MPZ gene variant site in Chinese patients with Charcot–Marie–Tooth disease

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Abstract
Background: Charcot–Marie–Tooth disease (CMT) is a hereditary monogenic peripheral nerve disease. Variants in the gene encoding myelin protein zero (MPZ) lead to CMT, and different variants have different clinical phenotypes. A variant site, namely, c.389A > G (p.Lys130Arg), in the MPZ gene has been found in Chinese people. The pathogenicity of this variant has been clarified through pedigrees, and peripheral blood-related functional studies have been conducted.

Method: Whole-exome sequencing and Sanger sequencing were used to detect the c.389A > G (p.Lys130Arg) variant in the MPZ gene in family members of the proband. Physical examination was performed in the case group to assess the clinical characteristics of MPZ site variants. The expression of MPZ and phosphorylated MPZ in the blood of 12 cases and 12 randomly selected controls was compared by RT-qPCR, Western blotting, and ELISA.

Results: The proband and 12 of her family members presented the AG genotype with different clinical manifestations. The expression of MPZ mRNA in the case group was increased compared with that in the control group, and the levels of MPZ and phosphorylated MPZ in peripheral blood were higher than those in normal controls.

Conclusion: The heterozygous genotype of the c.389A > G (p.Lys130Arg) variant in the MPZ gene mediated the increase in MPZ and phosphorylated MPZ levels in peripheral blood and was found to be involved with CMT.

KEYWORDS
Charcot–Marie–tooth disease, genetic, MPZ, phosphorylation, variant

Funding information
This work was supported by the Key Research and Development Project of Ningxia Hui Autonomous Region [grant number 2021ZDYF0557]
1 | INTRODUCTION

Charcot–Marie–Tooth disease is a hereditary monogenic peripheral nerve disease with high clinical and genetic heterogeneity and an incidence of approximately 1/2500 (Otani et al., 2020). As a hereditary motor and sensory neuropathy (HMSN) characterized by progressive muscular weakness in the distal extremities and muscular atrophy with a decreased or absent tendon reflex, CMT is also divided according to two main patterns: demyelinating patterns in CMT1, CMT3, and CMT4 and axonal patterns in CMT2, CMT5, and CMT6 (Reilly et al., 2011). At present, CMT is the most common familial peripheral neuropathy, accounting for approximately 90% of all hereditary neuropathies. More often, the onset is in childhood, adolescence or late adolescence; the onset is insidious; and the disease progresses slowly, showing obvious familial clustering and genetic patterns, including autosomal dominant, autosomal recessive and X-linked inheritance (Ramchandren, 2017). In the past few decades, the number of pathogenic genes found in CMT has sharply increased; thus far, more than 80 pathogenic genes containing more than 1000 variants have been found to be involved in the pathogenesis of CMT (Timmerman et al., 2014). In particular, in recent years, an increasing number of studies have suggested that MPZ plays an important role in the onset of CMT in Chinese patients (DiVincenzo et al., 2014).

MPZ, located on chromosome 1q22-q23, encodes the myelin zero P0 protein (P0, MPZ). P0, a type I transmembrane protein belonging to the immunoglobulin (Ig) superfamily, is mainly expressed in Schwann cells (SCs) during myelination to promote myelin formation and accounts for approximately 50% of the myelin structural proteins in the peripheral nervous system (Lemke et al., 1988). Previous research showed that MPZ variants most commonly affected the P0 immunoglobulin structural domain and the morphology and integrity of the myelin sheath, leading to peripheral neuropathies, including CMT, hereditary motor sensory neuropathy III, hereditary ataxia with muscle atrophy syndrome and congenital hypomyelinated neuropathy type 2 (CHN2) (Corrado et al., 2016). Moreover, both P0 protein deficiency and overexpression contributed to hypomyelination and peripheral neuropathy in a mouse model (Rücker et al., 2004); similarly, destruction of the endoplasmic reticulum and apoptosis were observed in an MPZ-overexpressing cell model (Chang et al., 2019).

