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Molecular Genetics of Charcot-Marie-Tooth Disease: From Genes to Genomes

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Abstract
Charcot-Marie-Tooth disease (CMT) is a heterogeneous group of disorders of the peripheral nervous system, mainly characterized by distal muscle weakness and atrophy leading to motor handicap. With an estimated prevalence of 1 in 2,500, this condition is one of the most commonly inherited neurological disorders. Mutations in more than 30 genes affecting glial and/or neuronal functions have been associated with different forms of CMT leading to a substantial improvement in diagnostics of the disease and in the understanding of implicated pathophysiological mechanisms. However, recent data from systematic genetic screening performed in large cohorts of CMT patients indicated that molecular diagnosis could be established only in ~50–70% of them, suggesting that additional genes are involved in this disease. In addition to providing an overview of genetic and functional data concerning various CMT forms, this review focuses on recent data generated through the use of highly parallel genetic technologies (SNP chips, sequence capture and next-generation DNA sequencing) in CMT families, and the current and future impact of these technologies on gene discovery and diagnostics of CMTs.

Charcot-Marie-Tooth Disease: Clinical and Genetic Heterogeneity

Charcot-Marie-Tooth disease (CMT) is a clinically and genetically heterogeneous group of sensorimotor peripheral neuropathies and represents probably the most frequent cause of inherited pathologies affecting the nervous system [Skre, 1974]. The current classification of these disorders is based on clinical, electrophysiological, genetic and pathological criteria.

Physical examination of patients with CMT reveals symmetric distal amyotrophy in lower limbs and hands, steppage gait, foot deformities (pes cavus, pes planus, pes valgus), distal sensory loss in a stocking/glove distribution and decreased or absent deep tendon reflexes [Harding and Thomas, 1980a, b; Thomas and Harding, 1993]. Patients may have impaired proprioception with balance difficulty. Neuropathic pain may or may not be present [Siskind and Shy, 2011]. Spine deformities are not a constant sign of the disease but are more frequently associated with specific subtypes such as CMT4C [Azzedine et al., 2006]. Onset is classically within the first to second decade of life, with initial symptoms affecting the lower limbs but occasionally also the hands (e.g. *GARS* and *GDAP1* mutations). However, there is a large spectrum of phenotypes, in terms of age of onset, disease progression and severity. Even among affected members of the same

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family carrying identical mutations, the phenotype may vary considerably. Other inherited peripheral neuropathies including hereditary motor neuropathies and hereditary sensory and autonomic neuropathies, which present a partially similar phenotype to CMT, are not covered by this review.

According to electrophysiological criteria including motor nerve conduction velocities (MNCV) and compound muscle action potential amplitudes, CMTs have been divided into 2 major subtypes; demyelinating (CMT1) and axonal (CMT2). In demyelinating forms, the MNCV in the upper extremities (median or ulnar nerve) are below 38 m/s. Conversely, patients with MNCV higher than 38 m/s but with a decrease in compound muscle action potential amplitudes are considered to be affected by axonal CMT. A third, less-defined subtype of the disease called intermediate CMT is characterized by MNCV values between 38 and 45 m/s (some authors report a larger interval: 25–35 to 45 m/s) [Thomas and Calne, 1974; Bouche et al., 1983; Rossi et al., 1985; Verhoeven et al., 2001; Patzko and Shy, 2011].

Earlier studies have shown that approximately two-thirds of CMTs are demyelinating and one-third is of primary axonal defect [Harding and Thomas, 1980a, b]. More recent studies in large cohorts of patients confirmed that most CMTs are demyelinating but lowered its prevalence to approximately half of all cases, while the prevalence of axonal forms was assessed to be less than one-third, i.e. 17.6–27.1% [Foley et al., 2012; Murphy et al., 2012]. Moreover, the proportion of axonal or demyelinating CMT seems to depend on the geographical area, the structure of the population and the frequency of CMT in the general population. For example, in isolated European populations CMT1 and CMT2 are equally distributed, accounting for 48.2 and 49.4% of all cases, respectively [Nelis et al., 1996; Braathen et al., 2011; Foley et al., 2012].

The variability of the disease is also prominent at the genetic level. The pattern of inheritance can be autosomal dominant, autosomal recessive, or X-linked. To add to the complexity of the classification, more than 30 loci and genes have been identified in various forms of the disease (table 1, for additional information, see also: http://neuromuscular.wustl.edu/time/hmsn.html and www.molgen.ua.ac.be/CMTMutations/). Dominant forms are the most frequent ones in Western countries (up to 90%). Recessive forms are conversely more prevalent (30–50% of all CMT cases) in countries or populations displaying a high rate of consanguinity (Mediterranean basin, Middle East, European gypsies) while they account for only ~4% of all cases in Europe [Dubourg et al., 2006; Patzko and Shy, 2011]. Importantly, depending on the nature and the position of the mutation, the same gene can be involved in demyelinating, axonal or intermediate forms, displaying autosomal dominant or recessive inheritance (tables 1 and 2).

