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Research Article

Identification of *MC4R* and *VCP* Genetic Variants in Two Pakistani Families Showing Symptoms of Diabetes, Hyperphagia, Seizures, and Obesity

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Abstract

Variations in the melanocortin 4 receptor (*MC4R*) are most commonly associated with serious early-stage monogenic obesity. The valosin-containing protein (*VCP*) gene, also referred to as p97, produces the ubiquitous, crucial, and multifunctional protein VCP, involved in a wide range of cellular processes, including endoplasmic reticulum-associated degradation (ERAD), degradation of lysosomal protein, and degradation of the proteasome-mediated protein. In the present study, we investigated two Pakistani families enrolled from Sibi, Pakistan, for variation in *MC4R* and *VCP* genes. Clinical symptoms involved obesity, hyperphagia, and diabetes in Family 1. Obesity with autism spectrum disorder (ASD), hyperphagia, diabetes, seizure, gastrointestinal, and sleep disorders were found in Family 2. Additionally, blood samples were collected and DNA extraction was performed. Subsequent molecular analysis was conducted to identify mutations. Clinical and genetic results were analyzed, and the segregation of *MC4R* and *VCP* gene variants in the families helped to make the diagnosis of the disease. For the identification of variations, we conducted whole exome sequencing (WES) and confirmed the findings through target sequencing. We divulged two previously reported heterozygous missense variations. Notably, WES revealed variants (c.307G>A, rs2229616) in the *MC4R* and (c.1360-35A>G, rs2258240) in the *VCP* genes in both families separately. The chromatogram illustrated homozygous unaffected siblings and parents, as well as heterozygous individuals with the disease. Both families segregate with the obesity disorder in an autosomal dominant manner. In Family 1, the change from 'G' to 'A' in the *MC4R* gene depicts the mutant allele 'A' segregating dominantly from one generation to another. Similarly, the change from 'A' to 'G' in the *VCP* gene illustrates the mutant allele 'G' segregating dominantly in Family 2.

Keywords: *MC4R*, *VCP*, genetic polymorphisms, diabetes, obesity, whole exome sequencing (WES).

1. Introduction

Chromosome 18q21.3 is the location of the melanocortin 4 receptor (*MC4R*) gene. The gene produces a 332-amino acid protein that plays a critical role in controlling body weight, food intake, and energy balance at the hypothalamus level (Oswal and Yeo 2007). *MC4R* is mostly found in the brain and is a G protein-coupled receptor. The most common kind of monogenic obesity, now recognized, is caused by variations in the *MC4R* gene. Although the incidence varies, it is reported that up to 6% of obese people have *MC4R* mutations. Notably, younger generations exhibit a higher penetrance of *MC4R*-related obesity, which results in heterozygous carriers developing severe obesity

