Association of Genetic Polymorphism in Leptin, Leptin Recptor, Resistin and FABP2 genes with type II Diabetes mellitus in patients of Rural South Western Maharashtra

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Abstract
India has the second highest number of diabetic patients in the world and the increasing trend of obesity poses a threat towards its working population and socioeconomic development. The inheritance pattern of type II diabetes mellitus (T2DM) is complex but recent advances in genetics have shed light on variations in novel genes as possible risk factors for development of T2DM. Our study aimed at studying the association of single nucleotide polymorphisms (SNP) in adipokine genes such as leptin, leptin receptor, resitin and fatty acid binding protein 2 (FABP2) which is involved in transport and metabolism of long chain fatty acids. The SNPs were genotyped using appropriate polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) conditions.

The results were analyzed using Chi-square test and Odds Ratio (OR) to show the association with T2DM among healthy non-diabetic controls and obese and non-obese T2DM patient groups. Leptin gene SNP (rs7799039) was found to be associated with occurrence of T2DM among non-obese diabetic patients (OR 2.153 (1.13-4.105) p = 0.028). Other SNPs of leptin receptor (rs1137101), resistin (rs3745368), FABP2 (rs1799883) were found not to be associated with T2DM.

Keywords: FABP2, Leptin, Leptin receptor, Resistin, Type II diabetes mellitus.

Introduction

Diabetes is estimated to affect 463 million people worldwide in 2019 [28]. India is the second most affected country globally with a case load of 77 million as of 2017 [24]. Additionally, increase in household income in urban and rural areas, sedentary lifestyle and higher consumption of fat-rich diet have contributed to an unprecedented increase in obesity and diabetes in India [31]. Type II diabetes mellitus (T2DM) is the most common form of diabetes and is caused by reduced secretion of insulin by β cells of islets of Langerhans in the pancreas and increase in insulin resistance of peripheral tissues. Obesity is a major factor contributing to progression of impaired glucose tolerance to T2DM. The increase in adipose tissue leads to imbalance in glucose metabolism mostly by decreasing insulin sensitivity. Hence, the normal functioning or expression of genes in adipose tissue is critical in maintaining glucose homeostasis. Some of the genes expressed by adipocytes include adipokines such as leptin, adiponectin and resistin[5].

Leptin gene is located on chromosome 7q32 and is expressed by white adipocytes and regulates energy metabolism by decreasing the expression of insulin, increasing uptake of fatty acids by adipocytes and promoting β oxidation of fatty acids. It is often termed as the 'satiety' hormone since it signals energy abundance via its interaction with leptin receptors in the hypothalamus leading to lowering of food intake. One of the important single nucleotide polymorphisms (SNPs) of leptin gene is G to A susbtituion at -2548 nucleotide upstream of the start codon (rs7799039)[5].

The leptin receptor gene is located on chromosome 1q and functions by the activation of the Janus kinase pathway which regulates transcription of other genes signaling stoppage of energy intake. The receptor protein is a single transmembrane polypeptide which forms homodimers on the cell membrane. One mutation is at the leptin binding site where glutamine is substituted by arginine due to A to G transition at exon 6 (rs1137101)[5,25].

Another important adipokine is resistin which has antagonistic effects to insulin and influences adipocyte differentiation. Resistin gene is located on chromosome 19 and its level in serum is elevated in obesity which may lead to insulin resistance and progression of impaired glucose tolerance to T2DM. +62 G->A mutation is located 62 bp downstream of the last codon for termination of exon 4 in its 3'UTR (rs3745368)[6,30]. The fatty acid binding protein 2 (FABP2) gene is expressed mostly by epithelial intestinal cells and is located on chromosome 4q26.

It is involved in the fatty acid absorption and intracellular transportation especially of long chain fatty acids. In obese individuals, higher levels of free fatty acids are found in the serum and intracellular absorption of higher levels of fatty acids may lead to insulin resistance. The SNP (rs1799883) is caused due to substitution of A by G at codon 163[1,34].

