Basic Sciences of Medicine 2012, 1(5): 40-45
DOI: 10.5923/j.medicine.20120105.04

Molecular Screening of Obesity Candidate Gene Variant Neuropeptide Y Receptor Type 2 (*NPY2R*) A172T among Malaysian Subjects

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Abstract The association of the A172T Neuropeptide Y Receptor Type 2 (*NPY2R*) gene with obesity and metabolic syndrome has not been widely studied in the Malaysian population. Therefore, the objective of this study was to investigate the association of *NPY2R* A172T gene variant with obesity, metabolic syndrome, related anthropometric measurements, glucose level, blood pressure and the demographic characteristics among Kampar Health Clinic subjects. In total, 168 subjects (68 males, 100 females; 66 obese, 102 non obese; 47 Malay, 89 Chinese, 32 Indians) were recruited in this study via convenience sampling and anthropometric measurements were taken. Genotyping was performed using *Hae*III Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) revealing 121 AA, 37 AT and 10 TT subjects; minor allele frequency 0.170. *NPY2R* A172T genotypes and alleles did not show any association with obesity (p = 0.982; 0.857, respectively), gender (p = 0.129; 0.245, respectively) and ethnicity (p = 0.581; 0.390, respectively). Besides, they did not show any association with the presence of metabolic syndrome according to 3 criteria in the modified NCEP ATP III (p = 0.447; 0.288, respectively). In conclusion, the *NPY2R* A172T gene variant was not associated with obesity, obesity-related traits and metabolic syndrome among Malaysian subjects in this study.

Keywords Neuropeptide Y Receptor 2, Single Nucleotide Polymorphism, Obesity, Metabolic Syndrome, Anthropometric Measurements, Malaysia

1. Introduction

Obesity is defined as a state of increased adiposity resulting from chronic nutrient excess and it has reached its epidemic levels in modern society. Malaysia is part of this epidemic too, whereby the prevalence rate of obesity has increased from 4.4% to 14.0% as reported by the Malaysian National Health and Morbidity Survey (NHMS) II in 1996 and NHMS III in 2006, respectively[1]. The increased prevalence of obesity has been accompanied by a parallel increase in the prevalence of the metabolic syndrome (MetS) or "syndrome X"[2]. The definition in diagnosing MetS was first introduced by WHO in 1998 whereby this definition focused on insulin resistance as a main criterion[3]. In addition, the National Cholesterol Education Program Adult Panel Treatment III (NCEP ATP III) and International Diabetes Foundation (IDF) introduced their own set of criteria in diagnosing metabolic syndrome in 2001 and 2005, respectively[4,5]. NCEP ATP III criteria are based on the presence of 3 out of 5 factors in diagnosing metabolic syndrome and the risk factors are waist circumference >102 cm in men and >88cm in women, blood pressure, serum cholesterol, plasma triglycerides and fasting blood glucose[4]. This definition is rather suitable in the US population than Asians due to the low prevalence of metabolic syndrome among Asians[6]. Thus, a modified version of NCEP-ATP III is made in waist circumference for Asians; > 80 cm for women and >90 cm in men is used to define central obesity[7,8].

The neuropeptide Y receptor type 2 (NPY2R) is one of the class of G-protein coupled receptors activated by closely related peptide hormones - neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide[9]. NPY2R has a heptahelix structure which comprises glycosylation site at the amino-terminal, two extracellular cysteines which form a disulfide bridge and a cysteine molecule at the cytoplasmic tail of the N-linked carbohydrate[10]. It binds selectively to Y2 receptor subtype agonists such as NPY_{3-36} and $PYY_{3-36[}$11]. The gene encoding for it, *NPY2R*, is located on chromosome 4q32.1 which consists 8447 kilobases including 2 exons and the coding region is located on the

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Published online at http://journal.sapub.org/medicine

second exon[12]. Wide expression of NPY2R in the CNS with a particularly high level in an area of the arcuate nucleus (ARC) with a permeable blood brain barrier makes the receptor a potential mediator of peripheral signals in the regulation of energy homeostasis[13] and may be relevant to feeding and body weight control[14]. Activation of the NPY2R reduces appetites while avoiding stimulation of the NPY receptor type 1 (NPY1R) and type 5 (NPY5R) that induces feeding, thus provides a pharmaceutical approach to modulate food intake[14].

