Research paper

The rs10830963 variant of melatonin receptor MTNR1B is associated with increased risk for gestational diabetes mellitus in a Greek population

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ABSTRACT

OBJECTIVE: To investigate the association between Gestational Diabetes Mellitus (GDM) and the variants rs10830963 and rs1387153 in the MTNR1B locus in a sample of the Greek population. DESIGN: One hundred seventy-five unrelated pregnant Greek women (77 with GDM and 98 non-diabetic control subjects) were enrolled and the SNaPshot method was employed in order to investigate the association between GDM and the variants rs10830963 and rs1387153 in the MTNR1B locus. Pregnant women were screened for GDM at the 26th week with the 75 g glucose oral glucose tolerance test according to the American Diabetes Association criteria. RESULTS: The GG genotype and the G-allele of the rs10830963 (C/G) variant was found to be positively associated with a significantly increased risk for GDM (p = 0.047 and p = 0.012, respectively). No differences in fasting glucose and insulin levels were found between GDM patients with and without the studied variants. The MTNR1B locus (rs10830963 C/G) seems to predispose for GDM in Greek pregnant women. CONCLUSIONS: Our study confirms the association of GDM with the rs10830963 (C/G) variant in a sample of the Greek population. Population based whole genome screening studies and larger studies with detailed phenotypic data in patients with GDM are needed to address the clinical significance of this finding.

Key words: Gestational diabetes mellitus, Melatonin receptor, MTNR1B, rs10830963, rs1387153

INTRODUCTION

Pregnancy is characterized by peripheral insulin resistance which is compensated by an increase of insulin secretion.[1,2] Gestational diabetes mellitus (GDM) occurs in about 2-5% of all pregnancies and develops when insulin secretion fails to overcome insulin resistance.[3] GDM shares many features with diabetes mellitus type 2 (T2DM), such as glucose intolerance, insulin resistance and impaired insulin secretion.[4] Uncontrolled GDM increases the risk

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for adverse neonatal outcome and long-term consequences during adulthood. Although no studies have evaluated the heritability of GDM, family studies suggest that GDM aggregates within families and is associated with a history of T2DM.[5] On the other hand, multiple genes, environmental and behavioural factors combine to cause T2DM. Because of the striking parallels between GDM and T2DM, it is likely that GDM is a multigenic disease related to T2DM. Recent studies on the etiology of GDM evaluate the role of common polymorphisms in genes involved in T2DM predisposition. Specifically, genome-wide association studies (GWAS) revealed that variations within or near the melatonin receptor 1B (MTNR1B) locus might be implicated among others in T2DM development.[6-9]

Melatonin is a circulating hormone secreted mainly from the pineal gland, while other endocrine cells may also synthesize and release it.[10,11] Antepartum maternal 24h integrated melatonin levels are significantly increased compared with postpartum levels, eventually influencing the concentration of receptors of gonadal steroids in the gravid uterus at term and the psychic perception of painful uterine contractions during labour.[12] It has been proposed that melatonin influences both insulin secretion and endogenous glucose production.[13] Melatonin exerts its effects *via* two distinct receptors, MTNR1A and MTNR1B, members of the G-protein-coupled receptor family.[14] Both receptors are expressed in human and rodent islets, with MTNR1A predominantly in glucagon-producing α-cells.[15] MTNR1B is also expressed in human pancreatic islets and pancreatic β-cells.[16,17] MTNR1B is coupled to an inhibitory G protein.[15] Addition of melatonin blocks cAMP formation in β-cells.[14] When MTNR1B expression on β-cells is increased, insulin secretion might be impaired due to diminished intracellular cyclic cAMP levels. Recent data from a meta-analysis of GWAS showed that variations in MTNR1B are a common genetic determinant of increased fasting plasma glucose and risk for T2DM.[9,17,18] A study in a European population revealed that rs1387153 was significantly associated with fasting plasma glucose (FPG) levels and T2DM, while another study in a European population found significant associations of rs10830963 with FPG and T2DM.[9] Specifically, in GWAS the rs10830963 variant of MTNR1B has been associated with increased expression of MTNR1B, reduced β-cell activity and increased fasting glucose levels.[9,17,18] A recent study by Kim et al confirmed the association between the variants rs10830963 and rs1387153 in the MTNR1B locus and GDM in Korean women.[19]

In the present study we investigated whether GDM has a genetic predisposition pattern similar to that of T2DM in a study of unrelated pregnant Greek women with or without GDM by assessing the variants rs10830963 and rs1387153 in the MTNR1B locus.

