Association of PPARγ2 (Pro12Ala) and neuropeptide Y (Leu7Pro) gene polymorphisms with obstructive sleep apnea in obese Asian Indians

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Abstract. Background: Obstructive sleep apnea (OSA) is prevalent in 7.5% in urban Asian Indians. Peroxisome proliferator activated receptor gamma2 (PPARγ2) has been implicated in adipocyte differentiation. Neuropeptide Y (NPY) is also considered as a candidate gene for excess body fat accumulation. The association of PPARγ2 (Pro12Ala) and NPY (Leu7Pro) gene polymorphisms with OSA has not been studied in Asian Indians.

Objective: To study the distribution of PPARγ2 (Pro12Ala) and NPY (Leu7Pro) polymorphism in Asian Indians with and without OSA.

Methods and results: This study was carried out in 252 obese subjects (body mass index (BMI) > 25 kg/m²); 142 with OSA and 110 without OSA. Measurements included anthropometric and biochemical parameters (fasting blood glucose, lipid profile, various circumferences and skin-fold thicknesses). PPARγ2 (Pro12Ala) and NPY (Leu7Pro) gene polymorphisms were studied in all subjects. The frequency of the variant allele (Ala12) of PPARγ2 gene was significantly higher in subjects with OSA (14.4%) when compared with subjects without OSA (5.5%; χ² = 9.7; p = 0.001). The distribution of the variant allele (Pro7) of NPY gene was comparable in subjects with OSA (3.5%) and without OSA (3.6%; χ² = 0.001, p = 0.94).

Conclusion: This study reveals a significantly higher frequency of PPARγ2 (Ala12) allele in obese Asian Indians with OSA when compared to obese Asian Indians without OSA.

Keywords: Obstructive sleep apnea, PPAR gamma, neuropeptide Y, gene polymorphism, Asian Indians, obesity
genetically determined [41]. Studies in subjects with elevated apnea hypopnea index (AHI) provide insight to the possible genetic overlap between obesity and AHI. Several investigators have shown familial aggregation of AHI level and symptoms of OSA in adults obese and non-obese subjects [29].

In humans, there are three isoforms of the peroxisome proliferator-activated receptor gamma (PPARγ) gene; PPARγ1, PPARγ2, PPARγ3. These are formed by alternative promoters and differential splicing [3]. Out of these three, PPARγ2 is expressed exclusively in adipose tissue [3]. PPARγ regulates adipocyte genes involved in adipogenesis and lipid metabolism. PPARγ activates adipocyte differentiation and mediates the expression of fat cell-specific genes [18]. Obesity and nutritional factors influence the expression of the PPARγ2 isofoms [11]. The most common variation involving C to A nucleotide substitution has been reported in exon-2 of PPARγ2 gene. This single nucleotide substitution results in an amino acid change, Proline-to-Alanine at codon 12 of this gene [6,15]. In vitro experiments have demonstrated that this variation is associated with reduced transcripional activity [12]. Association of PPARγ2 (Pro12Ala) polymorphism with body mass index (BMI) is conflicting. Few studies have shown that this polymorphism is associated with increased BMI [26] and others have even shown a negative or no association of this polymorphism with BMI [17,28]. Hsueh et al. (2001) reported that PPARγ2 (Pro12Ala) polymorphism accounted for 2–3% of the total variation of BMI in Mexican-American adults [19].

Neuropeptide Y (NPY) is a neurotransmitter that interacts with leptin in the regulation of sympathetic activity, body weight and energy balance. A recent study shows that NPY levels are increased in OSA independent of obesity [2]. Karvonen et al. (1998) have identified a Thymidine (1128) to Cytosine (1128) polymorphism (T1128C), which results in a substitution of Leu7 by Pro7 in the signal peptide part of pre-pro-NPY [24]. This polymorphism has also been found to be associated with increased birth weight [23]. Till date no study has been conducted on NPY (Leu7Pro) gene polymorphism in OSA subjects.

Previous studies on OSA conducted in Asian Indians have been limited to determining the prevalence of OSA and its relationship with road traffic accidents and public health awareness [16,37]. However, the status of PPARγ2 (Pro12Ala) and NPY (Leu7Pro) gene polymorphisms has not been investigated in Asian Indian subjects with OSA. We conducted a study in obese subjects with and without OSA, having comparable age, body mass index (BMI) and percentage body fat (%BF). In these two groups, we looked for the distribution of the polymorphic variants of PPARγ2 (Pro12Ala) and NPY (Leu7Pro).

