Research Article

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Biochemistry Characterization of Type 2 Diabetes in Tunisia and their Relationship with 4545G/C Polymorphism Located in Adiponectin Gene

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Abstract

Diabetes incidence is increasing worldwide. Many studies demonstrated that polymorphisms within the adiponectin gene could be associated with type 2 diabetes mellitus (T2DM). A case–control study was conducted to find the association between SNP 4545G/C and T2DM in Tunisian population. The study included 561 patients referring to diabetic and 437 healthy controls. DNA was extracted from blood and genotyped by PCR–RFLP by using (HinfI) enzyme. Prevalence of obesity (p <0.001), dyslipidemia (p <0.001), hypertension (p <0.001) were significantly increased in cases with report to witnesses. Also for the lipid parameters Urea, CRP and HDL-C were significantly (p <0.001) and HDL / LDL (p = 0.983) are decreased in diabetics compared to controls two types of alleles G (Absence of restriction site) and C (presence of the restriction site) respectively of frequencies (0.76 and 0.23) in the controls and (0.73 and 0.26) in the diabetics, as well as three genotypes GG, GC and CC In comparison with the GG genotype the relative risk (OR [95% CI]) for diabetes was 1.06 [0.86-1.31]; p = 0.275 for the genotypes (GC + CC) . Our results show significant variations for the anthropometric and lipid parameters in the type 2 diabetes mellitus (T2DM) but were not associated with the polymorphisms at site of 4545.

Keywords: Diabetes, Adiponectin, Genetic Variants, LDL, HDL.

1. Introduction

Type 2 diabetes (T2D) involves a complex interaction between genetic variants and environmental factors, and obesity can increase the risk of developing diabetes [1]. Adiponectin(also calledAPM1,ACRP30, ADIPOQ, and GBP28) is an abundant adipocyte-secreted protein in plasma. It can regulate glucose levels, insulin action and lipid metabolism [5-6]. The circulating adiponectin was significantly reduced in patients with type 2 diabetes and obesity [7,8]. Prospective studies showed that subjects with high adiponectin levels were protected against type 2 diabetes [9]. Moreover, administration of recombinant adiponectin decreased glucose and insulin resistance in mice of obesity or diabetes [6-10]. The plasma level of adiponectin is partly influenced by genetic factors which account for about 40–70% [11]. The human adiponectin gene is mapped to the 3q27 region where the metabolic syndrome and T2D loci were reported [12-14].

Some studies have found that the single nucleotide polymorphisms (SNPs) of this gene might increase the risk of T2D [15-17], whereas few reported the relationship between its SNPs and type 2 diabetes combined with obesity. As far as we know, there are only two teams who reported these associations with obese T2D. For example, +45 T/G and +276 G/T single nucleotide polymorphisms (SNPs) of adiponectin (ADIPOQ, 3q27) are highly involved SNPs as candidate risk variants for T2DM in Asian populations, whereas these same SNPs are not correlated with a risk of T2DM in Europeans, including Italian, French and Swedish individuals [18-21].

The 4545G/C polymorphism was chosen because they might influence the gene expression or change the protein function. cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) were determined using an automatic chemistry analyzer (Olympus AU5400, Japan) or an enzymatic kit (Roche Diagnostics GmbH, Basel, Switzerland). Fasting plasma glucose (FPG) was measured by a glucose oxidase or hexokinase reaction, and fasting serum insulin (Fins) was assayed by means of electro-chemiluminescence immunoassay (ECLIA, Roche Diagnostics, Rotkreuz, Switzerland). The homeostasis model assessment (the HOMA) indices for insulin resistance (HOMA-IR) and for beta-cell function (HBCI) were subsequently calculated by using the following formulas: HOMA-IR = Fins (mU/L) _ FPG (mmol/L)/ 22.5 and HBCI = 20 _ Fins (mU/L)/[FPG (mmol/L) _ 3.5], respectively.

The objective of this study is to evaluate the biochemical analyses in type 2 diabetic subjects and non-diabetic subjects and re-

search the relationship between 4545G/C polymorphism located in adiponectin gene and type 2 diabetes in Tunisia.

