



Case Report Distinct Phenotypic and microRNA Expression in X-Linked Charcot–Marie–Tooth Correlated with a Novel Mutation in the GJB1 Gene

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Abstract: We investigated genetic and clinical features in two siblings with an unreported frameshift mutation in the GJB1 gene, encoding connexin 32, to study CMTX-1 and its intrafamilial phenotypic variability. Connexin 32 is a gap junction protein that is located in paranodal regions and Schmidt-Lanterman incisures. Clinical features, family history, and genetic and microRNA information were collected. Genetic analysis determination was performed on genomic DNA from the two cases. Muscle-specific miR-206 was also investigated in serum. A muscle biopsy was conducted in one case, and EMG with conduction velocities was performed in both patients. In the first genetic analysis, no duplication of the PMP22 gene was found. A second genetic analysis of a panel of genes associated with inherited peripheral neuropathies was performed. We found a frameshift mutation in the connexin 32 (GJB1) gene, c.281_287del in hemizygosity, not previously reported, that segregated with the clinical phenotype. An X-linked hereditary sensory motor neuropathy was caused by the mutation in the connexin 32 gene. We found overexpression of miR-206 that was 4-fold up-regulated in the older brother and over 10-fold in the younger brother versus the controls; this might be correlated with a different muscle mass and regeneration. The two siblings presented differently evolving neuropathies due to environmental factors and lifestyles that caused nerve degeneration. We hypothesized that in this X-linked CMT, there is no expression of a truncated connexin 32 (Cx32) protein, with loss of function markedly reduced in the gap junction. In the peripheral nervous system (PNS), this might be mitigated by the presence of another connexin, Cx43. Such a reduction might affect not only gap junction formation but also myelination and muscle trophism, resulting in variable miR-206 expressivity.

Keywords: CMTX-1; connexin 32; new gene mutation in GJB1; miR-206; circulating microRNA

1. Introduction

Charcot–Marie–Tooth (CMT) disease is the most prevalent inherited disorder of the peripheral nerves [1,2]. It is a heterogeneous disorder both in terms of clinical phenotype manifestations and inheritance pattern: the most common types are autosomal dominant, X-linked, and autosomal recessive. There are several genes associated with CMT [3], but most (about 90%) of the CMT diagnosed cases are due to mutations in four genes (PMP22, MPZ, GJB1, and MFN2 genes) [4–6]. In the last decade, the next-generation sequencing (NGS) technique has seen increased use and represented an easier way to perform diagnostics, and it has also contributed to the understanding of the pathophysiologic mechanisms [7].

CMT patients manifest distal weakness with decreased grip strength, reduced fine motor function, and muscle atrophy with indolent, length-dependent, sensory motor polyneuropathy [4,6].



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). Electromyography is the best way to characterize the various types of CMT based on nerve conduction velocities (NCVs) and differentiate the two main subtypes: the demyelinating form (CMT1) with a reduced NCV, and the axonal form (CMT2) with a low normal NCV [6,8,9].

The X-linked dominant form (CMTX-1) is caused by pathogenic mutations within the gap junction beta 1 (GJB1) gene, which encodes the connexin 32 (Cx32) protein. The Cx32 protein forms gap junction channels that physically connect adjacent cells and allow the exchange of small-molecular-weight substances. Cx32 is localized in paranodal regions and Schmidt–Lanterman incisures [10,11]. Because of their key role in cell-to-cell communication, the tissue connexin proteins are regulated by post-transcriptional and post-translational modifications [12]. In the last two decades, the importance of microR-NAs (miRNAs)—small, single-strand, conserved RNA molecules—as a regulator of the expression of specific genes at the post-transcriptional level, typically by binding to the 3'-untranslated region of the mRNA sequence, has been highlighted [13]. Up-regulation of miRNAs results in translational repression/gene silencing, leading to decreased expression of the corresponding protein product. They also exert a role in connexin regulation and assembly [14].

miR-206 is a muscle-specific miRNA, a member of the myomiRNA family, that regulates, together with miR-1, myogenic differentiation, also playing an important role in skeletal muscle regeneration and maturation [15–17].

We report two CMT1-X siblings with different clinical phenotypes with an unreported frameshift mutation in the GJB1 gene. Muscle-specific miRNAs were investigated in serum and offered new insights into pathogenesis.

2. Results

2.1. Clinical Features and Family Pedigree

2.1.1. Family Pedigree

Clinical features and family history were collected. In Figure 1, we report the family pedigree. In the family history, one affected male cousin was reported but not examined.



