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Study of antibodies to PMP22, IL-6 and TNF-α concentrations in serum in a CMTX1 family

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Abstract

To further understand X-linked dominant Charcot-Marie-Tooth disease (CMTX1), we followed a family of 22 members in China, including 8 patients, 2 asymptomatic carriers and 12 normal family members. Twenty-two family members as well as 60 normal controls unrelated to this family were screened for point mutation by denaturing high performance liquid chromatography (DHPLC). All patients and asymptomatic carriers from this family, but none of the normal population controls, showed a T–C transition at position 266 in codon 89 of exon 2 of connexin 32, resulting in a leucine to proline (L89P) exchange. To study whether the immune system is involved in the pathogenesis of CMTX1 patients and asymptomatic carriers, we measured serum concentrations of antibodies to peripheral nerve myelin protein 22 (PMP22), interleukin-6 (IL-6) and tumour necrosis factor α (TNF-α) by ELISA. Serological results were also compared with those from GBS patients ($n = 11$) and with normal subjects ($n = 20$). Our analysis showed anti-PMP22 sera reactivity in 50.0% of CMTX1 patients, 63.6% of GBS patients and 10% of normal controls. Our results also indicated that anti-PMP22 antibodies in the CMTX1 family varied with sex. Anti-PMP22 antibodies were found in all male patients but not in all females, which may be one of the reasons that male patients usually have more severe clinical symptoms than that of female patients. There was no statistical difference in serum concentrations of IL-6 and TNF-α between CMTX1 patients and normal subjects. In conclusion, we identified a L89P mutation for the first time in a CMTX1 family in China and an associated response to PMP22 in males.

Keywords: Charcot-Marie-Tooth type X1; Immune; Peripheral myelin protein 22; Cytokines

X-linked dominant Charcot-Marie-Tooth disease (CMTX1) is the second most common cause of hereditary neuropathy, which accounts for 10–20% of patients with Charcot-Marie-Tooth (CMT) [7]. It is characterized by progressive distal extremity weakness, atrophy, sensory loss, and areflexia. Males are more severely affected than females, with usual onset of symptoms in adolescence. CMTX1 has been found to be associated with a mutation in the connexin 32 (Cx32) gene, mapped to chromosome Xq13, which codes for a 283 amino acid gap-junction protein found in myelinated peripheral nerve [1].

Mice mildly overexpressing human peripheral myelin protein 22 kD, expressing half of the normal dose of protein zero (P0± mice) or completely deficient for gap-junction protein connexin 32−/− mimic Charcot-Marie-Tooth (CMT) neuropathies type 1A, 1B and CMT type 1X, respectively [9,10,12,16]. In these models, almost normal myelin formation is observed during the first months of life, followed by a slowly progressing demyelinating neuropathy. There is a substantial increase of CD8+ T-lymphocytes and macrophages within the demyelinating nerves [2,8,9,10,12,16,17]. Elevated numbers of activated T-cells in the peripheral blood and up-regulated major histocompatibility complex (MHC) class II molecules in sural nerves in some CMT patients were found [19]. Some patients with CMT1 have a positive response to steroid treatment. It has been proposed that damage to peripheral nerves in CMT patients could be immune-mediated [13,20–23].

Immunological study of hereditary motor and sensory neuropathy type 1a (HMSN1a) showed some CMT1A patients may have an inflammatory, autoimmune component superimposed on the genetic condition [5]. The purpose of this study was to investigate whether an autoimmune mechanism was involved in the pathophysiologic process in the patients in a CMTX1 family.

All family members were serially examined. Neurophysiological studies were carried out on the proband. The pedigree includes 22 members, from Xinxiang county, Henan Province, China (Fig. 1).

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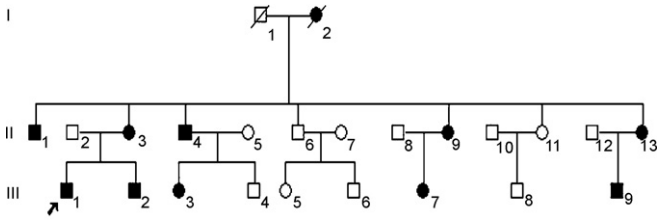

Fig. 1. Pedigree of the family showing no male-to-male transmission, consistent with X-linked dominant inheritance. All family members were recruited for clinical and genetic studies, of which 10 harbored the Cx32 mutation.