2. Materials and Methods

2.1. Study Population

In this study, a total of 1034 subjects were recruited, consisting of 561 type 2 diabetic subjects (mean age 55,44±9,15 years) and 473 non-diabetic subjects (mean age 54,35±9,35 years) at the research Unit in the Department of Endocrinology of Rabta University Hospital of Tunis.

The study population (561 subjects) was recruited from diabetic patients consulting in the endocrinology department of the Rabta hospital.

A group of controls (473 subjects) was recruited as part of a prospective survey on "Biochemical and genetic markers of atherosclerosis in the Tunisian population"

-A standardized questionnaire was carried out for all subjects comprising:

- Information relating to the subjects's personal and family history, their risk factors and their drug consumption.
- Part of the questionnaire is reserved for patients aiming to characterize diabetes and complications.

3. Biochemical Analyses

3.1 Assay of Biological Parameters

The blood samples were in the morning after 12 hours on EDTA tubes for the genetic study and lithium heparin for the lipid balance By using a standard enzymatic methods with commercial kits (Roche Diagnostics, Mannheim, Germany) on a Hitachi 912 analyzer. The serum was separated immediately from the pelled centrifugation at 3000 rpm for 15 minutes at 4°C. All the lipid parameters (total cholesterol,HDL cholesterol, triglycerides were assayed on ARCHITECT ci 8200 (Abbott). It allows the assays tobe carried out by: UV- visible spectrophotometry for TG, CT and HDL. LDL-C concentrations were calculated using Friedwald's formula : LDL= CT- (TG/ 5 + HDL) [22].

3.2 Statistical Analysis

All statistical analyses were conducted using the SPSS 11.5 (SPSS Inc., Chicago, IL, USA) statistical package. For all tests performed, a p value of less than 0.05 was considered as significant. Data are presented as means ± SD. Skewed continuous variables such as triglyceride including age, BMI was evaluated by multiple linear regression analysis, In order to explore the different genetic backgrounds of type 2 diabetes, we tried to genotype the SNP (4545G/C) in the adipose most abundant gene transcript-1 (APM1) gene in 561 type 2 diabetes (T2D) patients and 473 non-diabetic subjects by PCR–RFLP.).

4. Adiponectin Genotyping

4.1 DNA Extraction (DNA Concentration and Purity Assessement)

A dilution of 1/50 of the DNA extract by adding 50 µl of the DNA solution in 950 µl of double-distilled water is carried out. A first reading of the optical density (OD) at 260mm makes it possible to assess the DNA concentration of the sample, 1 unit OD 260 nm -> 50 µg / ml of double-stranded DNA.

DNA concentration µg / ml = OD value * 50 * dilution factor Asecond reading of the OD at 280 nm allows the protein contamination to be estimated and the calculation of the OD at 260 / OD at 280 ratio allows the purity of the DNA to be assessed. A DNA is considered pure when this ratio is between 1.8 and 2. The optical density is read by a conventional spectrophotometer. Thus, depending on the value of the report, possible contaminants such as proteins or RNAs are detected:

□ 1.8 <DO 260 nm / DO280nm <2 □ pure DNA

□ OD 260 nm / OD280nm> 2 □ contamination by RNAs (treatment with RN ases is recommended).

□ OD 260 nm / OD280nm <1.8 □ contamination by proteins (treatment with proteinase K is recommended).

4.2 Typing of Adiponectin

Typing of adiponectin was performed by molecular biology techniques. The first is PCR amplification "Polymerase Chain Reaction" followed by restriction enzyme HinfI digestion and 3% agarose gel electrophoresis.

4.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction or PCR is a cell-free cloning technique. It makes it possible to obtain, from a small sample, large quantities of a specific DNA fragment of defined length.

4.4 The Primers

The primers are synthetic oligonucleotides which represent short, conserved described sequences located on either side of the region to be amplified.

- The first oligonucleotide (oligo1) has the sequence of 5 "to 3":
  5'-TGGCTATGCTCACAGTCTCAC - 3'
- the second oligonucleotide (oligo 2) has the sequence 5' to 3' :
  5'- ACTTCAAAGCATCACAGGACC- 3'.

