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MOLECULAR ASSOCIATION OF MC4R AND PCSK-1 WITH OBESITY IN PAKISTANI COHORT

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Obesity; MC4R; PCSK-1; Genetic Screening; Monogenic Obesity; Consanguinity; Pakistani Population; Frameshift Mutation

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Abstract

Obesity is multifactorial disease with a high heritability, in which monogenic forms although uncommon provide very important information on energy homoeostasis pathways. Such monogenic forms may increase in consanguineous population such as that in Pakistan. The purpose of this study was to examine the role of pathogenic variants of two important genes of the leptin- melanocortin pathway, namely "Melanocortin-4 Receptor (MC4R) and Proprotein Convertase Subtilisin/Kexin Type 1 (PCSK-1) in severe obesity among Pakistani adults.

We performed a candidate gene screening in a well phenotyped cohort of ten unrelated Pakistani patients with severe obesity (Body Mass Index ≥ 30kg/m^2). The genomic DNA was isolated from the peripheral blood and targeted exons of MC4R and PCSK-1 were amplified using polymerase chain reaction (PCR) and bidirectional Sanger Sequencing. To identify the sequence variants, alignment with reference genome and validation with against public databases (dbSNP,ClinVar, gnomAD, COSMIC) was performed.

Interestingly, no pathogenic or probably pathogenic mutations were found on the PCSK-1 gene. The sequencing of MC4R gene on the other hand identified putative disease associated variants in 4 out of 10 subjects (40%). These were two frameshift deletions (one novel, c.498delC and one known pathogenic, c.431delT) and one missense variant (c.772T>G; p.Trp258Gly) in COSMIC and one synonymous variant (c.717C>G).

Our results show MC4R as the key determining factor of severe obesity in current Pakistani cohort, which its consistent contribution in appetite regulation. The lack of PCSK-1 variant indicate that it could be less chronic monogenic factor in this particular cohort. The study highlights the paramount role of genetic screening of MC4R in the clinical assessment of extreme obesity in consanguineous groups. It provides the basis of correct diagnosis, genetic counseling and provides the door to individualized treatment plans with specific therapies to correct malfunctioning melanocortin pathways becoming accessible.

INTRODUCTION

Obesity is described as a chronic, multifactorial disorder identified by the excessive adipose tissue accumulation and is linked with the increased morbidity and mortality worldwide (Kopelman, 2000; Ng et al., 2014). It significantly increases the risk of type-2 diabetes mellitus, cardiovascular diseases, hypertension, dyslipidemia and

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certain cancers, thereby effecting the global healthcare system (Hruby & Hu, 2015; Blüher, 2019). The etiology of obesity is based upon a complex interplay between the environmental factors, lifestyle behaviors and genetic susceptibility with the genetic factors contributing significantly to individual differences in body weight regulation.

Heritability counts derived from twin, adoption and family studies suggests that genetic factors consist of about 40 – 70% of the variance in the body mass index (BMI), underscoring the strong genetic part of obesity (Herskind et al., 1996; Maes et al., 1997; Locke et al., 2015). Advances in molecular genetics have facilitated the identification of many loci linked with obesity through genome-wide association studies (GWAS); however, these loci explain only a relatively moderate proportion of BMI variability. Highlighting the significance of rare monogenic variants in severe obesity phenotypes (Yengo et al., 2018; Loos & Yeo, 2022).

In the regulation of appetite, hypothalamic melanocortin pathway central role. It is also involved in the satiety and energy expenditure. With in this pathway, the melanocortin-4 receptor is the most important regulator of the food intake by mediating anorexigenic signals downstream of proopiomelanocortin (POMC) derived peptides (Cone, 2005; Farooqi & O'Rahilly, 2006). MC4R is a G protein coupled receptor which is predominantly expressed in hypothalamic nuclei associated with energy homeostasis. It also includes paraventricular nucleus. Disruption in the signaling of MC4R due to loss of function mutation causes hyperphagia, reduced energy expenditure and rapid weight gain (Vaisee et al., 1998; Huszar et al., 1997).

The most common cause identified to the date is MC4R mutations, which have been reported both in pediatric and adult population across the diverse ethnic backgrounds (Lubrano et al., 2003; Stutzmann et al., 2007; Yu et al., 2020). These mutations involve missense, nonsense, frameshift and synonymous variants, each applying different effects on receptor function, intracellular signaling and ligand binding affinity (Hinney et al., 2013; Tao, 2010). The phenotypic expression of MC4R considerably vary, depends upon the nature of the variant, genetic background and environmental influences.

Another important component of melanocortin pathway is protein convertase subtilisin/kexin type 1 (PCSK1), which encodes for the enzyme prohormone convertase 1/3 (PC1/3). PC1/3 has role in the post-translational processing of the several key hormones and neuropeptides i.e., POMC, insulin, glucagon and ghrelin all of which has very important role in the metabolic regulation and appetite control (Steiner, 1998; seidah & Prat, 2012). The deficiency or dysfunction of PC1/3 causes impaired hormone maturation which leads to severe onset of obesity including hypoglycemia and abnormalities in endocrine (Jackson et al., & Löffler et al., 2016).

Pakistan has particularly limited data for the genetic research on obesity as compared to European and East Asian populations. A rapid nutritional transition is undergoing in Pakistan which is identified by caloric intake, reduced physical activity and rising in the prevalence across all age groups (Nishtar et al., 2014; Rana et al., 2018). Given the genetic diversity and unique population structure of Pakistan, population specific studies are important to identify rare and potentially novel variants that are suspected to contribute to obesity.

The context of recent study is to identify the association of MC4R and PCSK1 gene variants with the obesity in Pakistani cohort. Using the polymerase chain reaction (PCR) amplification followed by Sanger sequencing in which the selected exonic regions of MC4R and PCSK1 were confirmed through sequence alignment and cross referenced with publicly available genomic databases. This study provides the insight onto the molecular spectrum of MC4R and PCSK1 variants in obese individuals in Pakistan and have contribution in better understanding of the population specific factors involved in obesity.

1. Materials and Methods

1.1. Study design and Participant selection

The research was started as initial step to discover the genetic links to obesity in the people of Pakistani cohort. Specifically, we

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wanted to identify closely the two genes MC4R and PCSK1, which are often associated with weight regulation. Our main purpose was to identify for any small changes or variations in these individuals living with obesity, rather than studying genetic patterns across the wider population.

Before starting the research, we made sure that all the methods are ethically approved. The research was fully approved by the ethical committee of the institution. Every person who took part was volunteer and we discussed the study with them in detail before the written consent. The privacy was out top priority after the collection of their blood samples, each sample was labeled with a unique code, so that the information of the participant remains confidential.

