Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder in women and genetic predisposition has been associated to FTO rs9939609, common variant in intron 1. The objective of the present study was to identify rs9939609 genotyping of FTO single nucleotide polymorphism and its association in PCOS women and healthy controls from Pakistani origin.

A total of 72 subjects consisting of 36 women with PCOS and 36 healthy controls were included. Whole blood was obtained and genomic DNA was extracted for PCR amplification of unique primers of SNP rs9939609 for FTO gene. Restriction digestion was carried out by Sca1 restriction enzyme. Digested products were resolved on 2% agarose gel to infer the allele distribution. Data obtained was analyzed using SPSS 23.0.

BMI status was 23.7±5.9 in PCOS women which is higher than 21.65±4.82 of non-PCOS women. A significant difference was found in genotype frequencies in patients and controls (p=0.010) while no significant association in allele frequencies (p =0.096) between patient and control women.

In this study, SNP genotype of FTO showed association with PCOS but no association with allele frequency. Further studies may explore the role of FTO involvement in the pathogenesis of PCOS.

Keywords: Polycystic Ovarian Syndrome; Obesity; FTO; SNP; Pakistan
syndrome; this revealed that FTO variants had an important role in obesity, hyper-androgenism and diabetes in these women [15]. Due to the variable role of FTO (fat mass and obesity associated gene) gene in the predisposition to PCOS in combination with obesity, it is important to explore the exact contribution of FTO associated to these phenotypes in different ethnicity. From Pakistani population, role of FTO gene, SNP rs9939609 has been studied and found link with BMI and risk of obesity in Pakistani women. Association of rs9939609 variant with metabolic biochemical parameters indicates that this polymorphism may disturb the metabolism in women and predispose them to obesity and type-2 diabetes [16]. In another study from Pakistan, association of type 2 diabetes SNPs which were not linked to lipid traits nut found associated to the obesity. In the Pakistani population the stated outcome of six SNPs for obesity is comparable to that associated for type-2 diabetes and taking a combination of risk alleles on obesity can be significant. The biochemical mechanism of this influence is uncertain, but seems not to be arbitrated by altering serum lipid chemistry [17]. Also from Pakistan, some SNP variants of FTO were connected to the obesity, and some biochemical and anthropometric measures and had higher frequencies of minor allele "T" in contrary to the previous reports for Asian populations. The risk allele of each SNP ensued in an escalation in BMI in a quantitative mode [18]. All the previous reports from Pakistan, association of SNPs linked to risk of type-2 diabetes and no study highlighted the role of common variant in this population. Therefore, aim of this study was to identify genotype of SNP rs9939609 of FTO in PCOS women and healthy controls and the phenotypic association of SNP rs9939609 variant of FTO in obese and non-obese women with PCOS.

Materials and Methods

Prior to start of the study approval was granted from Advance Studies & Research Board (AS&RB), University of Health Sciences, Lahore, Pakistan.

Study Population

A total of 72 subjects between 14-35 years of age consisting of 36 PCOS patients and 36 controls were recruited according to the criteria of Rotterdam 2003 i.e., hyper-androgenism, oligomenorrhea/amenorrhea and polycystic ovaries, from the government hospitals in Lahore [19]. The women having congenital adrenal hyperplasia, androgen secreting tumor, Cushing syndrome, thyroid dysfunction, and hyper-prolactinemia were excluded from the study. Furthermore, women with pregnancy, hormonal therapy and any medication for the treatment of PCOS were also excluded. All subjects in control group had normal menstrual cycle and ovarian morphology. They were explained with purpose and characteristics of the study and written informed consent were obtained.

For the PCOS patients and control group, the height in meters and weight in kilogram was measured by the same observer; BMI was calculated. Obesity was defined as BMI ≥ 30 Kg/m² according to WHO [20]. Ultrasonography was performed and then blood sample was collected. For genetic analysis, 3ml blood sample was collected in K3 EDTA 5.4 mg (BD Vacutainer) tube and mixed gently to avoid clot formation. The samples were labeled properly and transported to University of Health Sciences, Lahore for further processing.

Genetic Association by RFLP

DNA was extracted by standard phenol-chloroform method. Genotyping of rs9939609 at FTO gene was carried out (F-5’ AACTGGCTCTTGAATGAAATAGGATTCAGA-3’; R-5’ AGAGTAACAGAGACTATCCAAGTGAGATAC-3’) using specific primers by polymerase chain reaction-restriction fragment length polymorphism assay (PCR-RFLP).

