Research Communication

Effects of *MC4R*, *FTO*, and *NMB* Gene Variants to Obesity, Physical Activity, and Eating Behavior Phenotypes

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Summary

Obesity is a major contributory factor of morbidity and mortality. It has been suggested that biological systems may be involved in the tendency to be and to remain physically inactive also behaviors such as food and beverage preferences and nutrient intake may at least partially genetically determined. Consequently, besides environment, genetic factors may also contribute to the level of physical activity and eating behaviors thus effect obesity. Therefore the aim of this study is to investigate the effect of various gene mutations on obesity, physical activity levels and eating behavior phenotypes. One hundred patients and 100 controls were enrolled to the study. Physical activity levels were measured with an actical accelerometer device. Eating behaviors were evaluated using Three-Factor Eating questionnaire (TFEQ). Associations between eating behavior scores and physical characteristics were also evaluated. The information about other obesity risk factors were also collected. Mutations were investigated with PCR, direct sequencing and Real-Time PCR. rs1051168, rs8050146 −2778C > T mutations were found statistically significant in patients, rs1121980 was found statistically significant in controls. 21 mutations were found in *MC4R* and near *MC4R* of which 18 of them are novel and 8 of them cause amino acid change. In addition, it was found that, some obesity related factors and questions of TFEQ are associated with various investigated gene mutations. Any relation between gene mutations and physical activity levels were not detected. It is thought that, due to the genotype data and eating behaviors, it may be possible to recommend patients for proper eating patterns to prevent obesity. © 2016 IUBMB Life, 68(10):806–816, 2016

Keywords: *obesity; physical activity; eating behavior; FTO; MC4R; NMB*

Additional Supporting Information may be found in the online version of this article.

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Introduction

Obesity is a complex disease, which results from an imbalance between energy intake and expenditure, producing an excessive fat depot accumulation (1). There is convincing evidence that human obesity is a multifactorial disorder where both genes and lifestyle factors, including physical activity and diet are important factors (2). Obesity-related genes have reciprocal effects on energy intake and expenditure, although their primary effect appears to be on the regulation of appetite and satiety. In this sense, the melanocortin 4 receptor (*MC4R*) is a strong obesity candidate gene (1). *MC4R* gene is expressed in various sites in the central nervous system and has been implicated in mediating most of the effects of melanocortin on food intake and energy expenditure (3,4). MC4R is a seven trans-

membrane G protein-coupled receptor, consisting of 332 amino acids, and is encoded by a single exon located in chromosome 18q221 (5). In humans, *MC4R* mutations cosegregate with severe obesity and are related to defective binding or signaling properties of the variant receptors (6). MC4R plays a critical role in the leptin/melanocortin axis and regulates food intake (7,8). In addition, interruption of the melanocortin signaling in the hypothalamus may lead to reduced physical activity and obesity in mice (4).

Common intronic variants of the *FTO* [fat mass (FM) and obesity-associated] gene have been also found to be associated with obesity-related traits in humans (9). The *FTO* gene is composed of nine exons that span more than 400 kb on chromosome 16 (1). *FTO* encodes a protein with 2-oxoglutarate-dependent nucleic acid demethylase activity concerned with DNA repair, metabolism of fatty acids, and post-translational modifications (10). *FTO* is expressed predominantly in brain, pancreatic islet tissue, adipose tissue, and adrenal glands (11). Its high expression in the hypothalamus, pituitary, and adrenal glands indicates an important role in the hypothalamic-pituitary-adrenal axis, itself implicated in body weight and satiety regulation. Heritability studies have revealed that appetite and eating behavior associated with susceptibility to weight gain are also under genetic control. Food deprivation increases *FTO* expression in the mouse hypothalamus, consistent with the hypothesis that genes at the *FTO* locus play a role in governing eating behavior. However, specific eating behavior phenotypes associated with this genetic polymorphism have not yet been identified (12). In addition, *FTO* gene might participate in controlling energy expenditure; however, a study in twins failed to show evidence of an interaction between the *FTO* variant and the environment (13).

Neuromedin beta (*NMB*) is another obesity candidate gene. It is located on chromosome 15q24-q25. It is a member of the bombesin-like peptide family, a subfamily of ranatensins (14). The bombesin-like peptides family has many biological effects that may be associated wtih eating behaviors and obesity, including the modulation of the serotonergic (5-HT) system, the stimulation of pancreatic hormones such as peptide YY (PYY) and the regulation of thyrotropin secretion in the pituitary. These pathways may all be important for the NMB biological activity related to the control of eating behaviors. Therefore, by stimulating PYY, the *NMB* gene could increase the satiety signal or decrease the hunger signal (15). *NMB* mutations revealed many associations with dietary disinhibition, susceptibility to hunger and FM change over time. It was therefore considered possible that NMB could play a role in the regulation of eating behavior and might affect body weight. Therefore the goals of our study were to investigate the association between *MC4R*, *FTO,* and *NMB* gene polymorphisms with obesity, physical activity, and eating behaviors. In this study, we analyzed independent and combined effects of the rs9939609, rs1121980, rs1421085, rs1477196, rs1861868 ve rs805013 risk alleles in *FTO*, rs1051168 mutations in *NMB*, rs17782313 and rs7242169 risk alleles near *MC4R* and whole *MC4R* gene mutations on obesity, obesity related factors as well as their interactions with physical activity levels and eating behaviors in Turkish population.

