

Copy number variations are a rare cause of non-CMT1A Charcot-Marie-Tooth disease

Jia Huang · Xingyao Wu · Gladys Montenegro · Justin Price ·
Gaofeng Wang · Jeffery M. Vance · Michael E. Shy · Stephan Züchner

Received: 9 October 2009 / Revised: 12 November 2009 / Accepted: 13 November 2009 / Published online: 1 December 2009
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Abstract Hereditary peripheral neuropathies present a group of clinically and genetically heterogeneous entities. All known forms, including the various forms of Charcot-Marie-Tooth disease (CMT) are characterized as Mendelian traits and over 35 genes have been identified thus far. The mutational mechanism of the most common CMT type, CMT1A, is a 1.5 Mb chromosomal duplication at 17p12 that contains the gene *PMP22*. Only recently it has been realized that such copy number variants (CNV) are a widespread phenomenon and important for disease. However, it is not known whether CNVs play a wider role in hereditary peripheral neuropathies outside of CMT1A. In a phenotypically heterogeneous sample of 97 patients, we performed the first high-density CNV study of 34 genomic regions harboring known genes for hereditary peripheral neuropathies including the 17p12 duplication region, with comparative genomic hybridization (CGH) microarrays. We identified three CNVs that affected coding exons.

A novel shorter form of a *PMP22* duplication was detected in a CMT1A family previously tested negative in a commercial test. Two other CNVs in *MTMR2* and *ARHGEF10* are likely not disease associated. Our results indicate that CNVs are a rare cause for non-CMT1A CMT. Their potential relevance as disease modifiers remains to be evaluated. The present study design cannot rule out that specific CMT forms exist where CNVs play a larger role.

Keywords Copy number variation · Charcot-Marie-Tooth disease · CMT1A · Peripheral neuropathies

Introduction

Hereditary peripheral neuropathies comprise a clinically and genetically heterogeneous group of diseases with a prevalence of 1/2500 [1]. All subtypes with motor and sensory deficits are known as Charcot-Marie-Tooth (CMT) diseases and make up the majority of peripheral neuropathies. A clinical spectrum can be drawn from pure motor neuropathies, over motor and sensory, to pure sensory forms of peripheral neuropathies. Genetically more than 35 genes have been identified, though there is often not a strict genotype–phenotype correlation (Inherited Peripheral Neuropathies Mutation Database: <http://molgen-www.uia.ac.be>). With the identification of the underlying genes, the screening for mutations has become a mainstay in the diagnosis of peripheral neuropathies. Although current testing batteries are still limited to a few more common CMT forms it is generally expected that the scope of these tests will greatly increase in the near future. The state of the art approach for genetic testing in CMT is based on direct sequencing. This technique is suited to detect point mutations and small insertions/deletions with a high sensitivity

Electronic supplementary material The online version of this article (doi:10.1007/s00415-009-5401-2) contains supplementary material, which is available to authorized users.

J. Huang · G. Montenegro · J. Price · G. Wang ·
J. M. Vance · S. Züchner
Hussman Institute for Human Genomics,
Miller School of Medicine, University of Miami, Miami, USA

X. Wu · M. E. Shy
Department of Neurology, School of Medicine,
Wayne State University, Detroit, USA

S. Züchner (✉)
Department of Human Genetics, Hussman Institute
for Human Genomics, University of Miami Miller School
of Medicine, Biomedical Research Building, Room 523,
LC: M-860, 1501 NW 10 Ave, Miami, FL 33136, USA
e-mail: szuchner@med.miami.edu

and specificity. However, it is not possible to directly detect chromosomal gains or losses that extend beyond the reach of polymerase chain reaction (PCR) primers, usually a genetic distance of less than 1,000 bp. The majority of exons fall into this range, but copy number variations that include several exons or a large portion of an intron would be missed by conventional methods. Importantly, the major subtype of demyelinating CMT, CMT1A, is caused by a duplication of a 1.5 Mbp fragment of chromosome 17p12 that contains the causative gene *PMP22*. The opposite event, a loss of *PMP22* is associated with a different peripheral neuropathy, hereditary neuropathy with liability to pressure palsies (HNPP) [2, 3]. No other CMT form is known to be caused by copy number variations (CNV). With the recent availability of high-density custom microarrays, the opportunity arises to screen the complete set of CMT genes for CNVs. In this study we performed the first high density CNV screen on a clinically heterogeneous sample of 97 CMT cases.