1.2. Study Population

We worked with a group of ten unrelated individuals, all were adults of Pakistani background, who were living with obesity. For our study, the standard definition of obesity by the World Health Organization has been used, which means each person who has body mass index (BMI) of 30 or higher (BMI ≥ 30 Kg/m^2). We also made sure that none of the participants had any other syndrome or chromosomal disorder linked to obesity so that we can focus on more common genetic factors. Detailed anthropometric data which include age, weight and BMI were recorded (Table 1.1)

Table 1.1 Anthropometric data of the study subjects

Subject	Age	Weight (Kg)	BMI (Kg/m^2)
X1	40	93	32.6
X2	32	98	33.7
X3	53	96	31.4
X4	28	95	36.5
X5	36	100	30.8
X6	39	106	36.5
X7	24	98	31.2
X8	29	95	31.7
X9	20	101	32.3
X10	33	105	32.8

Sample Storage

For further DNA analysis, the blood samples were collected from each participant. The process was straightforward where small amount of blood 3 to 5 milliliters was drawn from a vein in the arm. The blood was collected in EDTA tubes (BD Vacutainer®, Becton, Dickinson and Company, USA) that contained anticoagulant to keep samples from clotting. To ensure the privacy of participants, each tube was immediately labeled with a code instead of a name. Right after the collection, the samples were placed under the cool temperature (4°C) in the container and transferred to lab for further processing. The DNA extraction was performed with in the next 24 hours

1.3. DNA Extraction and Quantification

For DNA extraction 200µl of the blood was taken from the whole blood sample and transferred to sterile microcentrifuge tube. After that, 20µl of proteinase K was added which was followed by the immediate addition of the lysis buffer. This mixture was thoroughly vortexed and then incubated at 56°C for 10 minutes. After incubation, 200 µl of ethanol (96-100%) was added and the entire lysate was transferred to the silica spin column. The columns were then subjected to centrifugation and subsequently washed in the order using AW1 and AW2 wash buffers. Finally, the purified DNA was eluted from the column in 100µl of nuclease free water.

The quality and quantity of the extracted DNA was assessed by using 1.0% agarose gel electrophoresis stained with ethidium bromide

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and then visualized by using Grab IT software and a gel documented gear (UV trans-illuminator) to visualize the gel slab.

1.4. Candidate gene selection and primer designing

The MC4R and PCSK1 genes were selected as candidate genes due to their well-established roles associated with monogenic obesity. Specific exons of these genes which were previously reported to harbor mutations in obese populations were targeted for sequencing. Primer 3 online software (http://primer3.ut.ee/) and the specificity of the genes was confirmed *in silico* by using UCSC Genome Browser's BLAST and In-silico PCR tools. The primers were synthesized by Invitrogen, USA. (Table 1.2)

Table 1.2 Primer sequencing designed for PCR amplification and Sequencing

Primer Name	Sequence (5'→3')	Target Gene
F_PCSK-1a	ATGAAGGTCTCCTGTTCCGGGA	PCSK-1
R_PCSK-1a	GGTTCTACACATCACAGGGAGT	PCSK-1
F_PCSK-1b	TCATGTGGGTCTGTGTAAGCA	PCSK-1
R_PCSK-1b	AGAGTATTGGGCTTTTGTCACTGT	PCSK-1
MC4R-1a	CCCAACCCGCTTAACTGTCA	MC4R
MC4R-1b	CTCCCTGACCCAGGAGGTTA	MC4R

1.5. PCR amplification and Electrophoresis

Polymerase Chain Reaction (PCR) was performed in 10µl reaction volume which contained 2.2µl genomic DNA, 0.4µl of each forward and reverse primer (10µM), 0.4µl of Green Taq master Mix and 3.0µl of nuclease-free water. The amplification was carried out in a thermal cycler BIO RAD T100™ under the below conditions: Initial denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 94°C for 60 seconds, annealing at 55.5°C for 60 seconds and extension at 72°C for 2 minutes which is then followed by the final extension at 72°C for 10 minutes. To confirm the successful amplification the amplified PCR products were resolved on 1.5% agarose gel.

1.6. DNA Sequencing and variant analysis

The PCR products were subjected to purification and then to Sanger sequencing in both directions. Same primers were used as for amplification. The resulting chromatograms were analyzed and assembled with the help of BioEdit sequence alignment editor. The reference sequences MC4R (ENSG0000016663) and PCSK-1 (ENSG00000175426) Sequences from Ensembl genome browser (www.ensembl.org) were used against which the sequences were aligned so that the variations are identified. The annotation tool in Ensembl was used to identify the nature and potential functional impact of the identified variants.

2. Results

The main purpose of the study was to identify the genetic variants within the MC4R and PCSK1 genes linked with the obesity susceptibility in Pakistani cohort. A total number of 10 obese individuals (BMI ≥ 30kg/m^2) were studied. Targeted sequencing of the selected exons of PCSK1 and MC4R was performed after which the PCR amplification was done. Genetic analysis showed that there were no pathogenic or likely pathogenic variants in the PCSK1 gene among the participants. Contrarily the analysis of MC4R gene identified distinct genetic alterations in 4 out of 10 subjects (40%). The following section is about the detailed workflow and the specific genetic findings for each of these four individuals (Subject X5, X7, X8 and X9).

2.1. DNA Extraction and Quantification

GF-1 blood DNA extraction kit was used to extract the genomic DNA effectively from whole 10 blood samples. To evaluate the integrity and concentration of the isolated DNA spectrophotometric analysis

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(NanoDrop™) and 1.0% agarose gel electrophoresis were used. The genomic DNA showed the sharp distinct bands which reflects that all the corresponded genomic DNA had high molecular weight DNA with little degradation (Fig.3.1). With an A260/A280 ratio of 1.82 ± 0.05, the average DNA content was found 85.4 ± 12.7ng/L (mean ± SD) showing that for the subsequent PCR amplification the purity is good.

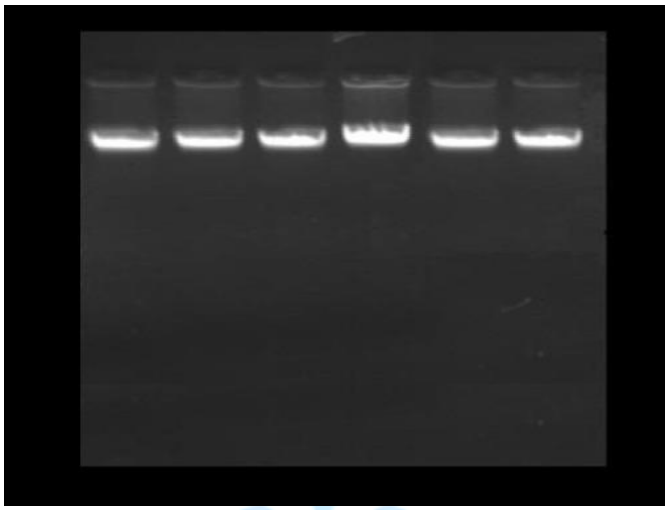

Figure 1 Agarose gel electrophoresis of extracted genomic DNA. Horizontal lanes represent samples from subjects. Horizontal Lane: 1 kb DNA ladder. Clear, high-molecular-weight bands indicate successful DNA extraction with minimal degradation.