However, the MPZ variant involved in the pathogenesis of CMT showed differences due to the different variant sites, which leads to considerable diversity in the severity of clinical phenotypes and pathological damage (Pareyson et al., 2017). Clinical studies have reported that more than 200 different MPZ variants present an association with a variety of subtypes of CMT and a wide range of clinical manifestations, from CMT3, in which severe Dejerine–Sottas syndrome is noted in infancy; to CMT1, in which the time of onset is in the adolescent period and the symptoms increase and gradually worsen until the patient becomes disabled; to CMT2, in which nerve conduction velocity (NCV) is not significantly affected until the later onset in adulthood with a benign phenotype in adolescence (Corrado et al., 2016; Epure et al., 2014; Ghanavatinejad et al., 2020).

CMT is a global genetic disease without any unified criteria for its diagnosis at present. Despite growing evidence indicating that MPZ participates in the pathogenesis of CMT, its specific pathogenesis has not yet been reported. In particular, relatively little information about MPZ variants in CMT patients in China is available, and only a few studies have reported the occurrence of CMT caused by pathogenic gene changes, while reports on the detection related to MPZ variants are even rarer. Therefore, a study of MPZ gene variants in Chinese patients with CMT is of great clinical value (He et al., 2018; Sun et al., 2017; Wang et al., 2015).

2 | MATERIAL AND METHOD

2.1 | Gene sequencing of the proband

In the early stage of the experiment on May 1, 2020, the General Hospital of Ningxia Medical University outpatient service collected peripheral venous blood from a 28-year-old female patient. Her main clinical symptoms were disturbance of superficial and proprioceptive sensation in both the upper and lower limbs with symmetrical muscular atrophy in the distal extremity, especially the thenar, hypothenar and gastrocnemius muscles, and weak and normal flexion of the extended fingers of the left hand. Walking steppage gait, talipes cavus and Romberg’s sign were positive. Electromyogram (EMG) showed that the bilateral median nerve, ulnar nerve, common peroneal nerve, sural nerve and posterior tibial nerve were severely involved, demyelination was present, and the axons were seriously damaged. However, cranial computerized tomography showed unspecified abnormal findings. Her blood samples were successively sent to Shanghai Xiangyin Biotechnology Co., Ltd. (http://www.xiangyin.org.cn) (Shanghai, China), for multiple links amplification technology sequencing (MLPA) and Chigene (Beijing, China) Translational Medical Research Center Co., Ltd., for whole-exome sequencing (WES) (https://www.chigene.cn) after obtaining her informed consent. The total exonic region of 20,000 genes in the human genome was analyzed in the peripheral blood from the proband by
WES, and it was found that the MPZ gene c.389A > G (exon 3) site variant was present in this case with a heterozygous genotype. There was an amino acid change of p.K130R (p.Lys130Arg) (NM_000530) caused by a single-nucleotide polymorphism (SNP) at rs281865127.

2.2 Sanger sequencing

There were 64 members of this family within four generations, 17 of whom had different clinical symptoms but 41 of whom were normal. Two members of the first generation had died, one of whom had clinical symptoms, and 5 members of the family had unknown status. Peripheral venous blood was taken from the 12 suspected patients with symptoms, including the proband, within the four generations as the experimental group, and peripheral venous blood was collected from the proband’s offspring, an 8-month-old boy. Additionally, informed consent was obtained from those members (informed consent for the child was signed by both parents on his behalf). At the same time, peripheral venous blood was collected from 43 normal volunteers at Ningxia Medical University and General Hospital of Ningxia Medical University, and the control group also provided informed consent. All blood samples were sent to Genesky Biotechnologies, Inc. (http://www.geneskybiotech.com) (Shanghai, China), for Sanger first-generation sequencing for MPZ rs281865127 genotypes in the experimental group and control group. Ethylenediaminetetraacetic acid (EDTA) solution was added to all blood samples.

2.3 Real-time quantitative PCR

Whole blood samples were selected from 12 patients in the case group and 12 randomly selected healthy controls. The RNAsimple Total RNA Kit (TIANGEN, China, Beijing) was used for RNA extraction with the following steps. First, a 3 volumes of TRizol reagent was added to 300 μl of each blood sample. Samples were mixed thoroughly, stored on ice for 10 min and then centrifuged at 12,000 rpm for 10 min at 4°C, and 0.5 volumes of anhydrous ethanol was added to the supernatant and mixed vigorously. Next, these samples were centrifuged for 30 s, the waste liquid was discarded, 500 μl of RD liquid was added, and the mixture was incubated for 2 min. After centrifuging for 30 s at 4°C, 500 μl of DW liquid was added, and the mixture was incubated for 2 min, followed by centrifuging for 30 s; this cycle was repeated once more. Then, 30 μl of ddH2O was added, and the mixture was centrifuged for 2 min; after drying, the liquid was collected to quantitate the RNA by measurement of the A260 using a NanoDrop. The PrimeScript™ RT reagent Kit with gDNA Eraser (Takara, Shiga, Japan) was used to reverse transcribe the RNA into cDNA.