Experimental Procedures

Study Participants

Totally, 100 obese cases and 100 healthy individuals were enrolled to the study from Marmara University Hospital, Sports Physiology and Endocrinology Departments from September 2013 to September 2015. The present study complies with the Declaration of Helsinki and was approved by the Institutional Ethics Committee of Yeditepe University, Istanbul, Turkey. All individuals gave written informed consent prior to study inclusion. Individuals were excluded if they had a significant medical condition, were taking medication known to affect body weight, had abnormal hepatic, renal, or thyroid function or had a psychiatric disorder that might impede protocol compliance. Also to avoid the confounding effect of professional dietary advice, subjects with prevalent diabetes were excluded.

Phenotypic Characterization

Body weight was measured to the nearest 0.1 kg with a digital balance, and height was measured to the nearest 1 mm with a stadiometer. The subjects removed their shoes and wore light clothing for these measurements.

The waist circumference (WC) was measured at the level of the trunk where the girth was minimal; this was the location where there was a noticeable indentation of the trunk when viewed from the front. If there was no such indentation, the measurement was made at the level that was midway between the lowest rib (laterally) and the iliocristale landmark. Hip circumference (HC) was measured at the widest protuberance across the pelvis in cm with a nonstretchable tape measure. Waist-hip ratio (WHR) was calculated by dividing WC with HC. Waist-height ratio (WHtR) was calculated by dividing WC with height. Percentage of body fat was estimated by bioelectric impedance analysis method via using Body Composition Analyzer Model BC-418 MA III (Tanita, Middlesex, UK). FM and fat-free mass (FFM) were calculated by the composition analyzer system, and was expressed as kg. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Subjects with a BMI less than 25 were defined as normal weight, and those with a BMI of 30 or greater were defined as obese. Systolic and diastolic blood pressure were measured at the right arm after a 10-min rest in the supine position using a calibrated sphygmomanometer (16).

Blood samples were collected after an overnight fast. Serum fasting triglycerides, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), glucose, HbA1c, and insulin levels were determined by commercially available test kits using a biochemical auto-analyzers (Hitachi 7060, Tokyo, Japan, Roche Diagnostics, Mannheim, Germany;

Boehringer, Mannheim, Germany; Ortho Clinical Diagnostics, Neckargemuend, Germany).

Physical Activity Estimation

Physical activity of subjects was estimated by using an activity monitor (Actical, USA). Activity monitor was used to measure the frequency, duration, and intensity of physical activity (6). The monitor was designed to measure whole body physical activity. It contained an omnidirectional accelerometer built from a cantilevered rectangular piezo-electric bimorph plate and seismic mass, which was sensitive to all directional movements. On the test day, the monitor was affixed above the iliac chest of hip with an elastic belt and adjustable buckle. The subjects were asked to wear the monitor for 72 h, and to behave in their usual manner. They were wanted to un-wear the monitor only during shower. The time sampling interval (epoch) was set at 15 sec (2, 17).

Acceleration of the device is integrated and expressed as a number—activity counts—for each 1 min recording interval that is stored in memory until the device is returned and data uploaded. Total activity represents the cumulative sum of accelerometer counts in 24-h (counts/d). Minutes in physical activity range was categorized into sedentary, light, moderate, and vigorous levels of physical activity according to thresholds defined in terms of energy expenditure (6,17). Thresholds of the device to distinguish light/moderate and moderate/vigorous are shown respectively, light/moderate cut-point: 0,031 kcal/min/kg, moderate/vigorous cut-point: 0,083 kcal/min/kg. According to this classification three groups were revealed, such as vigorous ($n = 18$), moderate ($n = 80$), and light ($n = 2$). Additional classification was used according to total activity counts. Two groups were revealed as, active ($n = 72$) and very active ($n = 26$). Cut-point between active and very active groups were determined by using ROC curve analysis (cut-point: 179713). When these two methods were compared with each other any statistically significant difference were not detected. Therefore all of the physical activity evaluations were carried out with the first method. Patients in moderate group were further divided according to total activity spent in intervals. In the first group, intervals were set at 10 min (total of 30 min: 3 $\times$ 10 min group; $n = 69$), in the second group interval was set at 30 min continously (1 $\times$ 30 min group; $n = 11$).

Resting Metabolic Rate (RMR) Measurement

The indirect calorimetry method was used for RMR measurements (18). The measurement of heat production by measuring oxygen consumption and/or carbon dioxide production is called indirect calorimetry. Subjects were instructed to refrain from caffeine, nicotine, strenuous exercise, and alcohol for one day prior to their scheduled morning appointment. After a 12-h fasting period, the subjects were fitted with an appropriately-sized air-cushioned face-mask and were asked to remain still, but awake, for 15 min. The expired air was collected continuously. Oxygen consumption levels (VO2) were measured by a metabolic measurement system (Fitmate, Cosmed, Italy). RMR was calculated automatically by the system according to Weir equation (18). RMR was evaluated as slow, normal, or fast according to manufacturer's categorization.

