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Article

Association of MC4R codon 42 polymorphism with obese/diabetic ethnic Kashmiri population

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Abstract: Our objective was to study the sequence variations of melanocortin 4 receptor (MC4R) gene in obese/diabetic populations of the Kashmir region and workout association of such variations (if any) with the disease phenotype. No such study has ever been taken up in the Jammu and Kashmir State, despite the existence of a significant population of obese/diabetic patients. A total of 420 samples were taken in the present study. Among them 200 were obese and 220 were normal. The samples were divided into three groups i.e., normal (220), obese with non diabetics (150) and obese with diabetics (50). Allele frequencies were tested for Hardy–Weinberg disequilibrium. The genotype and allele frequencies were evaluated using the $\chi 2$ tests or the Fisher exact tests. A novel heterozygous variation (Glu42Lys) was observed in all groups at a frequency of 10% in obese with non diabetics, 26% in obese with diabetics and 1.81% in healthy controls (BMI $\leq$ 23 kg/m^2) which was significantly ($p<0.05$) higher in both obese or obese with diabetes groups. G to A substituting glutamic acid with lysine appeared to associate with both diabetes and obesity significantly ($p< 0.05$). Insilico predictions show that substitutions likely have an impact on structure and functional properties of protein making it imperative to understand their functional consequences in relation with diabetes and obesity. These results indicate that this novel genotype may have a possible bearing in the genesis of obesity or diabetes in Kashmiri population.

Keywords: diabetes; obesity; MC4R; CSGE; genetic polymorphisms

1. Introduction

Obesity is a worldwide epidemic that predisposes to a high risk of premature mortality (Lee, 2009). The most powerful risk factor in the pathogenesis of diabetes mellitus is obesity. It is well established now that there is a direct co-relation between the emerging epidemic of diabetes mellitus and the obesity. The prevalence of overweight and obesity is increasing rapidly worldwide both in the developing as well as the developed countries (Ravussin and Bogardus, 2000). It affects approximately 50% of the adult and 25% of the pediatric population in the United States and is thus assumed to be the country's major health problem. Severe obesity in about 5% children has been linked to autosomal dominant mutations in the gene for the melanocortin 4 receptor (MC4R) (Vaisse *et al*., 2000; Branson et al., 2003; Farooqi *et al*., 2003). As the prevalence of obesity has steadily increased, it has rapidly emerged as a major public health concern (Mokdad *et al*., 1999; Must *et al*., 1999) due to significantly increased mortality (Allison *et al*., 1999) and a high risk of morbidity (Must *et al*., 1999). Because obesity disrupts lipid and glucose homeostasis, it is frequently associated with dyslipidemia, hypertension, diabetes mellitus, atherosclerosis and other cardiovascular diseases (Must *et al*., 1999; Vaisse *et al*., 2000; Kobayashi *et al*., 2002; Tao, 2005;Mackenzie, 2006) and often leads to a diagnosis of the metabolic