2.2. PCR amplification and Product verification

Amplification of PCSK1 and MC4R genes was done using the specific exons as shown below in Table 3.3. The optimization procedure for PCR showed a temperature of 55.5°C to be optimal in all set of primer. Amplification was endured successful by resolution of their product on 2% agarose gels. PCSK1a, PCSK1b, and MC4R amplicons of the appropriate sizes were obtained in all samples: approximately 450bp, 500bp and 600bp (Fig). There was no identification of non-specific amplification or primer-dimer linked to the primers in amplification process and therefore, the specificity of the templates in further Sanger sequencing was ensured.

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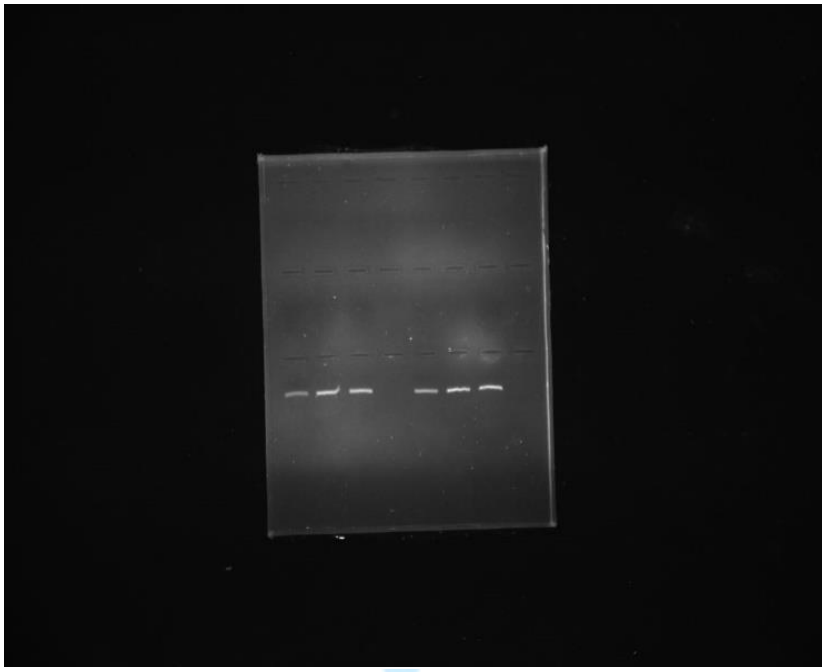

Figure 2 Verification of PCR amplification products. Gel image shows specific amplicons for MC4R and PCSK-1 (representative samples). Lane: 100 bp DNA ladder.

2.3. Sequencing analysis and variant identification

The PCR products were first subjected to purification and then a bidirectional Sanger sequencing was done. The quality of resulting chromatograms (Phred >30) was assessed using the BioEdit software and aligned to respective sequence of MC4R (ENSG00000166603) and PCSK1(ENSG00000175426) based on the ENSEMBL genome browser (GRCh38.p13). The name was given manually to the variants and matched with the public databases such as dbSNP, ClinVar and genomeAD so as to establish their novelty and their clinical importance.

2.3.1. Subject X-9: Frameshift deletion in MC4R

The sequence comparison showed a heterozygous single nucleotide deletion of a thymine (T) at cDNA position 431 (relative to reference NM_005912.3) at the genomic position of chr18:60,371,872(GRCh38). This deletion c.431delT leads to a frameshift mutation which is expected to lead a premature stop codon (p.Leu144CysfsTer6) (Fig 4.3& 4.4). Overlapping peaks (Dual fluorescence) was also observed in the forward and reverse chromatograms below the deletion site, which verified the variants. The presence of this variant (rs121913343) in was confirmed by ENSEMBL browser query, in the literature it has been previously reported to be associated with monogenic obesity (Fig 4.5). The mutation is loss-of-function which is pathogenic (ClinVar accession: VCV000002342).

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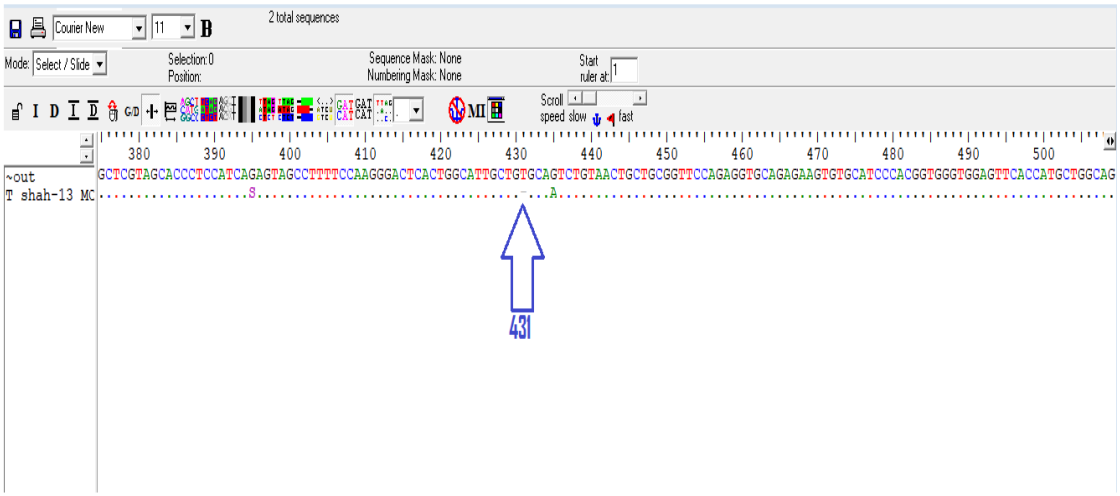

Figure 3 BioEdit alignment for Subject X-9. The arrow indicates the deletion of 'T' (dash) in the patient sequence (top) compared to the reference sequence (bottom) at position 431.

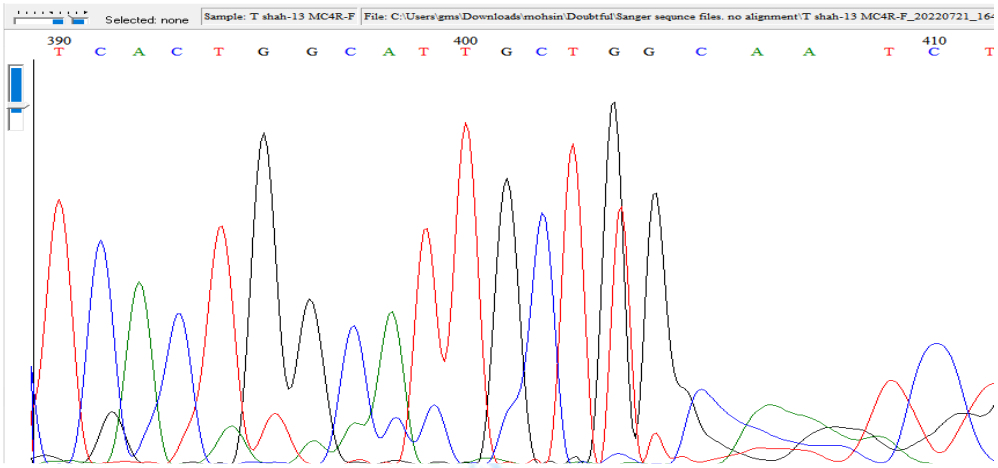

Figure 4 Chromatogram of the c.431delT variant. Overlapping peaks (black 'G' and red 'T') beginning at position 405 in the trace indicate a heterozygous deletion.