Eating Behavior Measurements

Eating behaviors of the individuals were assessed with the use of the Three-Factor Eating Questionnaire (TFEQ) to cases and controls. TFEQ is a widely used scale to quantify eating behaviors in normal-weight, overweight, obese, and in individuals with eating disorders (19). This questionnaire has 18 items, 13 true-false and the remainder asking for a rating on a scale of 1–4 or 1–8 (*20*). The 3 eating behaviors assessed by the 18 questions of the TFEQ are cognitive dietary restraint (2, 11, 12, 15, 16, and 18th questions), uncontrolled eating (1, 4, 5, 7, 8, 9, 13, 14, and 17th questions) and emotional eating (3, 6, and 10th questions) (21,22). In some researches, uncontrolled eating were divided into two categories as disinhibition (1, 7, 13, 14, and 17th questions) and susceptibility to hunger (4, 5, 8, and 9th questions) (15). To our knowledge, the TFEQ has not been used before in Turkish population. In our previous study, it was translated into Turkish and validated. The means and distributions of answers to each question as well as the overall scores for eating behaviors were investigated and similarly it was found that TFEQ measures cognitive dietary restraint (2, 11, 12, 15, 16, and 18th questions), disinhibition (1, 7, 13, 14, and 17th questions), susceptibility to hunger (4, 5, 8, and 9th questions) and emotional eating (3, 6, and 10th questions) as well (23). Therefore, it can be stated that this quesitonare measures four different factors. Then validated Turkish version of TFEQ was used in this study. Restrained eating is defined as the tendency to restrict food intake to control body weight. Disinhibition is loss of restraint resulting in overeating. It is the inability to resist emotional and social eating cues. Susceptibility to hunger expresses the need for food as perceived by the individual (8,20). Emotional eating is defined as inability to resist emotional cues (22). The TFEQ was administered at the same visit of individuals. TFEQ is shown in Online Resource 1 (Supporting Information).

Molecular Analysis

Total genomic DNA was extracted from peripheral blood leukocytes collected from each subject, into EDTA-tubes, using the High Pure PCR Template Preparation Kit (Roche, Basle, Switzerland), according to manufacturer's instructions. Mutations of *MC4R* were numbered 119 bp before the start codon as the beginning for mutation numbering, as well as with cDNA numbering by using Human Genome Variation nomenclature (http://www.hgvs.org/mutnomen/).

PCR Amplification

MC4R gene was amplified as 2 fragments and the region located 2696–3275 bp upstream of the *MC4R* start codon were amplified as one fragment by polymerase chain reaction (PCR) using 50–100 ng of total DNA. PCR amplifications were performed in a total volume of 50 μL containing 50–100 ng DNA template in 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 1.5 mM

$MgCl_2$, 100 mM each of dNTPs, 1.0U Taq DNA polymerase, and 1.0 mM of each primer. *MC4R* was amplified as 2 fragments. The conditions of PCR amplification for fragment 1 and 2 are similar except annealing temperatures. The conditions for amplifiying of fragment 1 are as follows: a denaturation step at 95°C for 3 min followed by 35 cycles at 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, a final extension at 72°C for 5 min, and a stop at 4°C. Forward primer 5′-ATGGCAATTTTAG CCTCACA-3′ and reverse primer 5′-TGGACATAGAGAGAA GCCATGA-3′ were used for amplification of fragment 1 (760 bp.) The conditions of PCR amplification for fragment 2 are also similar with fragment 1 except annealing temperature (58°C). Forward primer 5′-ATTGCAGTGGACAGGTACTTTA-3′ and reverse primer 5′-AGTACCCTACACGGAAGAGAAA-3′ were used for amplification of fragment 2 (680 bp.). The conditions of PCR amplification for the region located 2696 to 3275 bp upstream of the *MC4R* start codon are also similar with *MC4R* conditions which were described above except annealing temperature (56°C): Forward primer 5′-GGCATTTCTCCAAAGAT TAT-3′ and reverse primer 5′-CACCGCACCTTGCTAAT-3′ were used for amplification. All PCR products were fractionated by electrophoresis on a 2% agarose gel.

Purification of PCR Products and Direct Sequencing of MC4R and the Region Located 2696–3275 Bp Upstream of the MC4R Start Codon

All PCR products were purified by using the High Pure PCR Product Purification Kit (Roche), according to the manufacturer's instructions before direct sequencing. Then, purified PCR products were sequenced by using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham, Buckinghamshire, UK) in ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Real-Time PCR (RT-PCR)

RT-PCR were performed in a total volume of 30 μL containing 20 μL 2xPCR master mix, 1 μL genotyping primer prob mix and 4 μL distilled water and 5 μL DNA. The conditions of RT-PCR for detecting rs9939609, rs1121980, rs1421085, rs1477196, rs1861868, rs8050136 mutations in *FTO*, rs17782313 mutation which is located near 188 kb of *MC4R* and rs1051168 mutation in *NMB* gene are as follows: an enzyme activation step at 95°C for 8 min followed by 10 cycles at 95°C for 10 sec, 60°C for 1 min, and 35 cycles at 95°C for 10 sec, and at 66°C for 1 min. All mutations were detected with Qiagen rotorgene Q device (Germany).

Statistical Analysis

Statistical analyses were performed using SPSS software version 23 and MedCalc 16.2. by Department of Biostatistics and Medical Informatics of Yeditepe University. Descriptive analyses were presented using means and standard deviations for contionus data and frequecies and percentages for categorical data. The variables inverstigated using Kolmogorov Smirnov test to determine whether or not they are normally distributed. Since the variables were normally distributed, two independent samples t test was used to compare them. Since the variables are not normally distributed, Mann-Whitney U test was used to compare the patient and control groups. The Chi-Square and Fisher's exact test, where appopriate, was used to compare the proportions of the groups. Relationships between the genotypes were determined using contingency coefficient values. Since the variables are not normally distributed, Kruskal-Wallis test were conducted to compare these parameters among physical activity groups. Mann-whitney U test was performed to test the significance of pairwise differences using Bonferroni correction adjust for multiple comparasions. The daily energy intake values in predicting physical activity groups were analyzed using ROC (Reciever Operating Characteristics) curve analysis. A 5% type-I error level was used to infer a statistical significance.

Results

Comparision of Anthropometric and Metabolic Characteristics between Groups

All of the anthropometric and metabolic characteristics were found statistically significant between patient and control groups except height, RMR %, total cholesterol and HbA1c levels. Table 1 summarize all of the information about anthropometric and metabolic characteristics of individuals.

