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FREQUENCY DISTRIBUTION OF TCF7L2, MTNR1B, CDKAL1, SLC30A8 AND FTO GENE POLYMORPHISMS PREDISPOSING TO TYPE II DIABETES MELLITUS AND/OR OBESITY IN A GREEK POPULATION AND THEIR IMPACT IN PERSONALIZED MEDICINE

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SUMMARY

Diabetes mellitus type II(DMt2) is a chronic metabolic disease with a pandemic spread worldwide. Environmental influences and genetic background are included in the pathogenesis of the disease. More than 40 suspected loci have been associated with the risk of developing type II diabetes. Four of these (rs7903146, rs10830963, rs7756992, rs13266634) have been studied in a Greek population sample.Numerous SNPs have been associated with obesity, includingFTO rs9939609, which has been studied in the above population, is quite frequent (44% frequency of A), without showing any difference between genders and has also been associated with type II diabetes, although it is still unknown whether the association is independent or not to obesity. According to our findings the frequency of disease susceptible alleles of polymorphisms rs7903146, rs10830963, rs7756992 and rs13266634 ranges from 25% to 70%. No statistical significant differences in the frequency between the two genders in both genotype and allele level have been observed. Obesity represents the most important risk factor for type II diabetes. Although, the genetic basis of diabetes type 2 and obesity are now recognized and documented by several studies, including the present, and it is well understood that polymorphisms could likely explain differences in disease susceptibility and prevalence among groups of humans and lead to individualization of treatment, unfortunately, it is not yet transferred in daily clinical practice. However, it is expected in the following years to contribute significantly to prevention, diagnosis and treatment of these frequent diseases.
Key words: type II diabetes mellitus, obesity, TCF7L2, MTNR1B, CDKAL1, SLC30A8, FTO, polymorphism, personalized medicine

RÉSUMÉ

La fréquence de distribution des polymorphismes des gènes TCF7L2, MTNR1B,CDKAL1, SLC30A8 et FTO predisposant au diabete sucré de type II et/ou de l'obésité d'une population Grecque. Importance à la médecine personnalisée

Le diabète mellitus type II (DMt2) est une maladie métabolique chronique qui se répand dans le monde entier sous forme de pandémie. Les facteurs environnementaux et le support génétique sont inclus à la pathogenèse de la maladie. Plus de quarante épitropes génétiques se corrèlent avec le danger de développer diabète de type II. Quatre d'entre eux (rs7903146, rs10830963, rs7756992, rs13266634) ont été étudiés sur la population grecque. Beaucoup de SNPs sont corrèleés avec l'obésité. Le FTO rs 9939609 a été étudié sur cette population. Il s'est avèré assez fréquent (taux de fréquence d'apparition à 44%) sans apparaitre aucune différence entre les sexes. FTO n'est pas considèré en littérature d'être lié avec le diabète de type II bien que l'obésité soit un des principaux facteurs de risque de la maladie. Selon nos constatations, la fréquence d'apparition des allèles des polymorphismes rs7903146, rs10830963, rs7756992 et rs13266634 varie entre 25 à 70%. Aucune différence statistiquement significative concernant la fréquence des allèles ou du génotype n'est observée entre les deux sexes. Bien que la base génétique tant du diabète de type II et de l'obésité soient reconnues et répertoriées par différentes études, la présenté incluse, il est clair que les polymorphismes pourraient expliquer les différences sur la susceptibilité à la maladie et son traitement. Il serait tres important que des données génétiques soient obtenues dans la pratique clinique. Une telle procédure pourrait contribuer à la réalisation des traitements spécifiques même individuels.
Mots Clefs: diabete mellitus de type II, obésité, TCF7L2, MTNR1B, CDKAL1, SLC30A8, FTO, polymorphisme, médecine personnalisée

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INTRODUCTION

Diabetes can be classified into diabetes type I, diabetes type II (DMt2), gestational diabetes and specific types of diabetes. In the case of DMt2, which involves the majority of cases, there is either resistance to insulin or reduced insulin secretion in a proportion that varies from person to person but even in the same person depending on diabetes duration. DMt2 is a chronic metabolic multifactorial disease. The global burden of this disease will amount to 366 million patients by 2030, if its prevalence continues to increase at the current rate. [1,2,3] DMt2 is characterized hyperglycaemia in conjunction with disorders in metabolism of carbohydrates, lipids and proteins, as a result of inadequate insulin secretion and/or insulin action. Glycaemic control plays a key role in the management of DMt2. Individuals with DMt2 should receive personalized diet and training. Antidiabetic agents are indicated for the treatment of patients with DMt2 when diet alone is not sufficient enough. [4]