Primer 5.0 was used to design a pair of primers: MPZ, sense strand, CTATCCTGGCTGTGCTGCTCTTC and antisense strand, TGGACCTCCCTGTGGGTGTAAC. The above cDNA template was used to verify the results. Then, according to the manual included with TB Green Premix Ex Taq™ II (Tli RNaseH Plus) (Takara), each sample was mixed in the following proportions: cDNA: MPZ-F: MPZ-R: Mix: ddH2O = 2 μl: 1 μl: 1 μl: 12.5 μl: 8.5 μl, where the concentration of each primer was diluted to 10 μM. Three replicates were run for each sample. The CFX96 real-time PCR Detection System was used, and the reaction conditions were 95°C for 30 s, (95°C for 5 s, 61.5°C for 30 s) × 40 cycles, and 95°C for 10 s. In addition, the relative mRNA expression levels normalized to GAPDH were calculated using the ΔΔCT method.

2.4 Elisa

Whole blood samples from 12 cases and 12 controls were centrifuged at 5000g for 15 min at room temperature (RT) to separate the serum and blood cells, and the serum was collected in Eppendorf (EP) tubes for ELISA performed according to the protocol of the Human Protein Zero Myelin ELISA Kit (OM305274, Omnimabs, California, USA). Fifty microlitres of serum was added to each well, and three replicates were run per sample. The MPZ level of each sample was calculated by measuring the absorbance at 450 nm.

2.5 Western blotting

Three cases and three controls were randomly selected for Western blotting analysis of MPZ, and 4 cases and 4 controls were randomly selected for analysis of phosphorylated MPZ (p-MPZ). Precooled cell lysis buffer was added in the following proportion of lysis buffer: protease inhibitors: phosphatase inhibitors: 100 mM PMSF (all reagents were from TIANGEN) = 1 ml: 1 μl: 10 μl: 5 μl. A 7-fold volume excess of prepared lysis buffer was added to dilute the whole blood, and the mixtures were shaken vigorously before being placed on ice. Next, the samples were allowed to stand for 5 min and shaken for 30 s, and the previous step was repeated 4 times. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant was collected and placed into 5X SDS–PAGE loading buffer (Beyotime, Shanghai, China). Finally, the samples were boiled at 100°C for 10 min.

According to the manufacturer’s instructions for the SDS–PAGE Gel Preparation Kit (KeyGEN, Shanghai,
China) for the 5% concentrated gel and 12% separating gel, 15 μl of sample was loaded per well, and 6 μl PageRuler™ Prestained Protein Ladder (Thermo Fisher Scientific, MA, USA) was used as a reference. The gel was run at 70 V for approximately 50 min and then changed to 90 V until the dye reached the bottom. Samples were transferred to a membrane at 100 V for 45 min, after which, the blot was blocked in 5% BSA for 1.5 h with shaking at room temperature (RT) to block nonspecific binding sites. Blots were incubated with the following primary antibodies overnight at 4°C: anti-myelin protein zero antibody (Abcam, PR20383, Cambridge, England) (1:1000), phospho-PKC substrate antibody (Cell Signaling Technology, #2261, MA, USA) (1:1000) and GAPDH polyclonal antibody (Proteintech, 10,494-1-AP, Chicago, USA) (1:5000). Next, the membranes were washed 6 times for 5 min at RT and then incubated with goat anti-rabbit IgG H&L (IRDye® 800CW) preadsorbed (Abcam, ab216773) (1:2000) for 1.5 h with shaking at RT in the dark. The images were captured in the same field of view using an ODYSSEY CLx (LI-COR, Nebraska, USA) after rinsing the membrane.

3 | RESULTS

3.1 | Variant sites and the genotypes

MLPA suggested that the gene copy number and common pathogenic genes and loci of CMT, including PMP22 repeat variants, GJB1 variants and common variant sites of MPZ, were not changed in the proband (Figure S1). WES analysis revealed heterozygous genotype (AG) at the c.389A > G (p.K130R) variant in exon 3 of the MPZ gene (rs281865127). The rs281865127 genotype in other family members and normal controls was detected by Sanger sequencing. The genotype at rs281865127 of the family members with clinical symptoms was AG, while the genotype of the normal family members without clinical symptoms and controls was AA. Therefore, AG was confirmed to be the patient’s genotype, and rs281865127 was the variant site (Figure 1, Table 1).