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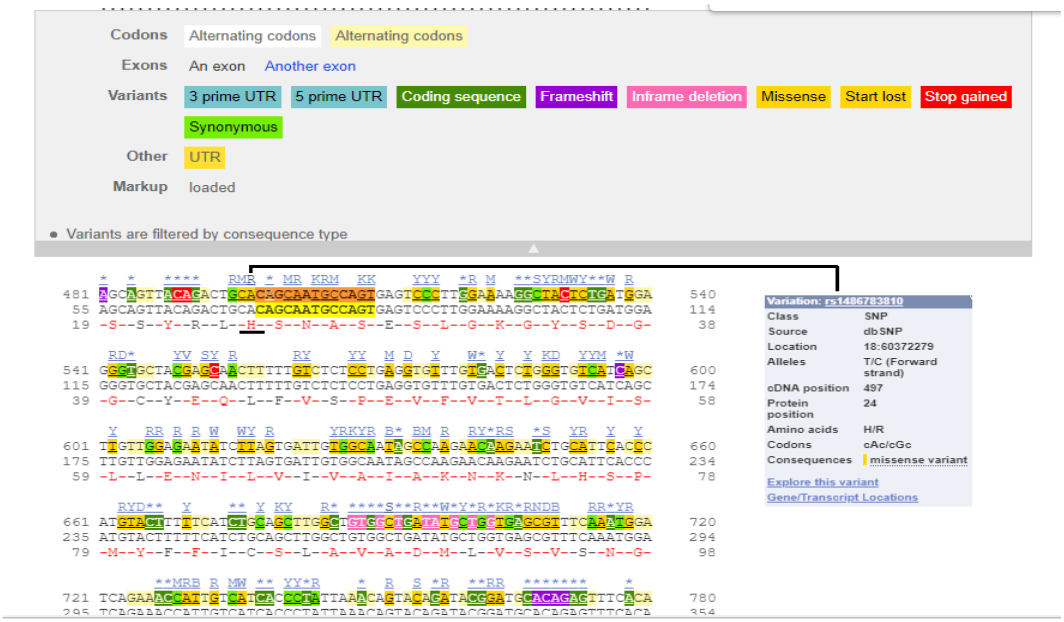

Figure 5 ENSEMBL genome browser report showing the recorded pathogenic variant at the identical genomic position (chr18:60,371,872).

2.3.2. Subject X-5: Synonymous Variant in MC4R

A heterozygous transversion C>G was identified by the analysis at cDNA position 717 (c.717C>G) in the MC4R gene, which is corresponding to thymine to guanine change (T>G) on the genomic sense strand a chr18:60,372,059. This variant leads to synonymous change, p.Val239Val. (Fig. 4.6 & 4.7). The amino acid sequence is not been changed by this variant, in silico analysis by using the tool like RegulomeDB indicates impact on splicing regulatory elements. In dbSNP, variant (rs134447331) is listed with a global minor allele frequency (MAF) of ~0.03 but in obese individual it was found in heterozygous state (Fig. 4.8 & 4.9).

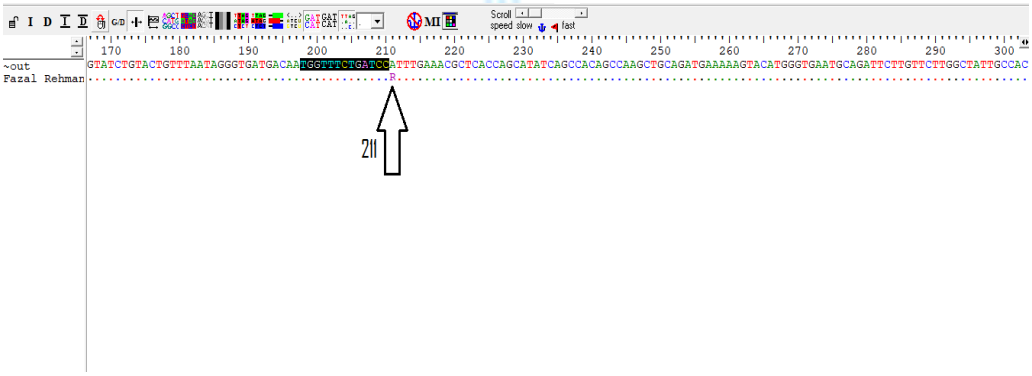

Figure 6 BioEdit alignment for Subject X-5. Arrow shows the nucleotide change from 'A' (reference) to 'G' (patient) at cDNA position 717.

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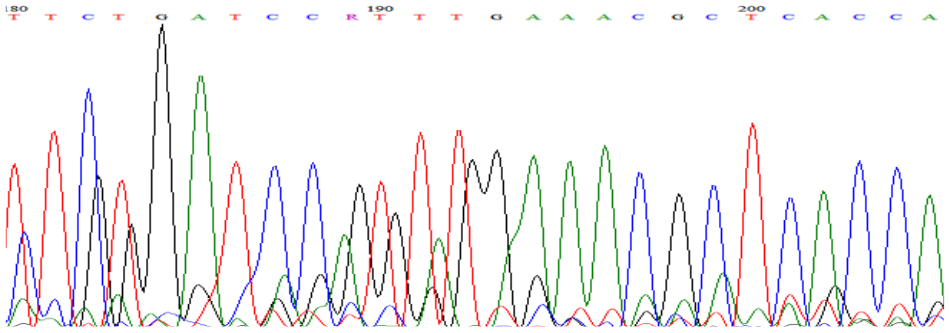

Figure 7 Chromatogram of the c.717C>G variant. Dual peaks (green 'A' and black 'G') at position 189 in the trace confirm heterozygosity.