Association studies of variants or SNPs with T2DM have not been consistent.[2,7–9,12,13,16,17] Most of the Indian studies have been reported form the south and north India.[4,9,33] These studies have reported association between leptin SNP (rs7799039) and T2DM while the other SNPs namely rs1137101, rs3745368 and rs1799883 were not associated with T2DM. Studies from rural south western Maharashtra population have been lacking. Hence, it is imperative to study the association of these candidate genes with T2DM since they are involved in obesity and insulin sensitivity in the stated population group.

Material and Methods

Sample Collection: Clinically diagnosed T2DM patients attending the out-patient and in-patient Department of Medicine at the Krishna Hospital and Medical Research Centre, Karad (KHMRC) were enrolled in the study. 120 obese and 120 non-obese patients were enrolled separately into two groups for the study. 120 non-obese non-diabetic subjects were enrolled as control group from the same hospital. Classification of obese and non-obese subjects was done based on the body mass index (BMI). Subjects with BMI $\leq$ 24.9 were termed as non-obese and BMI > 24.9 were termed as obese.

Exclusion criteria: Subjects currently using lipid-lowering drugs and known cases of hypothyroidism, hyperthyroidism, tuberculosis, malignancy, pregnancy, Cushing's syndrome were excluded from the study. After receiving informed consent, 3 ml whole blood was collected in EDTA-containing vacutainer. The study protocol was approved by the Institutional Ethics committee and written consent from patients and control subjects was taken.

Genomic DNA extraction: Following the provided manufacturer's instructions, genomic DNA was extracted with blood DNA extraction kit (Make: Qiagen). The blood and DNA samples were stored at -80 °C until required.

Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) analysis

Leptin (SNP rs7799039): Genotyping of Leptin (rs7799039) was performed by PCR-RFLP method using appropriate PCR and RFLP conditions represented in table I. A 252 bp PCR product was digested with restriction enzyme HhaI and analyzed on 3 % agarose gel. The restriction digestion yielded undigested homozygous wild-type allele (252 bp), homozygous variant-type allele 181 bp and 71 bp and the heterozygous allele 252 bp, 181 bp, 71 bp fragments[5].

Leptin receptor (SNP rs1137101): Genotyping of leptin receptor (rs1137101) was performed by PCR-RFLP method using appropriate PCR and RFLP conditions represented in table I. A 416 bp PCR product was digested with restriction enzyme MspI and analyzed on 3 % agarose gel. The homozygous wild-type allele was undigested (416), homozygous variant-type allele 229 bp, 187 bp fragments and the heterozygous allele 416 bp, 229 bp, 187 bp fragments[5].

Resistin (SNP rs): Genotyping of resistin (rs) was performed by PCR-RFLP method using appropriate PCR and RFLP conditions represented in table I. A 249 bp PCR product was digested with restriction enzyme BseRI and analyzed on 4 % agarose gel. The result of restriction digestion produced homozygous wild-type allele 249 bp, homozygous variant-type allele 238 bp and 11 bp and the heterozygous allele fragments of 249 bp, 238 bp and 11 bp[30].

Fatty acid binding protein 2 (SNP rs1799883): Genotyping of FABP2 (rs1799883) was performed by PCR-RFLP method using appropriate PCR and RFLP conditions represented in table I. A 180 bp PCR product was digested with restriction enzyme HhaI and analyzed on 3 % agarose gel. The homozygous wild-type allele fragments were 99 bp and 81 bp, homozygous variant-type allele 180 bp and the heterozygous allele fragments of 180 bp, 99 bp and 81 bp[34].

Statistical analysis: The demographic features of the control and patient groups were examined by applying Chi-square test using Instat software (Make Graphpad version 3.06). Level of significance of $p < 0.05$ was considered statistically significant. The allelic distribution of genes was studied by performing analysis of variance (ANOVA) and binary logistic regression modeling [Odd's ratio (OR) and adjusted Odd's ratio] using SPSS Statistics software (Make IBM version 20).

Results and Discussion

The leptin gene is involved in energy regulation, fatty acid oxidation in adipocytes and regulation of insulin expression. The leptin receptor binds to leptin and down-regulates expression of genes involved in energy uptake via intracellular signaling pathway[5]. Resistin acts counter to insulin; while FABP2 is involved in intracellular transport of long chain fatty acids[1,30]. Generally, SNPs in the intron or untranslated region of genes may affect their expression while SNPs in the exon may affect the structure and functioning of their protein products.