2. Methods

2.1. Subjects

A total of 252 obese subjects; 142 newly diagnosed subjects with OSA (109 male, 33 females) and 110 subjects without OSA (71 males, 39 females) were recruited from the Medicine Out Patient Department of All India Institute of Medical Sciences, a tertiary care referral hospital. The subjects were North Indians residing in New Delhi or surrounding areas. Polysomnography (PSG) was performed in all subjects. On the basis of AHI, subjects were categorized as with and without OSA (see definitions). All subjects were free of any acute or apparent chronic inflammatory disorders, chronic obstructive pulmonary disease (COPD) and clinically apparent coronary heart disease (CHD). Subjects with other sleep disorders such as upper airway resistance syndrome (UARS), central sleep apnea syndrome (CSAS), periodic limbs movements (PLMs), or narcolepsy were excluded. Clinical evaluation for all subjects included thyroid profile and an electrocardiogram (ECG). The study was approved by the institutional ethics committee and written informed consent was obtained from all subjects before their participation in the study.

2.2. Metabolic parameters

Blood samples were obtained after an overnight fast for estimation of fasting blood glucose (FBG) and blood lipoproteins. Levels of FBG, total cholesterol (TC), serum triglycerides (TG) and high density lipoprotein-cholesterol (HDL-C) were estimated using commercial kits (Randox Laboratory, San Francisco, CA, USA) with a semi-automated analyzer (Micro Semi-Autoanalyser 2000, C.L. Micromed, Italy).

2.3. Anthropometric measurements

The anthropometric measurements were carried out by a physician according to the methods described earlier [13]. Briefly, waist circumference was measured midway between the highest point of the superior iliac crest and lowest point of the costal margin; hip circum-
ference was measured at the maximum circumference of the buttocks. All measurements were taken in the standing position with feet placed together with subjects wearing only light clothes. Measurement of percentage body fat was carried out using leg-to-leg (two point contact) bioelectrical impedance method (Tanita TBF 300, TANITA Corp., Tokyo, Japan). For the estimation of bioelectrical impedance, subjects were evaluated after an overnight fast. They were instructed to avoid drinking fluids and void urine 1 hour prior to the measurements and just before the test. Gender and height details were manually entered into the system. The subject was instructed to stand on the apparatus so that both the feet were in firm contact with the surface of the apparatus and that hands were not touching any surface.

2.4. Polysomnography (PSG)

All subjects underwent overnight digital PSG (Medi palm; Braebon Medical Corp., Canada) and classified according to AHI. PSG channels included electrooculogram, electroencephalogram, electromyogram, an electrocardiogram and airflow (with an oro-nasal thermistor), chest and abdominal efforts, arterial oxyhemoglobin saturation by pulse oximeter. The recordings were analyzed with 60sec epoch, and sleep stages were scored according to the standard criteria of Rechtschaffen and Kales [33]. Obstructive apneas and hypopneas are typically distinguished from central events by the detection of respiratory efforts during the event.

2.5. Definitions

Obesity was defined as by criteria applicable to Asian Indians (BMI > 25kg/m²) [27]. Diagnosis of OSA was made on the basis of International classification of sleep disorders [34]. Apnea is defined as the cessation of airflow ≥ 10 s and hypopnea is defined as a recognizable, transient reduction of breathing ≥ 10 s associated with either an oxygen desaturation of ≥ 4% or an arousal [34]. The AHI was defined as the number of obstructive apneas and hypopneas per hour of sleep. Subjects with an AHI <5/hour were assigned to not having OSA. Subjects with an AHI ≥ 5/hour were considered to have OSA. Polysomnography was conducted in a single sleep laboratory and analysis was done by a single expert. The Epworth sleepiness scale (ESS) was used to check excessive daytime sleepiness [22]. A total score of less than 10 suggested that a person was not suffering from excessive sleepiness. ESS of 10 or more suggested the need for further evaluation to determine if an underlying sleep disorder was present [22].

2.6. Isolation of DNA from blood

Venous blood samples, 10 ml each, were drawn into EDTA containing tubes for genomic DNA isolation. Genomic DNA was extracted from leucocytes after lysing the erythrocytes from the cell pellet. DNA was liberated by sodium dodecyl sulphate and proteinase K digestion overnight at 37°C. Proteins were removed by addition of 5M NaCl, and the DNA was recovered by ethanol precipitation (Miller et al., 1983).