Materials and methods

CMT sample description

We studied 97 unrelated index patients with inherited peripheral neuropathies. Seventy patients were collected at the CMT clinic at Wayne State University and thirty patients came from the Miami Institute for Human Genomics. The majority (65) of these patients had an axonal phenotype, in accordance with the fact that the genetics of axonal neuropathies is least well understood (Table 1). We also included 17 demyelinating neuropathies, two dominant intermediate CMT patients, five sensory neuropathies, and three predominantly motor neuropathies. In five patients the neuropathy was not further categorized. The majority of these patients had been screened for the most frequent CMT genes. All participating individuals gave informed consent prior to initiating this study in agreement with the institutional review boards at Wayne State University and the University of Miami.

Table 1 Clinical phenotypes included in this study

Neuropathy phenotype	Number of patients
Axonal	65
Demyelinating	17
Dominant intermediate	2
Pure sensory	5
Pure motor	3
Unknown	5

Custom CGH microarray

CNVs were detected using NimbleGen custom 385 k 4-plex comparative genomic hybridization (CGH) microarrays. We selected 34 genomic regions harboring known genes for hereditary peripheral neuropathies, including the 17p12 duplication region (Table 2). Microarrays consisting of 72,000 probes per array were manufactured by Roche/NimbleGen. Probes had an average length of 54 bp and were designed to achieve isothermal hybridization conditions. The average distance between probes within each region is 64 bp. Hybridizations were performed by Roche NimbleGen as a standard service. A pool of eight anonymous DNA samples, four males, four females (Promega), served as reference DNA for the CGH reaction. CGH data were normalized with NimbleScan 2.4 (Roche/NimbleGen) and segmented with Nexus 3.1 (BioDiscovery) using the rank segmentation algorithm. The significance threshold for segmentation was set at 10^{-6} and the minimal number of probes required per segment was 100. Copy number gains and losses were defined with a \log_2 Cy3/Cy5 ratio of 0.3 and -0.3 , respectively; high gains and homozygous losses were defined with a ratio of 0.5 and -0.7 . The X-chromosome encoded gene *GJB1* (CMTX1) was excluded from the analysis as the reference DNA consisted of a pool of both genders. Data were further inspected manually by direct visualization using SignalMap (Roche NimbleGen).

Quantitative PCR assays

To confirm detected copy number variations we designed qPCR assays. Quantitative PCR was performed with Taqman assays and SYBR green-based quantification. A custom Taqman assay was designed for exon 15 of *MTMR2*. Sequences of the primers and probe are: *MTMR2*-ex15-PF: 5'-CCAGCATGCGCCACCTA-3'; PR: 5'-GTGGTTTC ATCCGTGGATTCCA-3'; Probe: 5'-CTGGGTGGGATA TTAC-3'. qPCR was performed using the Taqman Universal PCR Mastermix (Applied Biosystems) with the RNase P assay kit as endogenous control on a 7900HT Fast Real-Time PCR System (Applied Biosystems) with the manufacturer-recommended PCR conditions. For SYBR green-based detection, primers were designed for exon 2 of *ARHGEF10* and exon 8 of *IPO8* (endogenous control). qPCR was performed using the DyNamo HS SYBR Green qPCR Kit (New England BioLabs) with the following conditions: 50°C 2 min; 95°C 15 min; 40 cycles of 94°C 10 s, 60°C 30 s and 72°C 30 s. Reactions were performed in triplicate with 5 ng/ul DNA. Data were analyzed using the $\Delta\Delta C_t$ method. Sequences of the primers are: *ARHGEF10*-ex2-PF: 5'-TCCACCTGAACGGTGACAA G-3'; PR: 5'-TCTCAGCTCTGTTAAGGGAAGGA-3';