syndrome (Potoczna *et al*., 2004). Each year obesity causes at least 300,000 excess deaths in the U.S. alone (Allison *et al*., 1999), making it the second leading cause of unnecessary deaths. Even more alarming is the dramatic increase in childhood obesity and co morbidities (particularly T2DM) in industrialized countries (Dubern *et al*., 2001). Linkage studies have shown major obesity susceptibility loci to be located on chromosomes 2, 3, 4, 5, 6, 10, 11, 13, and 20 (Ravussin, and Bogardus, 2000; Lembertas *et al*., 1999; Stone *et al*., 2002). The possible candidate genes include leptin (Montague *et al*., 1997), leptin receptor (Clement *et al*., 1998), melanocortin-4 receptor (MC4R) (Vaisse *et al*., 1998; Yeo *et al*., 1998; Gu *et al*. 1999), prohormoneconvertase 1 (PCSK1) (Jackson *et al*., 1997) and proopiomelanocortin (POMC) (Krude *et al*., 1998; Barsh *et al*., 2000). Common obesity is generally a polygenic disorder, but monogenic causes have also been reported (Barsh *et al*., 2000;Korner and Aronne, 2003), that are associated with the mutations in genes that encode components of biochemical pathways involved in the control of appetite and eating behaviour. The melanocortin 4 receptor (MC4R) protein is one of these components and mutations in the MC4R gene have been found by some research groups in about 4-5% of severely obese people. Common genetic variation near MC4R is associated with risk of adiposity and insulin resistance (Chambers *et al*., 2008). Upto remarkable 6% of morbidly obese adults and children studied, possess single nucleotide polymorphisms (SNPs) of the MC4R (Vaisse *et al*., 2000;Branson *et al*., 2003; Farooqi *et al*., 2003; Xiang *et al*., 2006). This receptor integrates orexigenic and anorexigenic signals in the hypothalamus and elsewhere in the central nervous system to regulate food intake and energy expenditure. MC4R gene has only a single exon and thus, is amenable to mutation analysis.It is known to be a part of the central melanocortinergic system (Mountjoy *et al*., 1994; Lu, 2001). Within this pathway, binding of α-melanocyte stimulating hormone (α-MSH) to MC4R appears to form a central link. Stimulation of MC4R by alpha-MSH binding triggers the activation of inhibitory signals which, through a series of furthersteps, are believed to reduce food intake by creating perception of satiety. In cell culture studies, many of the obesity-linked MC4R mutations have been shown to render the receptor non-functional (Lubrano-Berthelier *et al*., 2003;Nijenhuis *et al*., 2003;Yeo *et al*., 2003). In context with these studies, mutations in MC4R appear to prevent the activation of inhibitory signals in response to alpha-MSH binding, so that individuals with obesity-linked mutations in MC4R may not experience the feeling of satiety. Common variations near MC4R were found to be associated with obesity, type 2 diabetes, and insulin resistance (Chambers *et al*., 2008; Loos *et al*., 2008; Morris *et al*., 2012; Speliotes *et al*., 2010; Willer *et al*., 2009). Much has been learned from studies on monogenic obesity; however, the role of variation at the MC4R locus in influencing inter-individual variation in body size and composition in the general population remains controversial (Sine *et al*., 1999; Lubrano-Berthelier *et al*., 2003; O'Rahilly *et al*., 2004). To date, MC4R mutations have proven to be of large individual relevancebut of small epidemiological relevance (Hinney *et al*., 2003). Therefore, further investigation, involving polymorphic variation and their correlation with the measures of adiposity in the general population, would help to elucidate the role of MC4R with regard to public health.

2. Material and Methods

2.1. Subjects

A total of 420 subjects (150 obese with non diabetic, 50 obese with diabetic and 220 healthy controls) were recruited from the Endocrinology Department of the Sher-i-Kashmir Institute of Medical Sciences, Soura (SKIMS) Srinagar, Jammu and Kashmir. Samples were divided into three groups i.e., controls, obese with diabetics and obese with non diabetics. Informed consent was obtained from the study subjects after an explanation of the nature and possible consequences of the study. Criteria for selection included a history of onset of obesity/diabetes in all affected subjects. The study was approved by Research Ethics Committee, SKIMS Srinagar. Blood samples of both diabetic and obese patients were collected after the complete clinical investigation. Subjects were encouraged to narrate all the details relevant to this study. This included age, gender, personal habits, dietary history, socio-economic factors, history of onset of obesity/diabetes, any associated complications and information regarding close work. Venous blood samples were collected in EDTA for DNA extraction. Samples were kept at -70°C until analyzed.

2.2. Polymerase chain reaction

Genomic DNA was extracted from whole blood samples using standard protocols like salting out and protinase k (Sigma Aldrich) methods (Nasiri *et al*., 2005; Sambrook and Russel, 2001). PCR reactions were carried out in a total volume of 50 µl, containing 50-100 ng of genomic DNA, 2-6 pmole of each primer, 1x of Taq polymerase buffer and 0.5 units of Taq DNA polymerase (Sigma Aldrich). The following primer sequences were used for amplification: 5'-AATAACTGAGACGACTCCCTGAC-3'(1F) and 5'-CTGATCCATTTGAAACGCTCACC-3'(1R); 5'-GCAGCTTGGCTGTGGCTGATATGC-3' (2F) and 5'-

GTGAAGCCTGGCCATCAGGAAC-3' (2R); 5'-CTCATGGCTTCTCTCTATGTCCAC-3' (3F) and 5'-CAGAAGTACAATATTCAGGTAGGG-3' (3R). Expected PCR products of 369, 416 and 459 bp were generated successfully. The PCR cycling conditions involved one cycle of denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 45 s, annealing at 60°C for 45 s and extension at 72°C for 45 s and one final 7 min elongation cycle at 72 °C. PCR products were then purified using GenElute^{TM} PCR Clean-Up Kit (Sigma Aldrich) or NaI.

2.3. Conformation sensitive gel electrophoresis (CSGE)

Purified PCR products were subjected to denaturation and renaturation procedures for generation of potential heteroduplexes and analyzed by CSGE strictly as described by Ganguly *et al*. (1993) (Figure 1). Samples with unusual mobility during these assays were finally sequenced to confirm the presence of sequence variations if present (Macrogen, Korea).