4.5 Amplification

The amplifications were carried out on an automatic thermal cycler (Appliedbiosystem 2720 Thermal cycler). The reactions were carried out in a final volume of 50 µl.

4.6 PCR Product Verification

- The PCR is checked on 1% agarose gel in 150 ml of Tris-Borate-EDTA buffer (TBE 1X) 1.5 g of agarose are added -2 µl (10 mg / ml) of ethidium bromide (BET) solution are added to the gel in each well of the gel is deposited 10 µl of PCR product.

4.7 Enzymatic Digestion

The nucleotide sequences recognized by the restriction enzymes are usually so-called palindromic sequences. Palindromic sequences are sequences where the sequence of nucleotides read in the 5 "to 3" direction for the first strand is identical to the sequence read in the right-left direction for the second strand (5 "to 3" direction). These palindromic sequences are most often made up of 4, 5 or 6 base pairs. A point mutation can lead to the appearance of a new restriction site (gain of restriction site) or the disappearance of an already existing site (loss of a restriction site). Following the PCR reaction, the product obtained is subjected to enzymatic digestion with a restriction enzyme HinfI. The polymorphic cleavage site of the adiponectin gene, corresponds to a substitution of G by C. It is located at position -4545

of the promoter of the adiponectin gene.

4.8 Agarose Gel Electrophoresis

Electrophoresis is a technique used for the separation and purification of DNA fragments. Nucleic acids are uniformly charged polyanionic macromolecules that can migrate in an electric field. The fluorescent bands allow the fragments to be identified A single band corresponds to the 198 bp fragment. This is the normal homozygous form. Two bands corresponding respectively to the fragments of 125 bp and 73 Pb. This is the mutated homozygous form. Three bands corresponding respectively to the fragments of 198 bp, 125 bp and 73 bp. This is the heterozygous form, the cleavage site is on one of the two strands of DNA.

4.9 Statistical Analysis

The search for a possible association between the polymorphism studied and the occurrence of diabetes is based on the comparison of the allele and / or genotypic frequencies of a population of patients with that of a population of healthy controls followed by calculation of the value of the ods ratio (OR). The calculation of the OR and the significance index "p" are performed by the SPSS software. The Hardy-Weinberg equilibrium Statement Over generations, the frequency of genotypes remains constant from one generation to the next if there is no selection, if the population is large, if there is no mutation, no migration and if unions are random. Odds Ratio (OR) It corresponds to the search for an association between alleles and / or genotypes and the occurrence of the disease,

OR = ad / cb

OR <1 The allele (or genotype) has a protective effect

OR = 1 The allele (or genotype) is neutral

OR> 1 The allele (or genotype) has an aggravating effect

4.10 Electrophoretic Profile

The amplification of DNA by PCR and its digestion with the restriction enzyme HinfI.

5. Result

5.1 Description of the Study Population

• Anthropometric Parameters of the Studied Population

Prevalence of obesity (p <0.001), dyslipidemia (p <0.001), hypertension (p <0.001) were significantly increased in cases with report to witnesses (table 1).

	Witnesses controls (N =473)	Case Diab (N =561)	P
Age (Years)	55,44±9,15	54,35±9,35	0,425
BMI	27,86±5,55	30,24±5,35	<0,001*
TAS	122,04±19,06	134,32±20,09	<0,001*
TAD	71,84±12,817	78,30±12,22	<0,001*
Obesity (%)	31,8	46,6	<0,001*
Dyslipidemia (%)	18,3	35,7	<0,001*
HTA (%)	16	54,1	<0,001*
Smoking (%)	80,1	83,2	0,117

Comparison of anthropometric characteristics between diabetic and control groups.

* P < 0.05.

Table 1: Anthropometric characteristics of the population

• Lipid Parameters of the Population

Comparison of lipids between the two groups studied did show a statistically significant increase in HDL-C and Urea, CRP are (p <0.001) in diabetics compared to controls (table 2).