Table 2 Regions included in the CGH array

Gene	Genomic region for array design	Size (bp)
ARHGEF10	chr8:1749556–1894214	144,658
BSCL2	chr11:62214323–62241395	27,073
CTDP1	chr18:75530789–75615495	84,708
DCTN1	chr2:74441790–74471472	29,683
DNM2	chr19:10679755–10803579	123,825
EGR2	chr10:64241763–64256133	14,371
FGD4	chr12:32536361–32684940	148,579
GARS	chr7:30590706–30640173	49,468
GDAP1	chr8:75415173–75441890	26,718
HSN2	chr12:837257–848561	11,303
HSPB1	chr7:75759859–75771546	11,688
HSPB8	chr12:118090978–118116934	25,957
IKBKAP	chr9:110669621–110746217	76,596
FIG 4	chr6:110109161–110253327	144,167
KIF1B	chr1:10183418–10364242	180,825
LITAF	chr16:11549357–11597730	48,374
LMNA	chr1:154341122–154376495	35,373
MFN2	chr1:11952956–11996152	43,197
MPZ	chr1:159541151–159556377	15,227
MTMR2	chr11:95205694–95306920	101,227
NDRG1	chr8:134318596–134388680	70,085
NEFL	chr8:24864386–24880043	15,658
NGFB	chr1:115630060–115692380	62,321
NTRK1	chr1:155042166–155118266	76,100
17p12 duplication region (including PMP22)	chr17: 13855443–15574662	1,719,229
PRX	chr19:45591511–45621111	29,601
RAB7	chr3:129917669–130016331	98,663
SBF2	chr11:9756905–10282312	525,408
SEPT9	chr17:72817197–73008273	191,075
SETX	chr9:134126648–134230193	103,545
SH3TC2	chr5:148341906–148432930	91,025
SOX10	chr22:36698265–36720485	22,221
SPTLC1	chr9:93833248–93927511	94,264
YARS	chr1:33013427–33066220	52,794

IPO8-ex8-PF: 5'-GGTTGCAATTGCTGCCTTGT-3'; PR: 5'-GGCAACTGAATTCGTTCTAAAGTATG-3'. Primers used to determine the breakpoints of the small duplication on chr17 are listed in Supplementary Table 1. We confirmed the chr17 duplication by qPCR using a custom Taqman assay designed for exon 2 of *PMP22* with the RNase P assay kit as endogenous control (Applied Biosystems). Sequences of the primers and probe are: PMP22-ex2 PF: 5'-CTCCTCCTGTTGCTGAGTATCA TC-3'; PR: 5'-GACGATCGTGGAGACGAACA-3'; Probe: 5'-CCAGCACCGCGACGTG-3'. PCR reactions were performed in quadruplicates with 5 ng/ul DNA on the 7900HT Fast Real-Time PCR System (Applied Biosystems).

Sequencing analysis

All exons of *MTMR2* were sequenced with BigDye V3.1 chemistry on an ABI 3730 capillary instrument. PCR was performed on genomic DNA from the appropriate subjects using Platinum Taq (Invitrogen). Primer sequences are listed in Supplementary Table 2. Sequence reads were analyzed with the Sequencher software package (Genecodes).

Results

In the present study we screened for copy number variations (CNV) in 34 genomic regions that are known to

harbor genes for hereditary peripheral neuropathies in 97 patients. We utilized high-density custom CGH microarrays for CNV detection. In each experiment, DNA isolated from patient blood samples was labeled with Cy3 and hybridized to high-density microarrays. As a control, a pool of DNA from eight anonymous individuals was labeled with Cy5, and hybridized simultaneously to the same microarray. The log ratio of the signal intensity from the patient relative to the control pool sample represents the DNA copy number ratio between the patient and the control sample. As an initial validation of this platform, we tested a CMT1A patient with a known chr17p12 duplication and were able to confirm the duplication with the CGH microarray. In 97 patients we detected three CNVs that affected coding exons (Table 3). All unique and potentially disease-causing CNVs were confirmed by qPCR analysis. In addition, we excluded additional compound heterozygous variants in samples that carried a CNV in the recessive *MTMR2* gene.

