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ARTICLE

Effect of six type II diabetes susceptibility loci and an *FTO* variant on obesity in Pakistani subjects

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The aim of the current study was to analyze the effect of six type II diabetes GWAS loci rs3923113 (*GRB14*), rs16861329 (*ST6GAL1*), rs1802295 (*VPS26A*), rs7178572 (*HMG20A*), rs2028299 (*AP3S2*) and rs4812829 (*HNF4A*), and an *FTO* polymorphism (rs9939609) on obesity. The probable mechanism of action of these SNPs was analyzed by studying their association with various biochemical and anthropometric parameters. A total of 475 subjects (obese = 250, controls = 225) were genotyped by TaqMan assay and their lipid profile was determined. Allele/genotype frequencies and an unweighted/weighted gene score were calculated. The effect of the gene score on anthropometric and biochemical parameters was analyzed. The minor allele frequencies of all variants were comparable to that reported in the original studies and were associated with obesity in these Pakistani subjects. Subjects with 9 risk alleles differ from those with <3 and overall there is no significant effect (*P*-value for trend 0.26). None of the SNPs were associated with any of the serum lipid traits. We are the first to report the association of these T2D SNPs with obesity. In the Pakistani population the reported effect of six SNPs for obesity is similar to that reported for T2D and having a combination of risk alleles on obesity can be considerable. The mechanism of this effect is unclear, but appears not to be mediated by changing serum lipid chemistry.
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INTRODUCTION

Obesity is defined as a medical condition in which excess body fat accumulation can impact health negatively.[1] It has become one of the leading disorders afflicting mankind worldwide.[2] Because of recent explosion of obesity, World Health Organization (WHO) has designated obesity a global epidemic. Overweight and obesity have been estimated to cause as much as 3.4 million deaths every year, with 4% of years of life lost and 4% of DALYs (disability-adjusted life years).[3] Obesity is a risk factor for various complication including diabetes, hypertension, cardiovascular diseases and so on.[4]

Obesity is a heterogeneous disorder involving a complex interaction of a multitude of factors including environmental, behavioral and genetic factors, none of which are completely understood.[5] Environmental factors include availability and abundance of food, place of living and socioeconomic status, and behavior in context to obesity largely refers to eating and physical activity that in turn are outputs of a complex integration of different nutritional, metabolic, neuronal and hormonal signals in the brain.[6]

Genetic studies on obesity started as a result of the observation that family clustering occurs in obesity. Early twin studies indicated that heritability estimates for twins are similar no matter whether they are reared apart or together and confirmed that 25–50% body mass index (BMI) variation observed within family studies and 40–90% within twin studies could be attributed to genetic factors.[7–9] A landmark discovery in the field of obesity genetics was the identification of mutations in the leptin gene in grossly obese children followed by identification of mutations in other genes involved in energy regulation pathways, laying down the basis for monogenic obesity.[10] However, it soon became clear that these mutations are very rare and could not explain the recent explosion of obesity and there must be some other mechanism responsible for common forms of obesity. The first genome-wide association study in 2007 opened the way to solve the issue, and in the same year, three independent GWA studies found the same gene, *FTO*, showing association with obesity.[11–13] This discovery laid the ground for the identification of many variants in other noncandidate genes and it is now accepted that common forms of obesity are product of the combined net effect of many risk variants in different genes.[14,15]

The association of common *FTO* variants, especially rs9939609, with obesity has been confirmed in Caucasians; however, the results in Asian populations are conflicting.[16–25] In Pakistan, there is limited research in the field of obesity genetics. A recent study in the Pakistani population indicated the association of this SNP with obesity in women and proposed that the SNP may play its role by affecting plasma glucose and leptin levels.[26] However, no study investigated the relationship between the variant and the lipid profile in obese individuals. A recent genome-wide association study in subjects of South Asian ancestry identified six new loci associated with type II diabetes and these associations have been replicated only in Chinese and Japanese populations.[27–29] As obesity is a risk factor for diabetes, it is possible that these SNPs may mediate their effect by affecting BMI. Because of this biological relevance and rationale, the aim of the current study was to analyze the role of these SNPs and the *FTO* variant rs9939609 in obesity in subjects from Pakistan and to correlate

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them with anthropometric traits and plasma lipid profile to elucidate the effect, if any, of these SNPs on lipid parameters.

MATERIALS AND METHODS

Study subjects

The study was a case–control observational type carried out at the Center for Cardiovascular Genetics, Institute of Cardiovascular Science, University College London, and Department of Microbiology and Molecular Genetics, University of the Punjab. The study included a total of 475 subjects (250 cases and 225 controls). Study subject recruitment was done by random sampling from hospitals and general population of Punjab, Pakistan. Subjects signed a written consent and filled in a detailed questionnaire regarding demographic information, lifestyle, exercise habits and family history of obesity. The inclusion criteria were BMI and waist-to-hip ratio (WHR) cutoffs defined for Asian populations previously (BMI $>23\,kg/m^2$ as overweight and $>26\,kg/m^2$ as obese).[30,31] Exclusion criteria included pregnancy, presence of malignancies and recent infections. The study was approved by the institutional ethics committee (Ethical Committee, School of Biological Sciences, University of the Punjab, Pakistan) and all procedures were carried out in accordance to Helsinki declaration.

