The Indian Journal of Pediatrics (February 2020) 87(2):105–110
https://doi.org/10.1007/s12098-019-03129-6

ORIGINAL ARTICLE

A Targeted Next Generation Sequencing Panel for Non-syndromic Early Onset Severe Obesity and Identification of Novel Likely -Pathogenic Variants in the *MC4R* and *LEP* Genes

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Received: 4 June 2019 / Accepted: 15 November 2019 / Published online: 11 January 2020

Abstract

Objectives To screen for variants in the *MC4R* and *LEP* genes in 46 patients with clinical suspicion of non-syndromic early onset severe obesity (NEOSO).

Methods Children with early onset obesity satisfying WHO criteria of obesity were studied. The *MC4R* and *LEP* genes were sequenced using a PCR amplicon based NGS on Illumina MiSeq next generation sequencer using an in-house developed protocol.

Results Of the 46 children tested, four were found to have novel pathogenic/likely-pathogenic variants (one in the *MC4R* gene and three in the *LEP* gene). In three out of the 4 families, the presence of the variants was confirmed using standard bidirectional capillary sequencing in the probands.

Conclusions Four children with novel likely pathogenic variants in the *MC4R* and *LEP* genes are reported. Genetic analysis is crucial in children with early onset obesity and should be considered.

Keywords Genetic obesity · Non-syndromic early onset obesity

Introduction

Childhood obesity is a serious problem in today's world and is increasing at an alarming rate [1]. The etiology is multifactorial, resulting from a complex interaction of genetic and environmental factors. Apart from well-known dietary and lifestyle factors, childhood obesity is also influenced by genetic factors [2]. In fact, genetic factors are reported to contribute to 40–70% variation in body mass between individuals [3, 4]. Genetic causes include chromosomal [Prader Willi syndrome (PWS)] and non-chromosomal obesity syndromes (Bardet Beidl syndrome, Cohen syndrome *etc*.) [5]. Another form of hereditary obesity is non-syndromic early onset severe obesity (NEOSO), resulting from pathogenic/likely-pathogenic variants in the genes involved in the hypothalamic-leptin-melanocortin pathway (*LEP, LEPR, MC4R, PC1/PC3/ PCSK1, POMC*) and hypothalamic formation (*SIM1, BDNF, NTRK2*) which are responsible for controlling food intake and body weight [6]. Genetic variants in the *LEP*, *LEPR* and *MC4R* genes are most frequent cause of monogenic forms of non-syndromic obesity [6].

Leptin is a protein secreted by adipocytes, and its concentration in blood positively correlates with body fat mass and body mass index (BMI). Mutations in *LEP* gene result in undetectable to low serum leptin levels resulting from improper protein folding, intracellular transport and secretion, or loss of biological activity of secreted protein [7]. Mutations in *LEPR* often lead to a truncated receptor, preventing leptin binding and signaling through hypothalamic-leptin-melanocortin pathway [8].

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MC4R is a seven-transmembrane G protein coupled receptor principally expressed in brain, including hypothalamus. *MC4R* gene mutations represent some of the most common monogenic causes of NEOSO and MC4R deficiency is the most common monogenic form of obesity [9].

Approximately 7% NEOSO are monogenic and are caused due to gene variants of major effect [10, 11]. The clinical presentation is non-specific. Identifying these genetic variants is important to plan personalized clinical management (for *example*, leptin replacement therapy for patients with *LEP* gene mutations) and provide genetic counseling to patient and family (these mutations are autosomal recessive and parents are usually carriers) [12].

Before the advent of Next generation sequencing (NGS), sequential capillary sequencing of *LEP*, *LEPR* and *MC4R* genes was the mainstay of molecular diagnosis, which was both time consuming and labor intensive. NGS has superseded capillary sequencing for molecular typing because of its huge multiplexing capability for genomic targets as well as number of samples, and reduced relative cost. The authors have developed a targeted NGS assay comprising of two NEOSO associated genes, *LEP* and *MC4R*. With this panel, authors screened for variants in the *MC4R* and *LEP* genes in 46 patients with NEOSO, and identified novel, likely pathogenic frameshift variants in *MC4R* and *LEP* genes in four patients.

