Journal of Clinical Case Reports

Case Report Open Access

# A Pro22Ser Mutation in *NEFL* Results in Charcot-Marie-Tooth Disease in a Chinese Family

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**Abstract**

The purpose of this study was to describe a pedigree with *NEFL* (c.64C>T, p. Pro22Ser, NM_006158) mutation which results in CMT (Charcot Marie Tooth) disease. This pedigree comprised 10 patients with 6 surviving cases over four generations. The clinical picture was characterized by *pes cavus*, distal limb weakness and atrophy and a steppage gait. Genetic testing was performed in a total of 12 subjects from this family (including 4 affected and 8 asymptomatic controls) to confirm this mutation. Electrophysiological findings revealed mixed demyelinating and axonal neuropathy. This mutation was well cosegregated with affected members in the autosomal dominant pattern. We have a pair of twins with vastly different phenotypes and genetic backgrounds in this family, giving robust evidence of genotype-phenotype correlation in CMT. A male patient was diagnosed with cancer, giving rise to the consideration of more function of this mutation.

**Keywords:** Charcot Marie Tooth Disease (CMT); *NEFL*; Cancer

## Introduction

CMT is an umbrella term which refers to a homogeneous group of clinical phenotypes, including progressive distal muscle weakness and atrophy, foot deformities, distal sensory loss and usually decreased tendon reflexes. CMT is genetically heterogeneous, caused by more than 1000 mutations in 80 disease-associated genes [1]. It is originally divided into two major subgroups, CMT1 and CMT2, according to electrophysiological appraisal and tissue biopsy, along with the molecular diagnostic, and each subgroup is further divided into several subtypes by genetic locus. CMT1 is a demyelinating peripheral neuropathy where myelinating Schwann cells are affected and median nerve motor nerve conduction velocity (MNCV) is below 38 m/s [2]. CMT2 is characterized by axonopathy with MNCVs normal (>40-45 m/s) or slightly reduced (30–40 m/s) [3]. Another term of intermediate CMT was coined to cover an overlapping situation between CMT1 and CMT2, with both myelin and axonal phenotypes and MNCVs between 25 and 45 m/s [2,4].

The mutations of *NEFL* gene cause either CMT2E or CMT1F phenotype, comprising about 0.8% to 2% in all CMT patients according to different research [5-7]. *NEFL* encodes neurofilament light-chain polypeptide, belonging to one of the neurofilament triplet subunits, which are *NEFL* (Neurofilament light chain), NEFM (Neurofilament middle chain) and NEFH (Neurofilament heavy chain) respectively [8,9]. Neurofilaments (NFs) are neuron-specific and crosslink with each other to form a highly stable cytoskeleton in large myelinated axons [10,11]. The widely extended network is responsible for maintaining the axonal diameter and transport [11,12]. Pro22Ser mutation of *NEFL* could cause CMT2E, which was first reported in a large Slovenian family by Georgiou in 2002 [13]. A case in a Japanese family showed that the symptom of CMT2E could be resulted from Pro22Thr mutation [14]. Another study by Shin reported Pro22Arg lead to CMT1F phenotype [15].

Here we reported a Chinese family, and ten of the family members were troubled by distal limb weakness and had difficulty in running as their normal counterparts in their second to third decades. CMT was diagnosed by electrophysiological examinations and typical clinical manifestations. Genetic analysis revealed that the mutation of *NEFL*, p. Pro22Ser, (c.64C>T, NM_006158) might be responsible for the disease.

## Materials and Methods

### Patient data

A single Chinese CMT family was recruited to participate in the study, including five patients and seven healthy controls (Figure 1). It should be noticed that II-14 and II-15 were twin brothers with distinctly different phenotypes while living in the totally same environment since their birth. The patients did not perform nerve or muscle biopsy yet.