Anthropometric measurements

The measurement of body weight (kg), height (m) and waist and hip circumference (cm) was according to standard procedures as described previously.[32] BMI (kg/m^2) and WHR were calculated for each study subject.

Blood sampling and biochemical analyses. Blood samples were taken after 8–12 h of fasting. Half sample was used for DNA isolation and the other half was used to obtain serum. Serum was separated by centrifuging gel vacutainers at 14 000 r.p.m. for 10 min, and samples were collected in sterilized eppendorf and screened for any infectious agents (HBV, HCV, HIV). Positive samples were discarded and safe samples were used for lipid profile determination. Serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were measured using commercially available kits (Spectrum Diagnostics, Obour, Egypt). Epoch, Biotek microplate reader (Biotek Instruments, Winooski, VT, USA) was used for all optical density measurements.

Genotyping

Genomic DNA was isolated from blood leukocytes using Wizard Genomic DNA purification kit (Promega, Madison, WI, USA). DNA was quantified using Nanodrop (ND-8000, Thermo Fisher Scientific, Waltham, MA, USA), made to a 5 ng/μl concentration and spotted on 384-well MicroAmp plates by liquid handling system robot (Biomerk FX, Beckman Coulter, Brea, CA, USA). Genotyping was done by TaqMan allelic discrimination assay using primers and probes given in Supplementary Table 1. The real-time PCR reaction mixture consisted of 1× KAPA probe fast qPCR master mix (KAPABiosystems, Wilmington, DE, USA), 100 nM of each primer, 100 nM of each probe, ROX high (0.4 μl/20 μl reaction mixture), 5 ng DNA and PCR grade water as needed. The PCR program consisted of the following steps: 50 °C for 2 min, denaturation at 95 °C for 10 min, 40 cycles of amplification at 95 °C for 15 s and 60 °C for 1 min. PCR was done on Bio-Rad C1000 thermal cycler and the results were called using sequence detection software (SDS) version 2.0 on ABI Prism 7900HT (Applied Biosystems/Life Technologies, Waltham, MA, USA).

Gene score calculation

The genotypes of the subjects were coded as 0, 1 and 2 for homozygous protective, heterozygous and homozygous risk genotypes, assuming that both alleles are codominant and the effect of one allele is not masked by the presence of another allele. The count for all SNPs was added to get the sum of risk alleles that each individual possesses, termed the unweighted gene score, that was multiplied by published effect size to get a weighted gene score.[28]

Statistical analysis

Data analysis was done using the Statistical Package for Social Sciences (IBM SPSS statistics, version 22, Armonk, NY, USA). Data were analyzed for mean, SD and normality of quantitative variables. Quantitative variables showing skewness were log transformed and results are presented as geometric means with approximate SD or SE. The study population was tested for Hardy–Weinberg equilibrium (HWE). Allele/genotype frequencies were calculated and compared between cases and controls using the χ^2 test. Odds ratio (OR) and 95% confidence intervals were calculated using logistic regression models. Age and gender association with obesity were checked by Mann–Whitney *U*-test and Pearson's χ^2 test, respectively, and independent sample *t*-tests were used to compare the other continuous variables by obesity. The association of variants with obesity was determined using logistic regression, whereas anthropometric (BMI, weight, waist and hip circumference and WHR) and lipid traits were analyzed by analysis of covariance (ANCOVA) with adjustment for age and gender. Linear regression was used to calculate rise/fall in lipid trait levels per risk allele with age and gender included as covariates in the models. In order to test for the hypothesis that the effect of T2D variants in disease progression may be mediated by BMI, a mediation analysis was performed. Mediation analysis was undertaken using the user written command binary_mediation in Stata version 13 (StataCorp, College Station, TX, USA) to estimate direct and indirect effects using the product of coefficients approach. This method uses logistic regression and ordinary least squares and hence the coefficients are rescaled to be comparable before the indirect effects are computed. The program is based on the methods described by MacKinnon and Dwyer.[33] To consider the effect of testing multiple genotypes, for each variable we calculated the false discovery rates (FDR), as shown in the tables for comparisons, that were significant at a *P*-value of <0.05. All but one comparison remained significant at a FDR of 10%. We did not include multiple phenotypes in this calculation as many of the examined variables are highly correlated (eg, BMI and weight and height, TC and LDL-C and TG and HDL are inversely correlated). The results should be considered as exploratory and interpreted in light of the number of tests conducted. Results will need confirmation in other studies. The allele/genotype frequency data have been submitted to GWAS central (http://www.gwascentral.org/).