The *NPY2R* gene variant, A172T has a G/A polymorphism predicting non-synonymous base change from Alanine to Threonine base at codon 172 in the coding region of *NPY2R* exon 2. The heterozygous genotype of this gene variant was first discovered in a 2-year-old Hispanic boy with hyperphagia and severe early-onset obesity in a British study[15]. This gene variant was also subsequently detected among adult Pima Indians subjects; however, was not associated with obesity[16]. Currently, there is limited data and evidence on this association worldwide, especially among the Malaysian population. As different populations show different associations in the existing research, the data on the association of *NPY2R* A172T variant with obesity in other populations cannot be used to extrapolate for the Malaysian population.

Therefore, the objective of this study was to perform genotyping of the *NPY2R* A172T gene variant among Malaysian subjects from a health clinic in Kampar, Perak to determine the prevalence of the mutated genotypes and alleles, and to investigate if there was any association with obesity. Demographic characteristics, anthropometric measurements, blood pressures and fasting plasma glucose level were also determined to investigate whether there was any association of these obesity and metabolic syndrome-related traits with the *NPY2R* A172T gene variant.

2. Methodology

2.1. Subjects, Questionnaire and Clinical Measurements

A total of 168 subjects (mean age: 54.3 ± 14.0; 68 males, 100 females; 47 ethnic Malays, 89 Chinese, 32 Indians; 102 non-obese, 66 obese) were recruited by convenience sampling from the Kampar Health Clinic from June to December, 2011. The exclusion criteria of the subjects include hyperthyroidism, pituitary diseases, chronic liver disease, chronic renal disease, acute infection, haematologic diseases and patients under medications that affect the glucose metabolism. The demographic data included in this questionnaire were age, gender and self-identified ethnicity; while blood pressures and anthropometric measurements consisting of systolic and diastolic blood pressures (SBP, DBP), pulse rate, weight, body mass index (BMI), waist and hip circumferences, total body fat (TBF), subcutaneous fat (SF), visceral fat level (VFL) and skeletal muscle (SM) were taken as described in our previous study[17]. Subjects with the BMI cut-off point of ≥ 27.0 kg/m^2 were considered as obese[18]. Overnight fasting peripheral blood drawing was conducted with the aid of a qualified phlebotomist. Fasting plasma glucose level was determined by using OneTouch® Ultra Easy™ blood glucose meter and test strips (LifeScan Inc., CA). The presence of MetS was based on the only three accessible criteria out of five risk factors in accordance to the modified NCEP ATP III definition of metabolic syndrome for Asians: WC > 80 cm for women and > 90 cm in men, hyperglycaemic/having elevated fasting plasma glucose of > 6.1 mM or currently being treated for diabetes, and having blood pressure of ≥ 130/85 mmHg. This study was registered under the National Medical Research Registry NMRR-09-826-4266 and the protocol was approved by the Medical Research and Ethics Committee, Ministry of Health, Malaysia. All individuals that have participated in this study signed informed consent forms and all samples were taken in accordance with the Declaration of Helsinki (revised in Seoul, 2008).

2.2. DNA Extraction and Genotyping

Five millilitres of blood sample was collected and genomic DNA was then extracted from the nucleated leukocytes using the Wizard® Genomic DNA Purification Kit (Promega Inc., Madison, WI) as mentioned in our previous study[17]. Each of the PCR reaction vial contained 20 µl of solution, containing forward primer (1 µM), reverse primer (1 µM), Taq buffer with ammonium sulphate (1×), *Taq* DNA polymerase recombinant (1 U/µl)(Fermentas, Lithuania), dNTP (75 µM) (Axygen Biosciences Inc., CA), $MgCl_2$ (1.25 mM) and 1 µl of dimethyl sulfoxide (DMSO). The PCR was carried out using Biometra T Personal Thermocycler (Biometra GmbH, Germany), beginning with a hot start cycle at 95°C for 5 min; denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec for 25 cycles; and a final extension cycle at 72°C for 5 min. The sequences for forward and reverse primers were 5'-CGG CAC AGG TGC ATC GTC TAC CA-3' and 5'-GAA GAT GGC CAG GGG ACT TGC CA-3', respectively[15]. Genotypes were determined by Restriction Fragment Length Polymorphism (RFLP), where restriction enzyme *Hae*III (isoschizomere *Bsu*RI) (Fermentas Inc., Lithuania) cut the PCR product (118 bp) and formed restriction fragments with the size of 73 bp and 45 bp. Hence, the NPY2R A172T genotypes were AA (digested, homozygous wild-type), AT (digested, heterozygous) and TT (undigested, homozygous mutant) (Figure 1). The fragments were resolved by 3 % agarose gel electrophoresis at constant 90 V for 45 min before staining with ethidium bromide and viewed under a UV transilluminator. The three genotypes were validated by sending to an outsourced DNA sequencing service.