Material and Methods

Children from a tertiary level care pediatric endocrine unit with NEOSO (age less than 5 y) were included in the study. Obesity was defined for <2 y of age as sex-specific weight for recumbent length > 97.7th percentile and 2–5 y as BMI > 95th percentile for age and sex [12]. A detailed history was recorded, anthropometric measures were noted and converted to Z scores [13] and clinical examination was performed. Children who appeared to have nutritional obesity were excluded from the study. Three milliliters of peripheral blood was collected from patients in K2 – EDTA vacutainers (Becton Dickinson, MD, USA). Blood samples were also collected from parents who were willing to check their carrier status. The institutional ethics committee approved the study and parents gave written informed consent.

Genomic DNA was extracted using DNeasy Blood and Tissue kit (Qiagen, Germany) as per the manufacturers' instructions. The *MC4R* and *LEP* genes were sequenced using a PCR amplicon based NGS on an Illumina MiSeq next generation sequencer. The broad steps involved were: generation of PCR amplicons, tagmenation of amplicons, ligation of Illumina specific index-adapter sequences and quantitation, purification and pooling of amplicons for sequencing. Primer sequences were designed using Primer Express v2.0 (Applied Biosystems, CA, USA) (Table 1). A long range PCR protocol using Q5 Polymerase (NEB) and an in-house master mix was used to amplify the 1.4 kb product of the *MC4R* gene. For the *LEP* gene, a multiplex PCR was designed to amplify both exons of the *LEP* gene using the Qiagen Quantitect (Qiagen) PCR mix (per the manufacturer's instructions). PCR amplicons were purified and diluted to 0.2 ng/μL before tagmentation. In one step, tagmentation protocol allows fragmentation of the PCR amplicons to about 250 bp length and integrates the Illumina adapters. An index PCR aids in ligation of the indices on the amplicons. These indices act as barcodes that allow processing of large number of samples simultaneously, and were incorporated through a secondary PCR step. Further, the PCR products were quantified using the Qubit fluorometric system. Concentrations were molar normalized for pooling of samples. The pooled library was purified using the Purelink PCR Purification Kit (Invitrogen) and diluted down to 4 nM final concentration using Resuspension Buffer (RSB – Illumina, CA, USA). The library was denatured using 0.2 N NaOH and further neutralized and diluted to a final concentration of 15pM using HT1 Buffer (Illumina, CA, USA). Further, a 5% phiX library spike (Illumina, CA, USA) was added as a control and diversity enhancer. The final library was loaded onto an Illumina MiSeq cartridge (Illumina, CA, USA) and run in 2*300 or 2*250 mode on an Illumina MiSeq (Illumina, CA, USA).

In all samples, the average coverage exceeded 10x with a sequence quality (>Q30) of more than 80% in all the targeted regions. Bioinformatics analysis was carried out using Illumina's BaseSpace cloud analytics platform along with an in-house developed pipeline. Generated .fastq files were trimmed for Q score ≥ 30 and adaptors followed by alignment to release GRCH37/ hg19 of the reference human genome assembly. Identified variants were analyzed using VarSeq (VarSeq™ v2.x, Golden Helix, Inc., Bozeman, MT).) and .bam files were visualized in the GenomeBrowse (GoldenHelix Inc., Bozeman, MT, USA) tool. Mutations were detected and identified manually.

Results

Targeted next generation sequencing was performed using a panel comprising of the *MC4R* and *LEP* genes (two exons) on 46 patients with NEOSO. The mean age of the children at the time of diagnosis was 25.2 ± 19 mo (n = 46, 22 girls, 24 boys). Fourteen children were younger than 2 y and 32 were > 2 y of

Table 1 The Primer sequences for PCR amplification of the *LEP* and *MC4R* genes

MC4R-F	5'-AGATTCGTCTCCCAATGGC-3'
MC4R-R	5'-ATTCTCAACCAGTACCCTACACG-3'
Leptin-1_F	5'-GATGCATTTCATTAATACATATGTAG-3'
Leptin-1_R	5'-GTTTCTTGGACTATCTGGGTCCAGTGC-3'
Leptin-2_F	5'-GCACTTGTTCTCCCTCTTCCT-3'
Leptin-2_R	5'-GTTCCTTCCCTTAACGTAGTCCT-3'

age when tested. Mean length for age Z score in children younger than 2 y was 6.6, weight for age Z score was 7.6, and weight for length Z score was 4.9 (all above +2). For children older than 2 y of age, height Z score was 2.1, weight Z score was 4.5 and BMI Z score was 4.4 (all above +2) as per WHO standard [10].