### Electrophysiological study

Needle Electromyography (EMG) was used to examine the right abductor pollicis brevis, left first interosseous muscle, left vastus

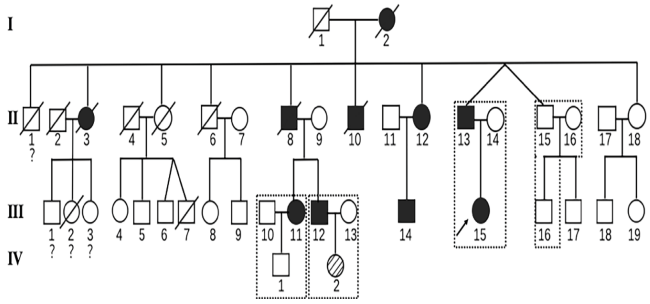


**Figure 1:** The open symbols represent unaffected males (open square) and unaffected females (open circle), and the filled symbols affected males (solid square) and affected females (solid circle). IV-2 with a slash-filled symbol was asymptomatic but genetically positive. The arrow indicates the proband, and dotted boxes indicate the available Deoxyribonucleic Acid (DNA) samples. The question mark means data is not available.

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**Received** February 25, 2020; **Accepted** March 05, 2020; **Published** March 12, 2020

**Citation:** Zhao W, Ye J, Liang Q, Hou Q (2020) A Pro22Ser Mutation in *NEFL* Results in Charcot-Marie-Tooth Disease in a Chinese Family. J Clin Case Rep 10: 1328

intermedius and left sternocleidomastoid muscle for the Motor Unit Potentials (MUPs) analysis at minimal and maximum voluntary efforts. Nerve Conduction Studies (NCS) on median, ulnar, peroneal and tibial nerves were performed under standardized conditions and in accordance with the requirements of the CMT neuropathy score (CMTNS) [16]. The skin temperature of wrist and ankle was at least 27°C.

### DNA extraction

5 mL EDTA-anticoagulated peripheral blood was collected from the proband. DNA samples was extracted from whole blood with DNA extraction kit (Bestnovo, Taizhou, China) following manual protocols.

### Sequencing and PCR

Genetic analysis was performed using an CMT and hereditary spastic paraplegia gene panel consisting of 122 genes by next-generation sequencing (Supplemental Table 1). The SNPs of *NEFL* were verified using Sanger sequencing. The primers used were as follows: Forward: 5' GAGCCGCACACAGCCATCCAT 3', Reward: 5' CTGCTCGTACAGCGCCCGGAA 3'.

The PCR were carried out in a final volume of 30 μL containing 1 μL template DNA (50 ng/μL), 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 15 μL 2 × Taq MasterMix (CWBIO, China) and 13 μL RNase-free $H_2O$ (CWBIO, China). PCR amplification conditions were 98 °C for 2 min, followed by 35 cycles at 98°C for 10 s, 65°C for 15 s and 72°C for 15s, and a final extension at 72°C for 3 min. All reactions were conducted using a thermal cycler (Veriti 96, Applied Biosystems, USA). The PCR products were separated by capillary electrophoresis using an ABI3730 lx DNA Analyzer (Applied Biosystems, Foster City, USA).

## Results

### Clinical features of the patients with *NEFL* mutation

III-15, the proband, complained of a difficulty in walking normally with a pair of slippers at the age of 12 years old. In a later stage after three years, she began to feel a weakness in the calf muscle and the weakness would be aggravated in winter. Shoes with heels of moderate height made walking easier. The patient was diagnosed with "peripheral neuropathy" at 19 years old. Neurological examination showed difficulty in heel walking, absence of knee jerk reflex and atrophy of thenar muscles and interosseus muscles. Tiptoe walking was relatively normal. Hypoesthesia and peroneal muscular atrophy were not observed. She did not display any signs of cranial nerve involvement, nor any of other affected members.

II-13, the father of the proband, showed a much severe feet deformity and peroneal muscular atrophy, and could only walk with the assistance of braces at the time of study (Figure 2). The atrophy of hand muscles resulted in claw hand deformity and difficulties in fine motor function, such as doing buttons. His fraternal twin brother II-14 exhibited neither amyotrophy nor deformity, and no neuropathic signs was found by physical examination. II-8, who died of heart failure at the age of 63 years old, showed slightly milder symptoms with muscular weakness and slow walking speed. The two offspring of II-8, III-11 and III-12 were also affected. The regular physical exercise helped them to alleviate clinical situation. Clinical features of all affected members are summarized in Table 1.