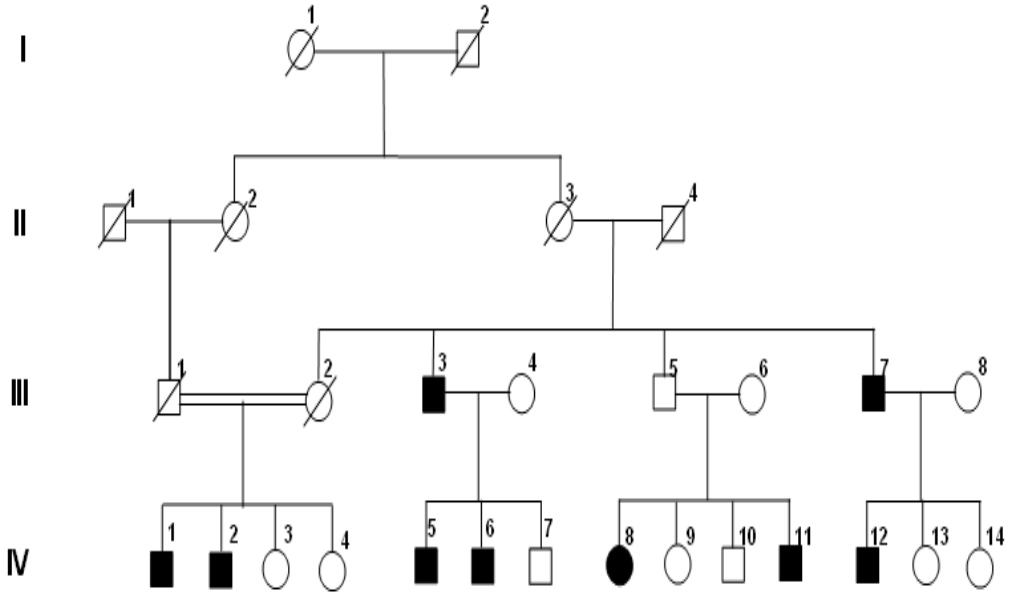

Figure 1: (A) Family 1 pedigree of obese patients penetrates in an autosomal dominant manner.

at an early age. However, to date, no systematic investigation has evaluated the incidence of *MC4R* and *LEP* variations in the Pakistani population. Additionally, whether *MC4R* is the more prevalent cause of monogenic obesity in this region of the world remains unclear (Saeed et al. 2012). Inheritance of *MC4R* gene variants typically follows a dominant pattern, with the majority presenting as heterozygous variants. However, both homozygous and heterozygous mutations have been reported. Moreover, *MC4R* mutations often result in the absent or diminished activity of *MC4R*. Over 150 missense variants have been documented so far(Doulla et al. 2014).

The valosin-containing protein (*VCP*) gene, also referred to as p97, is located on the 9p13.3-p12 chromosome. It produces the ubiquitous, crucial, and multifunctional protein VCP, which has shown significant evolutionary conservation (Papadimas et al. 2017, Lyupina et al. 2018). The VCP protein consists of four functional domains: an N-terminal ubiquitin-binding domain, D1 and D2 (2 ATPase domains), L1 and L2 (2 linker domains), and a C-terminal domain (van den Boom and Meyer 2018). VCP protein is a member of superfamily AAA-ATPase, a collection of chaperone-like proteins that are involved in a variety of cellular functions, like fusion of membranes, repair of nuclear envelope, reassembling post-mitotic Golgi, DNA damage responses, suppressing apoptosis, and ubiquitin-dependent degradation of proteins (Meyer and Weihl 2014). A total of 45 *VCP* gene mutations have been discovered so far (Saracino et al. 2018).

In the current study, we conducted investigations to identify *MC4R* and *VCP* genetic variations by screening two Pakistan families, belonging to the Sibi district in the Western region of Pakistan. These families exhibited symptoms of diabetes, hyperphagia, seizures, and obesity.