Copy number variations and validation

Overall we identified six CNVs in the vicinity of CMT genes (Table 3). Three of them are novel CNVs and have not been reported previously according to the Database of Genomic Variants (DGV, <http://projects.tcag.ca/variation/>):

- (1) A 6.1 kb duplication was identified in one patient at the *ARHGEF10* locus affecting exon 2. This occurred in a CMT patient with a demyelinating phenotype. The CNV was found to be common as it was present in six out of 57 controls (Fig. 1).
- (2) A heterozygous 6.7 kb deletion that included exons 13–15 of the *MTMR2* gene was identified in four patients, which all had an axonal phenotype. This deletion was confirmed to be a common CNV occurring in four out of 51 control samples. Sequencing of all coding exons of *MTMR2* in the four patients carrying the CNV did not reveal any point mutations (Fig. 1).
- (3) One patient showed a 5.3 kb heterozygous deletion in intron 5 in gene *SPTLC1* at 9q22.31. This individual also had an axonal dominant form of CMT. As this is a relatively small intronic deletion and is unlikely to be causative it was not further tested in controls.

The other three CNVs overlapped with known common CNVs reported in the Database of Genomic Variants (DGV: <http://projects.tcag.ca/variation/>):

- (1) A region of 5.4 kb in size in intron 11 of *ARHGEF10* at 8p23.3 showed copy number gains in 13 patients and loss in one patient. This region overlaps with previously reported CNVs in the DGV database, but is larger in size.
- (2) One patient with a CMT1 phenotype showed a 14.7 kb heterozygous deletion in an intergenic region at 17p12, which was found in the DGV database in multiple reports. This genetic variant did neither affect *PMP22* nor any other coding gene and is thus not likely a disease-causing CNV.
- (3) Three patients showed copy number gains with the size of 0.8 or 1.5 Mb at the 17p12 locus, which is discussed in the following section (Fig. 2).

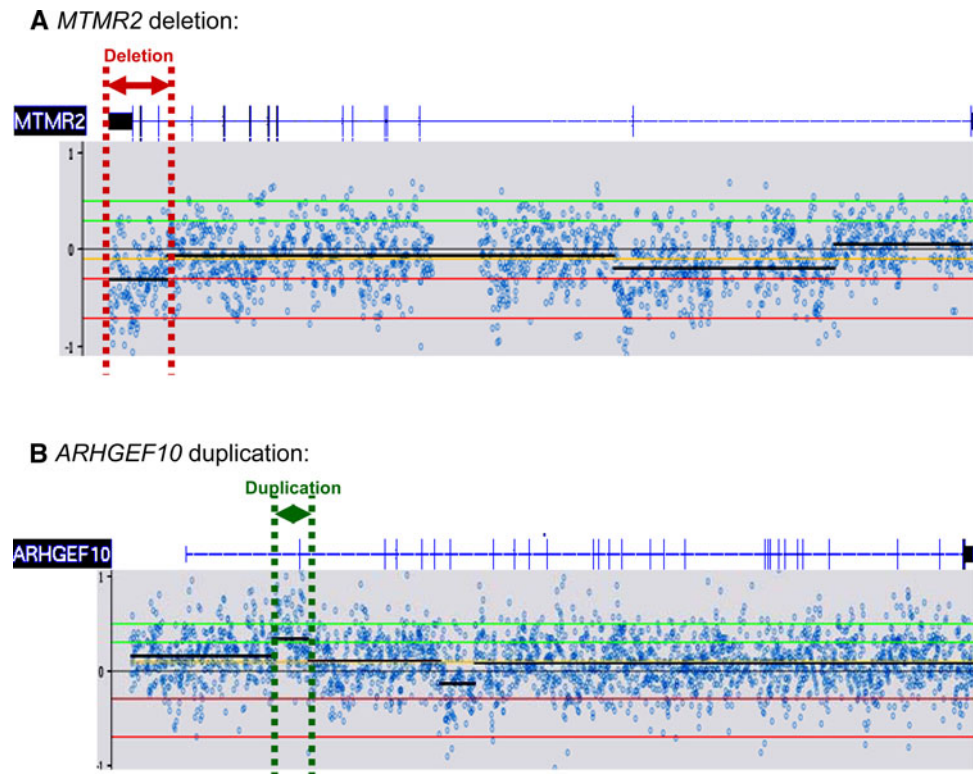
Chromosome 17 multiplications

Several changes were identified at the CMT1A locus at chromosome 17p12. First, we identified a classic 1.5 Mb duplication in a patient that was previously shown to carry this CNV. This individual served as a control sample and the investigator was blinded for its identity. Further, we identified 17p12 multiplications in two additional samples. Interestingly both samples were previously excluded for CMT1A in a commercial test. Both patients had a characteristic demyelinating clinical phenotype. Possibly explaining the divergent results, one of these patients showed a shorter duplication of only ~800 kb containing the *PMP22* locus (Fig. 2a), with a distal breakpoint between 14,497 and 14,499 kb and proximal breakpoint between 15,292 and 15,295 kb as defined by qPCR