Of the 46 patients tested, four patients were identified with pathogenic/ likely-pathogenic variants in the *MC4R* or *LEP* gene. Out of these 4 variants, one variant in the *MC4R* gene has previously been reported in heterozygous state in the ExAC database [14]. The three variants in the *LEP* gene are novel and have, to the best of authors' knowledge, not been described in published literature. The authors did not detect any clinically relevant small sequence variants in the other cases.

Description of Patients with Novel Mutations in the *MC4R* and *LEP* Genes

Patient 1 A 2.5-y-old girl weighing 28.7 kg (Z score = 6.9), height 101.2 cm (Z = 2.8SD) and BMI 27.9 kg/m^2 (Z = 7.2SD) was referred for severe obesity. The child was born to parents with 3rd degree consanguinity. Parents reported a delay in achieving early milestones. Hearing, vision and speech were normal. She did not have any history of frequent infections or comorbidities. Thyroid function tests and glucose concentrations were within reference range. Serum leptin levels were 3.7 ng/ml. Targeted NGS panel showed presence of homozygous deletion of two nucleotides *MC4R*:c.63_64delCA. This variant had been reported once in the ExAC database in a heterozygous form, but had not been reported, to the best of authors' knowledge, in the homozygous state prior to the diagnosis of this case [15]. This frameshift variant changes codon for tyrosine (Y) to a stop codon resulting in a premature termination of the amino acid chain at the 21st position (p.Tyr21Ter) (Fig. 1). Using *in silico* mutation prediction (MutationTaster), this variant was predicted to be disease causing. The parents were subsequently shown to be heterozygous carriers for the same variant. Targeted bidirectional capillary sequencing independently confirmed presence of variant in homozygous state in the child and in heterozygous states in each parent. The predicted loss of function in *MC4R* gene in the patient correlates with the phenotypic presentation of early onset severe obesity. The family was provided dietary advice, genetic counseling and the option of prenatal diagnosis for future pregnancies.

Patient 2 An 8-mo-old boy presented with severe obesity with weight of 14.4 kg (Z = 5.1), length of 80 cm (Z = 4.2) and weight for length Z score of 3.7. The child was born to parents with 3rd degree consanguinity. He had a birth weight of 3 kg and showed a rapid weight gain with age. Early developmental milestones were normal. Hearing, vision and speech were apparently normal. Thyroid function tests and glucose concentrations were within reference range. Serum leptin concentration was 1.1 ng/ml.

The NGS assay revealed the presence of a novel frameshift variant NM_000230.2:c.142_143delAC with a deletion of two nucleotides in exon 2 of the *LEP* gene. This frameshift variant results in a change in codon at the 48th position (of a 167 amino acid protein) from a Threonine residue to a stop (p. The48Ter) (Fig. 2a), causing a premature termination of the protein. *In silico* prediction software classified this variant as "disease causing" or "deleterious". The truncated gene was predicted to result in a protein that has limited or no activity, which correlates with clinical observation of reduced leptin levels. The mother was confirmed to be heterozygous carrier. The father's sample was not available. Further, bidirectional capillary sequencing confirmed the presence of the variant in the child and his mother. The family was advised dietary management and lifestyle changes as leptin therapy is not available in India.

Patient 3 A 6-mo-old girl presented with severe, early onset obesity with weight of 13.9 kg (Z score 5.7), length of 69 cm (Z score 1.4) and weight for length Z score of 6.3. The child was born to parents with 2nd degree consanguinity. She had a birth weight of 3.3 kg and showed rapid weight gain from 3 mo of age. Early developmental milestones, hearing, vision and speech were normal. Serum leptin concentrations were 0.9 ng/ml.

