Neurogenetics (2011) 12:51–58
DOI 10.1007/s10048-010-0272-3

ORIGINAL ARTICLE

Inheritance of Charcot–Marie–Tooth disease 1A with rare nonrecurrent genomic rearrangement

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Received: 20 September 2010 / Accepted: 13 December 2010 / Published online: 31 December 2010

Abstract Rare copy number variations by the nonrecurrent rearrangements involving *PMP22* have been recently suggested to be associated with CMT1A peripheral neuropathy. As a mechanism of the nonrecurrent rearrangement, replication-based fork stalling template switching (FoSTeS) by microhomology-mediated break-induced replication (MMBIR) has been proposed. We found three Korean CMT1A families with putative nonrecurrent duplication. The duplications were identified by microsatellite typing and applying a CGH microarray. The breakpoint sequences in two families suggested an *Alu–Alu*-mediated rearrangement with the FoSTeS by the MMBIR, and a two-step rearrangement of the replication-based FoSTeS/MMBIR and meiosis-based recombination. The two-step mechanism has still not been reported. Segregation analysis of 17p12 microsatellite markers and breakpoint junction analysis suggested that the nonrecurrent rearrangements are stably inherited without alteration of junction sequence; however, they may allow some alteration of the genomic contents in duplication across generations by recombination event. It might be the first study on the pedigree analysis of the large CMT1A families with nonrecurrent rearrangements. It seems that the exact mechanism of the nonrecurrent rearrangements in the CMT1A may have a far more complex process than has been expected.

Keywords *Alu* sequence · Charcot–Marie–Tooth disease 1A (CMT1A) · Copy number variation · FoSTeS · MMBIR · Nonrecurrent rearrangement · *PMP22*

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Electronic supplementary material The online version of this article (doi:10.1007/s10048-010-0272-3) contains supplementary material, which is available to authorized users.

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Introduction

Charcot–Marie–Tooth disease (CMT) is a genetically and clinically heterogeneous hereditary motor and sensory neuropathy with an estimated prevalence of 1/2,500. Based on the electrophysiological criteria, CMT is frequently classified into two forms, the demyelinating form (CMT1) and the axonal form (CMT2) [1]. Up to date, more than 50 genes or loci have been reported as the underlying cause of CMT at the Inherited Peripheral Neuropathies Mutation Database (http://www.molgen.ua.ac.be/CMTMutations/Mutations/). Of them, the nonallelic homologous recombination (NAHR) between proximal and distal homologous repeat elements (CMT1A d-REP and p-REPs) on chromosome 17p12 is the most frequent genetic cause of the demyelinating CMT, particularly CMT1A (MIM# 118220) [2–4]. Reciprocal deletion of

the same region by the unequal crossover is associated with the hereditary neuropathy with liability to pressure palsies (HNPP, MIM# 162500) [5, 6]. The two flanking CMT1A–REPs share 98.7% sequence identity, and the duplication comprises approximately 1.4-Mb genomic region which includes the dosage-sensitive myelin gene *PMP22* [7]. Duplication is found up to approximately 70% of patients with CMT1 [8].

Rearrangements of the human genome can be grouped into two types according to breakpoint sequences, recurrent rearrangements, and nonrecurrent rearrangements. The major mechanism of recurrent rearrangements is NAHR which is associated with several genomic repeated sequences, e.g., low copy repeats (LCRs), long interspersed repetitive sequences (LINEs), and transposons. Because significant regions of homology are required for recombination, short interspersed repetitive sequences (SINEs), such as *Alu*, are not usually substrates for the NAHR [9, 10]. The molecular mechanism of the nonrecurrent rearrangements by nonhomologous end joining (NHEJ) has been little understood. Recently, a replication-based mechanism of fork stalling template switching (FoSTeS) has been proposed to explain the nonrecurrent rearrangements of the NHEJ [11–15]. According to the FoSTeS DNA replication model, the active replication fork can stall and switch templates using complementary template microhomology to anneal and prime DNA replication (microhomology-mediated break-induced replication, MMBIR). Chen et al. suggested a serial replication slippage as a possible cause of smaller genomic complex rearrangements [16]. Template switching during replication may be exacerbated by the presence of cruciform or other non-B DNA structures [12, 17].