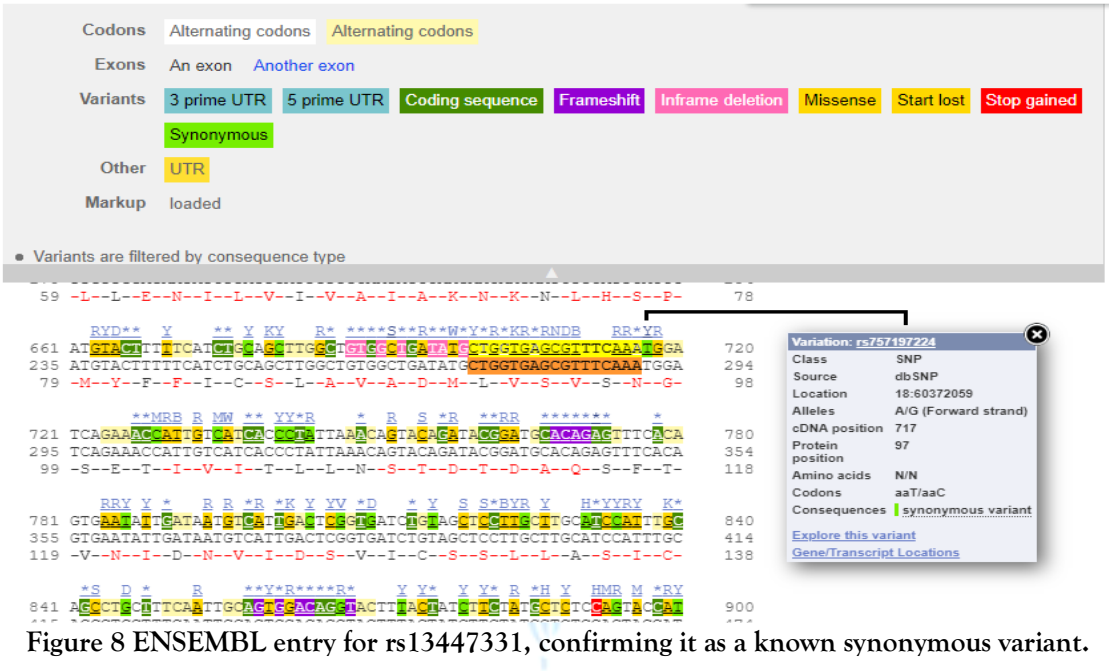

Figure 8 ENSEMBL entry for rs13447331, confirming it as a known synonymous variant.

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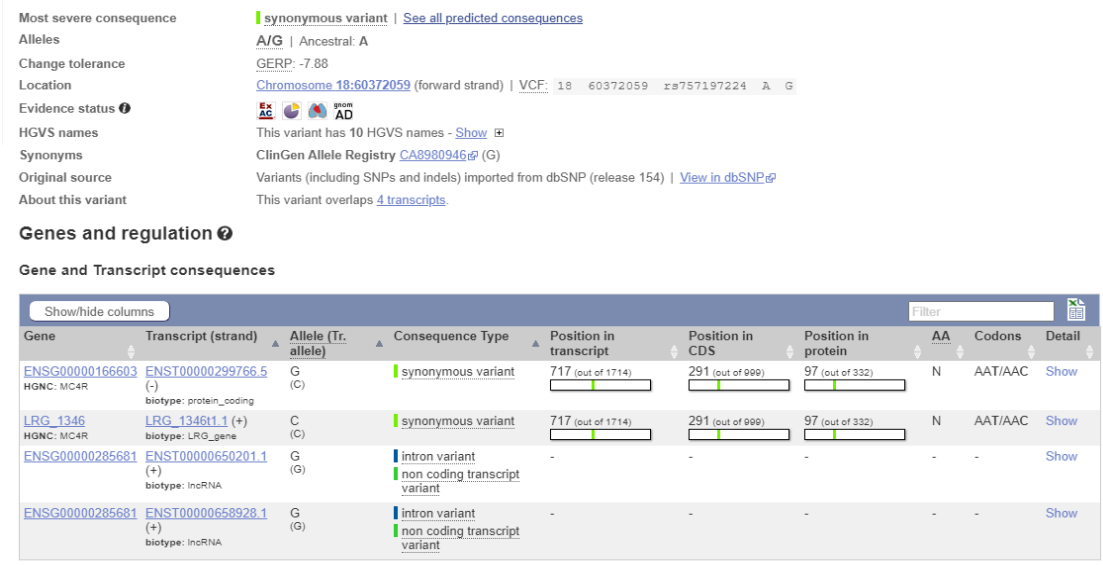

Most severe consequence	synonymous variant | See all predicted consequences
Alleles	A/G | Ancestral: A
Change tolerance	GERP: -7.88
Location	Chromosome 18:60372059 (forward strand) | VCF: 18 60372059 rs757197224 A G
Evidence status	
HGVS names	This variant has 10 HGVS names - Show
Synonyms	ClinGen Allele Registry CA8980946 (G)
Original source	Variants (including SNPs and indels) imported from dbSNP (release 154) | View in dbSNP
About this variant	This variant overlaps 4 transcripts.

Genes and regulation

Gene and Transcript consequences

Show/hide columns — Filter

Gene	Transcript (strand)	Allele (Tr. allele)	Consequence Type	Position in transcript	Position in CDS	Position in protein	AA	Codons	Detail
ENSG00000166603 HGNC: MC4R	ENST00000299766.5 (-) biotype: protein_coding	G (C)	synonymous variant	717 (out of 1714)	291 (out of 999)	97 (out of 332)	N	AAT/AAC	Show
LRG_1346 HGNC: MC4R	LRG_1346t1.1 (+) biotype: LRG_gene	C (C)	synonymous variant	717 (out of 1714)	291 (out of 999)	97 (out of 332)	N	AAT/AAC	Show
ENSG00000285681	ENST00000650201.1 (+) biotype: lncRNA	G (G)	intron variant, non coding transcript variant	-	-	-	-	-	Show
ENSG00000285681	ENST00000658928.1 (+) biotype: lncRNA	G (G)	intron variant, non coding transcript variant	-	-	-	-	-	Show

Figure 9 Variant consequence prediction from ENSEMBL, annotating it as a synonymous variant with a CADD score of 7.88.

2.3.3. Subject X-7: Novel Frameshift Deletion in MC4R

Sequencing identified a new heterozygous deletion of a cytosine ('C') at cDNA position at 498 (c.498delC), which is a genomic position chr18:60,371,941. This frameshift mutation is expected to lead to radical change in amino acid sequence of the protein and premature termination codon (p.Arg166GlyfsTer22) (Fig. 4.10 & 4.11). When the variant was being analyses, it was not present in dbSNP, gnomAD, ClinVar, idnicationg that the observation is novel (Fig. 4.12). It is not found in the population database and its functional impact is predicted to be severe, which indicates it is most likely a pathogenic variant that contribute to the obese phenotype of the individual.

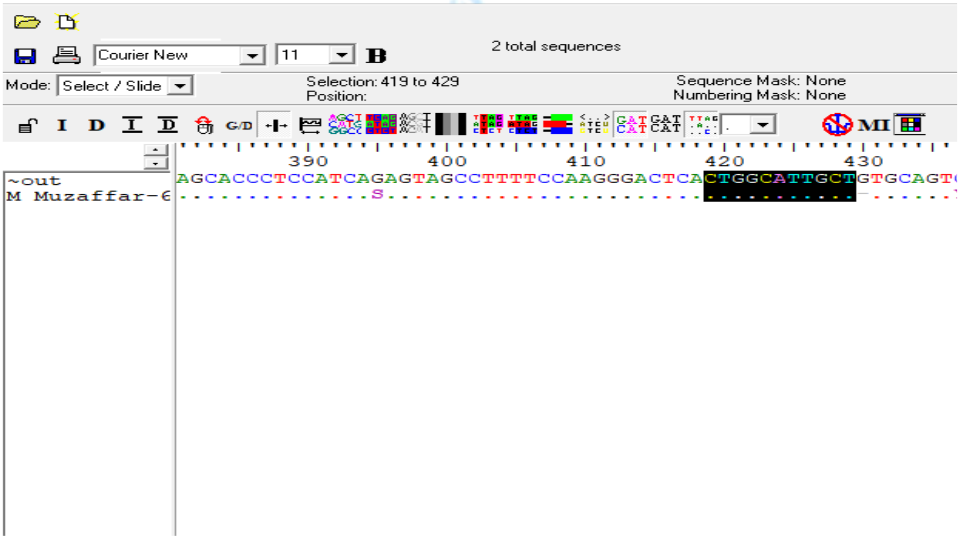

Figure 10 BioEdit alignment for Subject X-7. The arrow indicates the deletion of 'C' in the patient sequence.

