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Original Article

Genetics Section

Whole Genome Sequencing for Variant Detection in Charcot-Marie-Tooth Disease: A Cross-sectional Diagnostic Study

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ABSTRACT

Introduction: Charcot-Marie-Tooth disease (CMT) is one of the most common inherited Neuromuscular Disorders (NMDs), classified under peripheral neuropathies and characterised by progressive motor and sensory dysfunction. Although Whole-exome Sequencing (WES), gene panels, and conventional methods have improved detection rates, they often miss deep intronic, regulatory, and Structural Variants (SVs). Whole-genome Sequencing (WGS), with its comprehensive coverage of coding and non coding regions, enables the identification of variants that are often overlooked by other approaches.

Aim: To assess the diagnostic utility of WGS in CMT cases that remain unresolved by WES, analysing both coding and non coding variants.

Materials and Methods: The present cross-sectional diagnostic study was conducted between July 2023 and January 2025 at the Neuberg Center for Genomic Medicine (NCGM), Ahmedabad, Gujarat, India. WGS was performed on 31 clinically suspected CMT patients, including two who had previously tested negative by WES. Both coding and non coding variants including missense, nonsense, frameshift, in-frame, intronic, and 5' Untranslated Region (UTR) mutations were analysed. Variants were classified according to American College of Medical Genetics and Genomics (ACMG) guidelines, incorporating Combined Annotation Dependent Depletion (CADD) scores and Minor Allele Frequency (MAF) thresholds. They were interpreted based on pathogenicity, inheritance patterns, and genotype-phenotype correlations. Selected non coding variants in the Gap Junction Beta-1 (GJB1; c.-16-511G>C) and Lamin A/C (LMNA; c.-142C>A) genes were validated by Sanger sequencing.

Results: Sequencing data from 31 participants were processed using a standardised bioinformatics pipeline. Variants were classified according to ACMG guidelines, and their frequencies were calculated. WES and WGS results were compared to determine the additional diagnostic yield. WGS identified clinically significant non coding variants in GJB1 (intronic) and LMNA (5' UTR) in two cases, yielding a 6.5% increase over WES. Overall, 31 variants were detected: 11 (35.5%) classified as pathogenic, 2 (6.5%) as likely pathogenic, and 18 (58.0%) as Variants of Uncertain Significance (VUS), reflecting the genetic heterogeneity of CMT.

Conclusion: The WGS enhances diagnostic accuracy in CMT by detecting clinically relevant non coding variants often missed by WES. This is the first report from India confirming a GJB1 intronic variant and a Lamin A/C (LMNA) 5' UTR variant using WGS in CMT patients. These findings support the integration of WGS into routine diagnostic workflows and highlight the value of comprehensive variant analysis for early and precise genetic diagnosis.

Keywords: Genetic diagnostics, Genetic heterogeneity, Inherited neuromuscular disorders, Non coding variants, Peripheral neuropathy

INTRODUCTION

Charcot-Marie-Tooth disease (CMT) is a group of inherited neurological disorders that affect the peripheral nerves, which are responsible for transmitting signals between the brain, spinal cord, and the rest of the body. It is the most common form of inherited neuropathy, affecting an estimated 1 in 2,500 individuals, according to the National Institutes of Health (NIH). First described independently by Jean-Martin Charcot, Pierre Marie, and Howard Henry Tooth in 1886 [1], CMT is among the most prevalent inherited Neuromuscular Disorders (NMDs). It is characterised by progressive motor and sensory dysfunction, typically affecting the distal limbs.

The estimated global prevalence is 17.7 per 100,000 individuals, although earlier studies have reported higher rates [2,3]. The CMT is genetically and clinically heterogeneous, with over 100 known genes associated with the disease. These genes are involved in essential biological processes such as myelination, axonal transport, mitochondrial function, and Schwann cell biology [4-6]. Commonly implicated genes include Peripheral Myelin Protein 22 (PMP22), Gap Junction Beta-1 (GJB1), Myelin Protein Zero (MPZ), Mitofusin 2 (MFN2), SH3 Domain and Tetratricopeptide Repeat Containing 2, and Lamin A/C (LMNA). The subtypes are classified based on inheritance patterns and electrophysiological features into demyelinating (CMT1), axonal (CMT2), intermediate, and X-linked forms (e.g., CMTX1) [7,8].