The leptin SNP (rs7799039) is located in the upstream regulatory region and may affect the expression of the gene and has been reported to be associated with obesity and T2DM. The leptin receptor SNP (rs1137101) leads to substitution of Glutamine by Arginine in its extracellular domain leading to weak binding with leptin and reduced function of the leptin receptor. Several studies have linked this SNP with extreme obesity, glucose and energy imbalance among laboratory animals and humans [5,25].

The resistin SNP (rs3745368) located in the regulatory 3' UTR region may affect its activity during adipocyte maturation or expression. The regulatory role of resistin over insulin secretion is crucial in glucose homeostasis and its dysfunction may lead to decrease in insulin secretion [6]. The

FABP2 SNP (rs1799883) leads to a mis-sense substitution of alanine by threonine amoni acid residue at position 54 in the polypeptide. Several *in-vitro* and *in-vivo* studies have reported that the threonine variant of the FABP2 protein has higher affinity for binding long chain fatty acids and facilitates greater transport across the cell membrane leading to fat accumulation and affects activity and secretion of insulin [1].

The demographic features of patient and control groups were compared using Chi-square test. As obesity leads to progression of T2DM, our observations were in accordance with expectations [31]. Restricted physical movement or exercise resulting from sedentary life style and causal associations such as hypertension and other disorders were significantly more in T2DM patients. Other features such as family history of T2DM, alcohol and tobacco usage, education, economic standing were also different among patients and control group.

The genotype frequencies of leptin, leptin receptor, resistin, FABP2 genes among control and non-obese diabetic group were illustrated in table II. The results of the Chi-square test show that genotypes of leptin receptor (rs1137101) (p = 0.304), resistin (rs3745368) (p= 0.459) and FABP2 (rs1799883) (p= 0.34) genes were similarly distributed between control group and non-obese T2DM patients. Hence, it may be concluded that these SNPs have no association with occurrence of T2DM among the non-obese diabetic patient group.

Table I
Details of PCR and RFLP conditions used for SNP analysis

S. N.	Gene/ SNP	Primer sequence	PCR conditions	PCR product	RFLP conditions
1	Leptin (rs7799039)	FP:5'TTTCCTGTAATTTTCCCGTGAG 3' RP: 5'AAAGCAAAGACAGG CATAAAAA-3'	95°C for 5 minutes(min); 30 cycles of 95°C for 30 seconds(s), 52°C for 45s, 72 °C for 45 s; Final extension of 72 °C for 10 min	252 bp	1 unit (U) HhaI Incubation at 37°C for 16 hours (hrs)
2	Leptin receptor (rs1137101)	FP: 5'-GCCTAATCCAGTATTT TC-3' RP: 5'-GCCACTCTTAATACC CCCAGTAC-3'	95°C for 5 min; 30 cycles of 95°C for 30 s, 54 °C for 1 min, 72 °C for 1 min; final extension of 72 °C for 10 min	416 bp	1 unit (U) Msp1 Incubation at 37°C for 16 (hrs)
3	Resistin	FP: 5' AGAGTCCACGCTCC TGTGTT-3' RP: 5' –CATCTCCAGGTTTA TTTCCAGC-3'	95°C for 5 min; 30 cycles of 95°C for 30 s, 55 °C for 1 min, 72 °C for 1 min; final extension of 72°C for 10 min	249 bp	1 unit (U) BSeRI Incubation at 37°C for 16 (hrs)
4	FABP2 (rs1799883)	FP: 5'-ACAGCTGTTAATATA GTGAAAAG-3' RP: 5'-TACCCTGAGTTCA GTTCCGTC-3'	95°C for 5 min; 30 cycles of 95°C for 20 s, 55 °C for 20 sec, 72 °C for 20 sec; final extension of 72 °C for 10 min	180 bp	1 unit (U) HhaI Incubation at 37°C for 16 (hrs)

Table II
Genotype frequencies of leptin, leptin receptor, resistin, FABP2 genes polymorphisms in non-obese diabetic and control groups