2.4. Sequencing and sequence analysis

Samples that showed presence of heteroduplex bands were sent for sequencing to confirm the presence of sequence variations. Sequence results obtained in fasta and pdf formats were analysed using ClustalX version 2 software (Thompson *et al*., 1997; Larkin *et al*., 2007) and by Chromas Pro version 1.49 beta 2 software (Technelysium Pvt. Ltd. Australia) for the detailed inspection of individual chromatograms.

2.5. Statistical analysis

Genotypes were obtained by direct counting with subsequent calculation of allele frequencies. Statistical analysis was undertaken using the χ^2 test and probability p values. A p value of <0.05 was considered significant. Adherence to the Hardy-Weinberg equilibrium constant was tested using the χ^2 test with one degree of freedom. Odds ratio and confidence interval were also calculated. Genotype and allele distribution was compared between obese and non-obese subjects using χ^2 test. The independent segregation of alleles was tested for the Hardy-Weinberg equilibrium (HWE), comparing the observed genotype frequencies with those expected (χ^2 test). Genotypic frequencies were in accordance with the Hardy-Weinberg Equilibrium in all the groups.

2.6. Insilico analysis

The amino acid sequence of the protein in fasta format obtained from (NCBI) (www.ncbi.nlm.nih.gov) was submitted to an automated server (I-TASSER) (zhang.bioinformatics.ku.edu/I-TASSER) for 3D structure prediction (Zhang, 2007; Zhang, 2008). The server furnishes predicted 3D structure in a pdb format. Swiss PDB Viewer was used for viewing pdb files and computing the free energy of the predicted 3D structures (Camacho *et al*., 2000; Camachoand Gatchell, 2003).

3. Results

This study identifies sequence variants in MC4R gene in a pure ethnic Kashmiri population. Prior to DNA sequencing samples were screened for the presence of mutations by Conformation Sensitive Gel Electrophoresis (CSGE) and only those samples were sent out for commercial sequencing that showed differential migration i.e., heteroduplex bands by CSGE (Figure 1). Mutational screening revealed a total of one polymorphic variation i.e., G>A at codon 42 changing polar negatively charged amino acid Glutamic acid (Glu; E) to polar and positively charged amino acid Lysine (Lys; K) in the only exon of MC4R gene after sequencing (Table 1). Genotypic analysis of individual variant revealed the presence of only heterozygous genotypes. The genotypes of all the three groups were in accordance with the Hardy Weinberg Equilibrium. This variation was present in all the samples i.e., controls, obese with diabetics and obese with non diabetics with a frequency of 1.81%, 26% and 10% respectively. A statistically significant (Obese with diabetics: p allele < 0.01, p genotype < 0.01 ; χ^2allele = 35.20, χ^2genotype = 36.38; OR = 16.28 ; CI (95%) = 5.18-51.13; Obese with non diabetes: p allele < 0.01, p genotype < 0.01 ; χ^2allele = 11.93, χ^2genotype = 10.63; OR = 5.73 ; CI (95%) = 1.88-17.46) (Table 2) difference in the allelic and genotypic frequencies for codon 42 variant was indicative of its possible association with both diabetes and obesity.

MC4R was modeled by I-TASSER to obtain its PDB structure (Figure 2) and analysis (energy calculations) was done using PDB Viewer. The assessment of the I-TASSER predicted protein structure showed higher energy for mutant protein compared to wild type protein in MC4R gene (Table 3).

Table 1. Polymorphism detected in exon of MC4R gene in ethnic Kashmiri population.

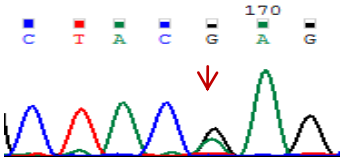
Codon position	rs no	Wild type nucleotide	Observed base pair change	Amino acid change	Chromatogram
Codon 42	Novel	G	A	Glu to Lys	

Table 2. Comparison of the distribution of alleles and genotypes of MC4R gene polymorphism at codon 42 in healthy and obese subjects.

Allele or Genotype	Controls (220) n (%)	Obese with diabetic (50) n (%)	χ^2	p value	OR	CI (95%)	Obese with non diabetic (150) n (%)	χ^2	p value	OR	CI (95 %)
G	436 (99.09)	87 (87)	35.20	< 0.01*	16.28	5.18-51.13	285 (85)	11.93	<0.01*	5.73	1.88-17.46
A	4 (0.90)	13(13)					15 (5)				
GG	216 (98.18)	37 (74)	36.38	< 0.01*			135 (90)	10.63	< 0.01*		
GA	4 (1.81)	13 (26)					15 (10)				
AA	0 (0)	0 (0)					0 (0)				

*Statistically significant

Table 3. Table shows the total energy of the I-TASSER predicted MC4R tertiary structures calculated by Swiss PDB Viewer. Model mutant has higher energy compared to wild type of MC4R.