SUBJECTS AND METHODS

Subjects and Protocol

The study involved 175 Greek pregnant women, 77 with GDM and 98 with normal glucose tolerance. The 77 women with GDM had no clinical or biochemical indication of T2DM prior to their pregnancy. Pregnant women were screened for gestational diabetes at the 26th week with a 75g glucose oral glucose tolerance test (OGTT) according to the criteria established by the American Diabetes Association (glucose levels at 0 min >95mg/dl, glucose levels at 60 min >180mg/dl, glucose levels at 120 min >155mg/dl, with at least two values over the normal ones) with onset or first recognition during pregnancy.[20]

All women with GDM were monitored for adverse metabolic and obstetric manifestations till delivery. They all delivered at term, with the exception of a few in whom additional obstetric complications set in. No women with GDM developed T2DM up to 12 months of follow-up postpartum. Age, pre-pregnancy body mass index (BMI), weight gain during pregnancy and family history for diabetes were recorded for all women (Table 1). Early, in the third trimester of pregnancy, a blood sample was drawn from all patients to determine fasting plasma glucose, fasting plasma insulin and genetic polymorphisms of the MTNR1B locus. Insulin sensitivity was estimated by calculating the Homeostatic model assessment of insulin resistance (HOMA-IR) index using the HOMA Calculator v2.2.2 software (http://www.dtu.ox.ac.uk).

Informed written consent was obtained from all participants and the Hospital Ethics Committee's approval was granted.

Table 1. Clinical and biochemical characteristics (mean±SD) of the studied Greek women with and without GDM

	GDM (n = 77)	Controls (n = 98)	P value
Age (y)	35.45±4.44	31.39±5.25	<0.001
BMI (kg/m^2)	25.83±5.13	26.76±6.26	0.279
Weight gain (kg)	7.19±5.22	7.71±3.96	0.457
Insulin therapy %	33.25	-	-
Third trimester			
Fasting glucose (mg/dl)	95.94±18.83	79.89±7.08	<0.001
Fasting insulin (mU/ml)	13.44±8.47	8.36±4.21	0.015
HOMA-IR[a]	1.81±1.12	1.11±0.49	0.015

[a]Homeostatic model assessment of insulin resistance

Genetic studies

To investigate the presence of MTNR1B rs10830963(C/G) and rs1387153(C/T), DNA was isolated from peripheral blood using the NucleoSpin blood kit (Macherey-Nagel, Germany). To confirm the integrity of the DNA, initially a 430-bp sequence in the human glyceraldehyde-3-phosphatate dehydrogenase gene was amplified.

Multiplex PCR primers and SNaPshot primers

The reverse and forward primer sequences that were used for the multiplex PCR and SNaPshot reaction are given in Table 2.

Multiplex PCR amplification

Multiplex PCR was performed in a volume of 25 μl containing 2.5μl 10x buffer (containing 670mM Tris-HCL, 165mM $(NH_4)_2SO_4$, 35mM $MgCl_2$, 100mM BSA and 290μl H_2O) 2.5 μl 10x dNTPs (5mM of each), 2.5 units HotStart Taq Polymerase, 2μl primer mix (10 pmol/μl of each primer previously described, stored at -20°C), 16μl H_2O and 2μl of template DNA. Thermal cycler conditions were: 95°C for 15 min, 30 cycles of 95°C for 15 sec, 57°C for 20 sec, 72°C for 30 sec and finally 5 min at 72°C. Multiplex PCR products were checked for quality and yield by running 5μl in 2% agarose-TBE gels. The remaining PCR products were treated with 25μl Ultra-Pure H_2O (Qiagen PCR Purification kit) to remove excess deoxyribonucleotide or dideoxyribonucleotide triphosphates (dNTPs or ddNTPs) and primers, respectively.