### Electrophysiological results

Needle EMG was performed on III-15 to examine the function of representative muscles. Results were summarized in Table 2.

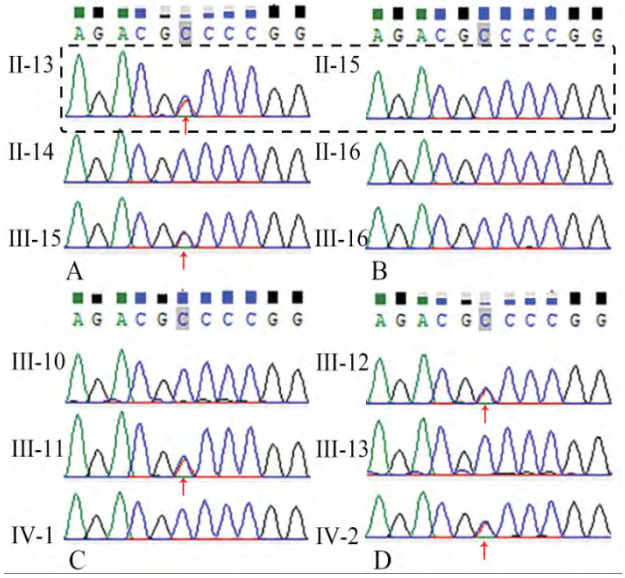


**Figure 2:** Peroneal muscular atrophy, *pes cavus*, curled toes and psoriasis in the distal lower limbs of II-13 (A, B) but not his twin brother II-14 (C, D).

The MUP duration of right abductor pollicis brevis was prolonged. High amplitudes were recorded in the right abductor pollicis brevis, left first interosseous muscle, left vastus intermedius but not left sternocleidomastoid muscle. Increased percentage of polyphasic MUPs was found in the right abductor pollicis brevis and left vastus intermedius, 37% and 20% respectively. Upon increasing the strength of muscle contraction, motor unit recruitment is reduced and MUPs manifest as simple pattern and giant potentials in all but the left sternocleidomastoid muscle. These results indicate neurogenic injuries in the four limbs.

Nerve Conduction Studies (NCS) were performed in III-15 and II-15. NCS results were suggestive of mixed demyelinating and axonal neuropathy (Table 3). III-15 showed generally prolonged terminal latencies in motor nerves except for left ulnar nerve while those of II-15 were in normal range, indicating demyelinating neuropathy. Motor conduction velocities of all tested nerves, except for left median nerve, were slowed to varying degrees. Sensory conduction velocities of left ulnar and right peroneal nerves were declined and the amplitudes of right median and left ulnar were reduced. Decreased F-wave ratio indicates demyelination. II-15 showed slightly reduced amplitudes in left motor peroneal nerve due to an accident trauma in left leg one year ago. These results indicate widespread demyelinating and axonal neuropathy in III-15 but not II-15.

### Molecular genetic analysis

The proband took a clinical NGS (Next Generation Sequencing) panel of CMT and hereditary spastic paraplegia, including 122 associated genes (Supplemental Table 1). The results indicated a heterozygous missense mutation of *NEFL* gene. The mutation of c.64 C>T located in the first exon of *NEFL* and resulted in a Pro22Ser amino acid substitution. To identify whether the mutation was responsible for the disease phenotypes manifested in the pedigree, twelve members from four families (FII-13, FII-15, FIII-10 and FIII-12, respectively, Figure 2, dotted box) were recruited to take genetic tests by sanger sequencing. The results verified that the mutation of c.64 C>T was