3.2 | Pedigree of the CMT family

The pedigree of this family with CMT in Ningxia, China, was constructed based on their medical history collection and genetic sequencing results (Figure 2) and showed that the variant exhibited an autosomal dominant inheritance pattern. Sixty-four members in this family spanning four generations had 17 members with known disease, consisting of 9 males and 8 females. Due to practical restrictions, we have thus far collected blood from 12 patients and an 8-month-old boy who was the proband’s son and did not carry the disease-causing variant. The disease status of 5 members of the fourth generation of this family is unknown because we failed to contact them (their average age was less

MPZ: c. 389 (exon3) A>G

![Figure 1](image-url) MPZ gene map of the variant site of the proband from WES. MPZ exon 3, c.389A > G site variant, SNP rs281865127
than 6 years old). Five male patients with an average age of 41.4 years old and 7 female patients with an average age of 42.3 years old were collected. According to their medical histories, the average age of onset was 6.5 years old for males and 6.3 years old for females, but within the fourth generation, the proband’s son, an 8-month-old boy, was healthy. Hence, it was concluded that there was no sex difference in CMT caused by the heterozygous variant c.389A > G (p.Lys130Arg) in the MPZ gene and that in general, the onset age was approximately 6 years old. Clinical symptoms and statistics suggested that the family’s main clinical symptoms were distal extremity musculature superficial and proprioceptive sensation disorder and lower extremity disorder, and they presented progressive aggravation at the same time. Some symptoms appeared with different degrees of symmetrical muscular atrophy. Most of the patients in this family showed walking steppage gaits with different degrees of numbness in the limbs, among which the most common involvement was the muscles of the distal extremities of the limbs; moreover, at the early stage of clinical manifestation. CMT was superficial and manifested as proprioceptive sensation disorder. However, as CMT progressed, the clinical symptoms of the patients gradually worsened, and more limbs were involved with increasing age (Figure 3, Tables 1 and S1).

### Table 1  Characteristics of study subjects in the entire cohort

| Characteristic                          | Case (n = 12) | Controls (n = 43) |
|----------------------------------------|---------------|-------------------|
| Age at examination (year)              | 41.8          | 29                |
| Age at onset (year)                    | 6.3           |                   |
| Missing                                | 3             | 0                 |
| Sex, n (%)                             |               |                   |
| Male                                   | 5             | 13                |
| Female                                 | 7             | 30                |
| Genotype                               | AG            | GG                |
| Initial site of clinical symptoms at onset |             |                   |
| Upper limb involvement                 | 9             |                   |
| Lower limb involvement                 | 0             |                   |
| The site of the lesion                 |               |                   |
| Upper limb                             | 5             |                   |
| Lower limb                             | 9             |                   |
| Trunk                                  | 0             |                   |
| Autonomic dysfunction                  | 2             |                   |
| Physical examination does not cooperate| 3             | 0                 |
| With other disease                     | Non           | Non               |

#### 3.3  The heterozygous genotype of the c.389A > G (p.Lys130Arg) variant in the MPZ gene resulted in increased mRNA expression of MPZ

RT–qPCR analysis of the 12 cases and 12 controls revealed that the expression of MPZ mRNA was increased in the case group compared with the control group, which suggested that the heterozygous genotype at the c.389A > G (p.Lys130Arg) variant in the MPZ gene resulted in an
increase in MPZ mRNA in the blood of patients relative to healthy people (Figure 4c).

3.4 Increased MPZ protein levels with the heterozygous genotype of the c.389A > G (p.Lys130Arg) variant in the MPZ gene

Three patients and three controls were randomly selected for comparison of the difference in the expression of MPZ protein in whole blood. The MPZ protein levels in Case 1, Case 2 and Case 3 were all higher than the levels in Control 1, Control 2 and Control 3, which indicated that the MPZ level in the whole blood of the patients was increased (Figure 4a,b).