SNaPshot analysis

SNaPshot analysis was performed using an Applied Biosystems SNaPshot Multiplex Kit as previously described.[21] Reactions were performed in a volume of 10μl containing 5μl of SNaPshot Multiplex Ready Reaction Mix, 1μl of probe mix (10 pmol/μl of each primer previously described, stored at 4°C) 3μl H_2O and 1μl of UltraPure H_2O-treated multiplex PCR product. Extension reactions were performed in a thermal cycler and consisted of 25 cycles of denaturation at 96°C for 10 sec, annealing at 50°C for 5 sec and extension at 60°C for 30 sec. 1μl of labelled extension products was mixed with 0.5μl FAST UP (UNIVERSAL, -20°C) and 10μl buffer (UNIVERSAL, -20°C), and it was incubated at 37°C for 10 min and at 75°C for 5 min. 1.5μl of the diluted extension product was mixed with 9μl of HiDi™ formamide and 0.5μl of Genescan-120LIZ size standard. Products were denatured at 95°C for 5 minutes, then separated using an ABI PRISM 3100 Genetic Analyzer with a 36 cm length capillary and POP-4™ polymer. Analysis was performed using GeneMapper 3.7 Software.

Statistical analysis

A comparison of clinical and biochemical characteristics between GDM subjects and controls was obtained *via* 2 independent samples t-test. Comparison

Table 2. Multiplex PCR and SNaPshot reaction, primer information

Multiplex PCR Primers	rs10830963F:	GCAGTCAGAAGCTGTGGTCTGACA
	rs10830963R:	GGCAAGGAACAGGGGCCACA
	rs1387153F:	GCCTGTCGACTTGGGTTGGTGT
	rs1387153R:	CCCCTGGGCCTAAGAGCCTCC
SNaPshot Reaction Primers	snap10830963F	AGTGATGCTAAGAATTCACACCATCT
	snap1387153R	GCTAGCAAATTAACTTACTGCCCTG

of the genotypes containing the polymorphism and the wild-type genotypes was performed with a 2x3 Pearson's x^2 test (3 genotypes for the 2 subject groups) and separate 2x2 Pearson's x^2 test (wild-type genotype and genotype containing the polymorphism for the 2 subject groups). Allele frequencies comparison was also performed in the same way (wild-type allele *versus* polymorphic allele for the 2 subject groups). Comparison of quantitative parameters among groups of GDM patients with different allelic profile were performed with one-way analysis of variance, followed by Fischer's *post-hoc* test. To test for the effect of possible confounders (age, pregnancy BMI and weight gain) upon variables of insulin resistance (HOMA-IR, fasting plasma glucose or fasting insulin), a backward stepwise regression analysis was performed taking as independent variables the age, the pregnancy BMI and the pregnancy weight gain, and as dependent variable the HOMA-IR, the fasting plasma glucose and the fasting plasma insulin values. All statistical analysis was performed with the STATISTICA 6 software (StatSoft, USA). The significance level was set to 5%.

RESULTS

1. Clinical and biochemical characteristics

The clinical and biochemical characteristics of the study subjects are reported in Table 1.

2. MTNR1B polymorphisms in women with GDM

The genotype and allele frequencies distributions of the investigated rs10830963 and rs1387153 MTNR1B polymorphisms as well as their statistical comparisons are presented in Tables 3a, 3b, 3c, 3d and 4. The Pearson's x^2 for the rs10830963 (C/G) polymorphism on a 2x3 contingency table was 6.01 which is significant for 2 degrees of freedom (P=0.049), while the same calculation for the rs1387153 (C/T) polymorphism was 0.72 which is non-significant for 2 degrees of freedom (P=0.698). Regarding the rs10830963 polymorphism, the GG genotype frequency was significantly greater in GDM as compared to control women (Table 3b), while no statistically

Table 3a. Comparison of the CG genotype of the rs10830963 MTNR1B polymorphism *versus* the wild-type genotype CC observed in the studied Greek GDM and Control pregnant women

rs10830963 (C/G) Genotype	GDM	Controls	
CC	30	56	$x^2 = 3.73$
CG	31	30	P = 0.053

Table 3b. Comparison of the GG genotype of the rs10830963 MTNR1B polymorphism *versus* the wild-type genotype CC observed in the studied Greek GDM and Control pregnant women

rs10830963 (C/G) Genotype	GDM	Controls	
CC	30	56	$x^2 = 4.35$
GG	16	12	P = 0.037

Table 3c. Comparison of the CT genotype of the rs1387153 MTNR1B polymorphism *versus* the wild-type genotype CC observed in the studied Greek GDM and Control pregnant women