RESULTS

The characteristics of the study population are summarized in Table 1. The proportion of individuals with a family history of obesity, hypertension, cardiovascular problems and diabetes in the obese group is 63.6%, 25.5%, 12% and 32%, respectively, whereas in controls the values are 8.4%, 0.4%, 0% and 1.3%, respectively. The diabetic patients were on medication, but the exact information on

Table 1 Study population anthropometric and biochemical characteristics

Characteristics	*Nonobese (n = 225)*	*Obese (n = 250)*	P-*value*
Weight (kg)	56.59 ± 4.00	90.66 ± 16.06	7.28×10^{-116}
Height (m)	1.61 ± 0.09	1.63 ± 0.13	0.12
BMI (kg/m^2)	21.93 ± 2.98	34.35 ± 6.13	1.17×10^{-100}
WC (cm)	71.95 ± 6.54	87.67 ± 12.11	2.083×10^{-52}
HC (cm)	78.22 ± 10.01	87.87 ± 12.56	1.354×10^{-18}
WHR (WC/HC)	0.92 ± 0.14	1.00 ± 0.07	1.458×10^{-12}
Total cholesterol (TC) (mmol/l)	4.55 ± 1.16	5.55 ± 0.91	3.010×10^{-23}
Triglycerides (TG) (mmol/l)	2.03 ± 0.71	2.44 ± 0.65	1.573×10^{-10}
HDL-C (mmol/l)	1.69 ± 0.49	1.11 ± 0.11	2.559×10^{-72}
LDL-C (mmol/l)	2.21 ± 0.69	2.99 ± 0.57	1.594×10^{-34}

Abbreviations: BMI, body mass index; WC, waist circumference; HC, hip circumference; WHR, waist-to-hip ratio; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol. The table summarizes the general characteristics of the study population. Values are indicated as mean ± SD. For TG, WHR and HDL-C, values are geometric mean ± approximate SD.

which medicine is being used could not be traced in the majority of patients as a large proportion of the samples came from the general population with insufficient literacy to remember the medicine's name. The number of males and females in the obese group ($n=250$) was 139 (55.6%) and 111 (44.4%), respectively, whereas in the control group ($n=225$) there were 118 (52%) males and 107 (48%) females.

Association of selected SNPs with obesity

The genotype distributions for all SNPs was in HWE ($P>0.05$) for all variants, and the minor allele frequencies of all variants were comparable to the original studies (Table 2). The effect of the gene score (the total number of risk alleles present in an individual) on obesity is shown in Figure 1. Compared with subjects with ≤ 3 risk alleles, those with ≥ 9 had an odds ratio for obesity of 5.7 (P-value $=0.03$). To explore which of the SNPs were contributing to this effect we examined the genotype frequencies of the six SNPs in cases and controls (Table 2). The genotype distribution differed significantly between cases and controls for rs1802295 (*VPS26A*; $P=0.02$), rs7178572 (*HMG20A*; $P=0.007$) rs2028299 (*AP3S2*; $P=0.04$) and rs4812829 (*HNF4A*; $P=0.02$), whereas risk allele frequencies differed significantly between cases and controls for rs1802295 (*VPS26A*; $P=0.03$) and rs9939609 (*FTO*; $P=0.01$; Table 2).

Association of SNPs with anthropometric traits

As shown in Table 3, rs3923113, rs2028299, rs4812829 and rs9939609 did not show a significant association with any of the anthropometric traits, rs16861329 showed an association with hip circumference, rs1802295 (*VPS26A*) was associated with BMI, weight and hip/waist circumference and rs7178572 (*HMG20A*) showed a significant association with weight and hip circumference. Even in the absence of a significant association for some traits, it was clear that the presence of risk alleles for some variants slightly changed the BMI and WHR values showing the quantitative effect of variants.

Association of SNPs with lipid profile

The association of selected SNPs with lipid traits (TC, TG, LDL-C and HDL-C) and their quantitative contribution per risk allele are shown in Table 4. When plotted against the unweighted gene score, rise or fall in lipid trait values is indicated in Figure 2 and Supplementary Figure 1. A mediation analysis for looking at the mediation effect of lipids on obesity showed that

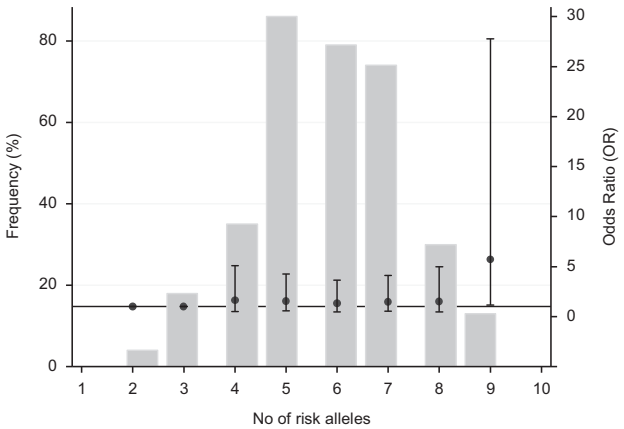

Figure 1 Figure showing the relationship of increasing number of risk alleles, the frequency of subjects carrying the respective number of risk alleles and odds ratios.