Settings Organic	Witnesses CONTROL (N = 473)	Diab (N=561)	P
Gly	0,91±0,12	1,77±0,77	<0,001*
CT (mmol / l)	1,89±0,38	1,9±0,41	0,928
TG (mmol / l)	1,22±0,79	1,3±0,72	0,029

C-HDL (mmol / l)	0,49±0,13	0,42±0,12	<0,001*
LDL-C (mmol / l)	1,21±0,37	1,2±0,36	0,895
Urea (g / l)	0,299±0,08	0,33±0,17	<0,001*
CRP (mg / ml)	3,68±7,19	6,25±6,43	<0,001*
CREAT (g)	9,00±1,72	9,19±4,60	0,419
CT / C-HDL	4,19±1,98	4,92±3,42	<0,001*
HDL / LDL	0,44±0,212	0,44±0,516	0,983

Comparison of lipid parameters between diabetic and control groups.
* P < 0.05.

Table 2: Average values of lipid parameters in the population studied

• Allelic Frequencies and Distribution of Genotypes

Using the RFLP method (PCR, enzymatic digestion and agarose gel electrophoresis) we were able to determine the genotypes of our subjects, and demonstrate two types of alleles G (Absence of restriction site) and C (presence of the restriction site) respectively of frequencies (0.76 and 0.23) in the controls and (0.73 and 0.26) in the diabetics, as well as three genotypes GG, GC and CC (figure 1), the frequencies of which are given in the table below.

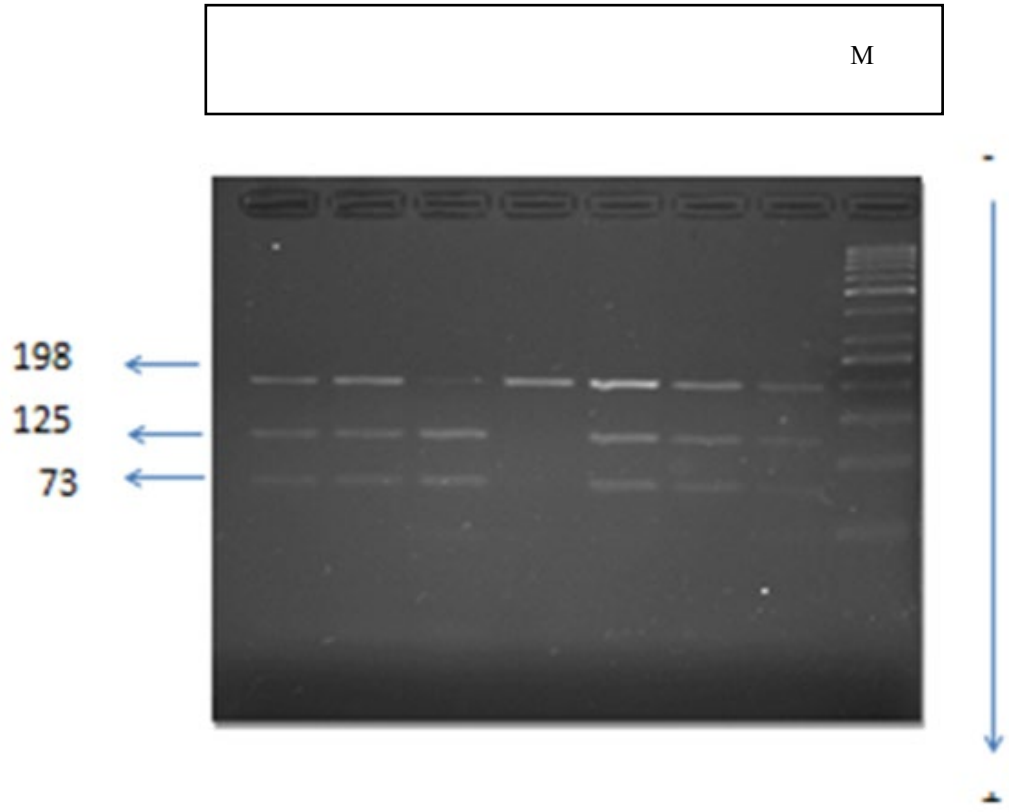

Figure 1: Electrophoretic profile of restriction fragments by the HinfI enzyme in a 3% agarose gel which identify 3 genotypes:
CC: a single 198 bp band. It is the wild homozygous form.
CA: 3 bands of 198bp, 125bp and 73bp. This is the heterozygous form.
AA: 2 bands of 125 bp and 73 bp. This is the homozygous mutated form.
M: mark size (50-1000 bp).