Direct Sequencing and RT-PCR Results

The results of direct sequencing and RT-PCR demonstrated many point mutations in the *MC4R* and the region located 2696 to 3275 bp upstream of the *MC4R* start codon, *FTO* and *NMB*. Representative DNA sequencing chromatograms of novel polymorphisms in the *MC4R* and at the region located 2696 to 3275 bp upstream of the *MC4R* start codon are shown in Figures 1 and 2, respectively.

At the end of the study, by direct sequencing, 16 mutations were found in *MC4R* of which 14 of them are novel and 8 of them cause amino acid change. Nucleotide numbering of *MC4R* begins 119 bp before the start codon. Also 5 mutations were found on the region located 2696 to 3275 bp upstream of the *MC4R* start codon in which 4 of them are novel. Nucleotide numbering for this region begins from start codon. Also they were named with cDNA numbering by using Human Genome Variation nomenclature (http://www.hgvs.org/mutnomen/). In addition, 1 mutation in *NMB*, 6 different mutations in *FTO*, and 1 mutation near 188 kb of *MC4R* were detected by RT-PCR. Table 2 summarize all of the information about mutations/polymorphisms which were found with direct sequencing and RT-PCR.

When mutatations were compared between groups, rs1051168, rs8050136 and $-2778C>T$ mutations were found statistically significant in patients whereas rs1121980 was found in controls ($P<0.05$). It was also found that the significances of rs1051168 and rs8050136 are related with homozygous genotype ($P=0.014$, $P=0.001$), the significance of $-2778C>T$ is related with hetereozygous genotype ($P=0.012$)

TABLE 1 *Anthropometric and metabolic characteristics of individuals*

Anthropometric and metabolic characteristics	*Groups*		
	Control group (n = *100)*	*Patient group* (n = *100)*	*P values*
Height (cm)	167,14 ± 8,64	164,7 ± 9,78	$P = 0{,}067$
Weight (kg)	61,4 ± 8,95	116,45 ± 26,83	$P < 0.001$[a]
Age	27,82 ± 8,28	33,74 ± 9,4	$P < 0.001$[a]
BMI (kg/m^2)	21,88 ± 1,8	42,65 ± 7,2	$P < 0.001$[a]
RMR (kkal/day)	1332,19 ± 331,57	1816,37 ± 509,37	$P < 0.001$[a]
RMR %	90,32 ± 16,79	91,97 ± 19,22	$P = 0{,}52$
WC (cm)	71,38 ± 7,27	112,27 ± 15,97	$P < 0.001$[a]
WHR	0,74 ± 0,07	0,85 ± 0,10	$P < 0.001$[a]
WHtR	0,43 ± 0,03	0,68 ± 0,08	$P < 0.001$[a]
FM %	22,35 ± 7,8	44,87 ± 6,32	$P < 0.001$[a]
FFM (kg)	47,55 ± 10,38	63,46 ± 14,69	$P < 0.001$[a]
FM (kg)	13,31 ± 4,49	52,68 ± 16,12	$P < 0.001$[a]
TFM (kg)	6,52 ± 3,75	25,17 ± 7,77	$P < 0.001$[a]
Systolic blood pressure (mmHg)	107,28 ± 17,42	118,28 ± 15,44	$P < 0.001$[a]
Diastolic blood pressure (mmHg)	69,87 ± 9,03	79,13 ± 12,53	$P < 0.001$[a]
Glucose (mg/dl)	80,82 ± 12,82	93,60 ± 15,28	$P < 0.001$[a]
Trigliseride (mg/dl)	67,99 ± 28,54	120,84 ± 58,52	$P < 0.001$[a]
Total cholesterol (mg/dl)	176,97 ± 36,15	186,96 ± 35,06	$P = 0{,}049$[a]
LDL (mg/dl)	102,02 ± 30,05	117,43 ± 31,07	$P < 0.001$[a]
HDL (mg/dl)	61,39 ± 17,31	45,5 ± 8,93	$P < 0.001$[a]
HbA1c (%)	5,1 ± 0,95	5,45 ± 0,82	$P = 0{,}0058$[a]
Insulin (uIU/ml)	7,06 ± 3,37	20,37 ± 12,72	$P < 0.001$[a]

[a] *$P < 0.05$.*
BMI: body mass index; RMR: resting metabolic rate; WC: waist circumference; HC: hip circumference; WHR: waist-hip ratio; WHtR: waist-height ratio; FM: fat mass; FFM: free fat mass; TFM: trunk fat mass; LDL: low density lipoprotein; HDL: high density lipoprotein; HbA1c: hemoglobin A1c.

and the significance of rs1121980 is due to wild type genotype ($P = 0.004$).

Comparision between Mutations and Obesity Related Factors

Some statistically significant relations were detected between mutations and weight, BMI, WC, WHtR, FM%, FFM, TFM, WHR, RMR, glucose, LDL, trigliseride, HDL, insulin, and diastolic blood pressure values. Details are shown in Online Resource 2 (Supporting Information).