Figure 1. Family pedigree. Index cases III-3 and III-5 (arrow). One affected relative was reported (III-1) but not examined. The pedigree tree follows the common conventions: squares, male; circles, female; filled, affected; open, unaffected; half-filled, carrier.

2.1.2. Patient 1: Clinical Features and Medical Examination

Patient III-3: A man, 55 years old, had diffuse hypotrophy in the upper and lower limbs since age 28 years and presented atrophic legs, bilateral pes cavus, and an ataxic gait.

Figure 2. Patient III-3 had lower limb atrophy since age 28, and flat feet. At age 55, he had an unsteady ataxic gait (**A**), difficulty dorsiflexing his hands (**B**), and atrophic interossei muscles (**C**).

Patient III-3's EMG showed an abnormally low motor nerve conduction velocity (VCM) of 25 m/s in the median nerve (normal \geq 40 m/s). The evoked potential amplitude in the abductor pollicis brevis was 0.4 mV, with a 5.4 m/s prolonged distal latency. The peroneal nerve was not excitable.

2.1.3. Patient 2: Clinical Features and Medical Examination

Patient III-5, 47 years old, was the younger brother of patient III-3 (Figure 3). He had distal weakness, a tremor in the upper extremities (right more than left), slight foot drop with a stepping gait, and thin atrophic leg muscles. He had atrophy of the ulnar eminence, and weakness of opposition and a slight deficit (4/5 MRC score) of the finger extensors in both hands, but his manual dexterity was not affected, and he could perform his job as a military pilot. His weakness was slowly evolving in his lower limbs.



Figure 3. Patient III-5, 47 years old, presented distal weakness, a tremor in the hands (right more than left), and slight gait unsteadiness (**A**). He had difficulty opposing the thumb to the small finger and could not dorsiflex his fingers (4/5 MRC) (**B**). His weakness was mostly in his hands, slowly evolving in his lower limbs (**C**).

He had difficulty grasping objects with his hands (Figure 2) and performing a fine motor function, such as opening zips or writing.



A muscle biopsy was performed and showed mild changes and a few type 1 atrophic fibers (Figure 4) with preservation of fast fibers.

Figure 4. Quadriceps muscle biopsy of patient III-5. H&E stain (**A**,**B**): the muscle fiber size is variable (**B**), and a small vessel is present. Gomori trichome stain shows one opaque muscle fiber (**C**–**E**), and PAS stain (**F**) shows few atrophic fibers (arrows), while PAS-positive fast fibers appear relatively spared.

MRI of the spinal cord in the cervical region showed a slight disc protrusion on C5/C6 with a rectified cervical cord.

The EMG of patient 2 (III-5) showed a preserved median motor nerve conduction velocity (VCM) of 40 m/s (normal \geq 40 m/s). The evoked potential amplitude in the abductor pollicis brevis was 0.3 mV, with a 4.2 m/s distal latency. The peroneal nerve was not excitable in the peroneus muscle, but excitable in the tibialis anterior muscle.

2.2. Genetic Analyses

Duplication analysis of the PMP22 gene by multiplex ligation-dependent probe amplification was normal. Analysis of a panel of genes associated with inherited peripheral neuropathies detected a previously unreported deletion of seven nucleotides in exon 2 of the GJB1 gene, encoding connexin 32: NM_000166.5:c.281_287del in hemizygosity. The mutation was confirmed by Sanger sequencing and is predicted to cause a frameshift and premature stop signal six codons downstream p.(His94Leufs*6) (Figure 5). The presence of the mutation was confirmed by Sanger sequencing in the brother. The mutation was submitted to the LOVD database (https://www.lovd.nl, accessed on 10 February 2022).



Figure 5. (**A**) Sequence of GJB1 exon 2 in the patient showing the deletion of seven nucleotides. Boxed is the premature TAG stop codon. (**B**) Predicted sequence of the resulting protein. In red are the five abnormal amino acids on the C-terminus. (**C**) Sequence of GJB1 exon 2 in a healthy control. Underlined are the deleted nucleotides in the patient. (**D**) The full-length connexin 32 protein. In green: the amino acids deleted in the patient. Underlined are the four transmembrane domains.

2.3. Circulating miR-206

The level of miR-206 in serum was significantly higher in the patients relative to the controls (Figure 6). The controls were unaffected individuals matched regarding age (age range 36–61 years) and sex (Supplementary Materials Table S1). Interestingly, when we compared the values in the two brothers, the overexpression of miR-206 was more relevant in the younger brother than in the older, which correlated with a more preserved muscle morphological pattern and bulk, both at the clinical and histopathological levels.