2.7. PPARγ2 (Pro12Ala) gene polymorphism

The polymorphic variants of PPARγ2 (Pro12Ala) and NPY (Leu7Pro) were determined by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) [14]. The upstream primer sequence used for PPARγ2 (Pro12Ala) was 5’GCC AAT TCA AGC CCA GTC 3’ and downstream primer sequence was 5’GAT ATG TTT GCA GAC AGT GTA TCA GTG AAG GAA TCG CTT TCC G3’. The reaction was carried out in a final volume of 25 µl containing 2.5 mM of each dNTP (Bangalore Genei, India), 0.1 µmol of each primer, 1.25 U of Taq DNA polymerase (Bangalore Genei, India). DNA was amplified during initial denaturation at 94°C for 1minute, followed by 34 cycles with 1min denaturation at 94°C, 1minute annealing at 60°C and 1minute extension at 72°C. At the end of 34 cycles, a final step of extension at 72°C for 10 minute was performed. The PCR product was digested with 0.5 µl of BstU1 restriction enzyme (New England Biolabs, UK). RFLP analysis yielded three bands, a 270bp fragment corresponding to the wild type allele and a set of 243bp and 23bp corresponding to variant allele.

2.8. Neuropeptide Y (Leu7Pro) gene polymorphism

For NPY (Leu7Pro) gene polymorphism, the upstream primer sequence was 5’CCC GTG CGT TGA GCC TTC TG3’ and downstream primer sequence was 5’CGG GCC CGC GGT CCC3’. The detailed method is mentioned elsewhere [24]. Briefly, DNA was amplified during initial denaturation at 94°C for 5min, followed by 34 cycles with 1min denaturation at 94°C, 1minute annealing at 62°C and 1minute extension at 72°C. A final step of extension was carried out at 72°C for 1minute. The PCR product was digested with 0.5 µl of BsiE1 restriction enzyme (New England Biolabs). The digested products were resolved by electrophoresis on a 2.5% agarose gel and visualized by ethidium bromide staining.
2.9. Statistical methods

Data were managed using an excel spreadsheet (Microsoft Corp., Washington, USA). Mean and standard deviation (S.D) were used as summary measures. Comparison between subjects with and without OSA was done by parametric ‘t’ test for continuous variables. The categorical variables were analyzed by using Chi²/Fisher exact test, wherever applicable. Multiple logistic regression analysis was done to see possible risk factors for OSA. The Hardy-Weinberg equilibrium was tested by using \( (p^2+2pq+q^2=1) \) formula and chi² analysis. In a preliminary study conducted by us, proportion of Pro12Ala genotype was 24% in OSA group and 10% in Non OSA group. With alpha = 5% and power 80%, 252 subjects were found to be appropriate for this study. STATA-9 statistical software was used for data analysis and \( p < 0.05 \) was considered as statistically significant.

3. Results

Table 1 shows the characteristics of subjects with and without OSA. There was no significant difference in the mean age, Body mass index (BMI), % body fat (%BF) and waist circumference (WC) between subjects with and without OSA. Diastolic blood pressure (DBP) was significantly higher in subjects with OSA but no difference was observed in systolic blood pressure (SBP) between these two groups. Neck circumference (NC), hip circumference (HC) and calf circumference (CC) were significantly higher in subjects with OSA as compared to the subjects without OSA. Fasting blood glucose (FBG) levels as well as blood glucose during 2hour (2h-BG) after 75grams oral glucose load was significantly higher in subjects with OSA when compared with subjects without OSA. TC, TG and HDL-C levels were comparable in both groups. Table 2 shows the ESS and polysomnography profile of subjects with and without OSA.
The frequency of $PPAR_\gamma^2$ (Ala12) allele was significantly higher in subjects with OSA (14.4%) when compared with subjects without OSA (5.5%; $\chi^2 = 9.7$; $p = 0.001$; Table 3). Allelic distribution of $PPAR_\gamma^2$ (Pro12Ala) in the subjects without OSA was in Hardy-Weinberg equilibrium. The distribution of the variant 'Pro7' allele in NPY gene was similar in subjects with OSA (3.5%) when compared with subjects without OSA (3.6%; $\chi^2 = 0.001$, $p = 0.94$; Table 3).

When OSAs subjects with $PPAR_\gamma^2$ (Ala12) allele were compared with subjects without OSA having $PPAR_\gamma^2$ (Ala12) allele, SBP and DBP, HC and CC were significantly higher in subjects with OSA having $PPAR_\gamma^2$ (Ala12) allele. Other variables (age, BMI, %BF, WC and NC, fasting as well as 2h-BG and lipid levels) were comparable in both of these groups (Table 4).