DNA template was amplified according to the optimized conditions for PCR. A 2X Amp Master Mix (Thermo Fisher Scientific) was used for amplification of DNA. 1 μl of DNA was amplified by the conventional PCR with the optimized conditions, using the synthesized forward and reverse gene specific primers. The reaction mixture was prepared in 200 μl of PCR tube. A 25 μl reaction volume containing 10 μl 2X Amp Master Mix, 1 μl of each forward and reverse primers (10 pmole) and dis.H₂O was prepared under safety cabinet. The reaction mixture was spin for few seconds for thorough mixing. The reaction mixture was taken through thermo-cycling conditions which were as follows; initial denaturation of template DNA for 5 minutes at 95 °C followed by 35 cycles of amplification each consisting, 30 seconds at 95 °C for denaturing DNA into single strands, 30 seconds for annealing at 58 °C for primers to hybridize or “anneal” to their complementary sequences on either side of the target sequence, and 30 seconds at 72 °C for extension of complementary DNA strands from each primer and final 5 minutes at 72 °C for Taq DNA polymerase (enzyme that catalyzes the reaction) to synthesize any un-extended strands left. PCR was carried out in Gene Amp PCR system 9700 (BIORAD, Germany) and T1 thermo cycler (Biometra, Germany).

After amplification, PCR products were resolved on 2% agarose gel with DNA ladder to check the product size. Digestion of PCR product was carried out by Sca1 (BioLabs, New England) restriction enzyme specific for rs9939609 variant of FTO gene which has AGT/ACT restriction site. Digestion of amplicon with restriction enzyme revealed two fragments of 154 bp and 28 bp (polymorphic) on 2% agarose when the digested product was resolved on agarose along with DNA marker (50bp ladder) on 2% gel electrophoresis while presence of a single fragment of 182 bp indicated no restriction on that strand of DNA.

Statistical analysis

Data was entered and analyzed using SPSS 23.0. The Mean±SD were given for quantitative variables and frequencies/percentages were given for qualitative variables. Pearson chi-square/Fisher exact were applied to observe association between FTO rs9939609
and PCOS. The associations were further evaluated by Odds ratio to find the odds of the diseased allele to be associated with desired clinical condition. A ‘p’ value of < 0.05 was considered as statistically significant.

**Results**

Demographic characteristics of the subjects are explained in Table 1. The population distribution of all the participants included women between 14 and 35 years of age. The BMI status of all the participants was 23.7±5.9 with PCOS women had greater BMI than non-PCOS including 27.8% and 13.9% obese PCOS and controls while 72.2% and 86.1% non-obese PCOS and controls respectively.

<table>
<thead>
<tr>
<th>Demographic Characteristics</th>
<th>Total Subjects Mean±SD</th>
<th>Cases (PCOS) Mean±SD</th>
<th>Controls (Non-PCOS) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>23.27±5.20</td>
<td>22.22±5.30</td>
<td>24.33±4.93</td>
</tr>
<tr>
<td>Unmarried</td>
<td>40</td>
<td>19 (52.8%)</td>
<td>21 (58.3%)</td>
</tr>
<tr>
<td>Married</td>
<td>32</td>
<td>17 (47.2%)</td>
<td>15 (41.7%)</td>
</tr>
<tr>
<td>BMI Status (Kg/m²)</td>
<td>23.79±5.92</td>
<td>25.93±6.20</td>
<td>21.65±4.82</td>
</tr>
<tr>
<td>Obese</td>
<td>15 (20.8%)</td>
<td>10 (27.8%)</td>
<td>5 (13.9%)</td>
</tr>
<tr>
<td>Non-Obese</td>
<td>57 (79.1%)</td>
<td>26 (72.2%)</td>
<td>31 (86.1%)</td>
</tr>
</tbody>
</table>

Table 1: Characteristics of the selected cases of PCOS and healthy controls from Lahore

SNP genotyping was done using specific primers for FTO rs9939609. The genotype distributions of the particular SNP for the PCOS patients and the control subjects were in Hardy-Weinberg equilibrium. A Single nucleotide polymorphism (SNP) was genotyped in 36 PCOS patients and 36 controls. Allele frequency is a measure of the relative frequency of an allele on a genetic locus in a population. Generally it is stated as a proportion or a percentage. In population genetics, allele frequencies display the genetic diversity of a species population or equivalently the richness of its gene pool. Genotype and allele frequencies of SNP rs9939609 are given in Table 2 and 3 respectively. As for rs9939609 a significant difference has been observed in genotype frequencies (p=0.010) in PCOS women while no association was found for allele frequencies (p=0.096) in PCOS patients and controls (Table 2 and 3). Frequency of A allele was more in PCOS patients than in controls and the “A” allele heterozygote genotype (AA and AT genotypes) were also greater in PCOS patients compared to the controls and the odds ratio for T allele was 0.338. For further analysis, the patients were divided into obese PCOS and non-obese PCOS sub-groups. Association analyses were conducted separately between the two sub-groups. There was no significant difference between genotype (p=0.979) and allele frequencies (p=0.1229) for rs9939609 genotype. There were more heterozygous alleles (A/T) and homozygous alleles (A/A) found in non-obese groups. Odds ratio (OR 1.100 95%CI) was proposed for A allele as a risk factor of PCOS.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Genotype rs9939609</th>
<th>p² (df=2)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TT</td>
<td>AT</td>
<td>AA</td>
</tr>
<tr>
<td>Total</td>
<td>31</td>
<td>37</td>
<td>4</td>
</tr>
<tr>
<td>Patients</td>
<td>10</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>Controls</td>
<td>21</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>22</td>
<td>4</td>
</tr>
<tr>
<td>Obese PCOS</td>
<td>3</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Non-obese PCOS</td>
<td>7</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 2: Genotype frequency of rs9939609 variant of FTO gene among patients and controls

