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POINT OF VIEW

Caveats in the Established Understanding of CMT1A

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Abstract

Charcot-Marie-Tooth disease type-1A (CMT1A) is one of the most common types of inherited peripheral nerve diseases. It is caused by the trisomy of chromosome 17p12 (c17p12), a large DNA segment of 1.4 Mb containing *PMP22* plus eight other genes. The size of c17p12 is formidable for any cloning technique to manipulate, and thus precludes production of models *in vitro* and *in vivo* that can precisely recapitulate the genetic alterations in humans with CMT1A. This limitation and other factors have led to several assumptions, which have yet been carefully scrutinized, serving as key principles in our understanding of the disease. For instance, one extra copy of c17p12 in patients with CMT1A results in a higher gene dosage of *PMP22*, thereby expected to produce a higher level of PMP22 mRNA/proteins that cause the disease. However, there has been increasing evidence that PMP22 levels are highly variable among patients with CMT1A and may fall into the normal range at a given time point. This raises an alternative mechanism causing the disease by dysregulation of *PMP22* expression or excessive fluctuation of PMP22 levels, not the absolute increase of PMP22. This has become a pressing issue since recent clinical trials using ascorbic acid failed to alter the clinical outcome of CMT1A patients, leaving no effective therapy for the disease. In this article, we will discuss how this fundamental issue might be investigated. In addition, several other key issues in CMT1A will be discussed, including potential mechanisms responsible for the uniform slowing of conduction velocities. A clear understanding of these issues could radically change how therapies should be developed against CMT1A.

There have been two studies done in a large cohort of patients with Charcot-Marie-Tooth type-1A (CMT1A) lately.[1,2] Both studies utilized human materials collected from CMT1A patients who participated in the ascorbic acid clinical trial. While the ascorbic acid trial failed to improve the disease of CMT1A, materials derived from the trials have been instrumental to several important neurobiological issues. Along with our published studies,[3,4] these have raised several critical concerns in the established understanding of CMT1A.

Uniform slowing of conduction velocity

Nerve conduction studies (NCS) in patients with CMT1A show an abnormal pattern, called uniform slowing. This pattern was initially described in a group of CMT patients with autosomal dominant inheritance, but genetic testing was not available in the 1980s.[5] Now, we know that this pattern is typically seen in patients with CMT1A caused by trisomy of chromosome 17p12 (c17p12) containing *peripheral myelin protein-22 (PMP22)* gene. The uniform slowing denotes that while conduction velocity is decreased in these patients, the values of conduction velocity are similar between different nerves of the same limb (for example, median vs. ulnar nerves in the right arm) or between different limbs of the same nerve (right median vs. left median nerve). Lewis et al. did not specify the maximal difference between different nerves that defined the "uniform slowing".[5] Based on our experience, the difference is typically less than 5 m/sec in a majority of CMT1A patients, but exceptions do occur.

Sural nerve biopsies from patients with CMT1A have consistently revealed numerous onion bulbs. They are

formed by membrane processes from multiple Schwann cells that circle around an axon but fail to form compact myelin. One of the Schwann cells does make contact with the axon and forms the compact myelin with reduced thickness.[6] It has been hypothesized that this pathology is caused by repetitive demyelination and remyelination. Demyelination has thus been considered to account for the slowed conduction velocity in CMT1A.[7,8]

However, there have been multiple lines of evidence against this assumption

(1) Multiple publications have quantified conduction velocities in a large cohort of patients with CMT1A. The mean of conduction velocities was always around 20 m/sec ± SD (~50 m/sec in normal controls). The SD is usually small.[9,10] In the study by Manganelli et al.[2], of 271 patients with CMT1A tested by NCS, the mean of CV from median, ulnar and peroneal motor nerves was 20.3 ± 4.5 m/sec. None of the 217 patients had temporal dispersion. Of 574 evaluated nerves, only 4.5% nerves had conduction block.
This finding is remarkable and suggests a robust biological determinant account for this highly consistent electrophysiological outcome in patients with CMT1A – uniform slowing. This highly reproducible observation is incompatible with the theory of "repetitive de-/remyelination". Active demyelination, seen in chronic inflammatory demyelinating polyneuropathy (CIDP) or Gillian Barre Syndrome (GBS), would predict highly variable conduction velocities from one case to another, plus temporal dispersion and conduction block. Yet, these electrophysiological demyelinating features are either absent or rarely present in patients with CMT1A.