Comparision of the Scores of TFEQ Questions between Groups

The a, b, c, and d question options were scored as 1, 2, 3, and 4, respectively. For the last question; 1-2th question options were scored as 1, 3-4th question options were scored as 2, 5-6th question options were scored as 3, 7-8th question options

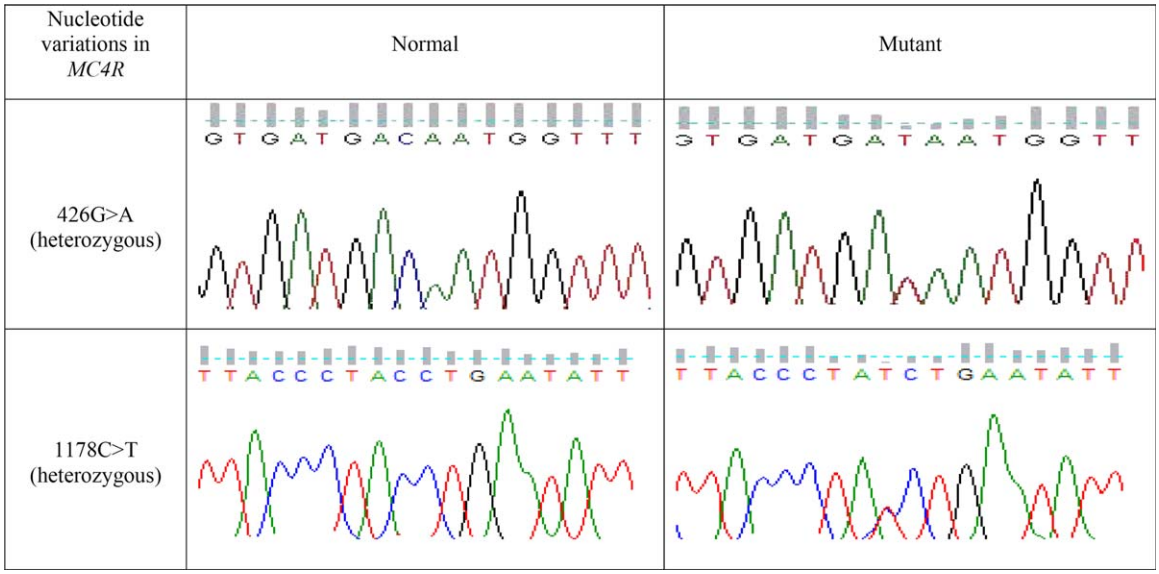

FIG 1 *Representative DNA sequencing chromatograms showing novel mutations/polymorphisms of* MC4R.

were scored as 4. When groups were compared with each other, all of the question mean scores were found statistically significant except 2, 11, 12, 14, 15, ve 18 ($P < 0{,}05$) Details are shown in Online Resource 3 (Supporting Information).

Comparision of Mean Scores of TFEQ Questions According to the Mutations Genotypes

The 2, 3, 5, 6, 7, 8, 9, 11, 13, and 17th questions were found statistically significant in rs1051168 (3, 6, and 9th questions), rs7242169 (17th question), rs1861868 (11th question), rs9939609 (17th question), 136insC (2, 7, 8, 13 th questions), 1029C > T (5th question), −3237A > G (5, 8, 11th question) according to the genotypes. Details are shown in Online Resource 4 (Supporting Information).

Association between TFEQ Factors and Mutations

136insC was found statistically significant between genotypes according to the cognitive dietary restraint and uncontrolled eating factors; 1029 C > T mutation was found statistically significant according to the uncontrolled eating factor.

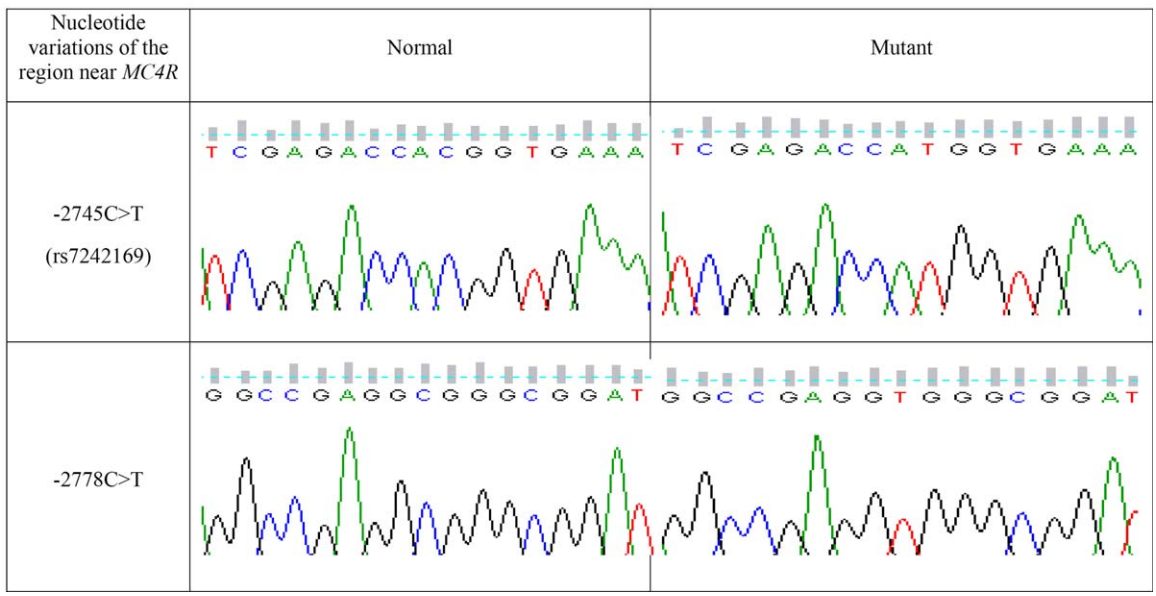

FIG 2 *DNA sequencing chromatograms showing mutations/polymorphisms of the region located 2696–3275 bp upstream of the* MC4R *start codon.*

TABLE 2 *Information of detected nucleotide variations and comparision of them between groups*