Table 3 Six CNVs identified

Region	Size	Chr.	Gene affected	Exon/Intron	Type	Number of patients affected	Detected in % controls	Novel
chr8:1,773,794–1,779,915	6.1 kb	8p23	ARHGEF10	Coding exon	Gain	1	10.5%	Yes
chr8:1,821,425–1,826,860	5.4 kb	8p23	ARHGEF10	Intron	Gain/Loss	13/1	NA	No
chr9:93,875,121–93,880,417	5.3 kb	9q22	SPTLC1	Intron	Loss	1	NA	Yes
chr11:95,205,705–95,212,389	6.7 kb	11q21	MTMR2	Coding exon	Loss	4	7.8%	Yes
chr17:14,984,484–14,999,226	14.7 kb	17p12	None	Intergenic	Loss	1	N/A	No
chr17:14–15.5 Mb	0.8–1.5 Mb	17p12	PMP22	Whole gene	Gain	3	N/A	No

Fig. 1 Two CNVs affecting coding exons. Two plots generated with the Nexus software are shown illustrating two novel CNVs affecting coding exons. The exon–intron structure for each gene is shown on the top. Each open blue circle represents a probe on the array. The y-axis represents the log₂ test-over-reference ratios of hybridization signals and the x-axis represents the location of probes corresponding to the gene above the plots. In the plot, the thresholds for calling single and higher copy number “loss” and “gain” are shown as red and green horizontal lines, respectively. The yellow line depicts a moving average value and the thick black lines are the output of the Nexus algorithm, defining segments. (a) A heterozygous deletion in *MTMR2* affecting exons 13–15; (b) A duplication in *ARHGEF10* affecting exon 2



(Supplementary Fig. 1), which correlated well with the CGH data. Moreover, the significantly higher ratio in qPCR with primers targeting the *PMP22* gene suggested a triplication of this region (Fig. 2b). Despite this patient having potentially four copies of *PMP22* instead of two, the phenotype appeared typical for CMT1A. The other patient showed the classic 1.5 Mb duplication. The 1.5 Mb variant was also reported in controls based on data from DGV (http://projects.tcag.ca/cgi-bin/variation/xview?source=hg18&view=variation&id=Variation_49816); however based on the high phenotypic penetrance of this mutation, it is highly likely the controls that carry the duplication have CMT1A. On the other hand, the 0.8 Mb variant was not found in the database.

Our sample of 97 patients also contained a patient clinically diagnosed as HNPP, but we did not identify a loss at 17p12, neither with CGH nor with qPCR.