(2) Active demyelination over years would result in a steady decline of conduction velocities, but conduction velocities showed minimal changes over decades in patients with CMT1A.[11–13] In the study by Manganelli et al.[2], CV even slightly increased during aging of CMT1A patients.

(3) Nerve pathology studies prior to the era of genetic testing are difficult to interpret. Fortunately, a series of pathological studies have been carefully done in sural biopsies from genetically confirmed CMT1A patients. Segmental demyelination was observed but mainly took place during the first decade and subsided thereafter. This observation is consistent with recent work showing that CV correlates with disease severities in pediatric patients with CMT1A better than the correlation in adults with CMT1A.[14] More importantly, detailed morphometric analysis demonstrated a significant decrease of g-ratio (an increase of myelin thickness in relation to the size of axons) in CMT1A.[15,16] This finding argues against active demyelination being a main pathological process in CMT1A since demyelination should increase the g-ratio (thin myelin), rather than decrease the g-ratio. Instead, these studies suggest a failure of developing the largest myelinated nerve fibers at the early stage of life in patients with CMT1A. This leaves a group of myelinated nerve fibers that are relatively "homogenous" in their sizes with small diameters. Based on the observations by Erlanger and Gasser,[17,18] nerve fibers with similar sizes would produce similar conduction velocities and explain the uniform slowing.

(4) We have utilized human skin biopsies to examine segmental demyelination in patients with CMT1A.[4,19] Skin biopsy has its limitations, including a difference of gene expression profile from that in human sural nerves.[4] However, with proper controls, important information has still been obtained via this approach. In a group of adults with CMT1A, no segmental demyelination was found. Interestingly, the internodal length was shortened in the CMT1A patients. This observation is also consistent with small diameters of CMT1A nerve fibers since nerve fiber diameters are positively proportional to the internodal length.

During the normal development, excessively produced Schwann cells have to be eliminated if they fail to make contact with axons and myelinate. It remains to be determined if the elimination process of Schwann cells is impaired in CMT1A, leading to onion bulbs.

Uniform nerve fiber sizes and shortening of internodal length would force one to consider a developmental defect in CMT1A. In other words, myelinating Schwann cells with the CMT1A mutation fail to reach their proper sizes both radially (nerve fiber diameter) and longitudinally (internodal length). Indeed, over-expression of PMP22 in rats decreases levels of Neuregulin-I,[20] a molecule known to instruct the development of myelin thickness (nerve fiber diameter).[21] This might not be the only altered signal since over-expression of Neuregulin-I failed to restore the sizes or myelin thickness of nerve fibers in rats with PMP22 overexpression.[20] Thus, CMT1A is probably better termed as a dysmyelinating disease, rather than a demyelinating disease. The former denotes a developmental abnormality of myelination, whereas the later indicates a removal of myelin from fully differentiated internodal myelin. The dysmyelination demands an early intervention during development if therapy is developed against CMT1A.

We recognize that sural nerve biopsies of humans with CMT1A have suggested some demyelinations in the early

phase of CMT1A.[22] By human skin biopsies, mild hemiparanodal asymmetry was found in myelinated nerve fibers of CMT1A patients, implicating insidious de-/remyelination.[19] However, the de-/remyelination process is unlikely primary or prevailing activity. Otherwise, NCS would not show the highly consistent uniform slowing.