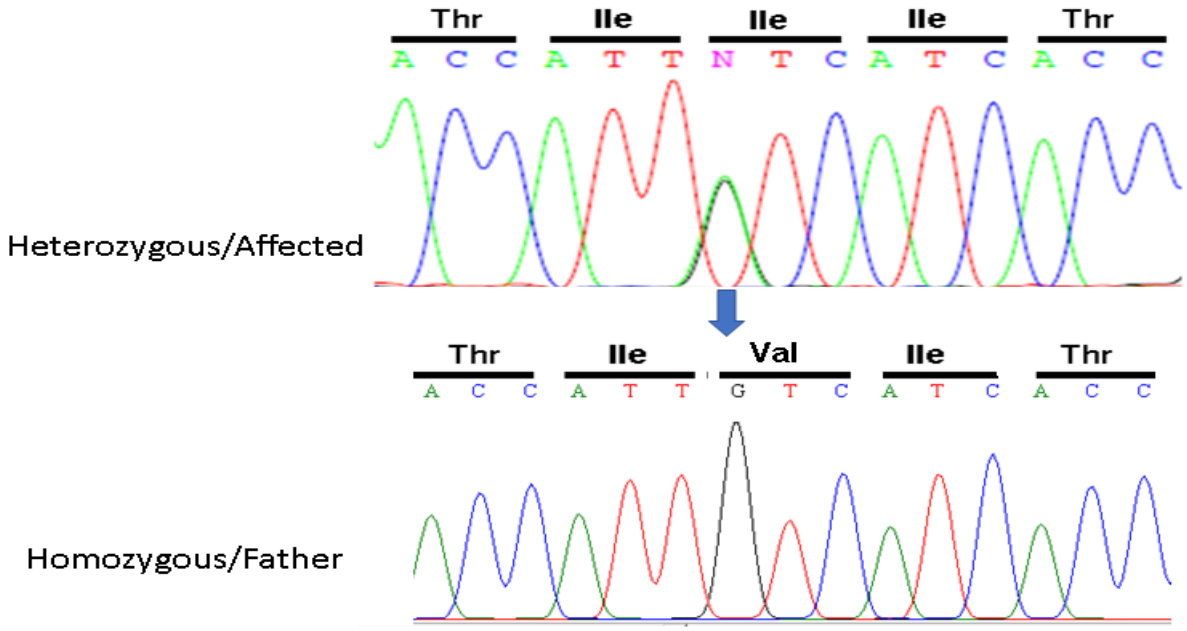

Figure 1: (B) Sequence analysis chromatogram shows heterozygous variant (c.307G>A) in *MC4R*.

2. Materials and Methods

2.1. Patients, Consents, and Ethical Approval

Two Pakistani families were enrolled from the city of Sibi, Pakistan (*Fig 1a & 2a*). Family 1 comprised nine affected individuals; seven affected and eight normal individuals were available at the time of blood sample collection. Family 2 comprised four affected individuals, and blood was collected from four affected and four normal individuals. Both families were visited, and clinical information was obtained by a questionnaire. The Institutional Review Board (IRB) at the Department of Biotechnology, Baluchistan University of Information Technology, Engineering & Management Sciences, Quetta, Pakistan, approved the study. Additionally, informed consent for genetic evaluations was taken from all affected patients and/or their guardians. Clinical information included, the age at which obesity first manifested (in years), any other significant chronic diseases, metabolic disorders that run in the family, dietary and exercise habits, and co-morbidities associated with obesity. Clinical phenotypes investigated in Family 1 involved obesity, hyperphagia, and diabetes; whereas, Family 2 presented clinical symptoms that included obesity with autism spectrum disorder (ASD), hyperphagia, diabetes, seizure, gastrointestinal, and sleep disorder.

2.2. Extraction of DNA

Genomic DNA was extracted from the patients' blood samples by using an inorganic genomic DNA extraction method (salting out and proteinase K treatment). Cell lysis was performed by using ethylene diamine tetraacetic acid (EDTA), TrisCl, and sodium dodecyl sulfate (nonionic detergent). Followed by centrifugation, used to remove cellular debris. Proteins were denatured by proteinase K treatment and were precipitated from the nucleic acid solution by using isopropanol. Subsequently, denatured proteins