Although the genomic disorders of CMT1A duplication and HNPP deletion are prevalently associated with recurrent rearrangements caused by NAHR events between two LCRs, recently, rare copy number variations (CNVs) on the chromosome 17p12 by the nonrecurrent rearrangements have also been suggested as the underlying cause of the CMT1A and HNPP [15, 18, 19]. Breakpoint sequence analyses have also revealed that various molecular mechanisms are involved in generating the nonrecurrent 17p12 rearrangements, such as NHEJ, *Alu-Alu*-mediated rearrangement, and FoSTeS and/or MMBIR mechanisms.

In the present study, we identified three Korean CMT1A families who are suggested to be associated with the nonrecurrent rearrangements by the replication-based FoSTeS. Analysis of the breakpoint junctions in each of the unique rearrangements suggested involvement of several mechanisms, such as *Alu–Alu* mediation, two-step process combining replication error and meiotic recombination, and complex rearrangement of two discrete duplications. The haplotype and breakpoint junction analyses also suggested that the nonrecurrent rearrangements may allow a slight modification of genomic contents by recombination event within duplication regions across generations.

Materials and methods

Patients

This study included three Korean CMT1A families (FC85, FC116, and FC388) with 34 individuals (19 affected and 15 unaffected individuals) (Fig. 1). This study also included four healthy controls for the CNV test. As the gain and loss controls, each two CMT1A duplication and HNPP deletion patients with the common 1.4 Mb recurrent recombination on 17p12 was involved. For the examination of clinical phenotypes, 149 CMT1A patients were also involved. All participants in this study provided written informed consent according to the protocol approved by the Institutional Review Board for Ewha Womans University Hospital. Total DNA was isolated from peripheral blood by using a QIAamp Blood DNA purification kit (Qiagen, Hilden, Germany).

Determination of duplication by microsatellite typing and real-time PCR

The nine microsatellites within the 17p12 CMT1A duplication region were amplified using the hexaplex PCR system that consisted of D17S921, D17S9A, D17S918, D17S4A, D17S122, and D17S2230, or single PCR method (D17S1296, D17S1357, and D17S9B) with fluorescent-labeled primers [20]. The PCR products were resolved with the automatic genetic analyzer ABI3100, and genotypes were determined by the Genotyper software (Applied Biosystems, Foster City, CA, USA). Duplication of *PMP22* was also determined by real-time quantitative PCR. The real-time PCR was performed on the RotorGene real-time PCR machine (Corbett Research, Australia) using the QuantiTech SYBR Green PCR kit (Qiagen). *GAPDH* was co-amplified for the control gene.

CNV determination by comparative genomic hybridization microarray

A high resolution of the rearrangement map and narrowed range of breakpoint junctions were obtained by determining the CNVs with a customer-designed high-density comparative genomic hybridization (CGH) microarray (HG18 CGH 2X 135K; Roche-NimbleGen, Madison, WI, USA). The mean probe size and spacing length of the array were