Gene names, nucleotide positions and variations	*Region*	*Amino acid change*	*Control group (n = 100)*			*Patient Group (n = 100)*			P *values*	*Reported in pubmed and other databases*
			Wild type	*Hom. mut.*	*Het. mut.*	*Wild type*	*Hom. mut.*	*Het. mut.*		
NMB										
rs1051168, c.217C>A	Exon 2	P73T	4	37	59	0	56	44	$P = 0{,}007^a$	Known
FTO										
rs9939609, IVS1-23525T>A	Intron 1	–	3	7	90	2	16	82	$P = 0{,}129$	Known
rs1477196, IVS1-35794A>G	Intron 1	–	53	9	38	66	6	28	$P = 0{,}134$	Known
rs1121980, IVS1-34805G>A	Intron 1	–	17	43	40	35	36	29	$P = 0{,}014^a$	Known
rs1421085, IVS1-43098T>C	Intron 1	–	33	13	54	22	19	59	$P = 0{,}17$	Known
rs1861868, IVS1 + 52261C>T	Intron 1	–	24	38	38	19	36	45	$P = 0{,}542$	Known
rs8050136, IVS1-27777C>A	Intron 1	–	34	13	53	25	33	42	$P = 0{,}003^a$	Known
MC4R										
26insT, c.−94insT	5′UTR	–	100	0	0	99	1	0	$P = 1$	New
136insC, c.17insC	Exon 1	H6P	98	2	0	98	2	0	$P = 1$	New
292G>A, c.173G>A	Exon 1	S58N	99	0	1	100	0	0	$P = 1$	New
426G>A, c.307G>A	Exon 1	V103I	96	0	4	96	0	4	$P = 1$	Known
835C>T, c.716C>T	Exon 1	A239V	100	0	0	99	0	1	$P = 1$	New
910A>G, c.791A>G	Exon 1	H264R	99	0	1	100	0	0	$P = 1$	New
1005A>T,c.886A>T	Exon 1	I295F	99	0	1	100	0	0	$P = 1$	New
1017C>T, c.898C>T	Exon 1	Silent mut.	99	0	1	100	0	0	$P = 1$	New
1018T>A, c.899T>A	Exon 1	L300Q	99	0	1	100	0	0	$P = 1$	New
1029C>T, c.910C>T	Exon 1	L304F	96	0	4	97	0	3	$P = 1$	Known
1091C>A, c.972C>A	Exon 1	Silent mut.	99	0	1	100	0	0	$P = 1$	New
1121insC, c.1002insC	3′UTR	–	98	2	0	100	0	0	$P = 0{,}5$	New
1145A>T, c.1026A>T	3′UTR	–	100	0	0	98	0	2	$P = 0{,}5$	New
1178C>T, c.1059C>T	3′UTR	–	99	0	1	100	0	0	$P = 1$	New
1199insA, c.1080insA	3′UTR	–	99	1	0	100	0	0	$P = 1$	New
1206insT, c.1087insT	3′UTR	–	99	1	0	100	0	0	$P = 1$	New
Near MC4R gene										
rs17782313, g.57851097T>C	Near 188 kb to MC4R	–	49	22	29	38	24	38	$P = 0{,}261$	Known
rs7242169, c.−2745C>T	5′UTR	–	66	4	30	51	8	41	$P = 0{,}08$	Known
c.- 3237A>G	5′UTR	–	95	0	5	97	0	3	$P = 0{,}72$	New
c.- 2794C>T	5′UTR	–	99	0	1	100	0	0	$P = 1$	New
c.−2793insT	5′UTR	–	99	0	1	100	0	0	$P = 1$	New
c.−2778C>T	5′UTR	–	75	5	20	61	3	36	$P = 0{,}04^a$	New

$^a P < 0{,}05$.

Comparision of Groups According to RMR

Any relation between groups according to the RMR levels were not detected.

Association between Nucleotide Variations and RMR

It was found that normal level of RMR's were mostly detected in individuals who had wild type genotype for rs17782313 ve 136insC ($P < 0.05$). The relation between mutations and RMR were also evaluated separately in each of the groups. No significant relations between them were detected.

Association between Mean Daily Energy Expenditure in Physical Activity Groups

When daily energy expenditure values were compared, it was found to be higher in vigorous group ($n = 18$; 798,75 ± 421,16 kcal/day) than moderate group ($n = 80$; 704,24 ± 432,99 kcal/day). But the difference was not found statistically significant ($P > 0,05$). Because of inadequate patient number in light group ($n = 2$), the values of this group were not compared with other groups.

Association between Total Count Values and Physical Activity Groups

When total count in physical activity groups were compared, it was found to be statistically higher in vigorous group ($n = 18$; 195.562,78 ± 99.580,04) than moderate group ($n = 80$) (112.474,68 ± 74.759,69; $P < 0.05$). Because of inadequate patient number in light group ($n = 2$), the values of this group were not compared with other groups.

Association between Daily Energy Expenditure Values in Subgroups of Physically Moderate Group

Patients in moderate group were further divided according to total activity spent in intervals. In the first group, intervals were set at 10 min (total of 30 min: 3 × 10 min group; $n = 69$), in the second group interval was set at 30 min continously (1 × 30 min group; $n = 11$). It was found that the values were statistically higher in 1 × 30 min group (977,91 ± 259,99 kcal/day) than 3 × 10 min group (619,15 ± 316,37 kcal/day; $P < 0.05$).

Association between Total Count Values in Subgroups of Physically Moderate Group

It was found that the values were statistically higher in 1 × 30 min group (236.993,82 ± 69.697,87) than 3 × 10 min group (92.623,80 ± 53.579,08; $P < 0.05$).