Accurate genetic diagnosis is crucial for confirming clinical suspicion, guiding family screening, informing reproductive decisions, and enabling access to gene-specific therapies where applicable [9,10]. However, conventional methods such as targeted gene panels and Whole-exome Sequencing (WES) predominantly cover coding regions. These approaches often fail to detect deep intronic, regulatory, or Structural Variants (SVs), leading to unresolved cases [11-13].

Whole-genome Sequencing (WGS) provides comprehensive coverage of both coding and non coding regions, allowing the identification of a broader spectrum of pathogenic variants, including those overlooked by WES [14,15]. With improving accessibility and declining costs, WGS is emerging as a highly relevant tool for diagnosing complex or genetically unresolved conditions [16-18].

Recent studies have demonstrated the added value of WGS in resolving cases missed by WES, particularly when non coding or SVs are suspected [19,20]. Its application in clinical practice offers

opportunities for early diagnosis, precise genetic counselling, and personalised care.

The present study evaluates the clinical utility of WGS in individuals with suspected CMT, including cases unresolved by WES. The diagnostic yield, variant spectrum, and detection of overlooked variants in under represented populations are highlighted. By capturing non coding and regulatory regions beyond the reach of WES, WGS provides a more comprehensive genomic view essential for diagnosing complex disorders. It is hypothesised that WGS improves the diagnostic yield in CMT- particularly in cases unresolved by WES- by detecting clinically relevant non coding and structural rearrangements.

MATERIALS AND METHODS

Sample processing, Deoxyribonucleic Acid (DNA) extraction, quality control, and sequencing workflow: The present cross-sectional diagnostic study was conducted at the Neuberg Center for Genomic Medicine (NCGM), Ahmedabad, Gujarat, India, between July 2023 and January 2025, involving a total of 31 clinically suspected CMT cases. The sample size was determined based on the availability of participants during the study period. In accordance with institutional policy, studies utilising de-identified residual diagnostic samples are exempted from additional ethical approval. Detailed information regarding the study's objectives and procedures was provided to patients and their families prior to blood sample collection, and written informed consent was obtained from all participants or their legal guardians. Participant confidentiality was strictly maintained, and no personal identifiers were disclosed or compromised at any stage of the study.

Inclusion criteria: Inclusion criteria encompassed individuals of any age presenting with clinical signs indicative of inherited peripheral neuropathy, such as distal muscle weakness, foot deformities (e.g., pes cavus), and reduced or absent deep tendon reflexes. Patients with prior WES results that failed to identify a definitive genetic diagnosis were also eligible.

Exclusion criteria: Exclusion criteria included individuals with evidence of acquired neuropathies (e.g., diabetic, toxic, or inflammatory) or insufficient clinical documentation.

Study Procedure

To ensure quality control and assess reproducibility of the sequencing pipeline, reference control DNA samples were used from the Genome in a Bottle (GIAB) Consortium. These controls, derived from well-characterised cell lines provided by the Coriell Institute for Medical Research (USA), served as benchmarking standards for variant calling and analysis consistency. Peripheral blood samples were obtained from participants based on neurological examinations and characteristic phenotypic features.

Non coding variants were specifically targeted in present study because they occur in regulatory regions, introns, and Untranslated Regions (UTRs), which are often missed by WES but can have clinically significant effects on gene expression, splicing, and disease manifestation in CMT. Genomic DNA was extracted using the QIAamp DNA Blood Mini Kit (Qiagen, Germany), quantified using the Qubit-4 Fluorometer (Invitrogen, USA), and assessed for purity with the NanoDrop One Spectrophotometer (Thermo Fisher Scientific, USA). Samples that passed quality control thresholds were subjected to library preparation, high-throughput sequencing, and bioinformatics analysis according to standardised protocols. Phenotypic features were annotated using Human Phenotype Ontology (HPO) terms to ensure consistency in clinical data interpretation across cases.

WGS and WES library preparation: WGS libraries were prepared using the Kapa HyperPlus Kit (Roche, South Africa) and sequenced on the Illumina NovaSeq 6000 platform with 150 bp paired-end reads, targeting a minimum of 30× coverage. The WES libraries were prepared using the Twist Exome 2.0 plus Comprehensive Exome Spike-in Kit (Twist Bioscience, USA), with a target coverage of 100×.