2.3. Statistical Analysis

The compiled data was analyzed using Statistical Package for Social Sciences, IBM® SPSS® Statistics Version 20.0

(SPSS Inc., IL). The normality of data was examined using One-Sample Kolmogorov-Smirnov Test whereby $p > 0.05$ indicates that the particular variable is normally distributed.

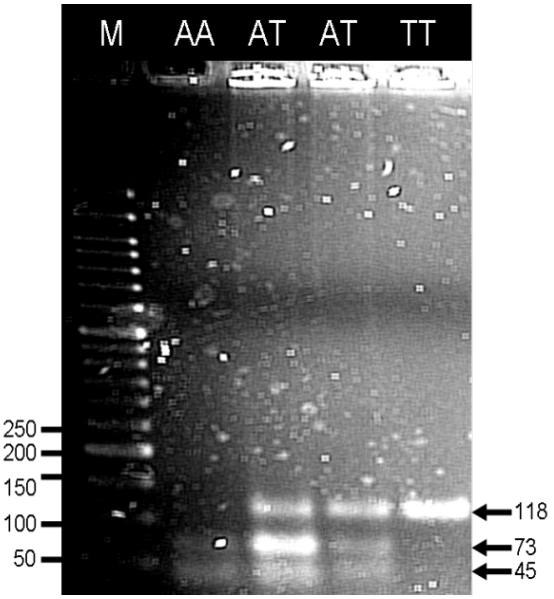

Figure 1. NPY2R A172T genotyping of subjects by *Hae*III Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis on 3.0% agarose gel. M - 50 bp DNA ladder (GeneRulerTM, Fermentas, Lithuania); homozygous wildtype AA (73 bp and 45 bp); heterozyogus mutated AT (118 bp, 73 bp and 45 bp); homozygous mutated TT (118 bp)

Descriptive statistics was used to compute frequencies and percentages for demographic data, genotype and allele frequencies. It was also used to calculate the means and standard deviations for anthropometric measurements. Besides, Pearson's Chi-square analysis was used to compare the genotype and allele frequencies between groups. Means of clinical parameters were compared by Student's *t*-test (between two variables) or using One-Way Analysis of Variance (ANOVA) (between more than two variables), except for VFL and fasting plasma glucose level whereby these two variables were compared using Mann-Whitney *U* test (between two variables) or Kruskal-Wallis test (between more than two variables). In all statistical tests performed, $p < 0.05$ was denoted as statistically significant.

3. Results

Based on Table 1, BMI status, gender and ethnicity did not show association with different *NPY2R* A172T genotypes and alleles. The allele frequency for mutated T allele (or known as Minor Allele Frequency, MAF) was 0.170 and most of the obese subjects carried AA genotype (72.1%) while only 4 had TT genotype. The distribution of genotypes did not deviate significantly from the Hardy-Weinberg Equilibrium. Besides, glycaemia status, hypertensive status (based on SBP or DBP) and MetS status were also all not associated with *NPY2R* A172T genotypes and alleles (Table 1).

With respect to different genotypes and alleles, there were also no significant differences between anthropometric and clinical measurements for different genotypes and alleles (Table 2). Last but not least, out of 68 males, only 3 had MetS while out of 100 females, only 19 had MetS. Therefore, presence of MetS was significantly associated with gender ($\chi^2 = 7.569$; $p = 0.006$).

Table 1. *NPY2R* A172T genotype and allele frequencies with respect to BMI status, gender, ethnicity, glycaemia status, hypertensive status (based on SBP and DBP) and MetS status