Highly variable levels of PMP22

Discoveries of genetic causes for CMT1A (trisomy of c17p12) and hereditary neuropathy with liability to pressure palsies (HNPP) (heterozygous deletion of c17p12 with only one copy of *PMP22*) have led to a hypothesis - CMT1A is caused by an increased dosage of *PMP22*, thereby the increase of *PMP22* mRNA and/or proteins results in the disease of CMT1A. In other words, this disease is resulted from over-expression of PMP22 from the trisomy of the *PMP22* gene.[23] Indeed, an early study on human sural nerve biopsies showed an increased average of PMP22 levels in patients with CMT1A and a decrease of PMP22 in patients with HNPP by immunological electron microscopy (immunoEM).[24] This theory has been the basis of animal model development and therapeutic exploration for CMT1A. For instance, strong effort has been made to develop cell-based models to over-express PMP22. The cells have been used to screen small molecular compounds that are capable of suppressing the levels of PMP22. The identified compounds will be used as the candidate drugs to be tested in animals and humans for therapy against CMT1A.

However, utilizing the human skin biopsy technique, we were able to revisit this issue by quantifying the PMP22 protein levels on myelin of peripheral nerves in a minimally invasive manner. Like previously published studies, the mean of PMP22 levels was increased in a cohort of patients with CMT1A, compared with that in normal controls (Fig. 1A). However, the levels of PMP22 in CMT1A patients were highly variable and fell into two subgroups – those higher than the level of controls and those comparable to the level of controls. The latter group would be incompatible with the increased dosage of PMP22 being the cause of the disease. This finding was replicated in two separated publications by different experimenters in our lab.[3,4] The fluctuation of PMP22 was not seen for another compact myelin protein–myelin basic protein (MBP). Nor was it shown in patients with HNPP.[3]

In line with this observation, mRNA levels of *PMP22* from skin and sural nerve biopsies of patients with CMT1A were not significantly different from that in normal controls and were not correlated with any functional outcomes or disease severity measurements collected through the ascorbic acid trial.[2] Again, the levels of *PMP22* mRNA were highly variable.[1,2] One might argue that these negative findings in *PMP22* levels were secondarily resulted from nerve fiber degeneration in patients with CMT1A. However, data by Katona et al 2009 have shown that this was not the case.[3] MBP levels were not decreased. The immunoEM technique measured PMP22 proteins only on the intact myelin. This measurement would not be affected by the nerve fiber degeneration.

Therefore, one would have to speculate that patients with CMT1A express PMP22 levels that highly fluctuate over time (Fig. 1B–D). Thus, at a single time point, patients may show a high or normal level of PMP22. These observations impose a serious challenge to the over-simplistic view of PMP22 dosage effect, particularly for those CMT1A patients with the PMP22 levels similar to that in normal controls.

Perhaps, a counter-argument is a study from Hirt et al.[25] In a single family with both HNPP and CMT1A alleles, HNPP and CMT1A alleles alone produced the expected diseases, whereas two sisters with compound heterozygous HNPP/CMT1A alleles are of no phenotype. Therefore, reducing the total *PMP22* copy number from three to two is sufficient to "cure" CMT1A in human.

However, this study was still not the approval that the increase of absolute PMP22 levels causes the disease versus the fluctuations of PMP22 levels (Fig. 1A–D). For instance, the duplicated 1.4 Mb DNA segment of chromosome 17p12 (c17p12) may have disrupted the normal genome structure. Intergene or interchromosomal interaction has been demonstrated to regulate genomic structure and expression of genes.[26,27] Normal trans interactions between two alleles of c17p12 (interchromatid) are likely altered by introducing an additional copy of c17p12 in CMT1A. Two tandem copies (copy-1 and copy-2) of c17p12 in allele-1 would compete respectively in their associations with the single copy of c17p12 in allele-2, leading to alterations of genome structure and unstable expression of PMP22. In other words, at one time point, copy-1 interacts with c17p12 in allele-2. At the next moment, copy-2 may interact with c17p12 in allele-2. Deletion of the c17p12 in allele-2 leaves two tandem c17p12 copies in allele-1. This gives no c17p12 in allele-2 for copy 1 and 2 on allele-1 to associate with and thereby eliminates the aberrant competition across the two chromatids. Removal of the aberrant competition may rescue the disease of CMT1A as reported by Hirt et al in the studied family.[25] This hypothesis may be further tested by comparing dynamic changes of genomic structure and trans-chromosomal/intrachromosomal interactions between cells from normal controls and cells from patients with CMT1A. The chromosomal structure derived from the trans/intrachromosomal interactions has been achieved using C3 technique.[28] More advanced