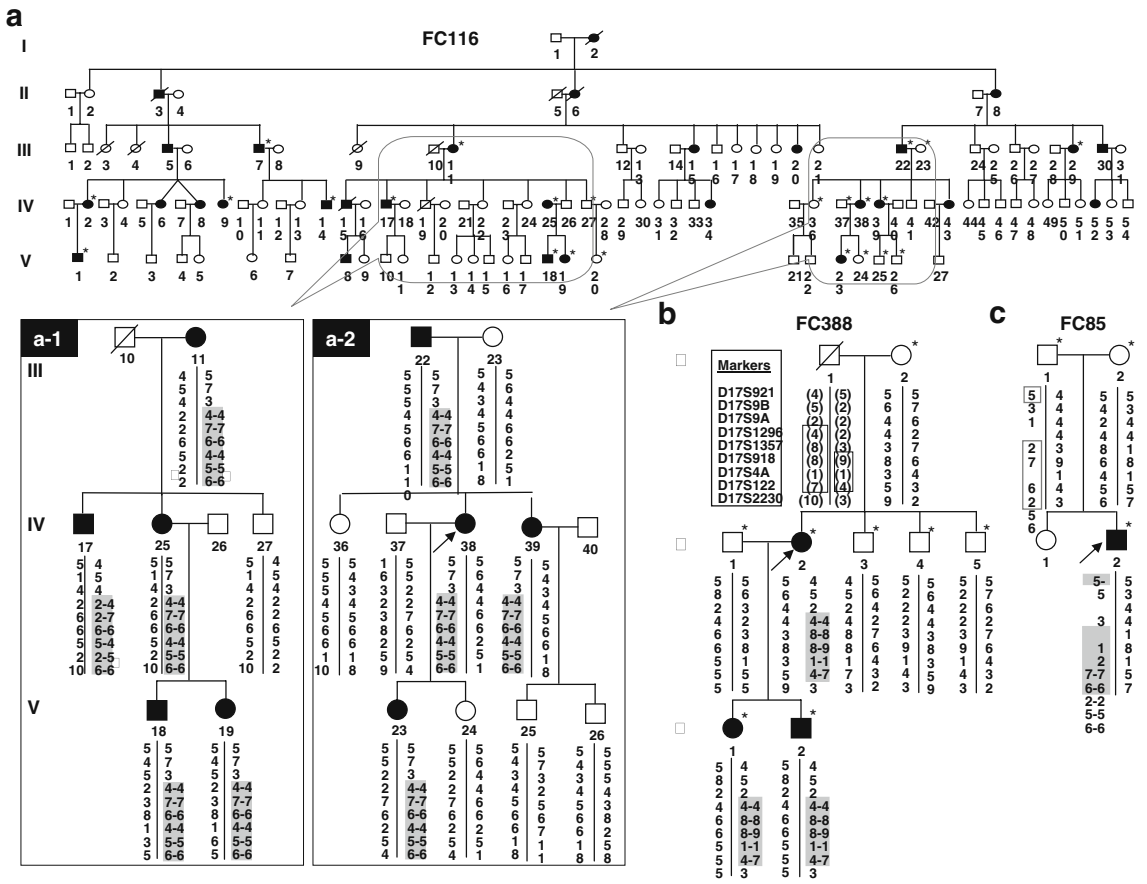

Fig. 1 CMT1A pedigrees with nonrecurrent duplication. Haplotypes (indicated below each member) were obtained by genotyping of nine microsatellites, but the genotypes in *parentheses* were deduced from the pedigree analysis. *Asterisks* (*) indicate individuals whose DNA were used for this analysis. The *gray boxes* on the haplotypes indicate the duplicated region, and *open boxes* indicate chromosomal regions before de novo event, respectively. *Arrows* (↗) indicate probands (*open squares and circles* unaffected individuals, *filled squares and circles* affected individuals). **a** FC116 family. Except for a putative crossover (III-11 to IV-17) on the duplication region (*black left-pointing pointer*) (*a-1*), the duplication was stably inherited without alteration (*a-2*). **b** FC388 family. Putative de novo rearrangement occurred from I-1 to II-2. **c** FC85 family. A de novo discrete rearrangement was observed (I-1 to II-2)

60-mer and 215 bp, respectively. The CGH microarray data were analyzed with NimbleScan (ver. 2.4) and SignalMap (ver. 1.9) software. The gain and loss threshold used in this study were $\log_2$ ratio >0.3 and <−0.3, respectively. Healthy individuals were used as normal control, and recurrent CMT1A duplication and HNPP deletion patients were used as gain and loss controls, respectively.

Long-range template PCR and determination of breakpoint sequence

Long-range template PCR was attempted to amplify the breakpoint junctions. PCR primers were designed as approximate intervals of 2 kb within about 10-kb ranges with either side of estimated breakpoints by the CGH analysis (Supplemental Table 1). The PCR was performed using the long-range template PCR kit (Roche, Mannheim, Germany). PCR products were sequenced on the automatic genetic analyzer ABI3100 using the BigDye terminator cycle sequencing kit (Applied Biosystems). DNA sequences were compared to reference sequences, UCSC hg18 at the UCSC Genome Browser (http://genome.ucsc.edu/) or Build 36.1 at the NCBI website (http://blast.ncbi.nlm.nih.gov/) by using the SeqScape (ver. 2.1, Applied Biosystems) and Chromas software (ver. 2.33; Technelysium, Australia).