When OSAs subjects with $PPAR_\gamma^2$ (Ala12) allele were compared with OSAs subjects with $PPAR_\gamma^2$ (Pro12) allele, there was no significant difference in age, BMI and %BF. AHI was significantly higher in subjects with OSA having $PPAR_\gamma^2$ (Ala12) allele [median (range); 33.5 (7-121)/h] when compared with subjects with OSA having $PPAR_\gamma^2$ (Pro12) allele [17.9 (5-88)/h, $p = 0.002$]. TG levels were significantly higher in subjects with OSA having $PPAR_\gamma^2$ (Ala12) allele [(198.2 ± 79.5) mg/dl] when compared with subjects with OSA having $PPAR_\gamma^2$ (Pro12) allele [(160.7 ± 89.9) mg/dl, $p = 0.03$]. All anthropometry measurements, blood pressure, fasting as well as 2h-BG and lipid levels were comparable in subjects with OSA having $PPAR_\gamma^2$ (Ala12) allele, when compared to subjects with OSA having $PPAR_\gamma^2$ (Pro12) allele. Logistic regression analysis showed that AHI [OR (95%CI); 1.01 (1.00–1.04), $p = 0.007$] and rapid eye moment (REM) sleep [0.88 (0.79–0.98), $p = 0.02$] were independent predictor for $PPAR_\gamma^2$ (Ala12) allele in subjects with OSA. Subjects with OSA having $PPAR_\gamma^2$ (Pro12Ala) genotype were having significantly higher serum TG levels when compared with subjects having $PPAR_\gamma^2$ (Pro12Pro) genotype (198.2 ± 79.5 vs. 160.8 ± 89.9; $p = 0.04$).

All parameters were comparable between OSA subjects carrying either the NPY (Pro7) allele or NPY (Leu7) allele. CC was significantly higher in OSA subjects with having NPY (Pro7) allele [(39.5 ± 3.0) cm], when compared with subjects without OSA having NPY (Pro7) allele [(35.6 ± 0.5) cm, $p = 0.008$]. NC (39.6 ± 3.6 vs. 34.8 ± 9.4; $p = 0.001$) and HC (107.8 ± 9.4 vs. 94.1 ± 28.3; $p = 0.001$) was significantly higher in subjects with OSA having NPY (Leu7Leu) genotype when compared with OSA having NPY (Leu7Pro) genotype.

### 4. Discussion

This study is the first attempt to determine the $PPAR_\gamma^2$ (Pro12Ala) and NPY (Leu7Pro) gene polymorphisms in OSA subjects. We found that the frequency of $PPAR_\gamma^2$ (Ala12) allele was three times higher in subjects with OSA when compared with subjects without OSA. Frequency of NPY (Pro7) allele was comparable in subjects with and without OSA. These findings suggest a possible role of $PPAR_\gamma^2$ (Ala12) allele in OSA.

In Asian Indians, previous studies on $PPAR_\gamma^2$ (Pro12Ala) gene polymorphism have been conducted in diabetes [31] and colorectal cancer [21]. Radha et al. (2006) have shown that $PPAR_\gamma^2$ (Ala12) allele is protective against type 2 diabetes mellitus (T2DM) in Caucasians but not in south Asians [32]. Jiang et al. (2005) has shown that C161T and not Pro12Ala polymorphism of the $PPAR_\gamma^2$ gene is related to the risk of colorectal cancer.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear hormone receptor superfamily that play a pivotal role in regulating inflammatory gene expression [10]. PPAR also plays an important role in the transcriptional regulation of lipid utilization and storage in several organs [1]. PPAR ligands are used for therapy of T2DM and hold the promise for treatment of inflammation [7].

$PPAR_\gamma^2$ (Pro12Ala) polymorphism has been studied in obese subjects in different populations worldwide (16–20). The role of this polymorphism is conflicting in obesity. Some reports show positive association whereas other show negative or no association with BMI [17,28]. Previous reports suggest the
Table 4