DMt2 is a heterogeneous syndrome resulting from the interaction of both genetic and environmental factors, which affect important parameters for glucose homeostasis such as insulin secretion and insulin action, the mass of beta cells, distribution of body fat and obesity. [5] Two main pathophysiological disorders contribute to the development of DMt2: inadequate insulin secretion by beta-cells of pancreas and insulin resistance, namely the inability of insulin to produce its usual biological effect in circulation, adequate for normal individuals.[6] Obesity may be the major risk factor for developing DMt2, but only a small percentage of obese people will get it eventually. Moreover, different prevalence has been observed in genetically distinct populations that share the same environment, suggesting a genetic basis. Furthermore, similar populations living under different environmental conditions may develop obesity, demonstrating the important contribution of the environment. [7,8]

In this study we have evaluated the frequency distribution of polymorphisms for 5 genes (*TCF7L2, CDKAL1, MTNR1B, SLC30A8* and *FTO*), reported to be related in previous studies with the development of DMt2 in a population of 513 subjects of Greek origin [9-13].

Transcription factor 7-like 2 (TCF7L2) regulates genes involved in cell proliferation and differentiation. The *TCF7L2* gene is located on chromosome 10q25 in a region of replicated linkage to type II diabetes. [14] It encodes a transcription factor which is involved in the regulation of expression for various genes participating in glucose homeostasis. [15] *TCF7L2* levels regulate apoptosis, proliferation and generally the function of beta-cells in pancreatic isles. [16] One of the most studied polymorphisms is the rs7903146, an intronic SNP, where the presence of T allele increases the risk for DMt2. [17]

Melatonin receptor 1B (MTNR1B) was proven to be the diabetogenic genes associated with the developing of Gestational Diabetes Mellitus (GDM). The gene *MTNR1B* encodes a receptor for melatonin which belongs to the G protein-coupled receptors. Melatonin receptors are expressed mainly in the brain, and *MTNR1B* has also been found in b cells, which implies that genetic variants in the *MTNR1B* might affect pancreatic glucose sensing, insulin secretion, and, conceivably, glucose tolerance. [10,18] *MTNR1B* gene associates with melatonin, a hormone involved in metabolic regulation and homeostasis of glucose and its variations are likely to modify gene expression at transcriptional level, which may be responsible for increased risk of DMt2. Pathophysiological relationship between rs10830963 and DMt2 is likely to take place in gene expression level, since the carriers of predisposing allele G show elevated mRNA levels for receptor *MTNR1B*. [19]

Cyclin-dependent kinase 5 regulatory subunit-associated protein 1-like (CDKAL1) is one of the novel DMt2 associated genes identified recently. *CDKAL1* gene, located in 6p22.3, spans 37 kb and encodes 579 amino acids. [20] It encodes a protein which is involved in the regulation of insulin secretion. Variants of *CDKAL1*, specifically rs7756992, affect the risk for DMt2, but are not associated with insulin secretion or obesity. [21]

Zinc transporter protein member 8 (ZnT-8), which is a member of the zinc transporter family, is only expressedin pancreatic beta cells and provides zinc to insulin maturation and/or storage processes in insulin-secreting pancreatic beta cells. *ZnT-8* (SLC30A8) is a 369-amino acidprotein coded by the gene SLC30A8 which is located in chromosome 8q24.11. [22,23] The non-synonymous single-nucleotide polymorphism (SNP) in *SLC30A8* (rs13266634) affects the amino acid sequence and the suspected allele C, which encodes for the amino acid arginine, instead of the wild type T encoding for tryptophan, is associated with reduction in the capacity of the carrier to adequately regulate zinc entrance in beta-cells, with consequent disturbed insulin secretion. [24,25]

Finally, a variation in the *fat mass and obesity–associated-gene (FTO)* has been linked to obesity in a recent series of genome wide association studies. A number of SNPs in tight linkage disequilibrium with rs9939609, and residing in the first intron of the *FTO* gene, have been associated with obesity in large populations of adults and children. These variants have also been shown to be associated with type 2 diabetes in an obesity-dependent manner. [26] *FTO* gene variants are related to increased energy intake and appetite, whereas they do not appear to exert any influence on consumption. [27] The association of *FTO* with DMt2 is a very controversial issue. A large number of GWAS have shown that *FTO* polymorphisms moderatelyincrease the risk for developing DMt2, however, many of them attribute the risk to indirect effects through increase in BMI, which is known to be a high risk factor. The fact that *FTO* protein is a potential pleiotropic factor is an element showing possible independent contribution in both DMt2 and obesity. [28]

MATERIALS AND METHODS

Patients

For the present study 513 volunteers were recruited, composed of 277 female and 236 male individuals. The mean age was 44 years, ranging from 18 to 94 years. All

volunteers were Caucasians of Greek origin, living in Greece and gave informed consent for sampling. The study was approved by the scientific ethics committee of Faculty of Pharmacy, National and Kapodistrian University of Athens.