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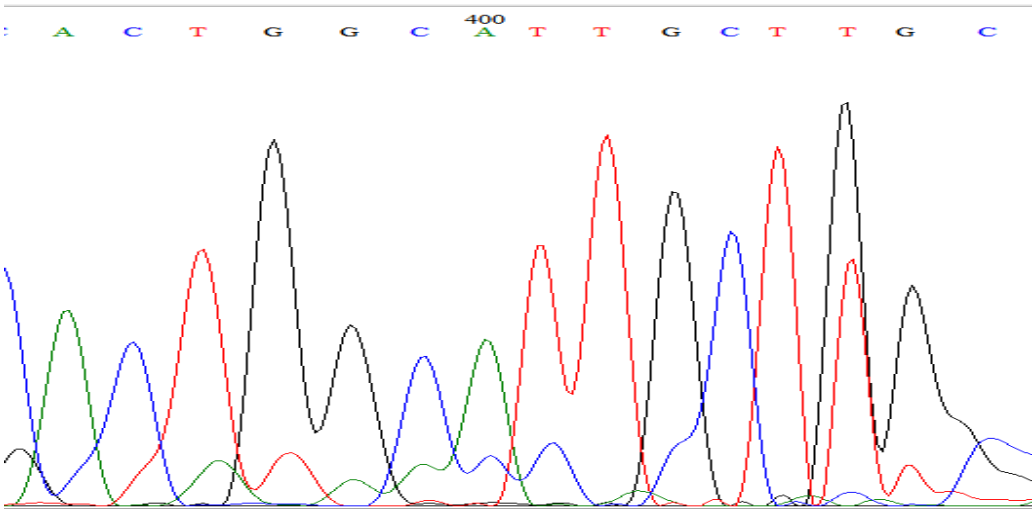

Figure 11 Chromatogram of the novel c.498delC variant. Overlapping peaks following position 430 confirm the heterozygous deletion.

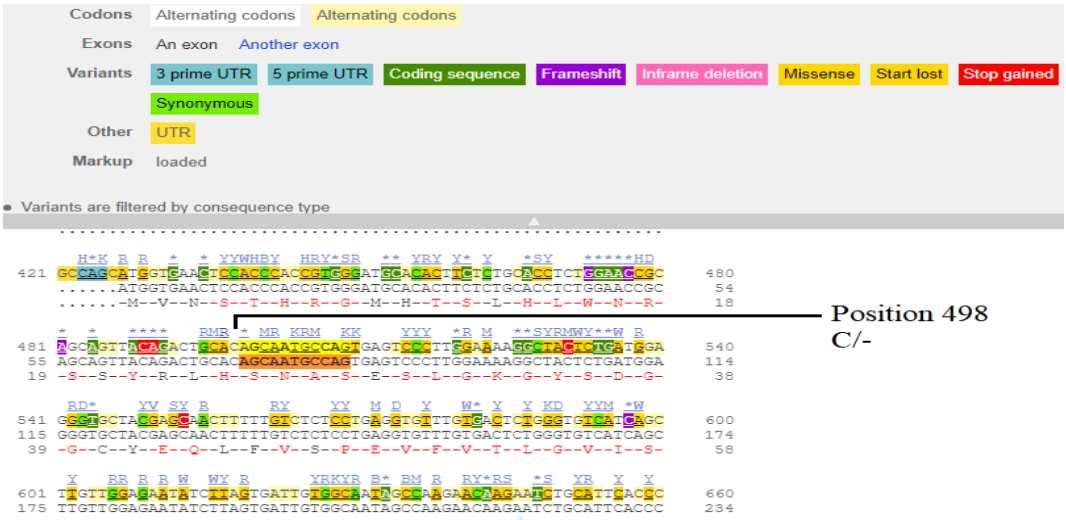

Figure 12 ENSEMBL browser output showing no previously recorded variation at this genomic coordinate, confirming its novel status.

2.3.4. Subject X-8: Missense Variant in MC4R

Missense mutation, p.Trp258Gly (TGG>GGG0) was found as a result of heterozygous T>G transversion at cDNA position 772 (c.772>G) (Fig 4.13 & 4.14). The variant was reported in COSMIC database (COSM4329034) and associated to somatic and germline observation in cancer, but few in obesity (Fig 4.15 & 4.16). The in-silico pathogenicity prediction tools (PolyPhen-2, SIFT) has predicted this variant being probably damaging and deleterious**.** The direct contribution of this variant to obesity still needs additional functional confirmation but its presence in a person with severe obesity should be considered as a variant of uncertain significance (VUS) with possible functional consequences.

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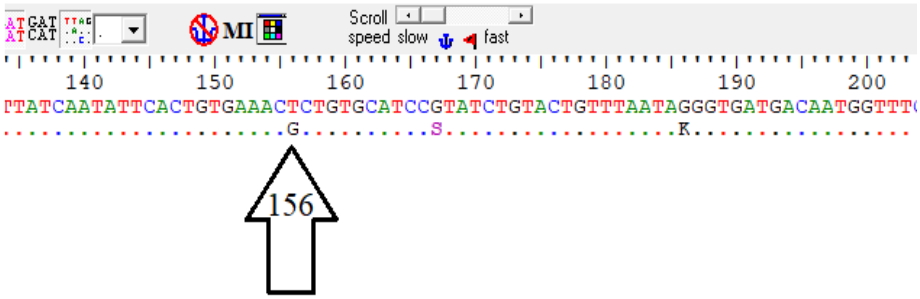

Figure 13 BioEdit alignment for Subject X-8. Arrow indicates the T>G change at cDNA position 772.