Protein model Name	Energy (Kj/mol)
Wild type	-14498.063
Mutant	-14290.602

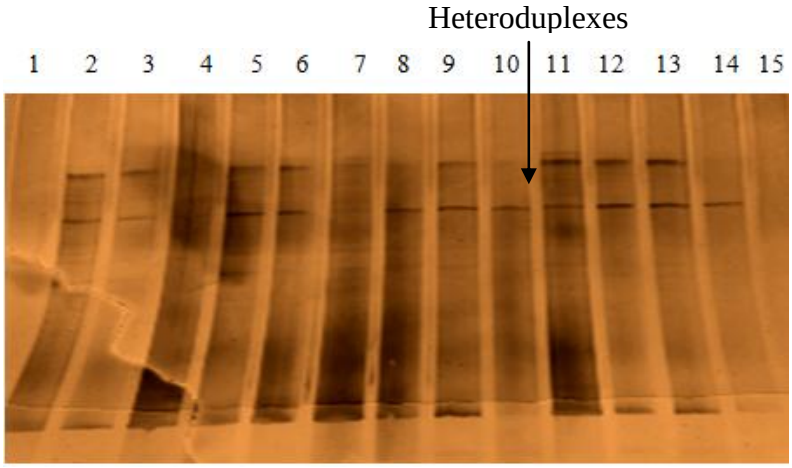
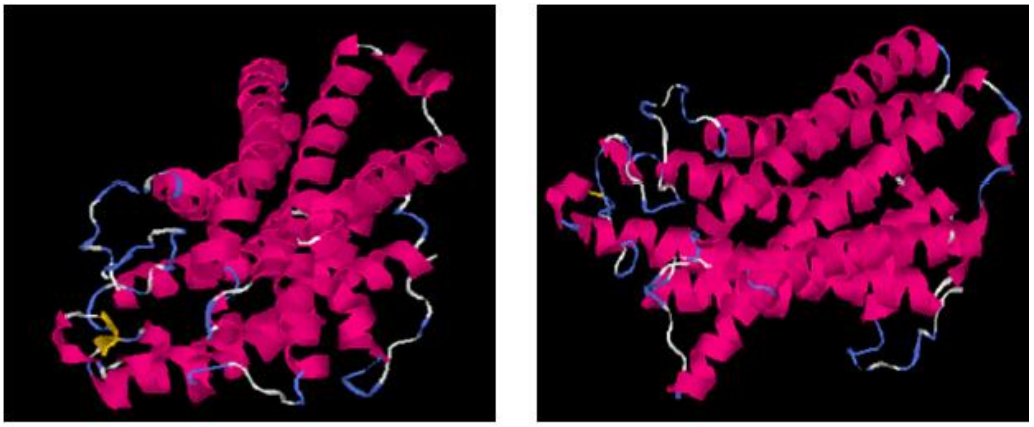


Figure 1. Heteroduplex analysis of MC4R amplicons by conformation sensitive gel electrophoresis. Heteroduplexes were observed in lanes 2, 3, 5, 6, 8, 9, 10, 11, 12, 13 and 14 and the samples showing heteroduplex bands were sent for sequencing for further confirmation. Lanes 1, 4, 7 and 15 show the analysis of normal samples. All the samples forming heteroduplexes on CSGE showed the presence of SNPs after sequencing.



(a) (b)

Figure 2. (a) Wild type and (b) mutant protein models of MC4R predicted by I-TASSER.