Variables	Genotype			Allele	
	AA	AT	TT	A	T
BMI status					
Obese	47 (71.2)	15 (22.7)	4 (6.1)	109 (82.6)	23 (17.4)
Non-Obese	74 (72.5)	22 (21.6)	6 (5.9)	170 (83.3)	34 (16.7)
χ^2; *p*		0.037; 0.982		0.033; 0.857	
Gender					
Male	48 (70.6)	13 (35.1)	7 (10.3)	109 (80.1)	27 (19.9)
Female	73 (73.0)	24 (24.0)	3 (3.0)	170 (85.0)	30 (15.0)
χ^2; *p*		4.089; 0.129		1.353; 0.245	
Ethnicity					
Malay	32 (68.1)	13 (27.7)	2 (4.3)	77 (81.9)	17 (18.1)
Chinese	68 (76.4)	16 (18.0)	5 (5.6)	152 (85.4)	26 (14.6)
Indian	21 (65.6)	8 (25.0)	3 (9.4)	50 (78.1)	14 (21.9)
χ^2; *p*		2.862; 0.581		1.882; 0.390	
Glycemia status					
Normal	60 (71.4)	18 (21.4)	6 (7.1)	138 (82.1)	30 (17.9)
Hyperglycemic	61 (72.6)	19 (22.6)	4 (4.8)	141 (83.9)	27 (16.1)
χ^2; *p*		0.435; 0.804		0.190; 0.663	
SBP class					
Normal	33 (66.0)	16 (32.0)	1 (2.0)	82 (29.4)	18 (18.0)
Hypertensive	88 (74.6)	21 (17.8)	9 (7.6)	197 (83.5)	39 (16.5)
χ^2; *p*		5.444; 0.066		0.108; 0.742	
DBP class					
Normal	71 (72.4)	22 (22.4)	5 (5.1)	164 (83.7)	32 (16.3)
Hypertensive	50 (71.4)	15 (21.4)	5 (7.2)	115 (82.1)	25 (17.9)
χ^2; *p*		0.311; 0.856		0.136; 0.712	
MetS status					
Absent	104 (71.2)	10 (6.8)	32 (21.9)	240 (82.2)	52 (17.8)
Present	17 (77.3)	5 (22.7)	0 (0)	39 (88.6)	5 (11.4)
χ^2; *p*		1.609; 0.447		1.127; 0.288	

Numbers in parenthesis are percentages out of the total cases for the variable in the same row. χ^2 and *p*-values by Pearson's Chi-square analysis, significant at $p < 0.05$

4. Discussion

Table 2. Mean and standard deviation of anthropometric and clinical measurements with respect to *NPY2R* A172T genotypes and alleles

Anthropometric and Clinical Measurements	Genotype				Allele		
	AA	AT	TT	*p*	A	T	*p*
SBP (mmHg)	143.3 ± 23.0	135.1 ± 18.6	149.0 ± 20.2	0.082	142.2 ± 22.6	140.0 ± 20.0	0.487
DBP (mmHg)	81.6 ± 12.2	81.7 ± 13.8	87.1 ± 14.5	0.416	81.6 ± 12.5	83.6 ± 14.1	0.277
Pulse Rate (bpm)	78.2 ± 13.4	79.5 ± 13.6	74.2 ± 8.0	0.527	78.4 ± 13.4	77.7 ± 12.1	0.721
WC(cm)	91.3 ± 11.7	92.6 ± 13.0	93.4 ± 119.9	0.792	91.5 ± 11.9	92.8 ± 15.4	0.452
WHR	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.246	0.9 ± 0.1	0.9 ± 0.1	0.072
Weight (kg)	65.4 ± 12.0	67.9 ± 16.9	71.4 ± 14.1	0.274	65.7 ± 12.7	69.1 ± 15.8	0.076
BMI (kg/m^2)	26.3 ± 4.6	27.3 ± 7.5	28.3 ± 6.0	0.377	26.0 ± 3.9	27.2 ± 6.3	0.122
TBF (%)	32.6±7.6	32.8±7.8	32.0±7.6	0.962	32.6±7.6	32.5 ± 7.6	0.907
SF (%)	26.5 ± 8.4	27.4 ± 8.9	25.5 ± 9.3	0.763	26.7 ± 8.5	26.8 ± 8.9	0.925
VFL (%)[§]	11.6 ± 5.9	12.1 ± 7.2	15.1 ± 8.4	0.563	11.7 ± 6.1	13.1 ± 7.6	0.455
RM (kcal)	1393.2 ± 215.4	1420.4 ± 248.8	1509.9 ± 219.6	0.261	1396.8 ± 219.5	1451.8 ± 239.1	0.091
SM (%)	24.8 ± 4.3	24.5 ± 3.4	25.6 ± 3.8	0.738	24.8 ± 4.2	24.9 ± 3.6	0.898
Fasting plasma glucose level (mmol)[§]	7.3 ± 3.0	7.1 ± 2.9	6.6 ± 2.1	0.830	7.3 ± 3.0	6.9 ± 2.7	0.541

p-values by Student's *t* test or One-way ANOVA for normally-distributed data or Kruskal-Wallis and Mann-Whitney *U* test for non-normally distributed data[§] ; significant at $p < 0.05$