Gene	Genotype	Control	Non-obese diabetic	Chi-squared	p value	Odd's ratio (95 % C.I.)	p value	Adjusted Odd's ratio (95 % C.I.)	p value
Leptin (rs7799039)	Wild Type	37 (30.8%)	21 (17.5%)	5.872	0.043	1		1	
	Heterozygous	54 (45%)	66 (55%)			2.153 (1.13-4.105)	0.0285	2.098 (1.089-4.039)	0.027
	Varient Type	29 (24.2%)	33 (27.5%)			2.005 (0.964-4.469)	0.0912	2.106 (1.003-4.424)	0.049
	Non-wild	83 (69.2%)	99 (82.5%)	5.82	0.016	2.102 (1.142-3.868)	0.0237	2.105 (1.14-3.889)	0.017
Leptin receptor (rs1137101)	Wild Type	36 (30%)	31 (25.8%)	2.38	0.304	1		1	
	Heterozygous	67 (55.8%)	63 (52.5%)			1.092 (0.604-1.972)	0.8876	1.15 (0.626-2.113)	0.653
	Varient Type	17 (14.2%)	26 (21.7%)			1.776 (0.8161-3.865)	0.2082	1.723 (0.779-3.811)	0.179
	Non-wild	84 (70%)	89 (74.2%)	2.518	0.417	1.23 (0.699- 2.166)	0.5649	1.293 (0.726-2.305)	0.383
Resistin (rs3745368)	Wild Type	87 (72.5%)	92 (76.7%)	0.55	0.459	1		1	
	Heterozygous	0	0			-		-	
	Varient Type	33 (27.5%)	28 (23.3%)			0.8024 (0.448-1.437)	0.5532	0.796 (0.434-1.461)	0.462
	Non-wild	33 (27.5%)	28 (23.3%)	0.55	0.459	0.8024 (0.448-1.437)	0.5532	0.801 (0.442-1.453)	0.465
FABP2 (rs1799883)	Wild Type	49 (40.8%)	58 (48.3%)	2.158	0.34	1		1	
	Heterozygous	52 (43.3%)	41 (34.2%)			0.666 (0.381-1.165)	0.1985	0.67 (0.379-1.188)	0.171
	Varient Type	19 (15.8%)	21 (17.5%)			0.9338 (0.4509-1.934)	0.8536	0.964 (0.458-2.03)	0.924
	Non-wild	71 (59.2%)	62 (51.7%)	1.36	0.54	0.737 (0.442-1.229)	0.2988	0.744 (0.443-1.249)	0.263

Table III
Genotype frequencies of leptin, leptin receptor, resistin, FABP2 genes polymorphisms in obese diabetic and control groups

Gene	Genotype	Control	Obese diabetic	Chi-squared	p value	Odd's ratio (95 % C.I.)	p value	Adjusted Odd's ratio (95 % C.I.)	p value
Leptin (rs7799039)	Wild Type	37 (30.8%)	33 (27.5%)	0.327	0.84	1		1	
	Heterozygous	54 (45%)	57 (47.5%)			1.184 (0.65-2.155)	0.69	1.149 (0.622-2.122)	0.657
	Varient Type	29 (24.2%)	30 (25%)			1.16 (0.579-2.132)	0.808	1.169 (0.57-2.398)	0.67
	Non-wild	83 (69.2%)	87 (72.5%)	0.323	0.57	1.17 (0.673-2.052)	0.6701	1.131 (0.64-1.996)	0.672
Leptin receptor (rs1137101)	Wild Type	36 (30%)	25 (20.8%)	2.882	0.837	1		1	
	Heterozygous	67 (55.8%)	73 (60.8%)			1.569 (0.853-2.884)	0.193	1.795 (0.946-3.409)	0.074
	Varient Type	17 (14.2%)	22 (18.3%)			1.864 (0.824-4.202)	0.192	1.954 (0.851-4.483)	0.114
	Non-wild	84 (70%)	95 (79.2%)	2.66	0.103	1.629 (0.904-2.934)	0.138	1.881 (1.02-3.469)	0.043
Resistin (rs3745368)	Wild Type	87 (72.5%)	92 (76.7%)	0.55	0.459	1		1	
	Heterozygous	0	0			-		-	
	Varient Type	33 (27.5%)	28 (23.3%)			0.802 (0.448-1.437)	0.553	0.661 (0.357-1.224)	0.188
	Non-wild	33 (27.5%)	28 (23.3%)	0.55	0.459	0.802 (0.448-1.437)	0.553	0.687 (0.375-1.257)	0.223
FABP2 (rs1799883)	Wild Type	49 (40.8%)	64 (53.3%)	5.413	0.052	1		1	
	Heterozygous	52 (43.3%)	35 (29.2%)			0.515 (0.292-1.09)	0.31	0.84 (0.861-1.267)	0.143
	Varient Type	19 (15.8%)	21 (17.5%)			0.846 (0.41-1.745)	0.789	0.792 (0.374-1.674)	0.541
	Non-wild	71 (59.2%)	56 (46.7%)	3.736	0.052	0.604 (0.362-1.007)	0.0702	0.559 (0.33-0.945)	0.03