Bioinformatics analysis: Base calling was carried out with bcl2fastq version 2.20, and sequencing reads were mapped to the GRCh38 human reference genome using BWA version 0.78. PCR duplicates were marked using Picard MarkDuplicates v3.1.1. Variant calling was conducted using Atlas v0.1 for WGS and GATK v3.5 for WES. Single Nucleotide Variants (SNVs) and Copy Number Variants (CNVs) were detected using Genome Analysis Toolkit-germline Copy Number Variation (GATK gCNV), and Repeat Expansions (REs) were assessed with Expansion Hunter v3.1.2. All identified variants were evaluated based on their genomic region (coding or non coding), type (missense, nonsense, frameshift, in-frame, intronic, or 5'UTR), zygosity, mode of inheritance, and genotype-phenotype correlation using HPO terms. All variants were annotated using ANNOtate VARiation (ANNOVAR) and prioritised based on Combined Annotation Dependent Depletion (CADD) scores and Minor Allele Frequency (MAF) from population databases such as Genome Aggregation Database (gnomAD). Classification of variants was performed according to the guidelines of the ACMG and the Association for Molecular Pathology (AMP) [21].

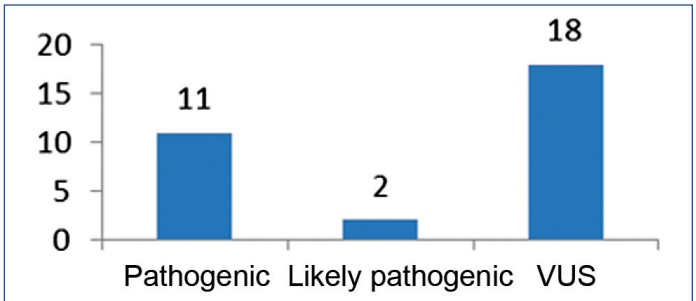
Sanger confirmation: Sanger sequencing was performed to confirm selected WGS-identified variants in GJB1 (chrX:71,223,181; c.-16-511G>C) and LMNA (chr1:156,114,777; c.-142C>A). Polymerase Chain Reaction (PCR) amplification was carried out using Takara Taq DNA Polymerase, Hot Start Version (Takara Bio Inc., Japan). Amplicons were sequenced using the ABI 3500 Genetic Analyser (Thermo Fisher Scientific, USA), and chromatograms were analysed with Mutation Surveyor v5.1 (SoftGenetics, USA).

STATISTICAL ANALYSIS

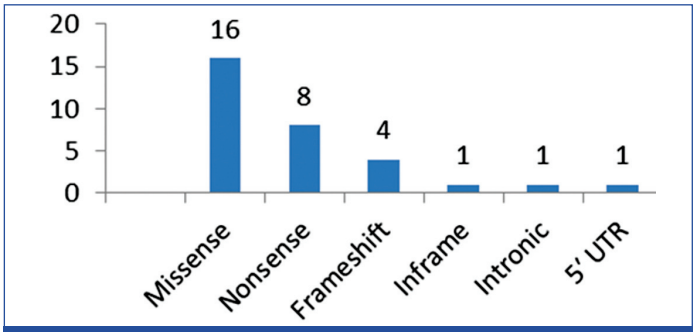
The diagnostic yields of WES and WGS were compared using Fisher's-exact test. A p-value of <0.05 was considered statistically significant. Analyses were performed using GraphPad Prism version 10.1.2.

RESULTS

The WGS identified 31 variants across various CMT subtypes, including 11 (35.5%) pathogenic, 2 (6.5%) likely pathogenic, and 18 (58.0%) variants of uncertain significance [Table/Fig-1]. The variant types comprised 16 missense, 8 nonsense, 4 frameshift, and one each of inframe, intronic, and 5' UTR variants [Table/Fig-2]. All missense, nonsense, frameshift, and inframe variants were detected by both WES and WGS, while the intronic (GJB1) and 5' UTR (LMNA) variants were uniquely identified by WGS [Table/Fig-3].