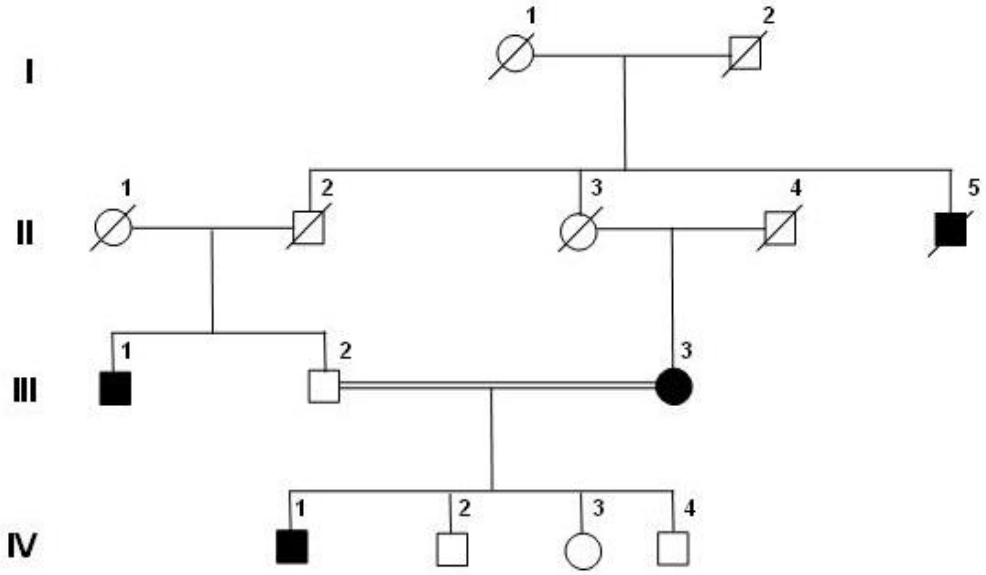

Figure 2: (A) Family 2 pedigree of obese patients penetrates in an autosomal dominant manner.

were eliminated through centrifugation and ethanol-washing steps. Afterward, DNA was concentrated by precipitation. The supernatant was removed and DNA was re-dissolved in double-distilled water or TE buffer.

2.3. Whole Exome Sequencing

Whole exome sequencing (WES) was carried out according to the manufacturer's instructions, SureSelect Exome target enrichment technology was used to enrich the DNA of both patients and normal individuals of both families. On a Genome Analyzer IIx (Illumina, CA), the enriched DNA was subjected to paired-end sequencing. Using the Genome Analysis Toolkit, sequence alignment and variation calling were carried out against the reference human genome (UCSC hg 18). Reads corresponding to the exon regions were gathered for further analysis and mutation detection. SNPs, insertion, and deletion data were collected and compared to the available Human Gene Mutation Database (HGMD) data.

2.4. Sanger Sequencing

To confirm the existence of variations in the other affected members of the families, Sanger sequencing was done using specific primers. Exons along with the introns of both genes were sequenced using an ABI 3730xl analyzer and Big-Dye Terminator v3.1 ABI, sequencer software version 4.2 was used for the analysis.

3. Results

In the current study, two heterozygous variants (SNPs) in *MC4R* and *VCP* genes were detected in patients of both families separately, penetrating in an autosomal dominant manner. A variant in the *MC4R* gene's coding exon identified is [c.307G>A p. Val 103 Ile, rs2229616] and intronic SNP [c.1360-35A>G, rs2258240] in the *VCP* gene, both have been reported previously. Clinical symptoms in both families were characterized by obesity, density of bone minerals, accelerated linear growth in the primary year of life, hyperphagia, and hypertension. Sequence analysis revealed the presence of mutant alleles, which were also identified in affected siblings