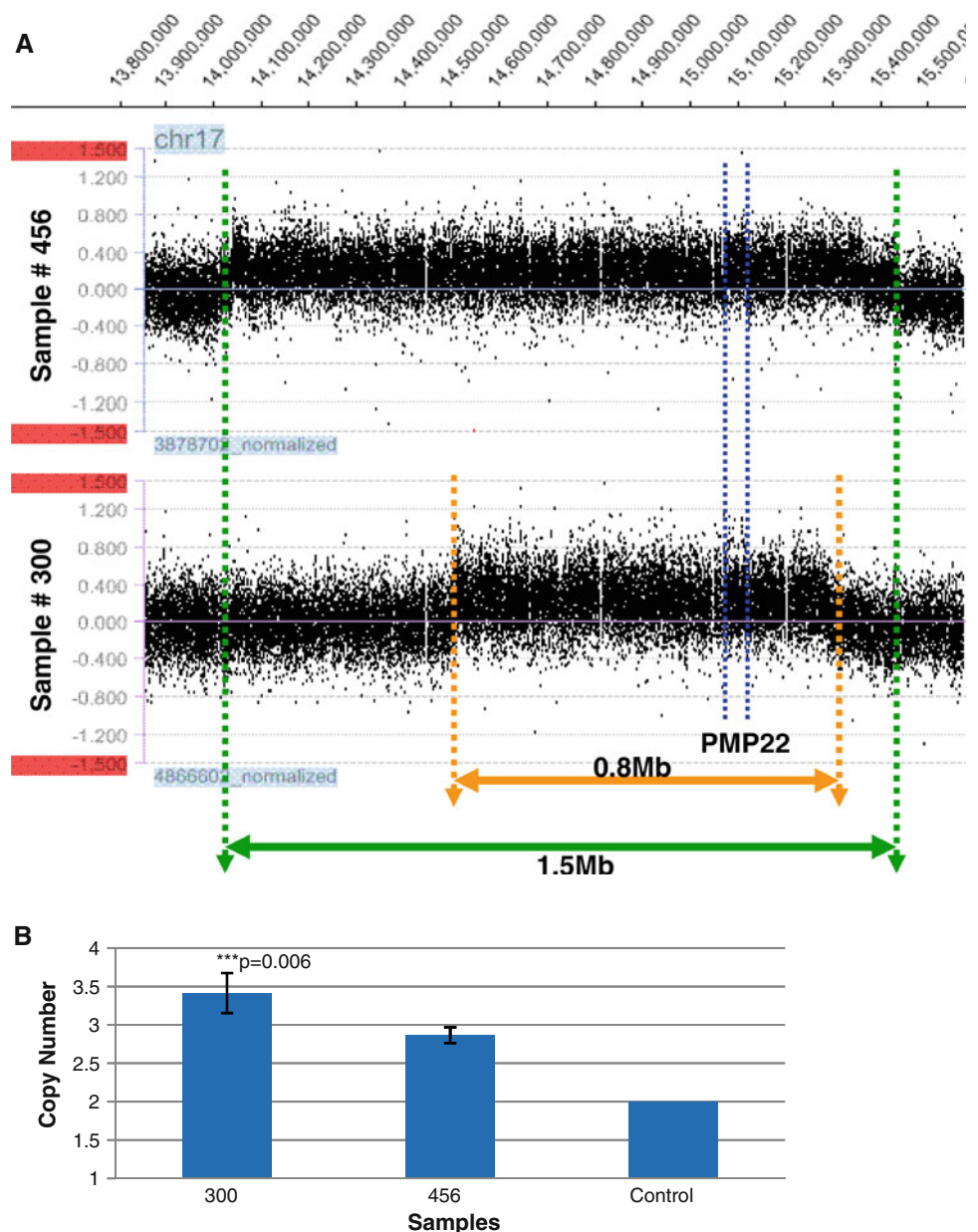
Discussion

Only recently has it been realized that CNVs play a much broader role in the human genome [4–6]. In fact, more base pairs vary between any two individuals as a consequence of CNVs than due to single nucleotide polymorphisms (SNP) [5]. Thus, CNVs are increasingly studied in the context of disease [7–14]. The first, and now classic, example of a disease causing CNV was the identification of a duplication

of chr17p12 containing the gene *PMP22* and leading to CMT1A [3]. In CMT1A only a minority of patients show a point mutation in *PMP22*. In contrast all other CMT genes appear to be caused exclusively by point mutations or small indels. To date it had not been comprehensively established whether additional CMT genes contain CNVs in CMT patients. Although it is desirable to perform whole-genome analysis of CNVs in CMT patients, the costs of such studies remain prohibitively high. The availability of custom microarrays allowed us to design an array that covers all known genes for hereditary peripheral neuropathies with a high density of probes. However, it is important to note that extra caution is needed when array CGH data are analyzed to detect smaller CNVs (<5 kb). As array CGH data are based on hybridization between probes and labeled DNA, sequence characteristics of probes, such as GC content, will have a significant impact on the hybridization. The smaller the region of interest, the greater the impact of local sequence content on hybridization dynamics, which could generate misleading results. In a high density array where probes are relatively close to each other, it is important to include a large number of probes when defining a CNV region, in order to reduce the influence of local sequence content characteristics. After intensive validation, we decided on a minimal CNV size of 100 probes (approximately 5 kb) to minimize false-positives in our results.