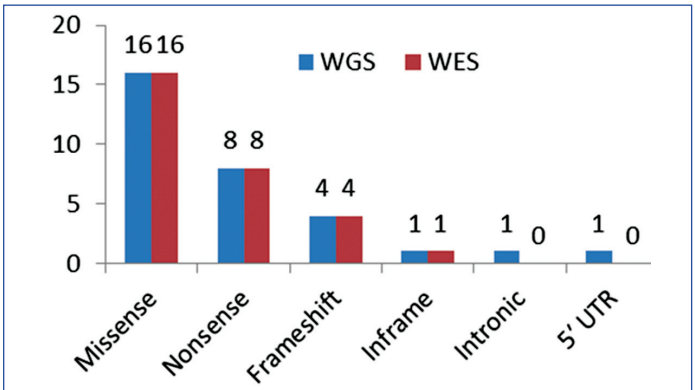


[Table/Fig-1]: Variant type distribution.
VUS: Variant of uncertain significance

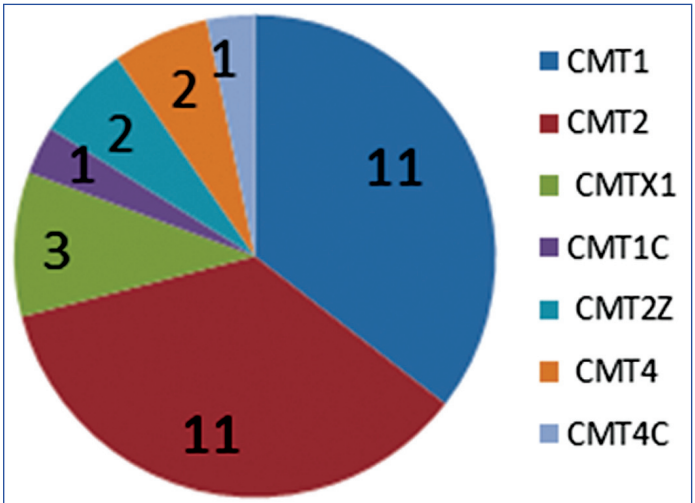


[Table/Fig-2]: Variant classification.

The distribution of molecular subtypes included CMT1 (n=11), CMT2 (n=11), CMTX1 (n=3), CMT1C (n=1), CMT2Z (n=2), CMT4 (n=2), and CMT4C (n=1) [Table/Fig-4]. Clinical features aligned with subtype phenotypes, such as distal weakness and sensory loss in CMT1, Central Nervous System (CNS) involvement in GJB1-related CMTX1, severe axonal degeneration in CMT2Z, and progressive demyelination in CMT4. Variants were further characterised by classification, pathogenicity scores (e.g., CADD), zygosity, gene-wise distribution, inheritance patterns (autosomal dominant, autosomal recessive, X-linked dominant), and associated subtypes.



[Table/Fig-3]: Comparisons of variations.



[Table/Fig-4]: Distribution of CMT subtypes.

Notably, Serial Numbers 4 (GJB1) and 8 (LMNA) [Table/Fig-5] were WES-negative but resolved by WGS, highlighting the added value of detecting non coding variants. Specifically, WGS detected an intronic heterozygous variant in GJB1 (c.-16-511G>C) [Table/Fig-6] and a 5' UTR homozygous variant in LMNA (c.-142C>A) [Table/Fig-7], both absent in gnomAD and with high CADD scores (33 and 15.9). These WGS-identified variants were confirmed by Sanger sequencing as orthogonal validation [Table/Fig-8] for GJB1 and [Table/Fig-9] for LMNA.

DISCUSSION

The present study highlights the significant clinical utility of WGS in the diagnosis of CMT, particularly in cases unresolved by WES. Unlike WES, which primarily targets coding regions, WGS provides comprehensive, unbiased coverage of the entire genome, including deep intronic regions, UTRs, and structural variants- areas often missed by probe-based methods. This extensive coverage is especially critical for genetically heterogeneous disorders like CMT, where pathogenic variants may reside outside traditional coding regions [22].

The identified pathogenic non coding variants in GJB1 and LMNA illustrate key diagnostic limitations of WES and targeted panels. These findings align with previous studies reporting regulatory region variants contributing to CMT pathogenesis [14,21]. Comparative literature reveals similar non coding variants in cohorts from the USA, UK, and South Korea, underscoring the importance of these findings in filling a critical gap in global genomic diversity and expanding the mutational landscape relevant to Indian populations [23].