Clinical assessment

Clinical information was obtained in a standardized manner, including motor and sensory impairments, and deep tendon reflexes. Histopathological examination of the affected individuals included the light and electron microscopic analyses of a sural nerve. Magnetic resonance imaging study was performed on the lumbar spine and lower legs of the patients using a supine position using a 1.5-T system (Siemens Vision, Erlangen, Germany). Physical disability was determined by two scales, the functional

disability scale (FDS) [21] and the CMT neuropathy score (CMTNS) [22].

Results

Identification of partial duplication of chromosome 17p12 in CMT1A families

From the genotyping of nine microsatellites on 17p12 in subjects with CMT1A families, we found three families with unusual partial duplication. CMT1A patients with common duplication showed 1.4 Mb duplication containing all the markers from D17S921 to D17S2230; however, FC116 and FC388 revealed duplication from D17S1296 to D17S2230 (Fig. 1a) and from D17S1296 to D17S122 (Fig. 1b), respectively. Particularly, FC85 showed a complex rearrangement with two discrete regions, D17S921, and D17S1357 to D17S2230 (Fig. 1c). The duplication regions included the *PMP22* gene in all three families, which was also confirmed by amplification of the *PMP22* using the real-time PCR. De novo rearrangements were suggested in two families with both paternal origins: father (I-1)-to-daughter (II-2) in FC388 and father (I-1)-to-son (II-2) in FC85. Although the proband's father (I-1) in FC388 was not directly tested in this study, we considered that a de novo rearrangement occurred during transmission because he was determined to be an unaffected individual by history taking.

The exact duplication regions were further narrowed into 14.84–15.44 Mb in FC116 and 14.59–15.31 Mb in FC388 by applying the CGH microarray (Fig. 2a). The complex duplication of FC85 included 14.00–14.46 Mb and 14.98–15.43 Mb. Figure 2b explains the genomic structure of the identified rare rearrangements.

Nonrecurrent rearrangements by *Alu–Alu* and microhomology mediation

The breakpoint junctions were successfully amplified by long-range template PCR from the patients in FC116 and FC388 (Supplementary Fig. S1). The range of distal breakpoints in FC116 and FC388 could be narrowed within 14,839,425–14,841,529 bp and 14,582,503–14,584,531 bp, respectively (hg18). However, we failed to obtain the PCR products from FC85 even though we tried to amplify the breakpoint junction by using various primer pairs.

The exact DNA sequences at the breakpoints and their surrounding regions were determined from the long-range template PCR products (Table 1). In FC116, the breakpoint junction was found within two different *Alu* sequence