3. Discussion

The MC4R is a G-protein coupled receptor that couples through G's to raise intracellular cyclic AMP (cAMP) (Mountjoy *et al*., 1997). MC4R, which is expressed in the hypothalamus, is stimulated by alpha-MSH (Fan *et al*., 1997) and antagonized by agouti-related protein (AGRP) (Ollmann *et al*., 1997). The melanocortin-4 receptor (MC4R) is a part of the melanocortin pathway controlling food intake and energy homeostasis (Adan and van Dijk, 2006). Recent research has shown that several genetic factors play a role in regulating energy balance via the neuroendocrine system and several candidate genes have been identified. The melanocortin system is important in the regulation of energy balance (Benoit *et al*., 2000; Fan *et al*., 1997). In particular, a functional MC4R has been shown to be necessary to prevent adiposity (Barsh *et al*., 2000).Transgenic knockout mice, lacking MC4R, show maturity-onset obesity, hyperphagia, hyperglycemia, increased linear growth (Huszar *et al*., 1997) and hyperinsulimenia(Farooqi *et al*., 2003) while heterozygous MC4R knockout mice show intermediate obesity, their average weight being between the homozygous knockouts and wild type mice, suggesting the importance of quality of receptors for weight regulation (Albuquerque *et al*., 2014). Moreover, studies conducted in both rodent and human subjects suggest more tendencies of females to gain weight or have a higher BMI than males with identical deletions or mutations (Huszar *et al*., 1997; Sina *et al*., 1999). Possible association of MC4R polymorphism with several obesity-related traits has been found in a sample of Portuguese children (Albuquerque *et al*., 2014).The prevalence of type 2diabetes and impaired glucose tolerance among humans with a defective MC4R gene are very similar to those in obese subjects (Vaisse *et al*., 2000). Farooqi *et al*. (2000) (Farooqi *et al*., 2000) reported normal fasting plasma glucose levels despite hyperinsulinemia in a group of patients with a MC4R mutation. The MC4R knockout mouse also displayshyperinsulinemia, but not diabetes (Huszar *et al*., 1997).

Mutations in MC4R gene are considered to be the most common form of monogenic obesity in humans (Farooqi *et al*., 2000; Farooqi *et al*., 2003; Hinney *et al*., 1999; Potoczna *et al*., 2004; Sine *et al*., 1999; Vaisse *et al*., 2000). Common obesity is thought to be a polygenic disorder (Korner *et al*., 2003)] with no firm evidence implicating MC4R loci to date (O'Rahilly *et al*., 2004). Five melanocortin receptor subtypes with different expression patterns in the brain and elsewhere have been cloned (Mountjoy *et al*., 1992). Most variants of the gene are found at very low frequencies and have only been identified in obese patients (Carroll *et al*., 2005) although a few variants have been seen with similar frequencies in patients and controls (Dempfle *et al*., 2004). Only two studies have been carried out to find variation outside the coding region of the gene. Possibility arises that polymorphic variations in the regulatory regions of the MC4R gene may contribute to the risk of developing obesity in the general population (Sina *et al*., 1999; Lubrano-Berthelier *et al*., 2003; O'Rahilly *et al*., 2004).

Ours is the first study to show an association between MC4R gene variation with measures of adiposity and diabetes in ethnic Kashmiri population. We screened the entire MC4R coding region in 420subjects (200 obese and 220 controls), from Kashmir valley identified one heterozygous novel missense variation (Glu42Lys). This variation was found in obese with non diabetics, obese with diabetics and healthy controls at a frequency of 10%, 26% and 1.81% respectively (Table 2).

Several misssense mutations in different transmembrane domains have been described. Furthermore, the data regarding intracellular transmembrane mutations are limited to the substitution of the asparagines at codon 62 with a serine, found to produce severe obesity exclusively in the homozygous state in five children from a

consanguineous pedigree (Farooqi *et al*., 2000). Yeo *et al*. (1998) and Vaisse *et al*. (1998) reported first MC4R mutation in humans in 1998. Later Gu *et al*. (1999) reported two missense mutations in 140 obese subjects investigated and Hinney *et al*. (1997) reported nine missense mutations and two nonsense mutations in 306 obese children. Recently Farooqi*et al*. (2000) reported six novel missense mutations while Vaisse*et al*. (2000) reported 8 novel mutations. Also the former (Farooqi *et al*., 2000) reported one homozygous mutation and the rest were heterozygous.

Here it was observed that the frequency of G allele at codon 42 was higher in both obese and obese with diabetic groups than in control group. People who have GG/GA genotypes at codon 42 may be at greater risk for developing diabetes and obesity (Table 2). Therefore, it could be concluded that MC4Rcodon 42 polymorphism is associated with both obesity and diabetes and is a candidate genetic marker of the diseases.

SNP presented here situated in the coding sequence may have a significant bearing on the biologic function of MC4R as predicted by in silico analysis (Figure 2). Predicted energy values for variant (-14290.602kJ/mol) exhibit a clear difference with the values obtained for wild-type protein (-14498.063kJ/mol) (Table 3). This may be construed as a possible influence of glutamic acid-lysine transition to alter physicochemical properties of the protein with compromised function. Further studies are however, needed to rule out the actual affect of these variations on protein structure and function.

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Conflict of interest

None to declare.

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