In our population, the genotypic distribution is in Hardy-Weinberg equilibrium.
Our results show that the frequency of the G allele is not significantly increased in diabetics compared to controls (table 3).

	WITNESS CONTROL (N= 473)		Diab (N= 561)		P	OR (95% CI)
	N	%	N	%		
Genotypic frequencies						
GG	286	60,5	312	55,6		
GC	154	32.6	202	36		
CC	33	7,0	47	8,4		
GC + CC	187	39,6	249	44,4	0,275	1,06(0,86-1,31)
Allelic frequencies						
G	0,76		0,73		0,565	
C	0,23		0,26			

GG: Wild homozygous; GC: Heterozygous; CC: Mutated homozygote

Table 3: Allelic frequencies and genotypic distribution of the -4545 G / C polymorphism in cases (diab) and controls.

In comparison with the GG genotype the relative risk (OR [95% CI]) for diabetes was 1.06 [0.86-1.31]; p = 0.275 for the genotypes (GC + CC) (Table 3).

• Lipid and Anthropometric Parameters According to Genotypes
Depending on the genotypes, our results do not show significant variations for the anthropometric and lipid parameters in the controls (table 4).

Genotypes	GG		GC		CC		P	
Anthropometric parameters	control	case	control	case	control	case	control	case
Age	55±9,24	55,24±9,30	55,87±8,95	54,98±9,31	55,56±9,60	53,28±9,85	0,765	0,408
BMI	27,85±5,57	30,17±5,80	27,48±4,93	30,32±4,70	29,66±7,45	30,39±4,95	0,163	0,935
Heap	121,11±19,62	133,87±21,39	122,11±19,17	133,36±17,90	130 ,00±17,32	139,35±19,87	0,753	0,209
Tad	70,00±12,63	77,89±12,55	73,68±13,82	78,27±11,69	76,67±5,77	81,20±12,16	0,512	0,232
Lipid parameters								
Gly	0,91±0,11	1,67±0,71	0,91±0,15	1,67±0,73	0,96±0,12	-	0,091	0,128
CT	1,90±0,36	2,05±1,64	1,90±0,36	1,91±0,41	1,86±0,44	-	0,873	0,132
TG	1,20±0,79	1,26±0,61	1,23±0,77	1,36±0,7	1,31±0,92	-	0,739	0,957

C-HDL	0,49±0,14	0,45±0,17	0,49±0,12	0,46±0,28	0,52±0,15	-	0,442	0,155
C-LDL	1,24±0,33	1,37±1,6	1,20±0,36	1,18±0,36	1,07±0,59	-	0,359	0,295
Urée	0,29±0,07	0,34±0,13	0,30±0,08	0,31±0,12	0, 31±0,08	-	0,243	0,974
CRP	3,80±8,56	5,64±5,41	3,47±4,01	6,06±4,65	3,57±5,27	-	0,9	0,950
CREAT	8,95±1,61	9,8±6,1	9,08±1,74	8,7±3,14	9,07±2,34	-	0,749	0,262
CT/HDL	4,15±1,30	5,07±4,79	4,27±2,74	4,71±1,67	4,11±2,64	-	0,803	0,844
HDL/LDL	0,42±0,19	0,43±0,62	0,45±0,23	0,54±0,76	0,45±0,22	-	0,202	0,535

Table 4: Average values of anthropometric and lipid parameters as a function of genotypes in controls (N=473) and in cases (Diab) (N=561)

Similarly, in diabetics, no variation in biological parameters depending on genotypes has been observed.