| Patient | Sex | Age at Study | Decade of Onset | Initial Symptoms | Gastrocnemius Atrophy | *Pes Cavus* | Aggravating Factors | Additional Disorders | Dying of |
|---|---|---|---|---|---|---|---|---|---|
| I-2 | F | 69 | NA | NA | Yes | Yes | NA | NA | Stroke |
| II-3 | F | 55 | NA | NA | NA | NA | NA | Diabetes | Diabetes |
| II-8 | M | 64 | NA | NA | NA | NA | NA | Cancer; HF | HF |
| II-10 | M | 49 | 1st | Abnormal gait | Yes | Yes | NA | NA | Stroke Diabetes |
| II-12 | F | 60 | 2nd | NA | Yes | Yes | NA | Diabetes | |
| II-13 | M | 57 | 2nd | Muscle weakness | Yes | Yes | Drinking | Diabetes; Hypertension; Psoriasis | |
| III-11 | F | 38 | 3rd | Muscle weakness | No | No | Fatigue;season | No | |
| III-12 | M | 36 | 2nd | Muscle weakness | Yes | Yes | NA | No | |
| III-14 | M | 33 | 2nd | Unsteady gait | Yes | Yes | NA | No | |
| III-15 | F | 29 | 2nd | Muscle weakness | Yes | Yes | Winter;weather | No | |
| IV-2 | F | 13 | ___ | No | No | No | No | No | |

NA: Not Available; HF: Heart Failure
Red number indicates the lifespan of deceased members

**Table 1:** Clinical features of affected members investigated in this study.

| MUPs / Muscles | Minimal effort (n=20) | | | | Maximal effort | |
|---|---|---|---|---|---|---|
| | Duration (ms) | ↑↓ | Amplitude (μV) | Polyphasic (%) | Pattern | Peak Amplitude (mV) |
| Right abductor pollicis brevis | 12.9 | 34%↑ | 4749 | 37 | Simple pattern | 6.0 |
| Left first interosseous muscle | 11.1 | 16%↑ | 4851 | 12 | Simple pattern | 7.3 |
| Left vastus intermedius | 13.6 | 14%↑ | 6991 | 28 | Simple pattern | 10.0 |
| Left sternocleidomastoid muscle | 10.7 | 22%↑ | 825 | 0 | Mixed pattern | 4.1 |

**Table 2:** Needle EMG test of representative muscles of III-15.

| Motor nerves | | Latency (ms) | | | | Amplitude (mV) | | | | Conduction velocity (m/s) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Terminal | | Proximal | | Terminal | | Proximal | | | |
| | | III-15 | II-15 | III-15 | II-15 | III-15 | II-15 | II-15 | II-15 | III-15 | II-15 |
| Median | L | 6.6 | 3.7 | 10.8 | 8.7 | 0.9 | 8.3 | 3.3 | 8.7 | 50 | 53.4 |
| | R | 5 | 4.2 | 10.8 | 9.1 | 3.8 | 10.3 | 2.6 | 9.4 | 34 | 50.9 |
| Ulnar | L | 3.4 | 2.5 | 8.4 | 7.3 | 4 | 8.1 | 3.2 | 7.2 | 42 | 54 |
| | R | 4 | 2.7 | 8.5 | 7.5 | 2.7 | 8.2 | 2.7 | 8.1 | 44 | 54 |
| Peroneal | L | NR | 3.9 | NR | 12.1 | NR | 3 | NR | 1.8 | NR | 41 |
| | R | 7.9 | 4.6 | 20.4 | 12.7 | 0.05 | 4.9 | 0.06 | 3.6 | 24 | 44.2 |
| Tibial | L | 10.4 | 3.8 | 24.1 | NR | 0.1 | 11.1 | 0.1 | NR | 23 | NR |
| | R | 16 | 3.3 | 30.8 | NR | 0.4 | 14 | 0.1 | NR | 25 | NR |