Among 12 cases and 12 controls, serum MPZ levels in the control group remained at a certain level, while the levels of MPZ in the case group were higher than those in the control group and were different for each patient. At the same time, given the medical history and clinical manifestations of all patients, we found that patients with more complicated clinical symptoms and greater organ involvement had higher serum MPZ levels, indicating that MPZ in serum was significantly correlated with the pathogenesis of CMT and associated with the progression of CMT (Figure 5c).
3.5 The c.389A > G (p.Lys130Arg) variant in the MPZ gene is accompanied by an increase in phosphorylated MPZ level in whole blood

We also compared the expression of phosphorylated MPZ and the protein kinase C (PKC)-dependent phosphorylation of MPZ in whole blood from four patients and four controls who were randomly selected. The results showed that the expression of PKC in whole blood was higher in the patient group than in the control group, indicating that MPZ was phosphorylated in a PKC-dependent region. The heterozygous genotype at variant c.389A > G (p.Lys130Arg) in the MPZ gene led to an increase in PKC-dependent phosphorylation of MPZ (Figure 5a,b).

4 DISCUSSION

Analysis of the c.389A > G (p.Lys130Arg) variant in the MPZ gene and related functional studies of this variant in peripheral blood of a CMT family in Ningxia, China, confirmed that the heterozygous genotype led to an abnormal increase in MPZ and phosphorylated MPZ levels and is involved in CMT.

At present, related records of c.389A > G (p.Lys130Arg) in CMT were not found in the 1000 Genome Project Database, Genome Aggregation Database (Genome AD), Genome AD of East Asia or dbSNP database. Moreover, this variant has not been reported among Chinese people in the Human Gene Mutation Database (HGMD, http://www.hgmd.cf.ac.uk/ac/index.php). There is only one case report involving a seven-year-old Japanese girl from 1995, but due to the lack
of relevant genetic testing information of other family members, it is not possible to identify the genetic cosegregation and pathogenicity of this variant (Kamholz & Shy, 2004). However, the current study of approximately 64 people from four generations of a family confirmed that the c.389A > G (p.Lys130Arg) variant is found in Chinese individuals. The pathogenicity of the heterozygous genotype at this variant and its autosomal dominant inheritance were determined according to ACMG genetic cosegregation.

Aetiological investigation has confirmed that genetics is the main pathogenic factor for CMT; thus, genetic testing has become the “gold standard” for the clinical diagnosis of this disease (Sun et al., 2017). MLPA was used to eliminate the possibility of common pathogenic genes of CMT, including PMP22 repeat variants, GJB1 variants and common variants in MPZ, in this family. Furthermore, the pathogenic heterozygous genotype of c.389A > G (p.Lys130Arg) in the MPZ gene was detected by WES, and MPZ rs281865127 was detected by Sanger sequencing of a large number of affected members in this family, which provided a research basis for the pathogenesis and genetic cosegregation of CMT caused by the heterozygous genotype of c.389A > G (p.Lys130Arg).

It has been reported that PKC increased the phosphorylation of L-MPZ in the sciatic nerve of L-MPZ mice compared with wild-type mice. The increase in PKC phosphorylation of L-MPZ may affect the phenotype of L-MPZ mice. In vitro, on the cytoplasmic side of each myelin layer, phosphorylated MPZ causes the accumulation of polypeptides that are negatively charged and mutually exclusive with the phosphorylation sites on the plasma membrane surface, resulting in nonfunctional myelin and leading to CMT-like neuropathy (Otani et al., 2020). Other studies have shown that PKC-mediated phosphorylation of specific residues in the cytoplasmic domain of MPZ is necessary for P0-mediated adhesion, which leads to demyelinating neuropathy in humans (Schenkel et al., 2016). In this study, PKC expression in the case group was higher than that in the control group; therefore, phosphorylated MPZ levels may be higher in the case group than in the control group. We will further confirm the reliability of this conclusion in future experiments.

Previous literature proved that most MPZ variants are associated with the CMT1 type, with fewer associated with the CMT2 type, while other types of CMT were rarely reported; nevertheless, considering the clinical symptoms of the proband, physical examination results, electromyography and symptoms of other family members with an onset age of approximately 6 years old, it was preliminarily determined that the c.389A > G (p.Lys130Arg) variant in the MPZ gene is a rare variant that is associated with coexisting demyelination and axonal involvement; intermediate CMT, with accompanying AD inheritance suggested that the form of CMT caused by c.389A > G (p.Lys130Arg) was autosomal dominant intermediate CMT (DI-CMT), the characteristics of which differ from the infantile onset characteristics of DSD affiliated with the CMT3 type (Xu et al., 2001).