Table 2 Minor allele frequencies and odds ratios of selected SNPs

	RAF calculated			*Genotype frequency (%)*							
				Obese			*Nonobese*				
SNP	*RAF*[25]	*Obese*	*Nonobese*	*Homozygous common*	*Heterozygous*	*Homozygous Minor*	*Homozygous common*	*Heterozygous*	*Homozygous Minor*	P-*value (FDR)*	*OR (CI)*
rs3923113 (*GRB14*) Chr 2: g.165501849**A**>C	0.74	0.755	0.781	58.7	33.5	7.8	59.9	36.5	3.7	0.20	0.88 (0.63–1.25)
rs16861329 (*ST6GAL1*) Chr 3: g.18694873 **C**>T	0.75	0.768	0.822	58.6	36.5	5.0	67.8	28.9	3.3	0.13	0.76 (0.53–1.08)
rs1802295 (*VPS26A*) Chr 10: g.69171718 C>**T**	0.26	0.294	0.230	52.5	36.2	11.3	58.1	37.7	4.2	0.02 (0.047)	1.42 (1.03–1.95)
rs7178572 (*HMG20A*) Chr 15: g. 77454848A>**G**	0.52	0.529	0.521	19.6	53.4	26.9	28.6	38.6	32.9	0.007 (0.046)	1.06 (0.82–1.37)
rs2028299 (*AP3S2*) Chr 15: g. 89831025A>**C**	0.31	0.332	0.269	47.1	39.5	13.5	52.4	41.4	6.2	0.04 (0.07)	1.33 (0.98–1.81)
rs4812829 (*HNF4A*) Chr 20: g. 44360627 G>**A**	0.29	0.310	0.248	50.5	37.2	12.4	55.2	40.0	4.8	0.02 (0.047)	1.34 (0.98–1.84)
rs9939609 (*FTO*) Chr 16: g. 53786615T>**A**	0.32*	0.351	0.275	42.3	45.3	12.4	51.5	41.9	6.6	0.06 (0.09)	1.54 (1.11–2.14)

Abbreviations: RAF, risk allele frequency; CI, confidence interval; FDR, false discovery rate.
Selected SNPs allele/genotype frequencies. Risk alleles are in bold and underlined. *P*-value indicates the significance of difference in genotype frequencies between cases and controls from χ^2 test. Odds ratios are for risk alleles. hg19 was the genomic reference used, *Reference 20.

rs1802295 was the only SNP associated with both obesity and any lipid trait.

Gene score

The total SNP score was calculated for controls and cases, adding the individual risk score and were compared by independent sample *t*-test. Histograms of unweighted gene score show that in controls 8% individuals had <4 risk alleles and only 57% individuals carried ≥ 6 risk alleles, whereas in cases only 5% subjects had <4 risk alleles and 58% individuals carried ≥ 6 risk alleles. In cases, mean gene score (5.93) did not differ significantly from mean gene score in controls (5.76) (Figure 3 and Supplementary Figure 2). The histogram of unweighted gene score is given as Supplementary Figure 3. The mediation analysis rejected the assumption of mediation of the T2D variants by affecting BMI, with neither the weighted or unweighted gene scores showing any

