.9	55.3	
	Divorce	1	165	-	
	Widow	7	198.3	21.6	

SD: Standard Deviation

The role of stress, particularly emotional, in the development of T2DM is well established [23]. In this study factors that may potentially contribute to the development of chronic state of stress such as unemployment, low family income, singleness, divorce, widow, exposure to different types of physical and emotional problems, living in potentially problematic areas, hospitalization and surgery all were positively associated with the development of T2DM.

In this context, people who were exposed to different types of problems were equally distributed among the cases and the controls. However, people who were able to deal with and solve their problems were significantly more in the control group

(P=0.000). This finding emphasizes the role of continuous rather than transient exposure to such problems as risk factors for T2DM.

The role of obesity as a risk factor for T2DM is also well established [24-28]. This fact was also present as an outcome of our study. The mean BMI of the case group was significantly higher than that of the control group (P=0.000).

The level of education also seems to be related to T2DM. This association might be explained by the fact that well educated people are usually more aware of the risks of having a disease like T2DM, and can take precautions accordingly. In support for this, our results show that the mean number of education years of the cases and the controls who regulatory practice sport (12.2 years) is significantly higher than among the cases and the controls with no practice of sport (6.9years) (P=0.000).

Practice of physical activity by itself seems to be protective against T2DM as evident from our study (99 % of the control versus 55% of the cases are practicing physical activity (P-value=0.000). This finding is in agreement with other studies [29, 30].
As explained earlier, the three E23K genotypes are significantly distributed among the cases and the controls. In an attempt to determine if the existence of E/K or K/K polymorphism by itself is sufficient to cause T2DM, we examined the cases for the age of onset of their T2DM. We found that the mean age of onset is not significantly different between E/E, E/K and K/K genotypes. In the case of direct relationship we would expect K/K patients to discover their T2DM at earlier age than E/K and E/E. Moreover, we would also expect to find a higher degree of consanguinity among K/K and E/K than E/E patients if the genotype alone positively influences the onset of T2DM. We found a significant relation between the consanguinity and the distribution of E23K genotypes (P=0.015), when taking the whole population. However, if taking the case alone, no such significant relation was seen, while a significant relation was detected in control group. Thus, the occurrence of the K/K or E/K genotypes was not sufficient alone to enhance development of T2DM. In order to have T2DM, the subject has to have other risk factors predisposing to T2DM, which might explain the significant distribution of consanguinity among the controls but not the cases. Other risk factors that can significantly influence the development of T2DM include physical conditions, environmental and stress related condition. This assumption leads us to focus on the three K/K cases of the control group who did not develop T2DM. We found that all of these three controls were practicing sport in a regular basis, they all have a normal BMI, they all work with a relatively high family income and they have not been exposed to any type of stressful condition. On the other hand, the 15 cases with K/K genotypes were all exposed to the upper mentioned physical and environmental risk factors. Such finding indicated that interaction between genetic predisposing factors together with other genetic risk factors and physical and environmental risk factors is of great importance in the development of T2DM. This is supported by the outcome of many studies that addressed this issue. [31-38]

Many types of chronic disease may be predisposing to T2DM [39]. We did not find in this study any causative relation between such disease and T2DM. However more than half of the patients with other chronic disease stated that they got these disease after they discovered their diabetes (P=0.000).

In order to assure the role of the previous discussed risk factors in the development of T2DM among our cases, we compared the mean FBG level between the different categories of risk factors. We found significantly higher mean value in case of K/K than E/K than E/E genotypes (p= 0.000). Likewise, the higher the BMI, the higher the mean FBG level was found (P=0.000). Similarly more stressful events are associated with higher levels of FBG (for P-value see table 4.14). Physical activity was associated with decreased risk for T2DM with lower FBG level (P=0.000).

Conclusions

a. The SNP E23K was found to be significantly distributed among the study population. More E/K and K/K were found among the cases than the controls.
b. Risk factors that are significantly implicated with T2DM in Gaza include the E23K polymorphism, obesity, stress, absence of physical activity, and low level of education.
c. These risk factors are significantly correlated with high level of FBG while protective factors (Education and practice of exercise) are significantly correlated with lower level of FBG.
d. The inheritance of the K allele predisposes for T2DM, provided that other genetic or/and physical and environmental risk factors to be present.

Conflict of interest

The authors declare that they have no conflict of interest.

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