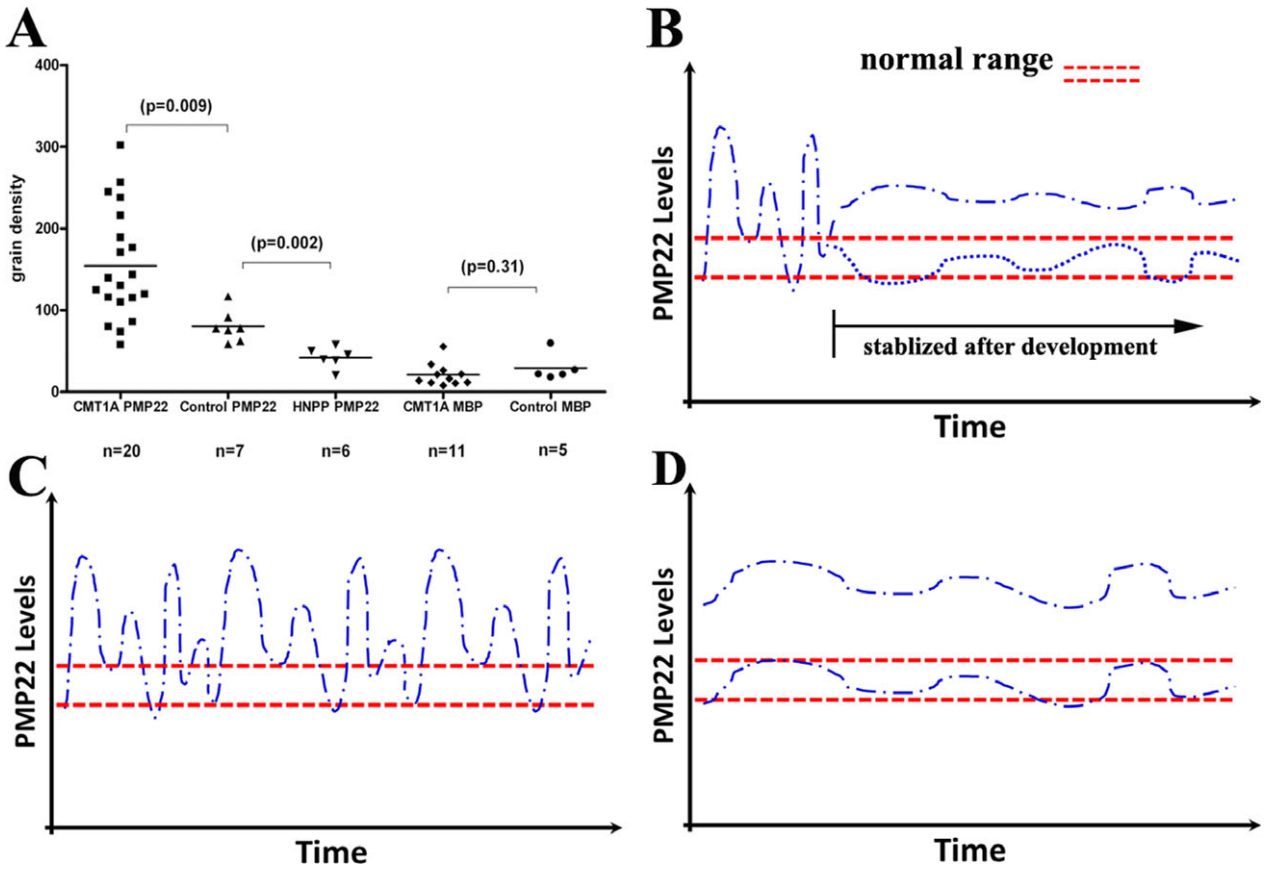

Figure 1. (A) Immunological electron microscopic measurement for PMP22 and myelin basic protein (MBP) grain densities (gold particles on compact myelin) in patients with CMT1A and normal controls. Human skin biopsy nerves were processed for immunological electron microscopic studies. PMP22 on the compact myelin of peripheral nerves was labeled with antibodies against PMP22 that were conjugated with gold particles. PMP22 grain densities in the dermal nerve myelin of CMT1A, but not HNPP or normal control, were highly variable. In contrast, levels of MBP, another protein on compact myelin of peripheral nerve, showed minimal variations. The densities were measured in grains/μm^2. This figure was cited from Katona et al. (Brain 2009; 132: 1734-40) with permission. (B) Model 1: PMP22 levels in CMT1A Schwann cells fluctuate during early development but stabilized to two different representative levels (higher, equal or near the low end of control level) after maturation. (C) Model 2: PMP22 levels in CMT1A Schwann cells fluctuate throughout entire life. At a given time point, immunoEM may show a level higher or comparable to the level of controls. (D) Model 3: PMP22 level in each patient is at different levels but relatively constant over time.

versions of the technique (C4 or C5) are now also available.[26,27] To determine if the dynamic changes of the duplicated c17p12 structure are pathogenic, single cell clones (such as fibroblasts from human skin biopsy or immature Schwann cells by iPSC technology) may be isolated from humans with CMT1A. The clones could be expanded into a sufficient amount of cells that are simultaneously used for C3 analysis and measurements of PMP22 transcripts by real-time PCR and proteins by Western blot. This will allow correlating fluctuation of PMP22 levels with the abnormally altered c17p12 chromosomal structure in homogeneous genetic background. If this mechanism is