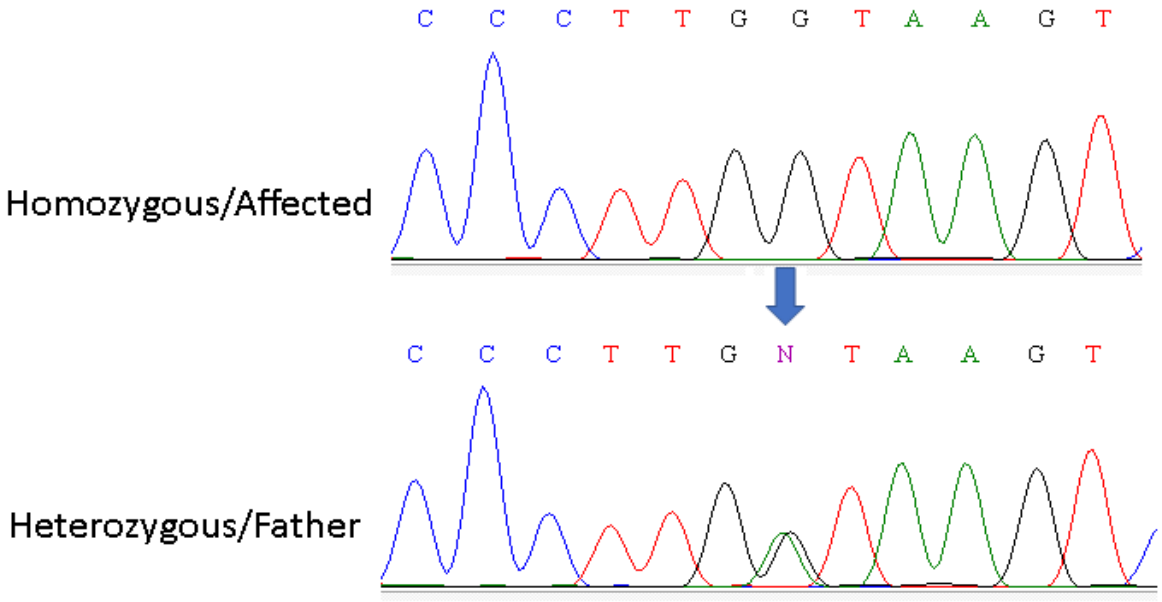

Figure 2: (B) Sequence analysis chromatogram shows heterozygous intronic SNP (c.1360-35A>G) in *VCP*.

and parents. The chromatograms show heterozygous affected patients, as well as homozygous unaffected siblings and parents (*Fig 1b & 2b*). Both families segregate with the obesity disorder in an autosomal dominant manner. Mutant alleles segregating from one generation to another may cause the disease phenotype, revealing a dominant genotype-phenotype correlation. We found that 'G' changes into 'A' in the gene *MC4R* depicting mutant allele 'A' segregates dominantly from one generation to another in family 1. Similarly, 'A' changes into 'G' in the gene *VCP* shows mutant allele 'G' segregates dominantly in family 2.

4. Discussion

MC4R is one of the crucial *MCRs* in the melanocortin pathway. Obesity is associated with a frame-shift mutation in the *MC4R* gene, first discovered in 1998. SNPs around this gene have been linked to a high risk of severe early-onset obesity in GWAS studies. Obesity has been linked to SNP (rs1778231) and SNP (rs129070134) in Europeans and Indian Asians (Singh, Kumar, and Mahalingam 2017). *MC4R* mutations are more severe in homozygous obese patients than in heterozygous obese patients. Val103Ile (rs2229616) is a frequent polymorphism linked to abdominal obesity, with a minor allele frequency of 2–4% in heterozygotes (Val103Ile) versus homozygotes (Val103Val) (Rosmond et al. 2001). We reported two missense variations in *MC4R* and *VCP* genes, previously reported in the Human Gene Mutation Database (HGMD), gnomAD, and ClinVar.

In one related study, (Rana, Rahmani, and Mirza 2018), enrolled 606 human participants of both sexes and ages ranging from 12 to 62. They showed an association of the *MC4R* variation rs17782313 with obesity. Several metabolic, anthropometric, behavioral, and physical characteristics associated with obesity were also examined in the Pakistani population in this study. The risk 'C' allele for this SNP in the

MC4R gene is estimated to have a minor allele frequency (MAF) of 42.1%. Likewise, the frequency of genotypes was wild-type TT (34%), homozygous carrier CC (18.2%), and heterozygous TC (47.8%). For overweight/obese the MAF for the 'C' risk allele was 43.8% and for normal-weight participants was 40.3%. They found that when comparing normal weight and overweight/obese participants, there was no discernible variation in the allelic and genotypic frequencies of the *MC4R* variant (rs1778313). However, when stratifying the entire sample group by gender, it was discovered that overweight/obese females had considerably higher frequencies of the *MC4R* variant (rs1778313) than normal-weight females.

However, the MAF of the *MC4R* variant (rs1778313) reported in this study (42%) was significantly greater when compared to other populations i.e., East Asian(Shi et al. 2010), Indian (Taylor et al. 2011), European (Lauria et al. 2016), and American (Liu et al. 2011), but similar to that of the Iranian population (Khalilitehrani et al. 2015). The variation rs17782313 (T > C) located at 188 kb downstream of the *MC4R* gene had the second greatest correlation with BMI (Rana, Rahmani, and Mirza 2018).