The difficulty of the clinical diagnosis of CMT is increased due to the differences in clinical manifestations and diversity of pathogenic genes of patients with CMT and the fact that there is not an exact supplementary examination to currently support the clinical diagnosis of CMT. Clinical diagnosis is therefore dependent only on some neuroelectrophysiological and imaging detection methods; hence, these challenges increase the difficulty of diagnosis of the disease and reduce the diagnosis rate of CMT (Dalby & Coffin, 2018). The MPZ variant contributed to the increase in MPZ levels in CMT patients compared with normal people. On the one hand, the pathogenicity of the c.389A > G (p.Lys130Arg) variant in CMT was confirmed, and on the other hand, the detection of MPZ in peripheral blood may provide a preliminary reference for the early-stage clinical diagnosis of CMT. It is possible to make an early diagnosis of CMT with a combination of clinical manifestations and a medical history examination when the MPZ level in peripheral blood exceeds a certain concentration. Furthermore, it is expected that peripheral blood MPZ quantification may become a method for prenatal and childhood early screening and diagnosis with the investigation of more samples and more accurate detection of pathogenic concentrations of MPZ in the peripheral blood of CMT patients, which is conducive to the early detection, diagnosis and prevention of CMT.

Due to the lack of unified diagnostic criteria for CMT and genetic differences among different ethnicities, the diagnosis of CMT varies in different regions; for example, the prevalence of CMT in Akshus County in eastern Norway is twice as high as the global average (1:1214), while the reported prevalence of CMT in Japan is lower (1:9200) (Barreto et al., 2016; Bis-Brewer et al., 2020). The diversity of MPZ variants and the clinical signs and symptoms of CMT have promoted in-depth diagnosis and treatment. With the improvement in human genome sequencing and related techniques, an increasing number of MPZ variants have been found worldwide, which is beneficial for comprehensively understanding and assessing the genetic background, occurrence and development of CMT, perfecting and deepening the understanding of CMT, and providing an effective reference for the early clinical diagnosis and prevention of CMT.

5 | CONCLUSION

The heterozygous genotype of the c.389A > G (p.Lys130Arg) variant in the MPZ gene results in an
abnormal increase in levels of MPZ and phosphorylated MPZ, which are involved in CMT.

ACKNOWLEDGMENTS
The authors thank the patients and volunteers for their participation and cooperation. Thanks for Ningxia Key Laboratory of Cerebrocranial Disease, Incubation Base of National Key Laboratory (Yinchuan, China) provided the experimental platform for this research, and also thanks for Dr. Hailiang Li, Dr. Lifei Xiao, MS. Jie Wu, MS. Sling Xv and MS. Zhangping Chen for helping in the experimental operation. The study was supported by the Key Research and Development Project of Ningxia Hui Autonomous Region.

AUTHOR CONTRIBUTIONS
All authors made great contributions and have permitted to submit this manuscript. X. H. was responsible for the detailed design of the whole experiment, specific operation and manuscript written; C. L., Y. L. and T. Z. played a crucial role in collecting the blood sample; H. T., Y. M. and J. D. finished the sample preparation and processing, reagent purchase and preparation; X. L., Y. W. and L. W. mainly completed statistic analysis; P. Y. contributed to the conception of this research, polished the article in written, improved the logical rationality of the article and ensured the final manuscript to submit. All the author worked to finish the discussion.

CONFLICT OF INTEREST
All the authors declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ETHICS STATEMENT
The ethics was approved by General Hospital of Ningxia Medical University Research Ethics Committee [No. 2020–627].

CONSENT FOR PARTICIPATE AND PUBLICATION
All the cases and controls signed the informed consent before we collected their blood, and the informed consent for the child was signed by their parents on their behalf (under the age of eighteen).

DATA AVAILABILITY STATEMENT
The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

**How to cite this article:** Hao, X., Li, C., Lv, Y., Zhou, T., Tian, H., Ma, Y., Ding, J., Li, X., Wang, Y., Wang, L. & Yang, P. (2022). *MPZ* gene variant site in Chinese patients with Charcot–Marie–Tooth disease. *Molecular Genetics & Genomic Medicine*, 10, e1890. https://doi.org/10.1002/mgg3.1890