rs1387153 (C/T) Genotype	GDM	Controls	
CC	39	52	$x^2 = 0$
CT	26	35	P >0.999

Table 3d. Comparison of the TT genotype of the rs1387153 MTNR1B polymorphism *versus* the wild-type genotype CC observed in the studied Greek GDM and Control pregnant women

rs1387153 (C/T) Genotype	GDM	Controls	
CC	39	52	$x^2 = 0.64$
TT	12	11	P = 0.424

Table 4. Comparison of the polymorphic (C and C) *versus* the wild-type (G and T) single alleles of the rs10830963 MTNR1B and rs1387153 MTNR1B polymorphisms observed in the studied Greek GDM and Control pregnant women and their frequencies

	Allele	GDM	Control	
rs10830963 (C/G)	**C**	91(59.09%)	142 (72.45%)	$x^2 = 6.91$
	G	63 (40.91%)	54 (27.55%)	P = 0.009
	Allele	**GDM**	**Control**	
rs1387153 (C/T)	**C**	104 (67.53%)	139 (70.02%)	$x^2 = 0.47$
	T	50 (32.47%)	57 (29.08%)	P = 0.493

significant difference was found regarding the CC and CG genotypes of this polymorphism (Table 3a). Regarding the rs1387153 polymorphism, no difference was found in the corresponding genotypes (CC, CT, TT) frequencies between GDM and control women (Tables 3c and 3d). When we compared the frequencies of the single alleles we found a statistically significant difference between the C and G allele frequencies for the rs10830963 (C/G) polymorphism, while there was no statistically significant difference between the C and T allele frequencies of the rs1387153 polymorphism (Table 4).

There were no significant differences in the clinical and biochemical characteristics among the GDM women with different genotypes of the tested polymorphisms (Tables 5a and 5b). There was no significant influence of age, pregnancy BMI and pregnancy weight gain when taken as independent variables upon insulin resistance variables (HOMA-IR, fasting plasma glucose and fasting plasma insulin) by backward stepwise regression analysis (data not shown).

DISCUSSION

In the present study, we investigated the rs10830963 and rs1387153 variants in the MTNR1B locus in unrelated pregnant Greek women with or without GDM. We found that the rs10830963 polymorphism of the MTNR1B gene locus is more frequent in GDM than in non-diabetic pregnant women, indicating a possible association of this polymorphism with GDM. No difference was found in the frequency of appearance of the rs1387153 MTNR1B polymorphism between GDM and non-diabetic pregnant women. In a recent study Kim et al found that both SNPs were associated with GDM and FPG and that the two genetic variants of MTNR1B were stronger risk factors for GDM in the Korean population compared to the previous results for T2DM in European populations.[19] The authors' explanation for this discrepancy is the inherent genetic differences of Asian and European populations.[19] This argument might stand as well for the explanation of the differences between this and the Korean study, while alternatively they could be attributed to the significantly smaller number of participants in this study.

In the past, the rs10830963 polymorphism was associated reproducibly in several studies of patients with T2DM.[9,10,22] More specifically, MTNR1B rs10830963

Table 5a. Comparison of the clinical and biochemical characteristics (mean±SD) in GDM pregnant women according to the different genotypes of the rs10830963 MTNR1B polymorphism (CC, CG, GG). Statistical significance is set at 0.05. P_1 and P_2 refer to the comparison of the wild-type genotype (CC) with the CG and the GG genotype, respectively. P_3 refers to the comparison between the CG and the GG genotypes

rs10830963 (C/G)	CC	CG	GG	P
Age (year)	35±4.15	35.83±4.39	34.4±4.76	P_1 = 0.188 P_2 = 0.844 P_3 = 0.193
Pregnancy BMI (kg/m^2)	25.05±3.79	25.27±4.88	26.84±5.91	P_1 = 0.065 P_2 = 0.061 P_3 = 0.852
Weight gain (kg)	6.67±4.19	7.67±6.41	7.03±4.79	P_1 = 0.752 P_2 = 0.987 P_3 = 0.780
Fasting plasma glucose (mg/dl)	96.58±14.16	96.45±25.24	95.28±14.94	P_1 = 0.123 P_2 = 0.053 P_3 = 0.432
Fasting insulin (μU/ml)	15.01±12.37	15.18±9.18	15.42±4.86	P_1 = 0.978 P_2 = 0.759 P_3 = 0.978
HOMA-IR[a]	2.5±0.95	2.65±0.92	2.35±0.49	P_1 = 0.889 P_2 = 0.581 P_3 = 0.628