The results showed that most of the Kampar Health Clinic subjects did not carry the mutated *NPY2R* TT genotype or T allele which has been identified as a candidate genotype/allele for obesity and metabolic syndrome in limited studies. This gene variant was first discovered in a 2-year-old Hispanic boy who had the heterozygous genotype, among 101 unrelated children less than 10-years-old with hyperphagia and severe early-onset obesity from the U.K. Genetics of Obesity Study, where 90% were Caucasians[15]. However, it was not detected in 100 unrelated adult white control subjects in the same study. Subsequently, this gene variant was also detected among 489 non-first degree relatives adult Pima Indians subjects (362 severely obese; 127 non-diabetic, non-obese), where the MAF was 0.015[16]. Out of the 11 gene variants or single nucleotide polymorphisms (SNPs) that they detected, the A172T variant (identified as SNP9) was the only coding missense substitution identified in *NPY2R* gene[16]. This gene variant, however, was not associated with obesity in the Pima Indian population[16].

Besides these two association studies specifically on the *NPY2R* A172T gene variant, other gene variants or single nucleotide polymorphisms (SNPs) in the exon 2 region of *NPY2R* namely rs1047214 and rs2880415, have been associated with obesity in Caucasian Danish subjects[19], obese Swedish men[20] and French White subjects[21]. This association is further supported by Hunt et al. (2011) who found that SNP rs2880415 showed strongest association with BMI in American Caucasian men while women did not show any significant association[22]. However, the same gene variants studied in the German population did not show any association with early onset obesity[23]. As different populations show different associations in the existing literature, the data on the association of the *NPY2R* gene variants with obesity in other populations cannot be used to extrapolate for the Malaysian population. Although we did not screen for the rs1047214 and rs2880415 SNPs in our study, we confirmed and replicated the findings of the British and Pima Indian population studies for the non-association of *NPY2R* A172T gene variant with obesity[15,16].

The NCEP-ATP III has a very flexible definition whereby the criteria can be modified according to different populations in terms of WC and is widely used in epidemiology studies to diagnose MetS[24]. In this study, the prevalence rate of MetS was 13.1%, whereby 1.8% males while 11.3% females had MetS - lower than a previous study conducted by Tan et al. (2008) where the prevalence rate of MetS in Malaysian subjects in their study was 16.5% (26.9%

males, 7.7 % females) in accordance to the NCEP-ATP III definition[24]. Furthermore, other Malaysian studies detected higher prevalence rates of MetS – for example, a study conducted by Mohamud et al. (2011) stated that 34.3 % of Malaysian adults had MetS[8], while Moy and Bulgiba (2010) found that 38.2 % out of 1494 Malay employees in Kuala Lumpur had MetS[25]. Therefore, the lower prevalence rate of MetS in our health clinic cohort in Kampar, a suburban town in the state of Perak, may indicate that the overall Kampar population has a better health status than other urban areas in Malaysia.

This study had some limitations whereby the respondents may not represent the whole Kampar population as only 168 subjects were studied. In addition, the small sample size in this study may limit the power for statistical analysis and extrapolation. In future, a larger sample size may help to prevent sampling bias. Future studies are also needed to assess the other *NPY2R* gene variants besides A172T and other main contributing genetic and environmental factors (like lifestyle factors and dietary habits) in associating obesity. Besides, evaluation of insulin level and lipid profile can be done to determine their association with obesity and *NPY2R* A172T genotypes and alleles.

5. Conclusions

NPY2R is a G-protein coupled receptor highly expressed in the ARC of the brain. It exerts anorexigenic or appetite-reducing effect when bound to its ligands. In this study, we screened for the prevalence of a coding missense SNP in the *NPY2R* gene, namely the A172T variant, to see if the SNP is associated with functional phenotypical effects manifested as obesity and MetS. In conclusion, we found that there was no evidence for an involvement of the *NPY2R* A172T gene variant in obesity, obesity-related traits and MetS in this multi-ethnic Malaysian study group. The distribution of the genotype and allele frequencies of this gene variant was also not significantly different among gender and ethnic groups. On the basis of the results available so far, the role of NPY2R especially its coding missense substitution A172T gene variant in obesity and MetS remains inconclusive.

ACKNOWLEDGEMENTS

This project was funded by the Universiti Tunku Abdul Rahman Research Fund (IPSR/RMC/UTARRF/C111/C35). We would like to extend our deepest gratitude to the Kampar District Health Office for granting us permission to carry out this study at the Kampar Health Clinic, the nurses who assisted with the blood sampling, and all the respondents who have volunteered to participate in this study.

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