6. Discussion

The aim of our study was to determine in a Tunisian population the allelic and genotypic frequencies of the 4545G / C polymorphism of the adiponectin gene and its possible association with biological parameters on the one hand and with type 2 diabetes on the one hand somewhere else. Most previous studies examining the association of ADIPOQ and T2DM were conducted on Caucasian, Asian, and African populations [23-25], The allelic and genotypic frequencies of our population are in Hardy Weinberg equilibrium. Our results show that the allele frequencies of the G and C alleles of the 4545G / C polymorphism of the adiponectin gene are 0.73 and 0.26 in diabetics, respectively, and 0.76 and 0.23 in controls, respectively. This result is contradictory with the result of Xizhen et al 2009 (26) with frequencies of the G and C alleles respectively in diabetics 0.68 and 0.31 and in controls 0.65 and 0.35. The 4545G / C polymorphism of the adiponectin gene is not associated with type 2 diabetes in our population. Our results are contradictory to those found in a Chinese population who described variant C as a predisposing marker for type 2 diabetes [26], The frequency of the C allele in our diabetic population (0.26) is comparable to those described in the literature in the Sweden population [27]. Several studies have investigated the effect of the 4545G / C polymorphism of the adiponectin gene on variation in biological parameters and BMI in the population.

Comparison of lipids between the two groups studied did not show a statistically significant increase in CT ($p = 0.928$) in cases compared to controls. As well as Prevalence of obesity, dyslipidemia, hypertension were significantly increased in cases with report to witnesses. Urea, CRP and HDL-C were increased in diabetics compared to controls. Our results show no significant variation in biological parameters as a function of the 4545G / C polymorphism in both cases and in diabetics and are in agreement with Harvest results in a Sweden population [26,27]. Our study show similar result with the study of Xizhen et al in 2009, which showed that HDL levels are significantly lower in diabetic subjects compared to controls. This disagreement between the different studies can be explained by the ethnic origin of the populations and / or by gene-gene and gene-environment interactions.

Regarding the association of the 4545G / C polymorphism with BMI, our results are consistent with the work published so far in the literature [26]. Variation in adiponectin expression has been reported to be involved in insulin resistance and therefore in the development of type 2 diabetes. In our subjects, adiponectin decreases insignificantly depending on the mutated C allele. It has been shown that androgens decrease plasma adiponectin levels (Arita et al., 1999) 28. Given the probable role of adiponectin in the regulation of mRNA stability or post-translational modifications which are of great importance in the biological activities of adiponectin [26]. exon 3 mutation intervention in type 2 diabetes [26–29],. Xizhen et al suggests that the modification of adiponectin at the β-cell level may be an important factor in the development of insulin resistance and type 2 diabetes [30].

An SNP 4545G / C of exon 3 of the adiponectin gene has been identified, this polymorphism is due to a change that affects the structure of the protein by replacing the following amino acids guanine by cytosine at position 4545 which is found in the variable region of the protein, this is explained by the presence of several uradylates and adenylates in and downstream of the RNA sequences. It potentiates the hypoglycemic effects of insulin in peripheral tissues, mainly in the liver and muscle, and modulates food intake in the central nervous system.

The absence of association of the 4545G / C polymorphism of the adiponectin gene with this pathology in our population could be explained as follows: at the level of exon 3, the repetitive sequences in lover and downstream of mRNA n 'have no direct influence on variable regions [26], therefore could not be involved in the modification of the protein conformation at the level of skeletal muscle and β cells (Chan et al., 2005; Zhou et al., 2004) [31,32] to affect its activity. Such as other study suggest that aerobic exercise affects adiponectin levels regardless of weight loss and this effect would not be influenced by SNP45 and SNP276 in the adiponectin gene [33].

7. Conclusion

In summary, the current study provides evidences that prevalence

of obesity, dyslipidemia, hypertension and the lipid parameters Urea, CRP and HDL-C were significantly were significantly increased in cases with report to witnesses. The polymorphisms at site of 4545 are not associated with type 2 diabetes, respectively. But these should be validated with more comprehensive and informative data and their mechanisms how the variants increase the risk of T2D should be clarified in further studies.

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Author Contributions

Hajer Kilani wrote original draft manuscript and conducted formal analysis; AK con-ducted and validated formal analysis; AK gave an idea and conducted data analysis AK conducted data analysis and correction of context; HK corrected in the context and gave an idea; RJ conceptualized and supervised whole manuscript. All authors have read and approved the final manuscript.

Declarations

Competing interests

The authors declare that they have no competing interests.

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