However, the SNP for leptin (rs7799039) was differently distributed among control and non-obese diabetic patients (Chi-square = 5.872; p = 0.043). The heterozygous genotype for rs7799039 was found to be associated with T2DM in this group (Odds ratio 2.153 (1.13-4.105); p = 0.0285). The genotype frequency of leptin, leptin receptor, resistin, FABP2 genes among control and obese diabetic group was mentioned in table III.

The Chi-square test results indicate that the genotype distribution of leptin (rs7799039) (p= 0.84), leptin receptor (rs1137101) (p= 0.837), resistin (rs3745368) (p= 0.459) and FABP2 (rs1799883) (p= 0.052) was not significantly different among control and obese diabetic patients. The observations indicated that these SNPs were not associated with occurrence of T2DM among obese diabetic patients. The association of SNPs and disease state depends on the interaction of several genes with each other and with environmental factors.

SNP (rs7799039) of leptin is located near the upstream SP1 transcription binding site and may contain inhibitory elements that down-regulate expression of leptin in adipocytes[10]. Several studies have reported association of this SNP with obesity and T2DM among Taiwanese, European, Brazilian and Finnish population[13,14,19].

More recently, rs7799039 was reported to be a risk factor for development of T2DM among Mongolian and Tunisian[8]. Other reports from Brazilian, Tunisian, Spainish populations failed to find any association between this SNP and T2DM.[3,20,27] The leptin receptor SNP rs1137101 has been reported to affect its leptin binding affinity and was found to be associated with T2DM among Chinese and Malaysian[9]. On the contrary, other research groups have reported no such association with T2DM among Iranian, Korean, Chinese Han and Taiwanese populations.[3,9,12,14,18–21,27]

The resistin SNP rs rs3745368 was reported to be associated with Taiwanese and Thai population [17,32]. However, no such association was reported for Chinese Han, German and Mexican populations[7,15,22]. The FABP2 SNP (rs1799883) leads to insulin resistance and was found to be associated with T2DM in Afro-Carribean, Kazakh and Spanish populations [16,29]. Some studies in Canadian and Chilean population failed to find any association[2,11,26]. Since T2DM is a polygenic disorder, interaction of SNP or genes with other variations and environment determines their phenotypic expression which can vary depending on physical attributes and ethnicity. A genetic polymorphism study of North Indian population with T2DM and leptin SNP (rs7799039) reported that the SNP was associated T2DM[4].

Another study in south Indian population of the leptin receptor SNP (rs1137101) did not find any association with T2DM [23]. No association of FABP2 SNP (rs1799883) and T2DM was found in another South Indian study [33]. In our study, we observed that only leptin SNP rs7799039 was associated with T2DM in non-obese diabetic patients whereas rs1137101, rs3745368 and rs1799883 were not found to be associated with T2DM. Our findings corroborate the above mentioned studies of Indian population and give information regarding western Indian population.

Conclusion

Our findings revealed that leptin SNP rs7799039 may be associated with development of T2DM among non-obese individuals. Other SNPs namely leptin receptor (rs1137101), resisistin (rs3745368) and FABP2 (rs1799883) did not show association with T2DM among the studied population.

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