Table 5b. Comparison of the clinical and biochemical characteristics (mean±SD) in GDM pregnant women with different genotypes of the rs1387153 MTNR1B polymorphism (CC, CT, TT). Statistical significance is set at 0.05. P_1 and P_2 refer to the comparison of the wild-type genotype (CC) with the CT and the TT genotype, respectively. P_3 refers to the comparison between the CT and the TT genotypes

rs1387153 (C/T)	CC	CT	TT	P
Age (year)	34.83±4.73	35.54±4.09	34.91±4.8	P_1 = 0.796 P_2 = 0.443 P_3 = 0.619
Pregnancy BMI (kg/m^2)	26.61±5.67	25.75±5.92	24.06±3.46	P_1 = 0.224 P_2 = 0.030 P_3 = 0.264
Weight gain (kg)	7.16±5.05	7.54±5.75	6.5±5.12	P_1 = 0.332 P_2 = 0.764 P_3 = 0.265
Fasting glucose (mg/dl)	96.09±16.86	94.5±23.48	99.09±14.15	P_1 = 0.130 P_2 = 0.208 P_3 = 0.865
Fasting insulin (μU/ml)	17.4±10.9	10.62±3.22	10.02±6.19	P_1 = 0.183 P_2 = 0.176 P_3 = 0.916
HOMA-IR	2.95±0.92	2.07±0.83	1.75±0.64	P_1 = 0.107 P_2 = 0.100 P_3 = 0.900

SNP was shown to predict the development of T2DM in 2,201 subjects among 16,061 Swedish (from the Malmoe Preventive Project, MPP) and 2,770 Finnish (from the Botnia study) subjects during a median follow-up period of 23.5 years.[10] The frequency of the risk G allele of SNP rs10830963 was higher in individuals from the MPP study who converted to T2DM compared to non-converters that yielded a modestly increased risk.[11] In GWAS, the rs10830963 variant of MTNR1B has been associated with increased expression of this receptor and reduced β-cell activity and subsequently with increased fasting glucose levels.[9,17,18] In this study no difference was observed in fasting glucose and insulin levels between GDM women carriers and non-carriers of this polymorphism at baseline. It is possible that the decreased insulin secretion associated with the presence of this polymorphism could be revealed more readily after β-cell stimulation by OGTT. Unfortunately, for the majority of the studied women the OGTTs were not executed in our institution and insulin levels were not measured.

In contrast to Bouatia-Naji et al, who have shown increased association of rs1387153 with increased fasting plasma glucose and T2DM risk in European populations, the frequency of this polymorphism in this study was not higher in GDM women as compared to non-diabetic pregnant women.[17]

MTNR1A and MTNR1B are members of the G-protein-coupled receptor family.[15] Both receptors are expressed in human and rodent pancreatic islets. MTNR1B is also expressed in human pancreatic islets and pancreatic β-cells.[16,17] MTNR1B is coupled to an inhibitory G protein.[15] Addition of melatonin blocks cAMP formation in β-cells.[14] Adenylate cyclase is the predominant mode of action for incretin hormones, such as GLP-1 and glucose-dependent insulinotropic polypeptide (GIP), while there is also evidence suggesting that glucose stimulation of the β-cell is exerted via rise of intracellular cAMP. Thus, increased expression of MTNR1B on β-cells leads to impaired insulin secretion. Interestingly, Lysenko et al have shown that individuals carrying the G allele in the rs10830963 polymorphism of the MTNR1B exhibit higher expression of this melatonin receptor on the β-cell as compared with carriers of the C allele.[10]

Currently, screening for GDM is based exclusively on biochemical criteria. Genetic screening is

not yet used, most probably because there is limited knowledge concerning GDM susceptibility candidate genes or glucose induced epigenetic alterations. To our knowledge, this is the first report of an association between GDM and the rs10830963 variant of the MTNR1B susceptibility locus in a population of pregnant Caucasian women. Population based whole genome screening studies and larger studies in European (Caucasian populations) with detailed phenotypic data in the future are needed to address the clinical significance of this finding because GDM is the cause of *in utero* originating adulthood diseases and future maternal metabolic disorders.

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