The observed genotype-phenotype correlations in this cohort further support the clinical relevance of non coding variants, which have historically been underappreciated due to challenges in variant interpretation. Detection of variants in Microrchidia Family CW Type Zinc Finger 2 (MORC2), PMP22 and MPZ further reflects the complex genetic architecture and heterogeneity of CMT [24]. WGS's ability to detect diverse variant types, including SNVs, CNVs, REs, SVs, and non coding mutations, positions it as a powerful diagnostic method. This is particularly valuable in clinical contexts where standard testing yields inconclusive or negative results [25,26]. Supporting this, a recent study reported the diagnosis of Spinocerebellar Ataxia Type 3 (SCA3) via WGS by detecting pathogenic REs, subsequently validated by Triplet-primed Polymerase Chain Reaction (TP-PCR) as an orthogonal method, further emphasising the utility of WGS in undiagnosed patients [27].

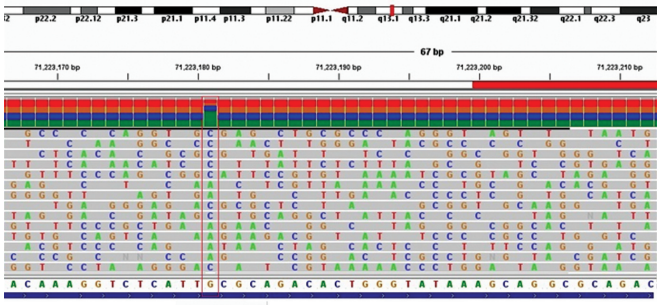
Notably, present study presents the first evidence of pathogenic LMNA 5' UTR and intronic GJB1 variants in Indian CMT patients, validated through Sanger sequencing. In total, 31 variants spanning multiple CMT subtypes were identified, resulting in a 6.5% increase in diagnostic yield compared to prior methods. This increase not only highlights the genetic diversity of CMT but also demonstrates the clear diagnostic advantage of comprehensive genome-wide analysis. These findings support the routine implementation of WGS in clinical workflows to improve diagnostic yield, reduce diagnostic odysseys, and enable timely, targeted therapeutic interventions.

Beyond diagnosis, WGS offers broad genetic insights that facilitate cascade screening of at-risk family members, guide reproductive counseling, and support personalised patient management. Orthogonal validation through Sanger sequencing remains essential to confirm clinically actionable variants and ensure diagnostic precision. Early molecular diagnosis also opens pathways to emerging genotype-specific therapies such as PXT3003 for PMP22-related CMT1A and the ongoing development of gene therapy strategies targeting GJB1 and LMNA mutations [28-30]. In populations with high rates of consanguinity or strong familial histories of NMDs, early genetic diagnosis via WGS can significantly reduce disease burden. This is

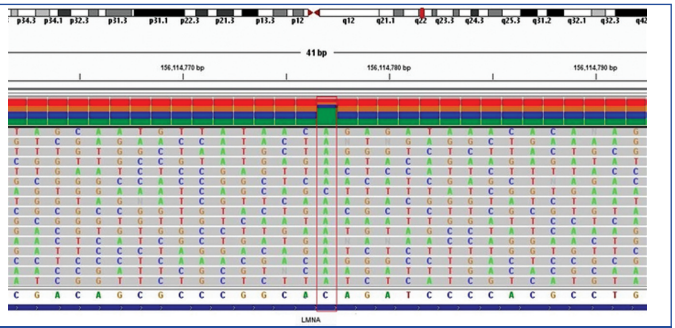
S. No.	Gene	MOI	Location	Variant type	HGVS notation	ACMG classification	MAF	Pathogenicity score	Disease
1	EGR2	AR	Exon 2	Missense	c.1366A>T (p.Ser456Cys)	VUS	Absent	CADD 22.5	CMT1
2	GARS1	AD	Exon 6	Nonsense	c.710C>A (p.Ser237Ter)	VUS	Absent	CADD 38	CMT2
3	GARS1	AD	Exon 6	Nonsense	c.733C>T (p.Gln245Ter)	VUS	Absent	CADD 43	CMT2
4	GJB1	XLD	Intron 1	Intron	c.-16-511G>C	P	Absent	CADD 33	CMTX1
5	GJB1	XLD	Exon 2	Missense	c.1A>T (p.Met1Leu)	VUS	Absent	CADD 22.8	CMTX1
6	GJB1	XLD	Exon 2	Nonsense	c.8G>A (p.Trp3Ter)	P	Absent	CADD 37	CMTX1
7	LITAF	AD	Exon 4	Missense	c.427G>T (p.Asp143Tyr)	VUS	Absent	CADD 29	CMT1C
8	LMNA	AR	5 UTR	UTR	c.-142C>A	VUS	Absent	CADD 15.9	CMT2
9	LMNA	AR	Exon 1	Missense	c.82C>T (p.Arg28Trp)	LP	0	CADD 26.7	CMT2
10	LMNA	AR	Exon 1	Missense	c.85C>A (p.Leu29Met)	VUS	0	CADD 23.8	CMT2