Table 3 Association of SNPs with anthropometric traits

Gene	SNP	Genotype	BMI	Weight (kg)	Height	Waist circumference (cm)	Hip circumference (cm)	WHR
Growth factor receptor-bound protein (*GRB14*)	rs3923113	AA	28.45±0.50	73.91±1.30	1.62±0.007	79.99±0.83	82.91±±0.83	0.964±0.008
		AC	27.79±0.65	73.82±1.69	1.63±0.010	80.13±1.09	83.46±1.07	0.959±0.010
		CC	29.97±1.61	76.27±4.16	1.61±0.024	85.41±2.67	88.54±2.65	0.965±0.024
		P-value	0.41	0.85	0.30	0.15	0.13	0.92
		FDR	–	–	–			–
S*ialyltransferase 6 galactosidase 1 protein* (*ST6GAL1*)	rs16861329	CC	28.21±0.47	73.78±1.22	1.62±0.007	79.14±0.76	81.79±1.04	0.967±0.007
		CT	28.59±0.65	74.55±1.70	1.62±0.009	81.95±1.05	85.68±1.04	0.954±0.010
		TT	28.10±1.84	79.87±4.78	1.68±0.026	81.02±2.95	86.12±2.93	0.938±.0.027
		P-value	0.0.89	0.46	0.11	0.09	0.007*	0.38
		FDR	–	–	–	–	0.035	–
Vacuolar protein sorting-associated protein (*VPS26A*)	rs1802295	CC	27.66±0.49	72.07±1.27	1.62±0.007	79.88±0.81	83.55±0.81	0.955±0.007
		CT	28.20±0.60	74.23±1.56	1.63±0.009	79.20±0.99	81.81±0.99	0.969±0.009
		TT	31.88±1.31	85.84±3.38	1.64±0.019	86.06±2.14	87.73±2.15	0.977±0.020
		P-value	0.01*	0.0008*	0.51	0.01*	0.04*	0.38
		FDR	0.07	0.006	–	0.07	0.10	–
High-mobility group protein 20A (*HMG20A*)	rs7178572	AA	27.42±0.76	71.19±1.97	1.62±0.011	79.79±1.24	80.15±1.2	0.998±0.012
		AG	28.91±0.55	76.92±1.42	1.63±0.008	80.66±0.89	84.73±0.88	0.950±0.008
		GG	28.17±0.68	71.98±1.77	1.60±0.010	78.99±1.11	82.87±1.10	0.953±0.010
		P-value	0.27	0.02*	0.06	0.50	0.01*	0.002*
		FDR	–	0.07	–	–	0.035	0.014
Adaptor-related protein complex (*AP3S2*)	rs2028299	AA	28.30±0.53	74.17±1.38	1.62±0.008	79.17±0.85	82.53±0.85	0.959±0.008
		AC	28.12±0.59	73.62±1.53	1.62±0.008	80.61±0.94	83.31±0.94	0.966±0.009
		CC	30.26±1.19	78.79±3.09	1.62±0.017	83.39±1.91	84.87±1.93	0.986±0.018
		P-value	0.26	0.32	0.95	0.11	0.52	0.40
		FDR	–	–	–	–	–	–
Hepatocyte nuclear factor 4A (*HNF4A*)	rs4812829	GG	28.18±0.51	73.59±1.34	1.62±0.007	80.69±0.84	83.22±0.84	0.968±0.008
		GA	27.91±0.60	73.61±1.56	1.63±0.009	78.70±0.98	82.48±0.98	0.954±0.009
		AA	29.86±1.27	79.16±3.31	1.64±0.018	83.62±2.08	87.64±2.07	0.952±0.019
		P-value	0.38	0.28	0.74	0.07	0.08	0.43
		FDR	–	–	–	–	–	–
Fat mass and obesity	rs9939609	TT	27.57±0.57	72.26±1.50	1.62±0.008	78.91±0.94	82.20±0.93	0.959±0.009
		TA	28.58±0.59	75.55±1.55	1.63±0.009	81.10±0.97	83.58±0.96	0.971±0.009
associated (*FTO*)		AA	29.39±1.26	74.88±3.33	1.60±0.018	82.36±2.09	85.96±2.06	0.955±0.019
		P-value	0.28	0.30	0.44	0.15	0.21	0.55
		FDR	–	–	–	–	–	–

Abbreviation: FDR, false discovery rate.
Values for different lipid parameters are indicated as mean±SE. Analysis of covariance with age and gender adjusted. *P*-values indicate the association pattern of the selected SNPs with different anthropometric traits.
*Indicates significant association.