substantiated, it would provide novel molecular targets to treat CMT1A by manipulating the trans-interactions. Under this mechanistic model, it is the fluctuation of PMP22 levels by the genome structural alteration or aberrant trans-chromatid interactions, not the absolute level of PMP22, causing the disease. Thus, simple suppression of PMP22 levels would not cure the disease.

It is unclear whether the fluctuation of PMP22 levels is at the intercellular or interpatient level. We thus reanalyzed the raw data used to produce Figure 1A from Katona et al.[3] The intercellular variations within patients were not larger than those in normal controls (Table 1). This leads to several possibilities (Fig. 1B–D) to be tested in the future: Model 1 (Fig. 1B) - Intercellular fluctuations of PMP22 level mainly take place at the embryonic stage but stabilize at a given level during adulthood; Model 2 (Fig. 1C) - PMP22 levels highly fluctuate

Table 1. Intercell variation of PMP22 levels.

	Control iSD	CMT1A iSD
	26.95	18.86
	14.31	33.92
	117.21	34.67
	44.67	18.15
	20.68	24.87
		7.29
		31.94
Ave	44.76	24.22
tSD	42.05	10.14

Each iSD was calculated from a patient who had at least 6 or more myelinated Schwann cells counted.
iSD , standard deviation from individual patient; tSD , standard deviation from all values of iSD.

throughout entire life but synchronize among Schwann cells within a patient; Model 3 (Fig. 1D) - A combination of trans-/intrachromosomal interactions and other factors intrinsic to each patient determines a specific level of PMP22 that may be higher, equal or even lower than that in normal controls but do not fluctuate over time. Those nongenetic factors could involve downstream mechanisms, such as an increase of calcium influx into CMT1A Schwann cells in reaction to the CMT1A mutation.[29] Longitudinal studies using immunoEM on human skin biopsies collected at different time points would be helpful in differentiating these possibilities.