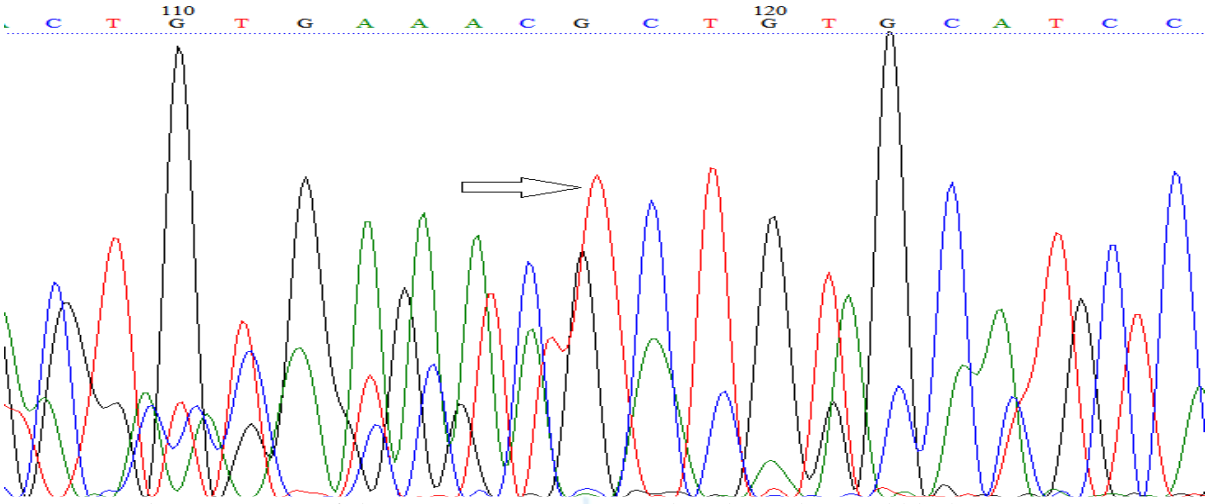

Figure 14 Chromatogram of the c.772T>G variant. Dual peaks (red 'T' and black 'G') at position 117 confirm heterozygosity.

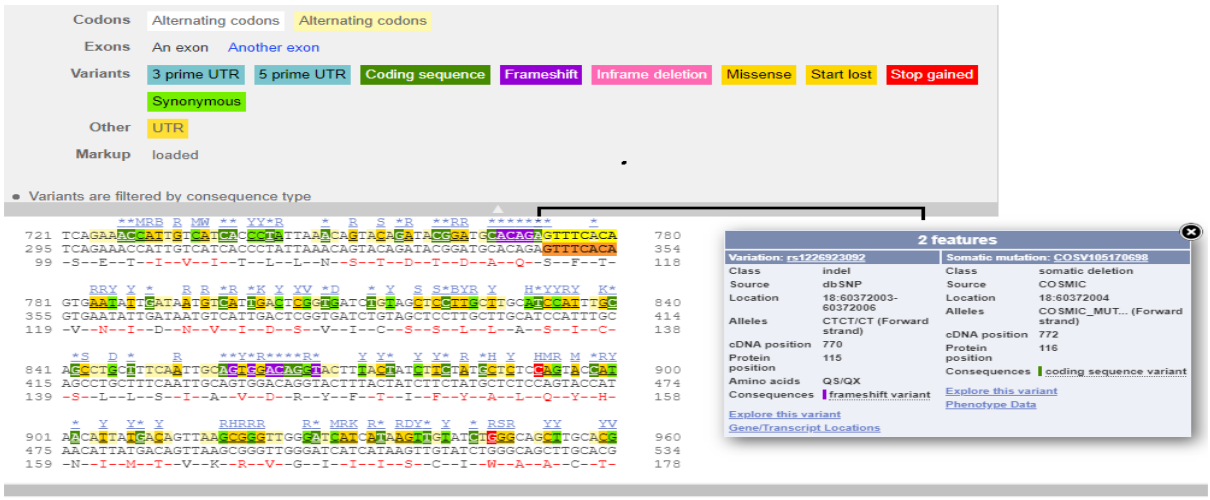

Figure 15 ENSEMBL report showing the COSMIC ID (COSM4329034) for this variant.

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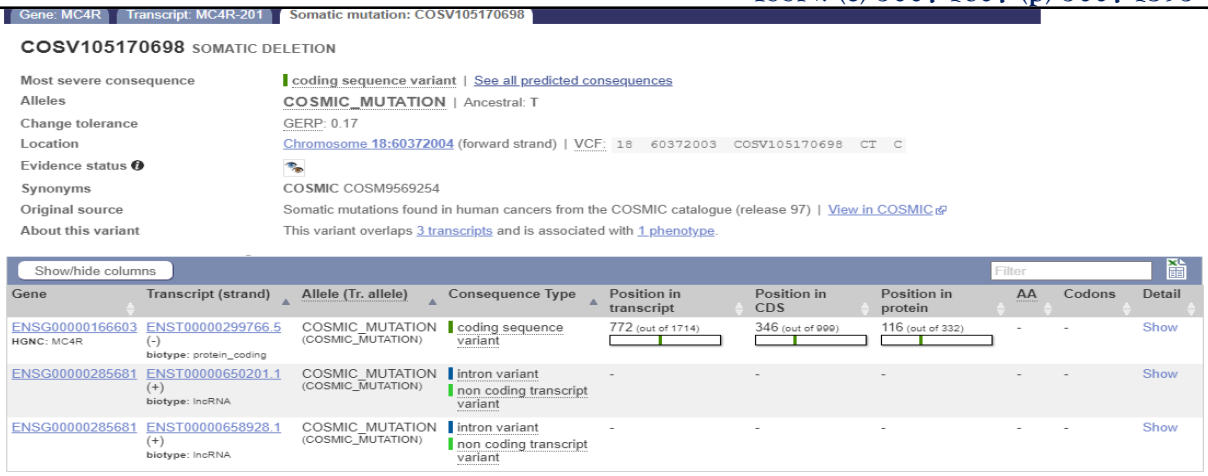

Gene: MC4R | Transcript: MC4R-201 | Somatic mutation: COSV105170698

COSV105170698 SOMATIC DELETION

Most severe consequence	coding sequence variant | See all predicted consequences
Alleles	COSMIC_MUTATION | Ancestral: T
Change tolerance	GERP: 0.17
Location	Chromosome 18:60372004 (forward strand) | VCF: 18 60372003 COSV105170698 CT C
Evidence status	
Synonyms	COSMIC COSM9569254
Original source	Somatic mutations found in human cancers from the COSMIC catalogue (release 97) | View in COSMIC
About this variant	This variant overlaps 3 transcripts and is associated with 1 phenotype.

Show/hide columns | Filter

Gene	Transcript (strand)	Allele (Tr. allele)	Consequence Type	Position in transcript	Position in CDS	Position in protein	AA	Codons	Detail
ENSG00000166603 HGNC: MC4R	ENST00000299766.5 (-) biotype: protein_coding	COSMIC_MUTATION (COSMIC_MUTATION)	coding sequence variant	772 (out of 1714)	346 (out of 999)	116 (out of 332)	-	-	Show
ENSG00000285681	ENST00000650201.1 (+) biotype: lncRNA	COSMIC_MUTATION (COSMIC_MUTATION)	intron variant, non coding transcript variant	-	-	-	-	-	Show
ENSG00000285681	ENST00000658928.1 (+) biotype: lncRNA	COSMIC_MUTATION (COSMIC_MUTATION)	intron variant, non coding transcript variant	-	-	-	-	-	Show

Figure 16 Variant consequences from ENSEMBL, highlighting its classification and predicted impact.

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Table 3.1 Overview of MC4R Gene Variants in the Obese Cohort.