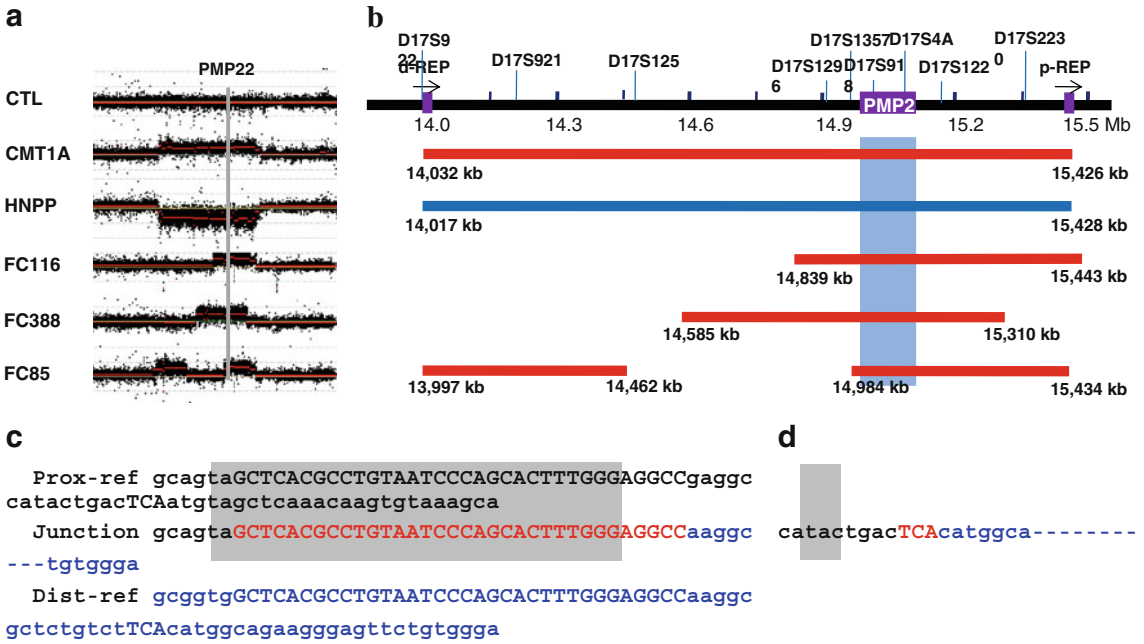

Fig. 2 Characterization of the rare nonrecurrent rearrangements in CMT1A patients. **a** Log_2 ratio plots obtained by the high-density CGH microarray (NimbleGen). Compared to healthy control (*CTL*), common CMT1A and HNPP patients showed duplication or deletion of 1.4 Mb on the 17p12 region. However, three families revealed unique partial duplications different from each other. **b** Diagram of genomic structure on 17p12 and rearrangements identified in CMT1A patients with partial duplication. All CNVs included the *PMP22* duplication. Map distances were from Map build UCSC hg18 (http://genome.ucsc.edu/). **c** Sequences of the break junctions in FC116 and **d** FC388. The proximal and distal reference sequences are given by *black* and *blue letters*, respectively. The sequences with microhomology are indicated by *red letters with gray boxes* (– deletion)

Table 1 Characterization of breakpoint sequences in the nonrecurrent rearrangements

Family	Size (kb)	Breakpoint junction		Microhomology	Putative mechanism
		Distal end	Proximal end		
FC116	607	*Alu*Y	*Alu*Sc	34 bp	*Alu–Alu*-mediated FoSTeS/MMBIR (NAHR may be involved)
FC388	725	*CDRT* (intron)	LTR (MALR)	3 bp (11 bp deletion)	2-step rearrangement with FoSTeS/MMBIR and crossover
FC85	465 450	d-REP ND	ND p-REP	ND	Complex FoSTeS/MMBIR

ND exact breakpoint sequences were not determined

families (distal end, *Alu*Y; proximal end, *Alu*Sc) with 34-bp exact microhomology (Fig. 2c). In FC388, each proximal and distal breakpoint was within a long terminal repeat (LTR, family MALR) sequence and an intron of *CDRT4* gene with 3-bp "TCA" microhomology. The breakpoint junction of FC388 also showed an 11-bp deletion, 7 bp after the microhomology (Fig. 2d). The 11-bp deletion was not observed in either non-rearranged regions corresponding to distal breakpoint end (presumptive ancestral sequence before de novo event) in proband (II-2) or same chromosomal regions of her mother (I-2) and unaffected siblings (II-3, 4, 5) (Supplementary Fig. S2). We also could not find the variation of 11-bp deletion from the dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?). Therefore, we considered that the 11-bp deletion is due to a complex nonrecurrent rearrangement during the de novo event. All the affected members in FC116 (n=15) and FC388 (n=3) showed exactly the same breakpoint DNA sequences in each pedigree.

When the microsatellite haplotypes were analyzed in FC116, the deduced two alleles by duplication (gray boxes) were always the same in six corresponding markers as shown in Fig. 1a-2 (4-4/7-7/6-6/4-4/5-5/6-6). Therefore, it seems that the rearrangement might be resulted from the FoSTeS by the MMBIR via 34-bp microhomology. The haplotypes of the duplicated region were the same in all the affected members except for IV-17 (Fig. 1a-1). The different haplotype of IV-17 might be due to a meiosis-based recombination event between D17S1357 and D17S918 during transmission of rearrangement region from mother (III-11) to son (IV-17) (Fig. 3a). The haplotype analysis of FC388 suggested a more complex two-step rearrangement process (Figs. 1b and 3b). First, a nonrecurrent rearrangement between D17S1296 and D17S122 might occur by the FoSTeS via 3-bp microhomology during DNA replication, which produced a presumptive intermediate haplotype, "4-4/8-8/8-8/1-1/7-7". Second, the crossover event between D17S1357 and D17S918 during meiosis might produce final rearrangement with the haplotype of "4-4/8-8/8-9/1-1/4-7".