Table 4 Association of SNPs with lipid traits

Gene	SNP	Genotype	TC Mean ± SE	TG Mean ± SE	HDL Mean ± SE	LDL Mean ± SE
Growth factor receptor-bound protein (*GRB14*)	rs3923113	AA	5.02 ± 0.07	2.25 ± 0.05	1.39 ± 0.03	2.62 ± 0.05
		AC	5.05 ± 0.09	2.24 ± 0.06	1.36 ± 0.03	2.61 ± 0.06
		CC	5.22 ± 0.23	2.14 ± 0.14	1.26 ± 0.08	2.72 ± 0.15
		P-value (ANOVA)	0.68	0.77	0.25	0.78
		FDR	–	–	–	–
		β (SE)	−0.069 (0.090)	0.015 (0.026)	0.039 (0.025)	−0.013 (0.061)
		P-value	0.45	0.57	0.12	0.83
Sialyltransferase 6 galactosidase 1 protein (*ST6GAL1*)	rs16861329	CC	5.04 ± 0.07	2.18 ± 0.04	1.38 ± 0.02	2.59 ± 0.04
		CT	5.10 ± 0.09	2.29 ± 0.06	1.34 ± 0.03	2.70 ± 0.06
		TT	4.77 ± 0.26	2.22 ± 0.17	1.36 ± 0.10	2.54 ± 0.17
		P-value (ANOVA)	0.47	0.34	0.57	0.36
		FDR	–	–	–	–
		β (SE)	0.023 (0.092)	−0.033 (0.027)	0.023 (0.025)	−0.051 (0.062)
		P-value	0.80	0.22	0.36	0.42
Vacuolar protein sorting-associated protein (*VPS26A*)	rs1802295	CC	5.01 ± 0.07	2.24 ± 0.05	1.38 ± 0.03	2.56 ± 0.05
		CT	5.06 ± 0.09	2.19 ± 0.05	1.39 ± 0.03	2.63 ± 0.06
		TT	5.38 ± 0.19	2.24 ± 0.12	1.22 ± 0.06	2.92 ± 0.13
		P-value (ANOVA)	0.19	0.80	0.06	0.03
		FDR	–	–	–	0.11
		β (SE)	0.126 (0.084)	−0.009 (0.024)	−0.029 (0.022)	0.132 (0.056)
		P-value	0.13	0.70	0.20	0.02
High-mobility group protein 20A (*HMG20A*)	rs7178572	AA	5.04 ± 0.11	2.21 ± 0.07	1.44 ± 0.04	2.53 ± 0.07
		AG	5.08 ± 0.08	2.24 ± 0.05	1.31 ± 0.03	2.67 ± 0.05
		GG	4.98 ± 0.10	2.21 ± 0.06	1.41 ± 0.04	2.59 ± 0.06
		P-value (ANOVA)	0.73	0.89	0.01	0.23
		FDR	–	–	0.07	–
		β (SE)	−0.030 (0.072)	−0.001 (0.021)	−0.008 (0.019)	0.024 (0.049)
		P-value	0.68	0.96	0.69	0.62
Adaptor-related protein complex (*AP3S2*)	rs2028299	AA	4.98 ± 0.08	2.22 ± 0.05	1.36 ± 0.03	2.58 ± 0.05
		AC	5.12 ± 0.08	2.20 ± 0.05	1.39 ± 0.03	2.64 ± 0.06
		CC	5.27 ± 0.17	2.48 ± 0.12	1.29 ± 0.06	2.68 ± 0.11
		P-value (ANOVA)	0.22	0.09	0.34	0.60
		FDR	–	–	–	–
		β (SE)	0.139 (0.080)	0.032 (0.024)	−0.005 (0.022)	0.055 (0.054)
		P-value	0.08	0.18	0.82	0.31
Hepatocyte nuclear factor (*HNF4A*)	rs4812829	GG	5.06 ± 0.07	2.23 ± 0.05	1.37 ± 0.03	2.63 ± 0.05
		GA	5.03 ± 0.09	2.17 ± 0.05	1.39 ± 0.03	2.51 ± 0.06
		AA	5.09 ± 0.18	2.50 ± 0.13	1.24 ± 0.06	2.88 ± 0.12
		P-value (ANOVA)	0.94	0.06	0.09	0.02
		FDR	–	–	–	0.10
		β (SE)	−0.006 (0.082)	0.024 (0.024)	−0.024 (0.022)	0.023 (0.056)
		P-value	0.95	0.33	0.27	0.68
Fat mass and obesity associated (*FTO*)	rs9939609	TT	5.02 ± 0.08	2.18 ± 0.05	1.41 ± 0.03	2.59 ± 0.05
		TA	5.02 ± 0.08	2.22 ± 0.05	1.37 ± 0.03	2.64 ± 0.06
		AA	5.19 ± 0.18	2.44 ± 0.13	1.31 ± 0.06	2.59 ± 0.12
		P-value (ANOVA)	0.65	0.15	0.34	0.79
		FDR	–	–	–	–
		β (SE)	0.052 (0.083)	0.041 (0.024)	−0.033 (0.023)	0.019 (0.06)
		P-value	0.53	0.096	0.15	0.74
Gene score		*Correlation*	–0.007	0.05	0.004	0.027
		P-value	*P* = 0.90	*P* = 0.38	*P* = 0.94	*P* = 0.62

Abbreviation: FDR, false discovery rate.
Values for different lipid parameters are indicated as mean ± SE. Data analysis for lipid trait association was done for cases and controls combined and covariates age and gender were adjusted.
β (SE) values indicate increase or decrease in a lipid trait per risk allele in mmol/l.

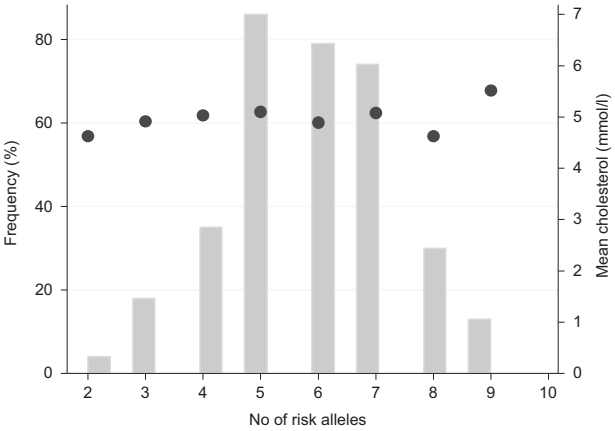

Figure 2 Effect of increasing number of risk alleles on mean total cholesterol.