Association between Mutations with Daily Energy Expenditure Levels

Only 1029C > T heterozygous mutation (313,67 ± 52,54 kcal/day) was found statistically lower than wild type genotype (727, 58 ± 423,34 kcal/day) in patients according to the daily energy expenditure levels ($P < 0.05$).

Association between Physical Activity Groups and Mutations

Any significant relation between physical activity groups and mutations were not detected. Details are shown in Online Resource 5 (Supporting Information).

Correlation Analysis

It was found that, there is a positive correlation between rs7242169 and −2778C > T mutations. In other words these two mutations were substantially found together in the same individuals ($P < 0,001$).

Discussion

According to our results, 16 mutations were found in *MC4R* gene of which 14 of them are novel and 8 of them cause amino acid change. Also 5 mutations were found on the region located 2696 to 3275 bp upstream of the *MC4R* start codon in which 4 of them are novel. Also 1 mutation in *NMB*, 6 different mutations in *FTO*, and 1 mutation near 188 kb of *MC4R* were detected by RT-PCR. rs1051168, rs8050136 and −2778C > T were found statistically significant in patients and contrary to expectations rs1121980 was found in controls ($P < 0.05$). It was suggested that further studies are needed to investigate the effect of rs1121980 on obesity. Also it was found that, there is a positive correlation between rs7242169 and −2778C > T mutations. Therefore it was considered that, these two mutations are substantially found together in the same individuals ($P < 0,001$). As expected, all of the anthropometric and metabolic characteristics were found statistically significant between patient and control groups except height, RMR%, total cholesterol and HbA1c levels.

Obesity is the result of complex interactions between environmental, behavioral and genetic factors that modulate individual responses to physical activity and diet. Although a large number of genetic variants and quantitative trait loci that potentially predispose to obesity-related traits have been reported, only few of these variants and loci have been confirmed (24).

Twin and family studies have shown that, besides environment, genetic factors also contribute to the level of physical activity. Therefore it has been suggested that biological systems may be involved in the tendency to be and to remain physically inactive. Most of the earlier-mentioned studies, physical activity levels were assessed by a self-reported questionnaire, but it is known that the assessment of physical activity by questionnaire may have lower accuracy (2). In this study, physical activity levels were assessed by an objective method (accelerometry). Animal studies have provided strong evidence for a role of the hypothalamus in the regulation of energy homeostasis (25). The MC4R deficiency is the most common genetic cause of obesity. Expression of the *MC4R* is especially high in the hypothalamus and the spinal cord. Interruption of the melanocortin signaling in the hypothalamus may lead to obesity and reduced physical activity in mice. It has been also shown that stimulation of *MC4R* increases physical activity/energy expenditure and leads to weight loss (4). Loos et al. found that −2745C > T (rs7242169) polymorphism showed significant associations with the physical activity scores. They suggested that DNA sequence variation in the

MC4R gene locus contributes to the variation in physical activity level and the tendency to be sedentary in humans (25). In this study, we could not found any association between physical activity levels with *MC4R* gene mutations. Similarly Jozkow et al. could not found any association between rs7242169 and physical activity levels (4). Alleles of common single nucleotide polymorphisms (SNPs) rs7242169 and rs17782313 located downstream of the *MC4R* were also shown to be associated with obesity and related traits (25,26). Contrary to these findings, Loos at al. did not find any association between BMI with the rs7242169 polymorphism (25). In this study, some of the obesity related factors were found to be associated with 426G > A (Diastolic blood pressure) 1121insC (weight, BMI, FFM, WC, WHtR), 1145A > T (insulin) mutations which were detected in *MC4R* and rs7242169 (weight, BMI, WC, WHR, WHtR, FFM), rs17782313 (TFM), −2778C > T (WHR) mutations which are found near *MC4R*. Details are shown in Online Resource 2 (Supporting Information). In additon, it was also found that normal level of RMR's were typically detected in individuals who have got wild type genotype for rs17782313 mutation near *MC4R* and 136insC ($P < 0.05$) in *MC4R*. Also some relations were found between physical activity groups as well as subgroups according to the daily energy expenditure and total count values. In addition, 1029C > T heterozygous mutation in *MC4R* was found statistically lower than wild type genotype in patients according to the daily energy expenditure levels ($P < 0.05$) therefore it was suggested that, this mutation may effect daily energy expenditure level. In some other studies, it was found that two *MC4R* variants are negatively correlated with the risk of obesity (27). These polymorphisms cause amino acid substitutions in codon 103 (V103I) and codon 251 (I251L) (27,28). Contrary to these findings, in our study V103I mutation was found equally in patients and controls and I251L mutation were not detected in both of the groups.

Although physiologic, environmental and psychological factors affect nutrient intake and appetite, various studies suggest that behaviors such as food and beverage preferences and nutrient intake are at least partially genetically determined (20). Similarly, heritability and linkage analysis of eating behavior measured by the TFEQ provides evidence that these behavior traits are heritable (19). Expression of the *MC4R* especially in the hypothalamus and the spinal cord, may regulates food intake and energy balance (4). In general, stimulated *MC4R* signaling may lead to a decreased food intake and an increased metabolic rate (25). In some studies, the rs17782313 C allele has been associated with increased snacking and food intake in obese and non-obese individuals (6). Similarly, rs17782313 has been linked to eating behaviors related to obesity such as low satiety responsiveness and increased enjoyment of food in obese patients (29). These data strongly suggest that this region downstream of *MC4R* may be important in modulating appetitive pathways to weight gain and obesity compatible with its functional role in monogenic obesity (30). In this study TFEQ was used to evalute eating behavior phenotypes. To our knowledge, the TFEQ has not been used before in Turkish population. In our previous study, it was translated into Turkish and validated (23). This validated verison was then used. At the end of the study, some TFEQ questions were found statistically significant between groups and some of them were found to be related with 136insC (2,7,8,13) and 1029C > T mutations (5) which are located in *MC4R* and rs7242169 (17), −3237A > G mutations (5,8,11), which are found near *MC4R*. In additon, some mutations were found to be associated with dietary restaint (136insC) and uncontrolled eating (136insC, 1029C > T) factors.