By studying a set of 97 CMT samples we identified a number of CNVs in different CMT genes. However, with

Fig. 2 (a) Two CMT1A patients with chr17p12 copy number gains. SignalMap software plots for two CMT1A patients with copy number gains at 17p12 are shown here. Each *black dot* represents a probe. The y-axis represents the log₂ test-over-reference ratios of hybridization signals and the x-axis represents the genomic location of probes. The *upper panel* shows a patient with the classic 1.5 Mb duplication (depicted by the *green arrow*) and the *lower panel* shows a patient with a shorter region of copy number gain of about 0.8 Mb. Note both CNVs involve the *PMP22* gene. (b) qPCR analysis of copy number at the *PMP22* locus for the above two samples. The y-axis represents the copy number of each sample calculated from the qPCR ratios. For each sample qPCR was performed five times in quadruplicates and the average copy numbers are shown here



the exception of *PMP22*, none of these changes appear to present a causative mutation. Three novel CNVs were found in our study. A heterozygous CNV in the recessive *MTMR2* gene included the loss of exons. The identification of such alleles is likely not disease causing, but could nevertheless be important in two ways [1]. A compound heterozygous inheritance in combination with a causative point mutation on the other chromosome is conceivable. In other words, the identification of a heterozygous point mutation in a genetic test does not rule out the co-occurrence with a CNV, which would result in a phenotype. Thus, we excluded additional mutations by direct sequencing of *MTMR2* in the four patients identified with this CNV. Importantly, CNV screens are not yet part of the

general genetic testing in CMT. The CNV in *MTMR2* might act as a modifying variant with consequences for age of onset, severity or other clinical characteristics. This possibility could not be proved in the current study, because the involved families were too small and the overall number of individuals carrying this change was limited. We also identified a novel duplication in *ARHGEF10* affecting a coding exon. A mutation in this gene has been shown to be responsible for autosomal dominant slowed nerve conduction velocity [15]; however, since the duplication was common in controls, it is unlikely to be causative for the disease. Finally, we detected an intronic heterozygous deletion in gene *SPTLC1*. Because this deletion affected neither the coding sequence nor the

splicing sites, it is unlikely to cause altered gene expression and a disease phenotype, though aberrations in intronic sequences can be disease-causing when they interfere with gene expression through altering DNA structure and stability, such as the GAA triplet repeats in the first intron of the gene FXN in Friedreich's ataxia [16].

We also identified a gain of *PMP22* that was suggestive of a triplication of the gene resulting in four gene copies in this individual. Gene dosage has been shown to be crucial for *PMP22* function. A loss of *PMP22* leads to HNPP; two copies represent the normal phenotype, while a duplication leads to CMT1A. The phenotype of the identified subject; however, was consistent with CMT1A. No especially severe clinical course was present. In addition, this individual had a shorter duplication of approximately 800 kb. Initial testing of this individual by a commercial laboratory came back with a negative result. This exemplifies that high density CGH could provide a more reliable technique for identification of small CNV or atypical CNV. The constant length of the chr17p12 CNV of 1.5Mbp has been attributed to two identical low copy number repeat (CMT1A-REP) sites that are flanking this chromosomal segment [17]. The occurrence of this shorter CNV would suggest an alternative mechanism not involving the REP sites, such as non-homologous end-joining (NHEJ) or Fork Stalling and Template Switching (FosTeS) [18].

Surprisingly, it appears that copy number detection in single cases can still pose challenges in quality-controlled commercial laboratories. The discrepancy between our data and the commercial analysis could result from different technologies used, i.e. CGH array vs. PCR-based MLPA. High density CGH arrays likely perform more consistently when detecting extended CNVs due to the large number of probes involved, as compared to PCR-based methods where typically only a few exons are being tested. However, the relatively high cost of CGH arrays remained (until recently) a major impediment for clinical diagnostic applications.

We conclude that CNVs play a rare role in CMT, with the important exception of *PMP22*. Nevertheless our study design does not exclude the possibility that a specific, relatively rare gene for CMT is characterized by CNV. The possibility of CNVs adds a considerable level of complexity to genetic testing, especially for recessive CMT genes. Further studies will be necessary to fully understand the contribution of CNV in the pathogenesis of hereditary peripheral neuropathies including CMT.

Acknowledgment This study was supported by the National Institute of Neurological Disorders and Stroke (5R01NS052767-04, to S.Z.) and the Charcot-Marie-Tooth Association (to S.Z.).

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