11	LMNA	AR	Exon 1	Frameshift	c.11dup (p.Ser5fs)	P	Absent	CADD 26.7	CMT2
12	LMNA	AR	Exon 1	Missense	c.1A>G (p.Met1Val)	P	Absent	CADD 24.9	CMT2
13	LMNA	AR	Exon 1	Missense	c.3G>T (p.Met1Ile)	P	0	CADD 26	CMT2
14	MFN2	AD	Exon 3	Missense	c.5C>T (p.Ser2Phe)	VUS	0.0000131	CADD 27.8	CMT2
15	MFN2	AD	Exon3	Missense	c.10C>T (p.Leu4Phe)	VUS	0.0000116	CADD 22	CMT2
16	MFN2	AD	Exon 11	Nonsense	c.1156C>T (p.Gln386Ter)	P	Absent	CADD 42	CMT2
17	MORC2	AD	Exon 26	Missense	c.3046A>G (p.Thr1016Ala)	VUS	0.00000205	CADD 25.3	CMT2Z
18	MORC2	AD	Exon 10	Nonsense	c.831C>G (p.Tyr277Ter)	VUS	Absent	CADD 36	CMT2Z
19	MPZ	AD	Exon 6	Missense	c.742A>G (p.Lys248Glu)	VUS	0.000000684	CADD 27.3	CMT1
20	MPZ	AD	Exon 6	Nonsense	c.742A>T (p.Lys248Ter)	VUS	Absent	CADD 39	CMT1
21	MPZ	AD	Exon 6	Missense	c.662C>T (p.Ala221Val)	VUS	0.000000684	CADD 29.8	CMT1
22	MPZ	AD	Exon 5	Missense	c.641G>T (p.Arg214Leu)	VUS	0.00000342	CADD 28.7	CMT1
23	MPZ	AD	Exon 5	Frameshift	c.613_616del (p.Pro205fs)	P	Absent	CADD 35	CMT1
24	MPZ	AD	Exon 3	Missense	c.403A>T (p.Ile135Leu)	LP	Absent	CADD 25.1	CMT1
25	MPZ	AD	Exon 3	Inframe	c.405_407del (p.Ile135_Val136delinsMet)	VUS	Absent	CADD 29.9	CMT1
26	MPZ	AD	Exon 4	Frameshift	c.491dup (p.Val165fs)	P	Absent	CADD 24.9	CMT1
27	PMP22	AD	Exon 5	Missense	c.449G>A (p.Gly150Asp)	P	Absent	CADD 26	CMT1
28	PMP22	AD	Exon 5	Nonsense	c.467T>A (p.Leu156Ter)	VUS	Absent	CADD 39	CMT1
29	SH3TC2	AR	Exon 1	Frameshift	c.43del (p.Arg15fs)	P	0.0000234	CADD 20.3	CMT 4
30	SH3TC2	AR	Exon 11	Nonsense	c.2599C>T (p.Gln867Ter)	P	Absent	CADD 35	CMT4C
31	SH3TC2	AR	Exon 1	Missense	c.43C>T (p.Arg15Trp)	VUS	0.0000291	CADD 20.3	CMT 4