DNA extraction

Epithelial cells were collected from the oral cavity of the volunteers using sterile buccal swabs. Genomic DNA was extracted with spin columns (Tissue Nucleospin, Machery-Nagel, Germany) according to manufacture's protocol and stored in-20oC until further use. The absolute measurement of DNA concentration was determined with the Biophotometer (Eppedorf, UK). All DNAs were of adequate quantity.

Real time – PCR

Genotypes of all polymorphisms were determined by real time polymerase chain reaction using Simple Probes Light-Snip-kits and Light Cycler Fast Start DNA Master HybProbe Kit (Roche Diagnostics, Germany). The reactions were performed on a Light Cycler 480 Real-Time PCR system (Roche Applied Science, Germany) in accordance to the manufacturer's recommendations. The hybridization is analyzed by melting curve analysis software provided in the instrument. The genotypes were classified as homozygote for wild type allele, heterozygote and homozygote for mutated allele.

Statistical analysis

Contingency tables 2x2 (1 degree of freedom) were constructed and odds ratio, the confidence interval and p value were calculated. The statistical test was performed with significance level a = 0.05 using Microsoft Excel.

RESULTS

Distribution of polymorphisms for TCF7L2 (rs7903146) gene

Distribution of *TCF7L1* in the study population have shown that 222 volunteers were homozygousthe wild type susceptible allele C:C, 214 heterozygous C:T and 58 homozygous for mutation T (T:T). From the total number of volunteers the results obtained were 45% C:C, 43% C:T 12% T and T:T (table 1).

Distribution of polymorphisms for MTNR1B (rs10830963) gene

The results of *MTNR1B* gene genotypes in the group of volunteers have shown that 273 volunteers were C:C homozygous, (allele C is the wild type), 189 C:G he-terozygous and 34 homozygous for the suspect allele G:G. From the total number of volunteers, 55% was C:C, 38% C:G and 7% G:G (table 2).

Table 1 - Frequency distribution of rs7903146 for TCF7L2 gene

Genotypes	Frequency	
	n	%
C:C	222	44.9
C:T	214	43.3
T:T	58	11.7
TOTAL	494	100.0

Table 2 - Frequency distribution of rs10830963 for MTNR1B gene

Genotypes	Frequency	
	n	%
C:C	273	55.0
G:C	189	38.1
G:G	34	06.9
TOTAL	496	100.0

Table 3 - Frequency distribution of rs13266634 for SLC30A8 gene

Genotypes	Frequency	
	n	%
C:C	273	54.8
C:T	180	36.1
T:T	45	09.0
TOTAL	498	100.0

Distribution of polymorphisms for SLC30A8 (rs13266634) gene

SLC30A8 gene genotypes in the study population were: 273 homozygous for the wild type predisposing allele C, 180 heterozygous C:T and 45 homozygous for mutant T:T. In total, 55% was C:C, 36%, C:T and 9% T:T (table 3).

Distribution of polymorphisms for CDKAL1 (rs7756992) gene

The results for CDKAL1 gene genotypes in the present study have shown that 250 volunteers (51%) were homozygous for the wild type genotype A:A, 195 (40%) were heterozygous A:G and 46 (9%) were homozygous for the mutant genotype G:G. (table 4)

Distribution of polymorphisms for FTO (rs9939609) gene

Genotype results for FTO rs9939609 in the study group have revealed 88 homozygous subjects for the predisposing allele A, which is the wild-type (A:A), 254 heterozygous A:T and 140 volunteers were homozygous for mutant allele T. In total, only 18% were homozygous for the susceptible wild type genotype A:A. 53% were heterozygous A:T and 29% homozygous for the mutated,

Table 4 - Frequency distribution of rs7756992 for CDKAL1 gene

Genotypes	Frequency	
	n	%
A:A	250	50.9
A:G	195	39.7
G:G	46	09.4
TOTAL	491	100.0

Table 5 - Frequency distribution of rs9939609 for FTO gene

Genotypes	Frequency	
	n	%
A:A	88	18.3
A:T	254	52.7
T:T	140	29.0
TOTAL	482	100.0

Table 6 - Results of statistical analysis of differences in allele frequency between the genders

Alleles	Female	Male	OR	95% CI	P	
rs10830963	C	400	335	0.97	0.73-1.29	n.s.
	G	142	115			
rs7903146	T	188	142	1.18	0.90-1.54	n.s.
	C	348	310			
rs13266634	C	388	338	0.86	0.65-1.15	n.s.
	T	154	116			
rs7756992	G	162	125	1.11	0.84-1.47	n.s.
	A	374	321			
rs9939609	A	232	198	1.00	0.78-1.29	n.s.
	T	288	246			

non DMt2 predisposing, genotype T:T (table 5).