| Variables                        | Subjects with OSA having Ala12 allele (n = 38) | Subjects without OSA having Ala12 allele (n = 12) | p value |
|----------------------------------|-----------------------------------------------|-------------------------------------------------|---------|
| BMI (kg/m²)                      | 31.0 ± 3.6                                     | 30.0 ± 2.2                                      | 0.24    |
| Age (y)                          | 44.3 ± 11.1                                    | 39.7 ± 7.6                                      | 0.12    |
| %BF                              | 35.7 ± 8.0                                     | 39.2 ± 11.5                                     | 0.36    |
| Blood pressure (mmHg)            |                                               |                                                 |         |
| SBP                              | 140.0 ± 19.6                                   | 125.8 ± 11.6                                   | 0.008   |
| DBP                              | 93.0 ± 12.5                                    | 83.5 ± 6.1                                      | 0.003   |
| Circumferences (cms)             |                                               |                                                 |         |
| WC                               | 105.5 ± 7.3                                    | 103.4 ± 3.7                                    | 0.19    |
| NC                               | 39.3 ± 3.8                                     | 39.5 ± 3.6                                     | 0.93    |
| HC                               | 107.4 ± 9.6                                    | 100.3 ± 6.0                                    | 0.005   |
| CC                               | 38.2 ± 3.1                                     | 35.6 ± 2.5                                     | 0.01    |
| Blood glucose during OGTT (mg/dl)|                                               |                                                 |         |
| FBG                              | 109.7 ± 21.2                                   | 100.7 ± 10.2                                   | 0.06    |
| 2h-BG                            | 151.2 ± 39.4                                   | 138.3 ± 35.7                                   | 0.30    |
| Lipid profile (mg/dl)            |                                               |                                                 |         |
| TC                               | 193.5 ± 42.8                                   | 204.3 ± 40.5                                   | 0.47    |
| TG                               | 198.1 ± 79.5                                   | 164.7 ± 79.4                                   | 0.24    |
| HDL-C                            | 45.9 ± 5.0                                     | 44.1 ± 4.6                                     | 0.31    |

Data have been represented as mean ± SD: BMI, Body mass index: %BF, percentage body fat: SBP, Systolic blood pressure; DBP, Diastolic blood pressure: WC, Waist circumference: NC, Neck circumference: HC, Hip circumference; OGGT, Oral glucose tolerance test: FBG, Fasting blood glucose; 2h-BG, 2 hours blood glucose: TC, Total Cholesterol: TG, Triglycerides: HDL-C, High density lipoprotein-Cholesterol.

PPARγ2 gene is involved in adipogenesis and presence of PPARγ2 (Pro12Ala) polymorphism decreases the expression of PPARγ gene [15]. This may be associated with lower BMI. In our study, the effect of obesity was ruled out by recruitment of subjects with comparable age, BMI and %BF. Furthermore, the mean AHI was significantly higher and other variables remained comparable in subjects with OSA having PPARγ2 (Ala12) allele, when compared to subjects with OSA having PPARγ2 (Pro12) allele.

PPARγ2 (Ala12) allele has also been found to be associated with increased inflammation [39]. In our previous studies, we have reported increased C-reactive protein and tumor necrosis factor alpha levels in OSA [4,5]. PPARγ2 (Ala12) allele has also been found to be associated with decreased carotid artery intima-media thickness [20]. The exact mechanism, how PPARγ affects OSA and whether PPARγ2 gene has some association in pathophysiology of OSA, needs to be investigated.

NPY has been considered as a thrifty gene due to its role in weight regulation and energy balance [35]. Previously, two reports have been published on serum NPY levels in subjects with OSA [2,8]. One study showed increased levels of NPY in OSA subjects in Spanish population [2] while the other study failed to show an increase in serum NPY protein levels in OSA subjects in Swedish population [8]. The NPY (Leu7Pro) polymorphism has not been reported in above studies. In our study, the distribution of NPY (Pro7) allele was similar between subjects with and without OSA. Our findings suggest that NPY (Leu7Pro) polymorphism may not have a role to play in the pathophysiology of OSA. This needs to be substantiated by studying more number of subjects.

The strength of our study is that the cases were carefully selected to avoid effect of any apparent acute or chronic inflammatory disease or infections. Age, BMI and %BF were matched carefully to see the independent effect of OSA. Pritchard and Roserberg [30] argued for the use of unlinked genetic markers to detect population stratification in association studies. Appropriate numbers of genetic markers were not looked in these subjects. Due to this, we were not able to check the effect of population stratification as suggested by Pritchard et al. [30]. Numerous studies have also looked at whether stratification really is a major issue in such studies and found the error rates to be extremely small [25].
Our study suggests increased frequency of Ala12 allele of PPARγ2 gene subjects with OSA. To conclude, PPARγ2 gene should be investigated as a candidate gene for OSA in a large population and also in different ethnic groups. We did not find any association of NPY gene polymorphism with OSA.

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