<table>
<thead>
<tr>
<th>Subjects rs9939609</th>
<th>Allele frequency</th>
<th>p² (df=1)</th>
<th>P-value</th>
<th>Odds ratio (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p (T)</td>
<td>0.69</td>
<td>0.31</td>
<td>2.76</td>
<td>0.0966</td>
</tr>
<tr>
<td>q (A)</td>
<td>0.58</td>
<td>0.42</td>
<td>2.38</td>
<td>0.1229</td>
</tr>
<tr>
<td>Controls</td>
<td>0.79</td>
<td>0.21</td>
<td>2.49</td>
<td>0.1146</td>
</tr>
<tr>
<td>Patients</td>
<td>0.58</td>
<td>0.42</td>
<td>2.38</td>
<td>0.1229</td>
</tr>
<tr>
<td>Obese PCOS</td>
<td>0.6</td>
<td>0.4</td>
<td>0.63</td>
<td>0.4274</td>
</tr>
<tr>
<td>Non-obese PCOS</td>
<td>0.58</td>
<td>0.42</td>
<td>1.77</td>
<td>0.1834</td>
</tr>
</tbody>
</table>

Table 3: Allele frequency of rs9939609 genotype in patients and controls in Pakistani women

**Discussion**

Polycystic ovarian syndrome and obesity shared genetic association due to high prevalence of obesity in polycystic ovarian
syndrome [21,22]. It is thought that polycystic ovarian syndrome and susceptibility of an individual to obesity are caused by interaction between genotype-phenotype characteristic and environment [23]. The mechanisms underlying the fat mass and obesity (FTO) gene polymorphism with PCOS risk remain unclear.

In present study, it was observed that all PCOS cases have greater BMI (25.93±6.20) than controls (21.65±4.82) which shows that increased BMI and obesity have significant association with PCOS which is comparable to the previous report of Chinese population which showed the association of PCOS women with higher BMI (24.81±4.29) as well as age and sex matched non-PCOS (22.73±3.15) women [23]. Another study reported a significant association of BMI or obesity and PCOS taking the standard BMI as ≥28Kg/m² while in the current study it has been ≥30Kg/m² according to WHO [24]. Also, a previous study showed the same association with almost the same BMI as in the current study [11].

In this study, the minor allele (A allele) frequency is greater in PCOS group as compared to controls and a significant association has been found in genotype distribution and PCOS but no association was determined with allelic distribution. Furthermore, when PCOS cases were further grouped as obese and non-obese subjects, no association was found between FTO variant and PCOS which is contrast to previous report investigated in Korean population which showed that no SNPs were significantly associated with PCOS [14]. From Pakistan, few studies described the association of SNPs with obesity to the risk of type 2 diabetes while no study has been conducted to highlight the role of SNP variation in PCOS women [15-17]. In a meta-analysis, it was reported a marginal association between FTO rs9939609 polymorphism and polycystic ovarian syndrome after the adjustment of BMI while there was a significant association in subgroup analysis in East Asians, but not in Caucasians due to different ethnic effect on associations [12]. Also another report frequently observed the obesity risk allele of FTO rs9939609 in women with polycystic ovaries than in patients without polycystic ovarian morphology [21]. It was also noted in Chinese Han population a significant association between FTO variant and polycystic ovarian syndrome has been established in obese and non-obese women [22]. The association of FTO and PCOS susceptibility has not been observed in Chinese women as well as in Caucasian and African population [25,26]. Yan, et al. reported that there was no meaningful association of rs9939609 with obesity related traits but a significant association between rs9939609 and PCOS in Chinese population was because of the effect of BMI [24].

It is concluded that SNP rs9939609 genotype of FTO showed association with PCOS but no association with allele frequency. Gene frequency and genotypic frequency are measured within a particular population, preferably in a micro-population. Frequency of “A” allele showed a weak pattern of association with obesity and polycystic ovarian syndrome. Over all homozygous genotype frequency “AA”, between the patients and controls differed statistically but the allele frequency was not significant in PCOS patients and controls. This may be due to the small sample size, environmental behavior and biological behavior of population. Further studies may explore the role of FTO involvement in the pathogenesis of PCOS.

Acknowledgment

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References