Subject	cDNA Change (NM_005912.3)	Protein Change	Variant Type	Genomic Position (GRCh38)	dbSNP / COSMIC ID	Predicted Pathogenicity	Frequency in Cohort
X-5	c.717C>G	p.Val239Val	Synonymous	chr18:60,372,059	rs13447331	Benign / Modifier	1/10
X-7	c.498delC	p.Arg166GlyfsTer22	Frameshift deletion	chr18:60,371,941	Novel	Likely pathogenic	1/10
X-8	c.772T>G	p.Trp258Gly	Missense	chr18:60,372,114	COSM4329034	VUS / Possibly damaging	1/10
X-9	c.431delT	p.Leu144CysfsTer6	Frameshift deletion	chr18:60,371,872	rs121913343	Pathogenic	1/10

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3. Discussion

The current research examined the role of genetic variations in MC4R and PCSK1 gene in predisposing obesity in a Pakistani cohort. The main result is the discovery of MC4R variants in 40% (4 out of 10) of these obese patients under study, whereas in PCSK1 gene no pathogenic variation was identified. The prevalence of MC4R variation linked it with its status of most frequent monogenic obesity etiology and highlight its important role in the etiology of the disease in this particular population. This prevalence may be increased due to high rate of consanguinity rate.

3.1. High Prevalence of MC4R Variants in Consanguineous Population

The identification rate of the MC4R in the well phenotyped cohort (BMI ≥30) is 40%, which is much higher than 0.5-5.8% typically reported in outbred European population with severe obesity (Dubern et al., 2007; Vollbacj et al., 2016). This observation is nevertheless in line with other consanguineous groups. Saeed et al., (2015) found that MC4R, LEP and LEPR variants are responsible for 30% of severe childhood obesity in a Pakistani cohort**.** The Middle Eastern studies have equally shown high rates of homozygous MC4R mutations. The hypothesis that the founder effects and genetic drifts in endogamous populations which also includes those found in Pakistan, may result in increased concentration of particular pathogenic alleles is reinforced by our observation. This observation suggests that monogenic obesity is more prevalent in endogamous populations than in heterogamous ones.

3.2. Spectrum and Functional implications of Identified MC4R Variants

The identified variants represent range of possible functional effects:

1. Loss-of-function frameshift mutation (Subjects X-7 and X-9): The predicted novel c.498delC and the pathogenic c.431delT mutations are predicted to result in premature termination codons, which can lead to truncated and non-functional MC4R proteins or can cause the nonsense-mediated mRNA decay (NMD). Total loss of MC4R can disrupt the leptin-melanocortin hypothalamic pathway that is a primary controller of the energy homeostasis. It results in impaired satiety signaling, hyperphagia and lack of energy expenditure which is a direct mechanistic connection to early-onset and severe obesity (Yeo et al., 1998; Farooqi et al.,2003).

2. A Missense Variant of Uncertain Significance (Subject X-8): In the literature of obesity c.772>G (p.Trp258Gly) has scanty evidence despite of being present in COSMIC. Tryptophane 258 is essential in the structure and binding of the receptor, because it is highly conserved residue in the transmembrane domain of MC4R. Deleteriousness In silico prediction predicts that the consequence of the variant is possible disruption of receptor trafficking or signaling. By functional studies its pathogenicity needs to be categorically classified which includes cAMP assays in transfected cells. Its comorbidity with obesity in this topic should be investigated further.

3. A Synonymous Variant (Subject X-5): The sequence of amino acid sequence didn't change by the c.717C>G (p.Val239Val), but the stability of mRNA. splicing or translational kinetics could be affected. The phenomenon is being supported by growing evidence. Its role is not well understood and it can be a harmless polymorphism or be in synergy with other genetic or environmental influences.

The absence of PCSK1 from our cohort is though surprising but it shows that MC4R might be the more significant monogenic factor in this particular subset. This should be, however taken with caution because of the small sample size focusing on particular exons; a whole-exon genome or genome sequencing method would help us to identify the variants in non-coding or other areas of the PCSK1.

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3.3. Contextualizing Findings within Pakistani Genetic Landscape

Due to high consanguinity (>60%) and large population substructure Pakistan has a distinctive genetic structure (Bittles & Black, 2010). This genetic environment enables homozygosity of rare recessive alleles. Although our research found heterozygous variants, the frequency is high which shows that the alleles could be more common in population gene pool. Moreover, the interaction of such monogenic risk factor with a polygenic background, which includes hundreds of common SNPs with a small effect, is likely to determine the ultimate obesity phenotype and its severity as suggested by Chami et al., (2020). In individual having a heterozygous MC4R mutation with a low polygenic risk score might remain lean but if the same mutations are combined with a high – risk polygenic background and an obesogenic environment, the result will be full disease expression.

3.4. Clinical and Mechanistic Insight

The deficiency of MC4R is important to diagnose clinically. This makes the diagnostic shifts out of a behavioral paradigm into a neurobiological paradigm. Although the present-day treatment is also based on lifestyle change, this genetic diagnostic may help reduce the stigma among the patients and their families which can gain more relevance with the introduction of the targeted treatment. For Example, Setmelanotide, an agonist of melanocortin-4receptor, has demonstrated impressive efficacy in patients with obesity with mutations in leptin-melanocortin pathway which includes PMOC and LEPR deficiency and has potential in certain MC4R defects that do not completely eliminate signaling (Roubert et al., 2010). The correlation of genotype and phenotype despite of being complicated also indicates that the mutations associated with total loss-of-function are generally linked with more severe and earlier – onset obesity as compared to the partial loss-of-mutation.

3.5. Limitations and future directions

This study has limitations. The sample size (n=10) is small which restricts the statistical power and generalizability. Although the candidate-gene method is focused, it might have overlooked other genes that are related to obesity (e.g., LEP, LEPR, POMC, SIM1). Future research must have:

1. **Expanded cohort study:** Generalizing these results to large, multi-center Pakistani cohort with additional information regarding phenotyping, including age of onset, hormonal profile and hyperphagia scores.
2. **Next Generation Sequencing (NGS):** It will help in using whole genome or exome sequencing to identify novel variants and explore polygenic risk score along with monogenic mutations.
3. **Functional Characterization:** In vitro experiments to establish the exact molecular implications of the novel and VUS variants identified, especially the c.772T>G and c.717C>G variants.
4. **Family Segregation Studies:** Identifying the variants of the identified un the pedigree of probands to determine the co-segregation with the obese phenotype and modes of inheritance.

4.4. Conclusion

In conclusion of the study the evidence of a significant genetic role of MC4R gene variants in obesity in a simple Pakistani adult with a significant prevalence rate of 40%. The identification of known pathogenic and new variants indicates genetic heterogeneity of obesity and influence of population specific genetic architecture. The lack of PCSK-1 in the cohort shows that MC4R may be a dominant monogenic factor in the group. These results support the need to incorporate genetic screening into the diagnostic workup of severe obesity in consanguineous group such as the one in Pakistan. It may be possible through such an approach to make accurate diagnosis, inform genetic counseling and open the path to individualize the treatment approaches as these targeted therapies are continuously developed. The extensive future genetic research is

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important to completely develop the genetic map of obesity in Pakistan and translate the results into better clinical outcomes.

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