Genomic DNA was extracted from peripheral blood lymphocytes by the salting-out method. DNA was diluted in TE to a concentration of 100 ng/μl and stored at 4 °C. The entire coding region of exon 2 in the Cx32 gene was amplified in three PCR fragments. Primers were designed to yield amplicons that had optimal melting profiles estimated with version 4.1 of the Wavemaker software (Transgenomic, Omaha, USA) supplied by the DHPLC manufacture (Table 1). Genomic polymerase chain reaction (PCR) was carried out in 30 μl reaction volume containing 100 ng genomic DNA, 0.2 μM primers, 100 μM dNTPs, 3 μl reaction buffer (100 Mm Tris pH 8.3, 500 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) and 1.5 U Taq (Takara) with the following cycling profile: 3 min denaturation at 94 °C and 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s, followed by a 7 min final extension step at 72 °C. All thermal cycles were run on a Gene Amp® PCR 2700 (Applied Biosystems). Amplicons were checked by 1.5% agarose gel electrophoresis before DHPLC analysis, to make sure that only the specific product was amplified and that no additional band occurred that could lead to artificial heteroduplex conformation.

DHPLC was carried out on a WAVE DNA fragment analysis system (Transgenomic, Crewe, UK). PCR products were examined for heteroduplexes by subjecting 2–5 μl of each PCR product, containing 50–100 ng DNA, that was denatured for 5 min at 95 °C and then gradually reannealed by decreasing sample temperature from 95 to 65 °C over a period of 30 min. In males, duplexes for DHPLC analysis were created by mixing, denaturing, and reannealing a PCR product from a normal subject with the corresponding amplicon of the CMTX1 patient. The PCR products were then separated (flow rate of 0.9 ml/min) through a 2% linear acetonitrile gradient. The starting concentrations of buffer B were selected by WaveMaker software version 4.1 (Transgenomic, Omaha, USA) (Table 1). The standard buffers were prepared from concentrated triethylammonium acetonitrile (TEAA) to give Buffer A = 0.1 M TEAA and Buffer B = 0.1 M TEAA plus 25% acetonitrile. Wash buffer was 75% acetonitrile. Analysis per each amplified sample took 8 min, including column regeneration and equilibration. The oven temperature for optimal heteroduplex separation were determined using the WaveMaker software version 4.1, which gives a computer-assisted determination of melting profile and analytical conditions for each fragment. For each abnormal elution profile, genomic DNA was re-amplified with DHPLC primers and the PCR products were directly sequenced in both directions using a 377 Genetic Analyser (ABI 100, USA) according to PRISM Dye Terminator and Dye Primer Cycle Sequencing chemistries.

Serum samples from the 22 family members above, together with those from 11 patients with GBS and 20 normal controls, were collected and stored at −70 °C. Titers of anti-PMP22 antibodies were measured by ELISA in the serum of all patients and controls as previously described [4,5]. PMP22 first and second extracellular domain peptides (ECD1 and ECD2) were synthesized, cross-linked to bovine serum albumin (BSA) and used to coat ELISA plates. Plates were incubated with patient's serum and bound antibody was detected with horse radish peroxidase (HRP) conjugated rabbit anti-human IgG. Plates were developed with *o*-phenylenediamine dihydrochloride and H_2O_2 and the optical density read at 490 nm. Negative controls (without serum) and sera from normal control subjects were included on each microtiter plate. Sera with an optical density (OD) value greater than three standard deviations above the mean of the normal control sera were considered positive. Titer was defined as the greatest dilution of sera to give a positive value. Serum interleukin-6 (IL-6) and tumour necrosis factor-α (TNF-α) concentrations were measured in CMTX1 patients and normal control, with a quantitative sandwich enzyme immunoassay technique (Quantikine™, R and D Systems, Minneapolis, USA). Optical densities were read at 450 nm with 570 nm wavelength correction, and concentrations were calculated by creating a standard curve with the standards provided.

Calculations were performed using CurveExpert 1.3 software with two tailed tests of significance. Statistically significant differences between two groups were determined by a rank sum test.

The pedigree of the family studied by us is shown in Fig. 1. The proband was 20 years of age. In the clinical diagnosis we distinguish four CMT phenotypes: (1) asymptomatic car-

Table 1
Primers sequences for the exon 2 of Cx32 gene and DHPLC gradient buffer and column temperatures for optional resolution of PCR products

Fragment	Primer name	Sequence	Ampl. size (bp)	DHPLC oven temp. (°C)	Start % buffer B
1	1F	GGTGTTTTGCAGGTGTGAATG	288	62	56
	1R	GGGGTGGAAACTAGGATGAG			
2	2F	TGTGCGGCTGTGGTCCCTG	360	62	58
	2R	AAGACGGTGAAGACGGTTTTC			
3	3F	TGGTGCGGCTGGTCAAGTG	413	63	61
	3R	AGGGCAGGGTCGGGGGTG			