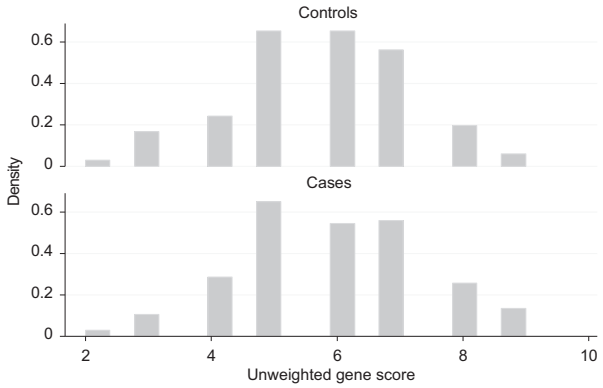

Figure 3 Obesity by unweighted gene score. Mean unweighted gene score in cases *vs* controls, mean (SD) = 5.76 (1.45) in cases *vs* 5.93 (1.54) in controls, $P = 0.29$.

significant association with diabetes between cases and controls. However, when tested individually, only rs1802295 showed a significant association with diabetes, but in the direction opposite to the published effect.

DISCUSSION

Pakistan, as a low-income country, initially faced the problem of malnutrition and undernutrition, but an improvement in life standards and availability of a variety of palatable foods at relatively low price resulted in an increase in overall body weight that became more evident because of decrease in physical activity. Owing to illiteracy and psychological stigma, obesity has not been considered a disease until recently, hindering research to understand what factors, biochemical as well as genetic, are involved in the development of obesity in Pakistan. In this context, it is needed to establish a genetic panel representing common variants predisposing to common forms of obesity in this region of the world.

The study aimed to seek insight into the possible mechanism through which six of the reported type II diabetes susceptibility loci and one well-established *FTO* variant may lead to obesity and subsequently to diabetes. As obesity is a risk factor for diabetes, we genotyped these variants in obese as well as nonobese subjects in order to clarify whether or not these variants are associated with obesity. The extensively studied variant rs9939609 in the *FTO* gene was selected in order to clarify the inconsistent association patterns observed for the variant with obesity and its minor allele frequency in previous studies.[26] The SNP is reported to have a high minor allele frequency in Caucasians (~0.4) but very low in Asian population (~0.1), whereas its association with obesity and type II diabetes has been confirmed in many populations but not in Chinese Han subjects.[11,18–26,34–38] The six GWAS SNPs were found to be associated with type II diabetes and have been replicated for Chinese and Japanese populations only for association with type II diabetes.[27–29] However, these SNPs have not been previously studied with respect to obesity, and the Pakistani population represents an interesting ethnic group because of the many cultural and social restrictions and with an increasing rate of obesity and type II diabetes. It was important to study whether or not these SNPs have any role in obesity in the Pakistani population.

The results of our study, presented in Table 2, indicate a modest association of the selected variants with obesity, with odds ratios ranging from 1.06 for rs7178572 (*HMG20A*) to 1.54 for rs9939609 (*FTO*), with 2/7 of the SNPs also showing association with weight and 1/7 with BMI. However, it is evident that the mechanism of the effect of these SNPs on obesity is only partly through influencing plasma lipid levels, with only 2/7 showing an association with LDL-Cl levels and 1/7 an association with HDL-C, most notably with the *FTO* gene showing no association with any plasma lipid trait. These results are supported by the function and differential expression of the genes in which these SNPs are located in specific tissues.

We used the unweighted and weighted gene score to study the combined effect of risk alleles on obesity. This is a robust approach, particularly when sample size is small, as it gives information regarding the additive effects of multiple variants in different genes in the same individual. As the weighted gene score takes into account the published effect sizes from large studies, the effect size assigned to each variant is independent of the effect estimated from the current small study such that the power problem is somewhat overcome. We had selected 7 variants in 7 different genes, and this means that an individual could have a maximum of 14 risk alleles. By using a gene score approach, we found that there is a threshold of having at least >4 alleles in order to have a considerable effect on body weight. This may be due to the fact that if only ≤ 4 risk alleles are present, even the combined effect is so small that the body's homeostatic mechanisms can maintain an ideal body weight, but if ≥ 5 alleles are present, the combined effect is pronounced and the result is an increased risk of weight gain and obesity. The effect may be accounted for by the small sample size, and these results need replication in a larger sample size.