**Table 3:** Nerve conduction studies of representative family members.

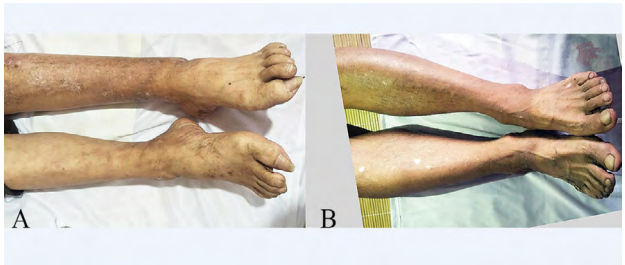


**Figure 3:** Sequencing results of the neurofilament light chain polypeptide (*NEFL*) gene in FII-13 (A), FII-15 (B), FIII-10 (C) and FIII-12 (D). c.64C > T, p. Pro22 Ser mutation was found in II-13, III-11, III-12, III-15 and IV-2, but not in the other asymptomatic subjects. Dashed box represents the results of the twin brothers.

found in all the patients (III-15, II-13, III-11, and III-12), and absent in the other healthy family members. The mutation was well cosegregated with affected members in the autosomal dominant pattern (Figure 3). It was notable that IV-2, the granddaughter of II-8, was a mutation carrier without displaying any symptoms. It was consistent with CMT process since most patients developed symptoms during or after their adolescence. Continuous observation of clinical situation would be necessary for IV-2.

## Discussion

We reported a missense mutation of *NEFL* (c.64C>T, p. Pro22Ser) was linked with CMT (Charcot-Marie-Tooth) disease in a Chinese pedigree. The mutation was cosegregated with the affected members of the pedigree obviously according to the results of the genetic tests. Distinctly different genetic and clinical backgrounds of the twin brothers (II-13 and II-15) further strengthened this conclusion. Previous research had been reported that the mutation was defined as "pathogenic" in the Clinvar Database and could lead to different subtypes of CMT disease [13-17]. Our electrophysiological detects supported a mixed demyelinating and axonal alteration with prolonged terminal latencies and moderately slowed motor conduction velocities. These results were consistent with previous study by Fabrizi's group [17], but different with "an axonal pathology" result made by Georgiou's group [13].

The onset ages were different among the affected members of the pedigree although the mutation was the same. II-10 had an earliest onset age in the first decade while most onset age was at the second decades of the patients. The severity of clinical symptoms varied significantly. The onset age of II-8 and II-13 was similar, however, the symptoms of II-13 progressed more quickly and certain physical support was necessary for his ambulation. Their different lifestyle could be one of reasonable explanations. II-8 was a factory worker with manual labor, while II-13 rarely did exercise in daily life with much more alcoholic intake than II-8. Frequently falling down and necrosis of the femoral head aggravated II-13's condition.

On the other hand, the offspring of II-8, especially III-11(F/38), showed milder symptoms than her peers. She didn't show peroneal muscular atrophy and curled toes at the time of study but complained of weakness and atrophy of thenar muscles and occasional falling. It is likely that some protective genetic factors exist in this branch. And we also found that affected males seem to have a more severe situation than females. The two affected members with an earliest onset age were II-10 and III-14, who are both males. This observation was consistent with the previous study by Fabrizi's team where the proband (F/38) was almost asymptomatic but her two sons developed CMT disease early in their childhood [17].

Neurofilaments (NFs) belonged to the family of intermediate filaments(IFs)with a diameter of 10 nm between actin and myosin filaments, which were neuron-specific and composed of three subunits of *NEFL*, NEFM and NEFH [18]. *NEFL* was indispensable for NF assembly. It could both self-assemble or assemble with other subunits, while NEFM and NEFH could only assemble in the presence of *NEFL* [19,20]. It was revealed that the Pro22Ser mutation of *NEFL* gene spoiled the ability of *NEFL* to form a filamentous network [21,22], resulting in a compromised function [23-30].

## Conclusion

Beyond its role in maintaining a normal neurological function, *NEFL* had been reported to be associated with cancer development as a tumor suppressor gene. Accumulating evidence supported that loss of heterozygosity (LOH) of *NEFL* was involved in the carcinogenesis of several kinds of cancers. One of CMT patients (II-8) in our study was diagnosed with esophageal cancer and cardia cancer at age of 63-years-old. It remained obscure and would be valuable to explore whether the functional role of *NEFL* could be linked between the development of CMT and cancer.

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