Another gene which may related with obesity is *FTO*. mRNA of *FTO* is highly expressed in the hypothalamus, an area that is known to be involved in the regulation of appetite. Associations between weight and *FTO* polymorphisms may be due to differences in eating behavior. This could be consistent with findings in monogenic obesity disorders, which are almost all characterized by an increased desire to eat (31). FTO may contribute to obesity by down regulating adipocyte production of leptin (19). Several SNPs were identified in *FTO* gene which are mostly found in the first intron of the gene, a region where the sequence is strongly maintained across species (1). The most frequently studied SNP is the rs9939609 variant, located in intron-1 and present at a high allelic frequency (30). It has been associated with higher body weight and higher risk of obesity in different studies and populations (1) and is associated with satiety responsiveness (19). Children and adolescents with 1 or 2 *FTO* rs9939609 obesity-risk alleles report more frequent loss of control eating episodes and select foods higher in fat at a buffet meal (12). In this study, 17th question of the TFEQ were found to be related with rs7242169 and rs9929609 mutations, also 11th question was found to be associated with rs1861868 mutation in the *FTO* gene.

There was also an interaction between the *FTO* rs9939609 genotype and physical activity, by which physically inactive homozygous risk A-allele carriers had an increased BMI compared with homozygotes for the T-allele. Therefore, low physical activity seems to accentuate the effect of *FTO* rs9939609 on body fat accumulation (32). Rampersaud et al. found that two *FTO* gene variants, rs1477196 and rs1861868 which are also found in the first intron of *FTO*, were associated with obesity and BMI, only in those subjects with a low level of physical activity (33). Vimaleswaran et al. found that T risk allele of rs1121980 which are also found in the first intron of *FTO*, was associated with WC and BMI, but physical activity level was able to attenuate this effect (9). Contrary to these findings, we couldn't found any association between physical activity levels and *FTO* mutations.

Genome-wide association scans identified common polymorphisms, in intron 1 of *FTO* that modulate BMI and associate with increased risk of obesity (24).

For the obesity related traits, Ruiz et al. found that, rs9939609 mutation was associated with higher levels of BMI, body fat percentage, and WC (2). Xi et al. found that rs9939609 variant is strongly associated with BMI and the risk of obesity (34). Ahmad et al. found that carriers of the *FTO*

rs8050136 risk allele have an increased risk of CVD, mediated by BMI in individuals who are less active (35). Cauchi et al. demonstrated that rs1421085 mutation which are found in *FTO* were also increased obesity (24). Also in some studies, it was demonstrated that, individuals who have rs1861868 and rs1477196 mutations were at increased risk of being obese and of being overweight. The A allele of rs1861868 was associated with greater WC and weight. Similar associations were observed with the C allele of rs1477196. Each risk allele for rs1477196 was associated with a mean increase in BMI (32). Similarly in this study, some obesity related factors were found to be associated with rs9939609 (trigliseride, HDL), rs1477196 (BMI, RMR, FFM, glucose), rs1121980 (weight, BMI, WC, FM%, FFM, WHtR, glucose), and rs8050146 (weight, BMI, WC, FM%, FFM) mutations, which were detected in the first intron of *FTO* gene. Details are shown in Online Resource 2 (Supporting Information).

Zobel et al. found that of the various polymorphisms found both in the *FTO* and in the *MC4R* loci, the most significant were rs9939609 and rs17782313, respectively (36). A few studies have also jointly analyzed the *FTO* and *MC4R* polymorphisms, describing their additive effects on obesity related variables (1,9,12,30). Cauchi et al. found that, compared to participants carrying neither *FTO* nor *MC4R* risk allele, subjects with three or four risk alleles had a 1.8-fold increased obesity risk (24). In this study, 9 known mutations in *FTO*, *NMB*, and located near *MC4R* as well as whole *MC4R* gene mutations were investigated together according to the obesity, obesity related factors, physical activity levels as well as eating behavior phenotypes.

Several studies showed the importance of eating behaviors in the context of weight-loss programs (15). Meal size, meal frequency, macronutrient intake, and eating behavioral phenotypes such as restraint, hunger, uncontrolled, and emotional eating also under genetic control (30). Although much remains to be understood about the genes regulating eating behaviors, genetic influence of disinhibition has been linked to neuromedin, a factor mediating satiety (19). Neuromedin beta (NMB) is a member of the bombesin-like peptides family, which have many biological effects that may be related to eating behaviors and obesity (14). Bouchard et al. found a significant association between the missense mutation P73T (rs1051168) in exon 2 of the *NMB* and levels of disinhibition and susceptibility to hunger, increased body weight, BMI, WC, and FM (15). Similarly in this study rs1051168 mutation in *NMB* gene was found to be associated with BMI, WC, and WHtR. Also 3, 6, and 9th questions of TFEQ were found to be related with rs1051168 mutation. Nevertheless we couldn't found any association between *NMB* gene mutations and physical activity level.

This study offers some clues to the mechanism by which *MC4R*, *FTO,* and *NMB* influences changes in BMI and may have important implications in targeting personalized lifestyle recommendations to prevent obesity in genetically susceptible individuals.

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