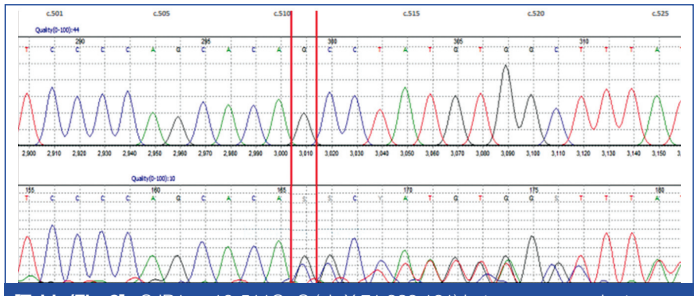
[Table/Fig-5]: Total variants identified in CMT.
HGVS: Human genome variation society; MOI: Mode of inheritance; AR: Autosomal recessive; AD: Autosomal dominant; XLD: X-linked dominant; VUS: Variant of uncertain significance; P: Pathogenic; LP: Likely pathogenic; CMT1: Charcot-marie-tooth disease type 1; CMT2: Charcot-marie-tooth disease type 2; CMTX1: X-linked charcot-marie-tooth disease type 1; CMT1C: Charcot-marie-tooth disease type 1c; CMT2Z: Charcot-marie-tooth disease type 2Z; CMT 4: Charcot-marie-tooth disease type 4; CMT4C: Charcot-marie-tooth disease type 4C; Absent: Indicates the variant is not found in population databases; MAF 0: The variant was observed but with zero frequency



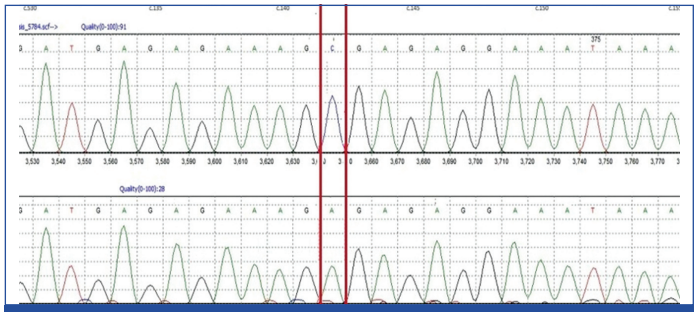
[Table/Fig-6]: GJB1 c.-16-511G>C (chrX:71,223,181) heterozygous variant detected by WGS.



[Table/Fig-7]: LMNA c.-142C>A (chr1:156,114,777) homozygous mutation detected by WGS.



[Table/Fig-8]: GJB1 c.-16-511G>C (chrX:71,223,181) heterozygous variant detected by WGS was confirmed by Sanger sequencing as an orthogonal method.



[Table/Fig-9]: LMNA c.-142C>A (chr1:156,114,777) homozygous variant detected by WGS was confirmed by Sanger sequencing as an orthogonal method.

achievable through informed reproductive decisions, including prenatal and preimplantation genetic testing, which can prevent the transmission of pathogenic alleles.

Within the Indian context, these genes remain commonly mutated, consistent with global patterns. The novel detection of a variant in this study underscores the necessity of developing population-specific variant databases and interpretation frameworks. Such initiatives will not only improve diagnostic accuracy but also inform personalised patient care, enhance variant reclassification efforts, and support regionally relevant genomic research and therapeutic development [31,32].

Limitation(s)

The present study is limited by a relatively small sample size, which may affect the generalisability of the findings across diverse populations. Although WGS improved the diagnostic yield and enabled the detection of novel non coding variants, functional validation of these variants was not conducted, thereby limiting insight into their pathogenic mechanisms. Furthermore, the interpretation of non coding variants remains challenging due to evolving classification guidelines and limited annotation resources.

CONCLUSION(S)

The present study highlights the diagnostic advantage of WGS in CMT, particularly in cases unresolved by conventional methods

such as WES. By enabling comprehensive detection of both coding and non coding variants, WGS overcomes the inherent limitations of capture-based approaches. The identification of pathogenic non coding variants in GJB1 and LMNA among Indian patients, confirmed through orthogonal validation, expands the mutational spectrum of CMT and underscores the clinical relevance of genome-wide analysis. With a 6.5% increase in diagnostic yield, these findings support the integration of WGS into routine diagnostic workflows, especially for genetically heterogeneous disorders like CMT. Beyond diagnosis, WGS facilitates cascade screening, informs reproductive counselling, and enables early access to emerging targeted therapies, offering significant clinical and public health benefits.

Acknowledgement

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