Clinical manifestations

The clinical phenotypes of 19 patients with the rare duplication showed no significant different features from the common CMT1A patients with 1.4-Mb recurrent duplications (Table 2). However, FC116 family revealed broad intrafamilial phenotypic variations, e.g., age at onset: 4 to 60 years, CMTNS: 2/30 to 27/30, FDS: 0/8 to 5/8, and median nerve conduction velocities: 0 to 30.7 m/s. The histological examination of a sural nerve biopsy in a 52-year-old woman (FC116—IV-25) showed severe loss of myelinated fibers of all calibers and revealed diverse features of onion bulb and pseudo-onion

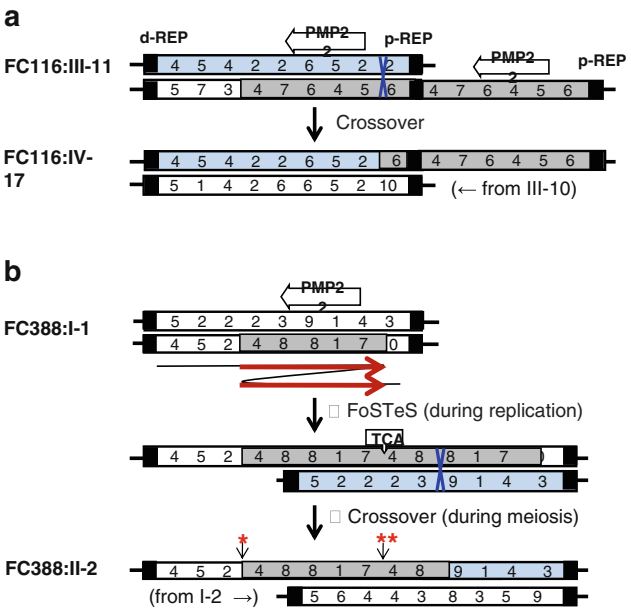

Fig. 3 Putative crossover and two-step process of a rearrangement (*blue X* crossover). **a** Crossover on the duplication region in FC116. A crossover event might occur between D17S1357 and D17S918 during transmission from III-11 to IV-17 (Fig. 1a-1). **b** De novo rearrangement by putative two-step process in FC388. The FoSTeS/MMBIR event might be preceded during DNA replication (*red horizontal arrows*), and then crossover occurred during meiosis between D17S1357 and D17S918 during transmission from II-2 to III-1 (Fig. 1b). The regions indicated by *single asterisk* and *double asterisk* were used for sequencing analysis to determine the 11-bp deletion

Table 2 Comparison of clinical phenotypes between nonrecurrent duplication and reciprocal duplication

Examination	Nonrecurrent dupl[a]	Current dupl[b]	*P* value
No. of patients	19	149	–
Age at onset (years)	18.3±15.7	15.7±10.1	ns
Asymptomatic patients	2 (11%)	10 (7%)	ns
FDS	1.8±1.5	2.0±1.0	ns
CMTNS	11.2±6.9	11.2±5.7	ns
Muscle weakness	15 (79%)	118 (79%)	ns
Sensory impairment	15 (79%)	102 (69%)	ns
Abnormal DTR	16 (84%)	134 (90%)	ns
Foot deformity	16 (84%)	131 (89%)	ns
Median MNCV (m/s)	22.2±5.4	20.8±4.9	ns
Median CMAP (mV)	7.3±3.2	7.1±4.1	ns
Median SNCV (m/s)	17.7±2.3	18.5±4.7	ns
Median SNAP (μV)	9.3±6.7	6.6±3.2	ns

FDS functional disability scale, *CMTNS* CMT neuropathy scale, *DTR* deep tendon reflex, *MNCV* motor nerve conduction velocity, *CMAP* compound muscle action potential, *SNCV* sensory nerve conduction velocity, *SNAP* sensory nerve action potential, *ns* not significant

[a] CMT1A patients with rare nonrecurrent duplications

[b] CMT1A patients with common 1.4 Mb duplications by unequal crossover on 17p12

bulb formations as the characteristics of common CMT1A patients (data not shown).