The NGS assay revealed presence of a single nucleotide deletion NM_000230:c.453delG [NP_000221.1:p.Ser153fs] in a homozygous state in exon 3 of the *LEP* gene (Fig. 2c). This variant is predicted to result in a prolonged protein; *in silico* prediction (MutationTaster) classified this variant as "likely pathogenic". Further, bidirectional capillary sequencing confirmed the presence of the variant. The family was provided genetic counseling; the parents, however, did not consent to testing of their blood. To best of authors' knowledge, this variant has never been reported in literature.

Patient 4 A 4-mo-old girl presented with severe, early onset obesity with weight of 12 kg (Z score 4.8), length of 68 cm (Z score 1.9) and weight for length Z score of 4.7. The child was born to parents with 2nd degree consanguinity. She had a birth weight of 3.4 kg and showed a rapid weight gain from 3 mo of age. Early developmental milestones including hearing, vision and speech were normal. Serum leptin levels were 0.7 ng/ml.

The NGS assay revealed presence of missense variant NM_000230.2:c.461 T > C [NP_000221.1:p.Leu154Pro, ClinVar submission ID 448905] in homozygous state in exon 3 of *LEP* gene (Fig. 2c). In silico prediction (Sift, Polyphen and MutationTaster) classified this variant as "disease causing" or "pathogenic". Further, bidirectional capillary sequencing confirmed the presence of the variant. The parents did not consent to genetic testing. To best of authors' knowledge, this variant has also never been reported in literature.

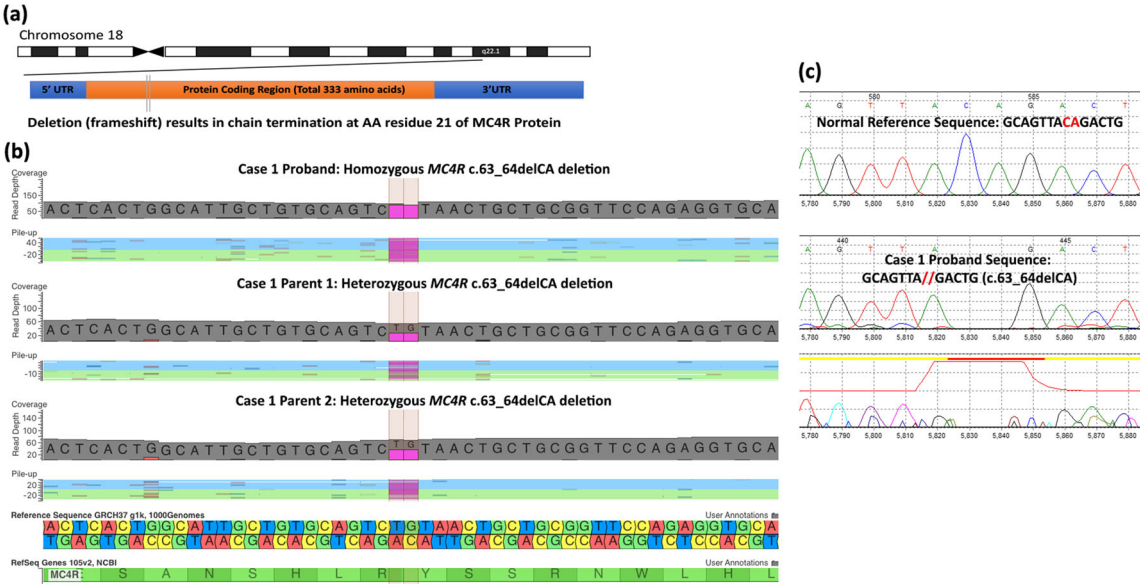

Fig. 1 *MC4R* gene c.63_64 delCA homozygous frameshift mutation. (**a**) Schematic representation of location of *MC4R* frameshift deletion in chromosome 18. **(b)** NGS read pile up showing *MC4R*:c.63_64delCA (These NGS data are in reverse complement orientation) in the proband (homozygous) and in the carrier parents (heterozygous) (**c**) Capillary DNA sequencing showing patients' c.63_64delCA GCAGTTA//GACTG compared to normal sequence.