In another investigation, 62 children with extreme obesity were screened. Ten (16.1%) of the 62 probands with early-stage severe obesity had homozygous *LEP* variants, while two (3.2%) participants had homozygous *MC4R* variants (I316S and M161T), which were linked to extreme obesity, hyperleptinemia, hyperphagia, and hyperinsulinemia. These children were all the offspring of first-cousin couplings(Saeed et al. 2012). In another case, two heterozygous coding variants in the *MCR4* gene, C293R and S94N, were found. The p.S94N variant has been linked to the obese phenotype reported previously, but the p.C293R mutation has not yet been published. Loss of *MC4R* function has been linked to the variation p.S94N (Doulla et al. 2014).

VCP is involved in a wide range of cellular processes, including ERAD, degradation of lysosomal protein, and proteasome-mediated protein (Ide et al. 2019). Additionally, ER stress is widely established to play a role in metabolic disorders including obesity, and diabetes mellitus (Wang et al. 1999, Harding et al. 2001, Ozcan et al. 2009). Using the CRISPR/Cas9 technique, A232E *VCP* knock-in mice have already been produced (Shibuya et al. 2022). One of the human *VCP* pathogenic variants that has been demonstrated to induce multisystem proteinopathy is A232E (Manno et al. 2010). Since newborn homozygous A232E (A232E/A232E) mice passed away shortly after delivery, heterozygous A232E (A232E/+) mice were used for analysis. Mice on a high-fat diet had a reduced incidence of diet-induced fatty liver; however, A232E/+ mice had an increased intra-abdominal fat mass (Shibuya et al. 2022).

Previous studies showed that *VCP* is associated with obesity in syndromic forms. (Shmara et al. 2023) identified variant (R159H) in the *VCP* gene in five Hispanic families. Among them, a 27-year-old girl from one family was suffering from a syndrome characterized by frontotemporal dementia, myopathy, and obesity. In another study, a genetic analysis revealed loss-of-function mutations in 20 genes, including the *VCP* variant (dnLGD) that described children with autism spectrum disorders who were highly obese and had low IQ scores. In that study, the investigators detected genetic lesions and attempted to find perturbed biochemical processes in a group of ASD children with obesity(Cortes and Wevrick 2018).

Numerous issues, such as seizures, sleep difficulties, obesity and metabolic diseases, hormonal dysfunctions, and nutritional deficiencies have been linked to a greater number of adolescents suffering from ASD

(Bauman 2010). Similarly, another study showed adults with autism were much more likely to have nearly all clinical symptoms, including obesity, diabetes, seizure, immunological disorders, gastrointestinal and sleep disorders, dyslipidemia, and hypertension (Croen et al. 2015). Several other studies also showed obesity with ASD (Curtin, Jojic, and Bandini 2014, Ho, Eaves, and Peabody 1997, Pashankar and Loening-Baucke 2005).

5. Conclusions

In the current study, two previously reported variants of *MC4R* and *VCP* were detected in patients with diabetes, hyperphagia, seizures, and obesity. Our research demonstrated the value of genetic testing in identifying individuals who may benefit from molecular diagnosis, genetic counseling, and specialized medical care. Genes and variants for complex disorders sequenced through WES and/or Sanger will make it easier to identify precise genotype-phenotype associations and provide genetic counseling to families with affected members.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

There was no separate funding available for this project.

Ethics Approval

Yes. The study was approved by the Institutional Review Board & Ethics Committee Baluchistan University of Information Technology, Engineering & Management Sciences, Quetta, Pakistan

Consent Forms

Yes. Consent forms were obtained from the participating patients.

Data Availability

All the data relevant to this manuscript is available with the authors.

Authors Contribution

SN and SD were involved in the conceptualization of the study, FKB, and MUH, did sample collection and experimentations, HT, and SM performed the data analysis, and AA, MT, and SD wrote the manuscript. All authors read and approved the final version of the manuscript.

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