Figure 6. The relative expression level of miR-206 in III-3 and III-5 cases versus the controls. There was a 4-fold up-regulation of miR-206 in the serum of the older 55-year-old patient which was over 10-fold in the younger brother versus 7 controls (CTRL). The higher level of microRNA in the less affected patient might be correlated with a relatively preserved muscle regeneration and muscle tropism, with less neurogenic atrophy. Pt: patient; *: *p*-value < 0.05.

2.4. Quality of Life in the Two Brothers

Patient 1: He is a depressed man and unable to work. The disease has changed his life: he has become discouraged and frustrated. He does not want help from anyone, especially from his brother, and is a heavy smoker.

Patient 2: He is in the military with a positive behavior and temperament—the opposite of his brother. He continues to be active in the military. The disease does not affect his lifestyle.

3. Discussion and Conclusions

Our current results show the frameshift connexin 32 mutation in two siblings with discordant phenotypes. In CMT disease, the complexity is not limited to the identification of genetic mutations, but to the interpretation of the same variant in the intrafamilial phenotypic variability of the two cases reported, in order to identify the correlation with the mechanism of the hereditary disease CMTX-1. The challenge, therefore, was first to determine whether the role of the genetic variant identified by next-generation sequencing (NGS) is responsible for the disease phenotype [18].

In CMT, symptoms typically begin between age five and twenty-five years, and also the condition is sometimes slowly progressive. Signs and symptoms include distal muscle weakness, hand wasting (atrophy), sensory loss, and a slow nerve conduction velocity [1]. It is typically related to pes cavus foot deformity (high arch) and bilateral foot drop [19].

Less than 5% of individuals with CMT become wheelchair-dependent, and both of our cases are still able to walk.

In this type of Charcot–Marie–Tooth, as shown in the muscle biopsy report, atrophy caused by the denervation/reinnervation process affects the grouping of slow muscle fibers more than fast fibers, which may be related to the different metabolic requirements of different types of muscle fibers and motor neurons. The slow-twitch fibers are mainly related to aerobic oxidation, and the fast-twitch fibers are mainly related to glycolysis and anaerobic performance. Under the circumstances of oxidative stress, the function of intracellular mitochondria will be inhibited or even damaged, affecting the aerobic oxidative energy supply.

The treatment of CMT hereditary neuropathology is symptomatic and supportive [20,21]. The life expectancy for individuals affected by Charcot–Marie–Tooth is usually normal. Although people with CMT have a normal life expectancy, the degree of their disability varies widely; in some cases, CMT disease affects every area of life quality.

A cure is not available, so it is important to minimize or antagonize the symptoms. Comprehensive treatments include physical therapy, shoe orthotics, leg braces, and surgery to correct foot deformities [22,23].

Additional medical care could also be psychological help to alleviate pain and discomfort and improve overall quality of life [24]. Vocational or occupational therapy, anticipating the progression of the disorder, could also be helpful for young patients.

In this study, we described two siblings with different late-onset phenotypes correlated with the same novel mutation and a different overexpression of serum miR-206. There was evidence from the family history of an X-linked hereditary sensory motor neuropathy that was confirmed by a frameshift mutation in *GJB1* which causes a deletion of seven nucleotides, and the formation of a premature stop signal six codons downstream. The deletion occurs in the final exon of the gene; therefore, nonsense-mediated decay probably does not occur in this case. Nevertheless, the mutation truncates the protein just after the second transmembrane domain (Figure 5); thus, it is unlikely that any functional channels are formed [25].

The GJB1 mutation encoding a truncated Cx32 protein caused a chronic denervation/reinnervation process in the muscles. The two affected patients had different personal adaptations to the neuropathy disability. While the older sibling, an inactive, smoking, depressed man, had gait difficulty and presented a chronic invalidating neuropathy, both in the upper and lower extremities, the younger brother was a military pilot and had minimal weakness, a slight tremor mostly in the right hand, and preserved fine motor function.

We hypothesized that in this X-linked CMT, there is no expression of a truncated connexin 32 (Cx32) even if the Cx32 protein is expressed. It is highly unlikely to be functional, and the protein with loss of function might be markedly reduced in gap junctions. Such a reduction might affect not only gap junction formation but also myelination and muscle tropism, resulting in variable miR-206 expressivity. The presence of another connexin, Cx43, was observed in the nerves of Cx32 null mice [26], which is down-regulated by miR-206 [14]. This might exert a negative feedback or cause serum miR-206 accumulation. We hypothesized that connexins form channels that transport bioactive molecules. A well-documented control mechanism of protein regulation is microRNAs. Moreover, dysregulation of circulating myomiRNAs, especially miR-206, has been previously described in several neurodegenerative and neuromuscular disorders, and in such a condition, they could be proposed as biomarkers of disease progression to monitor muscle atrophy and the reinnervation process [18,27–29]. The mutation we found likely produces a truncated protein with a reduced ability to transport ions and metabolites that induce miR-206. We suggest that the level of miR-206 might reflect the different muscle mass observed, with less neurogenic atrophy in the younger brother.