The clinical characteristics of cases in which variants were detected are summarized in Table 2.

Discussion

The authors have used a custom in-house developed NGS assay to analyze the *LEP* and *MC4R* genes in 46 patients with clinical diagnosis of NEOSO, and identified novel, previously unreported, pathogenic variants in the *MC4R* and *LEP* genes in four patients. The variant in the *MC4R* gene was reported only once in ExAC database in a heterozygous state, but had not been reported in a homozygous state. In all families there was a history of consanguinity and presence of variants was confirmed in heterozygous form in two sets of parents. The mutations in the *LEP* gene are novel variants that, to best of authors' knowledge, have not been reported in published literature. The *LEPR* gene is also one of the common genes associated with monogenic non-syndromic early onset obesity, however, authors were not able to include it in the current panel.

The eight important genes causing NEOSO are *LEP, LEPR, MC4R, PC1/ PC3/ PCSK1, POMC, SIM1, BDNF* and *NTRK2* which are mainly involved in satiety control, energy homeostasis and development of the hypothalamus. The *LEP*, *MC4R* and *LEPR* genes are probably the most significant, contributing to 4–7% of all cases of childhood obesity (70–80% of all cases of NEOSO) [10]. There are very few clinical clues for presence of pathogenic variants in a particular gene. Children with mutations in the *LEP* gene are known to have endocrine (hypogonadotropic hypogonadism and hypothyroidism) and immunological abnormalities [14]. Identification of the pathogenic/ likely pathogenic variants in the *LEP* gene helps in management, counseling, prediction of recurrence risk and providing prenatal diagnosis in any subsequent pregnancy. Further, patients with *LEP* mutations are known to respond to dietary management and leptin therapy [16].

Variations in the *MC4R* gene (OMIM *155541) have a population prevalence of at least one in 2000 (0.05%), and are found in 0.5–1% of obese adults and are accountable for 6% of all severe cases of disease starting in childhood [9, 17]. Pathogenic variants in the *MC4R* gene have been reported to be associated with both autosomal dominant and recessive forms of NEOSO with dominant form being much more common [18]. In index case reported (Case 1), patient had a homozygous frame shift mutation and parents were heterozygous.

Variants in the *LEP* and *MC4R* were classified as "likely pathogenic" based on available evidence from the published literature and databases and through *in silico* mutation prediction, according to guidelines laid down by American College of Medical Genetics and Genomics [19]. The clinical features of the four patients were consistent with previous reports of loss of function mutations in the *MC4R* and *LEP* genes [20, 21]. Pathogenic variants in the *LEP* or *LEPR* genes are inherited in an autosomal recessive form, hence homozygous mutations result in the disorder [7]. In the cases reported above, homozygous mutations were found in patients 2, 3 and 4. However, some reports suggest that even heterozygous mutations in the *LEP* gene (ΔG133 LEP) lead to low leptin levels and are responsible for obesity [22].