The SNP rs3923113 is close to the *growth factor receptor-bound protein 14* (*GRB14*) gene, a strong candidate for the observed association with obesity and type II diabetes. The gene product is an adapter protein that binds to insulin and insulin-like growth factor receptors inhibiting tyrosine kinase signaling.[39,40] *GRB14*$^{-/-}$ mice are lean and have improved insulin sensitivity. The rs3923113 risk allele is associated with reduced insulin sensitivity and T2D in South Asians, indicating a gain of function.[28] This SNP is in LD with another SNP rs10195252 that has been shown to be associated with central adiposity and *GRB14* expression in adipose tissue.[41]

The rs16861329 is an intronic SNP in *sialyltransferase 6 galactosidase 1 protein* (*ST6GAL1*) gene, the product of which is an enzyme located in the Golgi apparatus. Although this gene was not linked to T2D previously, glycosylation due to addition of sialic acid is reported to influence glucose metabolism via affecting insulin signaling and trafficking.[42] Alternatively, an association with obesity may arise due to the fact that this SNP is located close to the *adiponectin* (*ADIPOQ*)

gene that encodes adiponectin, a hormone known to increase insulin sensitivity.[43]

The SNP rs1802295 is in the *vacuolar protein sorting-associated protein 26A* (*VPS26A*) gene that encodes a subunit of a large multimeric protein complex involved in transport to trans-Golgi complex.[44,45] This gene is expressed in adipose, pancreatic and some other tissues, and although its relationship with glucose metabolism has not been clearly understood, it is considered as a candidate gene for susceptibility to obesity and T2D because of abundant expression in adipose tissue.[46]

The fourth SNP rs2028299 is in close proximity to the *adapter-related protein complex* (*AP3S2*). This gene is expressed in pancreatic and adipose tissue, and the product is involved in vesicle transport and sorting in these tissues.[47] The SNP may directly affect body weight by disturbing vesicular transport in smooth endoplasmic reticulum in adipose tissue or can act indirectly by affecting other genes, that is, it is used as a marker for variation in the *perilipin 1* (*PLIN-1*) gene. The product of *PLIN-1* is involved in coating of fat droplets in adipose tissue and regulation of lipolysis, as this gene lies close to *AP3S2*.[48] Many variations in *PLIN1* have been found to be associated with obesity in mouse models.[49,50]

We have observed a significant association of the SNP rs7178572 with weight, HC and WHR but with serum HDL-C only. This is an intronic SNP in the *high-mobility group protein 20A* (*HMG20A*) gene that encodes a regulatory protein controlling gene expression by histone modification and is important in neural development. The SNP may play its role by affecting the expression of genes involved in lipid metabolism, thereby leading to dyslipidemia and obesity.[51,52]

The SNP rs4812829 is strongly associated with LDL-C only. It is intronic in the *hepatocyte nuclear factor 4* (*HNF4A*) gene, a strong candidate for association with obesity and T2D. The product of the gene is a transcription factor strongly expressed in liver.[53] *HNF4A* mutations have been reported to cause mature-onset diabetes of young (MODY) type I, involving impaired β-cell function and insulin sensitivity.[54] The risk allele of rs4812829 has been found to be associated with reduced β-cell function in subjects of South Asian ancestry.[28]

The most extensively studied variant of *fat mass and obesity* (*FTO*) gene is an intronic SNP, rs9939609. This SNP is known to predispose to obesity, type II diabetes and cardiovascular diseases and has also been linked to psychological disorders.[55–58] A longitudinal study in Brazil found a 0.07 kg/m^2/year weight gain for each additional risk allele.[59] It has been previously reported that the overall risk of obesity is increased ~1.3-fold per risk allele. This variant is known to affect body weight by disturbing glucose metabolism.[26] Interestingly, we have found no association of this variant with any of the lipid traits, indicating that the variant has a direct role on energy regulation to increase body weight.

The study had the limitation of small sample size and therefore limited power. This problem was overcome by using a gene score approach. Although we observed a linear effect of the obesity gene score on lipid traits, the effect on obesity was not linear, which may be due to power problems, and therefore the observed association should be replicated in larger studies in the future. The lead GWAS SNP at a locus may not itself be functional, but rather may act as a marker of a functional variant in one of the nearby genes. Correctly identifying the functional SNP and the gene will be valuable as it will improve the size of effect for risk prediction and correctly identify novel targets for future therapeutic interventions.

CONCLUSION

The SNPs in the current study had high minor allele frequencies and moderate effect sizes. In future, studies with larger sample sizes should be conducted to not only clarify the effect of already known SNPs but also discover additional novel variants that are involved in affecting this group of related health problems, including obesity, diabetes, hypertension and cardiovascular disorders, and to find at least some common variants with moderate/large effect sizes in the Pakistani population in order to establish a risk score for obesity. Individuals having risk alleles greater than the threshold may be given advice to modify lifestyle, diet and exercise habits so that the genetic predisposition may not 'switch on'.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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