Finally, statistical analysis was carried out to identify possible differences in allele frequency between female and male subjects. No statistically significant difference in allele frequency was observed between genders (table 6).

Discussion and Conclusions

The aim of the present study was to evaluate the frequency of 5 single nucleotide polymorphisms which have been associated with the risk of developing DMt2 and obesity, in a Greek population. Four of these polymorphisms, located in different genes, appear to affect the function of pancreatic beta-cells, causing disturbance in insulin secretion, while they did not seem to significantly affect insulin resistance. [17] The fifth polymorphism, on the FTO gene, has been associated with the elevated risk of developing both obesity and DMt2. [29]

There were no significant differences in the frequency of predisposing alleles between other European populations and the Greek population sample examined. Furthermore, in our statistical analysis it was clear that there was no statistically significant difference in genotype frequency between female and male volunteers.

TCF7L2 gene is involved in various genes' expression regulation, participating in glucose homeostasis and perhaps for this reason its polymorphisms reflect the greatest risk (at least in Europe) of all known susceptible diabetogenic genes. The presence of the T allele may increase the risk for heterozygous and for homozygous T:T carriers. The effect appears to be more frequent in Caucasians. Based on the T allele frequency in Caucasians, Population Attributable Risk (PAR) is estimated to be 23.2, a number particularly important as it shows that if we could eliminate the effects expressed by the polymorphism, the incidence of diabetes type 2 in Caucasians would be reduced approximately by a quarter. [17] The homozygous mutant T genotype occurs in 6.83% of global population and in 11.53% of the European population. This study revealed that the homozygous mutant T genotype in the Greek study population is 11.7% (tables 7 and 8).

Table 7 - Comparison of Greek and Northern European population for rs7903146 of TCF7L2 gene

Populations	TT+CT	CC	Odds Ratio	95% CI	p value
Greek n=494	271	222	1,13	0.88-1.45	n.s.
Northern European n=503	261	242			

Table 8 - Comparison of Greek and Global population for rs7903146 of TCF7L2 gene

Populations	TT+CT	CC	Odds Ratio	95% CI	p value
Greek n=494	271	222	1.93	1.59-2.35	<0.0001
Global n=2504	970	1534			

Table 9 - Comparison of Greek and Northern European population for rs10830963 of MTNR1B gene

Populations	GG+GC	CC	Odds Ratio	95% CI	p value
Greek n=496	223	273	0.83	0.64-1.06	n.s.
Northern European n=503	250	253			

Table 10 - Comparison of Greek and Global population for rs10830963 of MTNR1B gene

Populations	GG+GC	CC	Odds Ratio	95% CI	p value
Greek n=496	223	273	1.08	0.89-1.81	n.s.
Global n=2504	1078	1426			

[http://browser.1000genomes.org/Homo_sapiens/Variation/Population?r=11: 92708210-92709210;v=rs10830963;vdb=variation;vf=6107653]

Although there is no significant difference in the allele frequency between southern (Greek) and northern Caucasian populations (OR=1,13) (table 7), the odds ratio (OR=1.93) indicated a significant TT+CT overrepresentation in the Greek volunteer group (n=494), when compared to the global population group (n=2504). Non Caucasian populations seem to be of reduced risk to develop DMt2 ($p<0.0001$).

The rs10830963 intronic polymorphism of the MTNR1B gene is associated with an increased risk of DMt2. The homozygous mutant G genotype occurs in a similar frequency in all three populations studied (8.99% of global, 7.95% of European and 6.9 of the Greek Caucasian population).[30] Accordingly, the risk association of rs10830963 G with DMt2 is estimated to be small for the 38.1% (heterozygous) and 6.9% (homozygous) of Greek subjects. (tables 2, 9 and 10).