Rodent models for CMT1A

Rat CMT1A model and C22 or C3 mouse models with over-expression of *Pmp22* have all developed slowed conduction velocities and dysmyelination.[30–32]However, duplicated c17p12 in humans with CMT1A is a 1.4 Mb DNA segment. This giant piece of DNA is too long for making a "humanized" rodent model of CMT1A by any current technology. Most rodent models were produced by a random insertion of *PMP22* cDNA into the rodent genome. This results in a persistent high level of PMP22 in Schwann cells. It does not reflect the altered genome structure and the variable levels of PMP22 in humans with CMT1A.[3]

Interestingly, a conditional transgenic mouse has been made to over-express PMP22 under specific temporal and spacial control.[33] Over-expression of PMP22 only at the embryonic stage resulted in dysmyelination. Over-expression of PMP22 after myelin developed caused demyelination in only about 9% of myelinated nerve fibers. This small fraction of demyelinated nerve fibers did not significantly affect nerve conduction velocity. These observations are in line with the findings in humans with CMT1A. Dysmyelination was found in children with CMT1A,[15,16] but conduction velocities were stable for decades[2] and segmental demyelination was minimal in the dermal myelinated nerve fibers of adult patients with CMT1A.[19] However, there were undesirable features in the mouse model. For instance, nerve conduction velocities were decreased by 22% in the transgenic mice[33] but usually are reduced by 50% in patients with CMT1A.[2]

Gain-of-function point mutations of *PMP22,* such as *Trembler* or *Trembler-J* (*TrJ)* mutation, also result in severe dysmyelinating polyneuropathies.[34,35] Experiments using microarray have shown distinct pathogenic mechanisms between mice with the gain-of-function *Trembler* mutation and mice with *Pmp22* over-expression. Those of the former show transcriptional changes of stress response, and those of the latter demonstrate alterations in Schwann cell proliferation.[36] Interestingly, studies have shown excessive numbers of protein aggregates in Schwann cells with the *TrJ* mutation.[37–41] In contrast, sural nerve biopsies from patients with CMT1A (*3 copies* of *PMP22*) showed no aggregates in myelin, but aggregates were identified in sural nerves from patients with missense mutations of *PMP22.*[42] Thus, mouse models with *Pmp22* missense mutations (also called CMT1E) are not models of CMT1A.

Why are these issues important?

There has been increasing effort in development of CMT1A therapy. In an *in vitro* study, high levels of ascorbic acid have been shown to repress *PMP22* expression by affecting intracellular cAMP level through adenylate cyclase.[43] Addition of ascorbic acid to the medium in neuron-Schwann cell coculture promotes myelination.[44,45] The application of vitamin C to decrease PMP22 has improved the pathological changes and clinical deficits in the C22 mouse model with *PMP22* over-expression.[46,47] However, ascorbic acid trials in humans with CMT1A failed to show any significant effect.[48,49]

Toward the same biological principal, recent studies have developed cell models by random insertion of *PMP22-report* transgenes to identify small molecule suppressors for the over-expression of PMP22. These cells were then used for the high throughput screening of small molecular compounds to suppress the levels of PMP22.[50] With the caveats discussed above, this approach may not necessarily normalize the levels of PMP22. For instance, using an antisense oligo to suppress the transcription of *PMP22* mRNA would not necessarily produce a stable level of PMP22. Moreover, the critical therapeutic window could be at the embryonic or early stage of development and the suppressive treatment of PMP22 may not be feasible.

Instead, researchers need to be open-minded about all these potential caveats. We may have to be redirected ourselves to investigate how the duplicated long DNA segment of c17p12 disrupts the homeostasis of PMP22 expression. Understanding this upstream mechanism may give us better opportunity to normalize PMP22 levels in CMT1A, rather than a simple suppression of PMP22 expression.

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Conflict of Interest

We have no conflict of interest to disclose.

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