Discussion

Human genomic disorders are caused by an alteration of the genome architectures which are mostly resulted from recurrent genomic rearrangements between region-specific LCRs. However, replication-based nonrecurrent rearrangement has been also proposed to be one of the important underlying causes of genomic disorders. Several CMT1A patients have been recently reported to be associated with the nonrecurrent rearrangements [15, 18, 19, 23]. This study also identified three CMT1A families with the rare nonrecurrent rearrangements. It might be the first haplotype analysis of large CMT1A families with the NHEJ. The exact breakpoint junctions were determined by the long-range template PCR after analysis of the CGH microarray in FC116 and FC388. However, we failed to obtain PCR product from FC85 with two discrete duplicated regions. The rearrangement of FC85 may contain more complex events such as inversion or microduplication/deletion. Two paternal originated de novo rearrangements were observed (FC388 and FC85). This is comparable to the predominant paternal origin of general CMT1A de novo duplications by the unequal crossover even though the rearrangement mechanisms might be considerably different between the two events [24–26].

A careful examination of clinical phenotypes for all the affected individuals showed no significant difference compared with the common CMT1A patients, which is consistent with the idea that the copy number of the *PMP22* gene may contribute to the phenotype. However, the broad intrafamilial phenotype variation in FC116 (e.g., broad range of age at onset from 4 to 60 years) might suggest an existence of a genetic modifier or environmental factor(s) which influence on the expressivity of the clinical symptoms rather than incomplete penetrance of the phenotype. Kleopa et al. suggested that mutations in the *PMP22* gene are relevant with the broad phenotypic spectrum [27].

The *Alu–Alu*-mediated rearrangement in the FC116 could be explained by either of two mechanisms, meiosis-based nonallelic homologous recombination (unequal crossover) or replication-based FoSTeS by MMBIR [15, 28, 29]. However, *Alu* sequences usually do not act as the preferring substrates for the homologous recombination because significant regions of homology are required for recombination [9]. When we performed the segregation analysis of 17p12 markers, both duplicated alleles were always the same. This segregation analysis indicated that the rearrangement might result from the FoSTeS by the MMBIR via 34-bp microhomology. If the rearrangement has occurred by NAHR mechanism via the unequal crossover between two homologous chromosomes, two duplicated alleles would be different. However, the evidence of the identical alleles could not completely exclude the potential involvement of NAHR in this rearrangement because an unequal crossover event between sister chromatids might generate identical alleles on the duplication region [30, 31]. The involvement of the FoSTeS mechanism via decades-bp microhomology for the *Alu–Alu*-mediated rearrangement was also suggested by Zhang et al. [15].

Although the rare rearrangement in the FC388 was regarded as by the FoSTeS/MMBIR via 3-bp microhomology, the haplotype analysis suggested that a more complex mechanism might be involved in the rearrangement. The de novo duplication from I-1 to II-2 might be achieved by two different events, FoSTeS/MMBIR during DNA replication and recombination during meiosis (Fig. 3b). This two-step rearrangement mechanism has still not been reported. The "serial replication slippage" suggested by Chen et al. might

involve in the nonrecurrent rearrangement of FC388 since the breakpoint sequence also revealed an 11-bp deletion [16].

Little has still been understood whether the nonrecurrent rearrangements are stably transmitted to the next generation. As expected, this study revealed that the breakpoint junctions are stably inherited with no sequence modification since all the 15 affected members revealed the same breakpoint sequence in the FC116 family including five generations. Three affected members in the FC388 also showed the same breakpoint sequence. But the other concern is whether the duplicated genomic contents of nonrecurrent rearrangements are stably transmitted across generations. One of the interesting findings of this study is the occurrence of meiotic recombination within the duplication. The recombinations may exchange the contents between duplicated allele and wild-type allele in genomic duplications. Moreover, this reminds us that the duplicated *PMP22* copies might be different in the same family (like FC116), although the copy number of *PMP22* is the same. It may also potentially contribute to variability in gene expression that could partially explain some of the broad phenotypic spectrum in the same CMT1A family.

Considering the combined replication error and meiotic recombination events, it seems that the exact mechanisms of the rare partial duplications may have far more complex and delicate processes than has been expected. It also seems that the genomic contents in duplication may be unstable across generations to some extent, although the breakpoint junction is stable. We believe that this study provides a clue to discover the molecular mechanisms of the nonrecurrent rearrangements in many other human genomic disorders with rare CNVs.

Acknowledgments This study was supported by Mid-career Researcher Program through NRF grant funded by the MEST (R01-2008-000-20604-0 and KRF-2008-313-C00750), Republic of Korea.

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