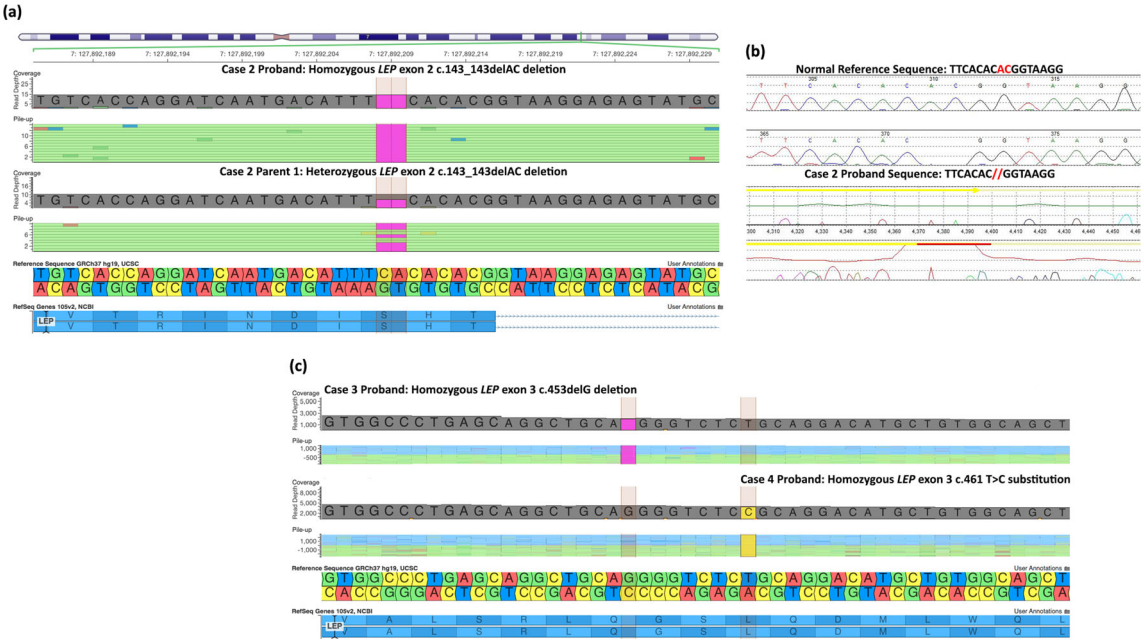

Fig. 2 Data of patients with novel likely pathogenic variants in the *LEP* gene. **(a)** NGS read pile up in patient 2, showing *LEP*:c.142_143 homozygous deletion in patient and heterozygous deletion in the parent (mother). **(b)** Capillary DNA sequencing showing patients' c.142_143 delCA CATTTCACAC//GGTA compared to normal sequence seen in patient 2. (Note that the 'CA' deletion is shown in different positions due to the NGS data being left aligned and the capillary sequence data being right aligned) **(c)** NGS read pile up showing *LEP*: NM_000230:c.453delG single nucleotide deletion in the patient 3 (*upper lane*) and *LEP* c.461 T > C / p.Leu154Pro variant in the patient 4 (*lower lane*)

With advent of whole exome sequencing, a large number of genes and genetic variants are being newly identified to be associated with NEOSO. The identification of these newer variants has contributed significantly to pathophysiology of non-syndromic obesity and has potential to develop novel therapeutic options.

In conclusion, authors report 4 children with NEOSO with novel likely pathogenic variants in the *MC4R* and *LEP* genes. Genetic analysis is crucial in children with early onset obesity despite not having any syndromic features and can be considered. This will help in prognosis, treatment and genetic counseling and option of prenatal diagnosis.

Table 2 Profile of children with novel mutation in *MC4R* and *LEP* gene

	Case 1	Case 2	Case 3	Case 4
Gender	Girl	Boy	Girl	Girl
Age at referral	2.5 y	8 mo	6 mo	4 mo
Consanguinity	3rd degree	3rd degree	2nd degree	2nd degree
Developmental delay	Delay in motor milestones +	Appropriate for age	Appropriate for age	Appropriate for age
Vision, speech, hearing	Normal	Normal	Normal	Normal
Height/ Length Z score	2.8	4.2	1.4	1.9
Weight Z score	6.9	5.1	5.7	4.8
BMI Z score	7.2	–	–	–
Weight for Height Z score	7.1	3.7	6.3	4.7
Leptin levels	3.7 ng/ml	1.1 ng/ml	0.9 ng/ml	0.7 ng/ml
Mutation	Frameshift mutation in the *MC4R* gene	Frameshift mutation in the *LEP* gene	Homozygous single nucleotide deletion in the *LEP* gene	Missense mutation in the *LEP* gene

BMI Body mass index

Authors' Contribution VK: Concept, patient management and manuscript draft; NP: NGS assay design; NG, PG, RLO: Data collection and manuscript draft; NP, MA, AK, NL: Patient management and manuscript draft. SR, TR, KP, AP, SA, AB: Manuscript draft. KK: Data collection, analysis and manuscript writing. AK is the guarantor for this paper.

Compliance with Ethical Standards

Conflict of Interest None.

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