[http://browser.1000genomes.org/Homo_sapiens/Variation/Population?r=11:92708210-92709210;v=rs10830963;vdb=variation;vf=6107653]

The rs7756992 polymorphism is located at the fifth intron of CDKAL1 gene. The homozygous mutant G genotype occurs in 19.29% of global population and in 9.94% of the European population. This study revealed that the homozygous mutant G genotype in the Greek study population is 9.36% (tables 11 and 12). Interestingly, there is a significant difference between the European/Greek and the world wide frequency distribution, (OR=1.19) possibly due to the influence Asian and African participation (table 10a). [http://browser.1000genomes.org/Homo_sapiens/Variation/Population?r=11:92708210-92709210;v=rs10830963;vdb=variation;vf=6107653], [30,31]

The occurrence of the rs7756992 mutant G allele in both southern and northern European population seem to be equally distributed (OR=1.12). When compared to the global population, our findings suggest that homozygous GG and heterozygous AG carriers of CDKAL1 gene are underrepresented in the Greek population and may be of slightly reduced DMt2 susceptibility risk (tables 11 and 12). [http://browser.1000genomes.org/Homo_sapiens/Variation/Population?r=6:20679209-20680209;v=rs7756992;vdb=variation;vf=4623442#373430_tablePanel]

The intronic polymorphism rs9939609 of the *FTO* gene is known to express the highest risk for developing obesity, compared to other genetic variations. Individuals carrying one A allele have the 1.3 x higher tendency to become overweight while homozygous A allele carriers are at increased risk (1.9x) to develop obesity. [29] It is estimated

Table 10a - Cumulative comparison of Greek and Northern Europeans with a Global population for rs10830963 of MTNR1B gene

Populations	GG+GC	CC	Odds Ratio	95% CI	p value
Greek+ Northern European n=999	473	526	1.19	1.03-1.38	0.023
Global n=2504	1078	1426			

Table 11 - Comparison of Greek and Northern European population for rs7756992 of CDKAL1 gene

Populations	GG+GC	CC	Odds Ratio	95% CI	p value
Greek n=491	241	250	1.12	0.87-1.43	n.s.
Northern European n=503	233	270			

Table 12 - Comparison of Greek and Global population for rs7756992 of CDKAL1 gene

Populations	GG+GC	CC	Odds Ratio	95% CI	p value
Greek n=491	241	250	0.56	0.46-0.68	<0.0001
Global n=2504	1584	920			

Table 13 - Comparison of Greek and Northern European population for rs9939609 of FTO gene

Populations	AA+AT	TT	Odds Ratio	95% CI	p value
Greek n=482	342	140	0.61	0.45-0.81	0.0011
Northern European n=503	403	100			

Table 14 - Comparison of Greek and Global population for rs9939609 of FTO gene

Populations	AA+AT	TT	Odds Ratio	95% CI	p value
Greek n=482	342	140	2.05	1.66-2.54	<0.0001
Global n=2504	1360	1144			

that approximately 1 billion people weigh about 3 kg more due to the action of *FTO* genetic variants. [28,27] The homozygous mutant T genotype occurs in 45.69% of global population and in 37.18% of the European population. [http://browser.1000genomes.org/Homo_sapiens/Variation/Population?r=11:92708210-92709210;v=rs10830963;vdb=variation;vf=6107653].

This study revealed that the homozygous mutant T genotype in the Greek study population is 29% (table 5). Within the European populations, a so-called north to the south downward slope seems to develop: The frequency of southern European Caucasian homozygous wild-type and heterozygous allele-A carriers, including Greeks, are underrepresented as compared to northern Europeans. Therefore, Greeks seem to be more protected from development of FTO gene initiated overweight (AT) or obesity (AA), than northern Europeans (table 13).

In contrast, comparison of the Greek and Global population for rs9939609 of FTO gene revealed that the odds ratio is equal to 2.05, which means that the risk exposure of a Greek person to develop overweight or obesity is 2.05 times greater than the risk exposure the global population. This significant result ($p<0.0001$) places Greece among the countries with high prevalence to develop DMt2 (table 14).

Although the genetic basis of DMt2 and obesity is well documented, its introduction in daily clinical practice is being incorporated very slowly. However, in the following years DMt2 genetics and pharmacogenetics is expected to significantly contribute in the prevention, diagnosis and treatment of this very common disease in several ways. Genetics and pharmacogenetics may help to understand pathogenic pathways of the disease and eventually define novel pharmacological targets, such as the zinc transporter 8 or melatonin receptor antagonists. [4] In addition, different subtypes of DMt2 with distinct genetic basis could potentially be identified, which may be diagnosed by simple diagnostic analysis (e.g. by a simple gene test). For each DMt2 subtype different therapeutic response to pharmacotherapy would be expected and different therapeutic approaches could be proposed, depending on the individual genetic background of each patient (personalized therapy). Our findings may significantly contribute to DMt2 early diagnosis and prevention, and may lead to individualization of treatment. Such development would be clinically and financially important for both individual patient and community health care system.

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