This study presented the contribution of molecular genetics to the diagnosis of CMT disease and its consequences on microRNA levels as observed in this family. We suggest that Cx–miRNA interactions may offer the potential for investigating new aspects of the clinical phenotype. Since foot or hand deformities might be an important clue and reduce gait and manual dexterity, they should be followed over time by a neurologist with physical examination and study of microRNAs combined with electrophysiology to differentiate the disease evolution.

In conclusion, we herein described the clinical features and evaluation of chronic neuropathy in two patients with CMTX1 and further expand the phenotypic spectrum associated with the disease. Multiple clinical, molecular, and investigative tools are needed to differentiate this neuropathic condition.

4. Materials and Methods

This study involved two CMTX-1 siblings. As clinical diagnostic procedures, EMG with nerve conduction velocities, muscle biopsy, and MRI were performed. Clinical features and family history were collected during the medical examination.

This study was performed in accordance with the ethical principles outlined in the Declaration of Helsinki, and all genetic analyses were performed in accordance with the quality management system UNI EN ISO 9001:2015. All patients and controls signed a written informed consent form. Bioinformatic software was used to analyze the effect of mutations on Cx32 protein function and structure.

Genetic analysis was performed in the 2 cases by the Clinical Genetics Laboratory, Department of Women and Children's Health (University of Padua). DNA was extracted from the peripheral blood with a QIAamp DNA Blood kit (QIAGEN) following the manufacturer's protocol. A panel of genes (BSCL2, EGR2, FGD4, FIG4, GARS, GDAP1, GJB1, HSPB1, IGHMBP2, LITAF, LMNA, MFN2, MPZ, MTMR2, NEFL, PMP22, PRX, SH3TC2) associated with peripheral neuropathies was analyzed by next-generation sequencing with Illumina MiSeq using a Haloplex HS kit (Agilent Technologies, Santa Clara, CA, USA). The procedure and subsequent bioinformatic analyses have been detailed elsewhere [30].

The Sanger analysis of Cx32 was performed with the primer: 5'-CCTTCATCTGCA ACACACTCC-3', 3'-GAAGACGGCCTCAAACAACA-5'. The PCR conditions were: 3 min at 94 °C; 35 cycles at 94 °C for 30 s, at 55 °C for 30 s, and at 72 °C for 30 s; 7 min at 72 °C. We used Taq DNA Polymerase (Roche). We used the Abi Prism 3500 (Applied Biosystem) sequencing instrument following the manufacturer's protocols.

Circulating miRNAs were extracted from serum by an miRNeasy extraction kit (Cat.NO/ID 217004, QIAGEN), following the manufacturer's specifications. Reverse tran-

scription to cDNA was performed using a TaqMan microRNAs reverse transcription kit (Thermo Fisher Scientific) according to the manufacturer's protocol and using a specific hsa-miR-206 probe (Thermo Fisher Scientific). Real-time PCR experiments were conducted using TaqMan Universal Master Mix. Data were obtained from at least 3 real-time PCR experiments and in duplicate. The level of miR-206 was normalized to hsa-miR-16 and U6 snRNA, as internal controls, and the miR-39-3p of *Caenorhabditis elegans*, as spike-in miRNA, as previously reported [17]. Supplementary Table S2 shows the details of the nomenclature, miRNA sequences, and specific Assay IDs. The relative amount of the miRNA expression level was calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as "relative expression of controls".

miRNA values were expressed as the mean \pm standard deviation versus the controls. Statistical analysis was performed using the Mann–Whitney/Wilcoxon test, where values were considered statistically significant at *p* < 0.05.

Supplementary Materials: The following supporting information can be downloaded at: https: //www.mdpi.com/article/10.3390/muscles1010007/s1, Table S1: Healthy controls; Table S2: TaqMan microRNA primer sequences.

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Institutional Review Board Statement: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the institutional ethics committee (protocol code AFM22392/2020, 2 March 2020).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish their pictures in this paper.

Data Availability Statement: Not applicable. All patient data is provided in this article anonymously.

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