Data from recent studies in relatively large cohorts of patients with CMT indicated that molecular diagnosis could be established in 60–70% of them [Saporta et al., 2011; Murphy et al., 2012]. The large majority of mutations (~90%) were identified in *PMP22*, *GJB1*, *MFN2*, and *MPZ*, all genes involved in dominantly inherited CMTs. Concerning demyelinating CMT forms, molecular diagnosis was established in approximately 80% of patients and classical *PMP22* duplications alone were responsible for ~70% of all CMT1 cases. In the group of axonal CMT cases, molecular diagnosis was achieved in ~25% of cases, the majority caused by mutations in *MFN2* or *GJB1*.

Molecular Pathology of CMTs

Recent identification of a number of genes that were mutated in various forms of CMTs provided unprecedented insights into the biology of both Schwann cells and neurons. Although genes mutated in CMTs encode proteins involved in a relatively wide range of cellular functions, there are some emerging themes that can be identified.

Axonal forms of CMT (see table 2 for the complete list and references) affect both dorsal root ganglion sensory neurons and spinal motor neurons. Both of these neuronal cell types are extremely polarized, axons extending up to 1 m away from their soma to sensory receptors in muscle or skin and to neuromuscular junctions with skeletal muscles [Gentil and Cooper, 2012]. Since the majority of neuronal proteins are synthesized in the cell body, this particular cellular architecture requires intensive transport of proteins between the soma and the axonal extremity. Accordingly, mutations affecting *DYNC1H1*, which encodes the cytoplasmic dynein heavy chain 1 protein, a critical subunit of dynein1 responsible for axonal retrograde transport, lead to CMT. In addition, *NEFL* (encoding neurofilament protein) and *LMNA* (encoding lamin A/C) are both involved in neurofilament homeostasis, which, if disrupted, will affect axonal transport. HSPB1 and HSPB8 (small heat shock proteins acting as ATP-independent chaperones in protein folding) also play a role in the architecture of the cytoskeleton. Among the most dynamic organelles functionally dependent on proper axonal transport are mitochondria. Mutations in

Table 1. Genetic classification of CMT

Inheritance	Demyelinating forms			Intermediate forms			Axonal forms		
	form	gene	OMIM	form	gene	OMIM	form	gene	OMIM
Autosomal dominant	CMT1A/CMT1E	*PMP22*	601097	DI-CMTA	*?*		CMT2A1/2	*MFN2/KIF1B*	608507/605995
	CMT1B	*MPZ*	159440	DI-CMTB	*DNM2*	602378	CMT2B	*RAB7*	602298
	CMT1C	*LITAF*	603795	DI-CMTC	*YARS*	603623	CMT2C	*TRPV4*	605427
	CMT1D	*EGR2*	129010	DI-CMTD	*MPZ*	159440	CMT2D	*GARS*	600287
	CMT1F	*NEFL*	162280	DI-CMTE	*INF2*	610982	CMT2E	*NEFL*	162280
				Slow SNV	*ARHGEF10*	608136	CMT2F	*HSP27 (HSPB1)*	602195
							CMT2G	*?*	
							CMT2I/2J	*MPZ*	159440
							CMT2K	*GDAP1*	606598
							CMT2L	*HSP22 (HSPB8)*	608014
							CMT2M	*DNM2*	602378
							CMT2N	*AARS*	601065
							CMT2O	*DYNC1H1*	600112
							CMT2P	*LRSAM1*	610933
Autosomal recessive	CMT4A	*GDAP1*	606598	RI-CMTA	*GDAP1*	606598	ARCMT2A2	*MFN2*	608507
	CMT4B1	*MTMR2*	603557				CMT2B1	*LMNA*	150330
	CMT4B2	*MTMR13 (SBF2)*	607697				CMT2B2	*MED25*	610197
	CMT4C	*SH3TC2*	608206				CMT2H	*GDAP1*	606598
	CMT4D (HMSNL)	*NDRG1*	605262				CMT2P	*LRSAM1*	610933
	CMT4E	*EGR2/MPZ*	129010/159440						
	CMT4F	*PRX*	605725						
	CMT4G (HMSNR)	*HK1*							
	CMT4H	*FGD4*	611104						
	CMT4J	*FIG4*	609390						
X-linked dominant				CMTX1	*GJB1*	304040			
X-linked recessive				CMTX2	*?*				
				CMTX3	*?*				
				CMTX4	*?*				
				CMTX5	*PRPS1*	311850			

Genes *KARS* [McLaughlin et al., 2010] and *CTDP1* [Varon et al., 2003] were not considered because they are associated with syndromic conditions for which peripheral neuropathy represents only a part of the pathology. ? = Disease gene is unknown.

2 different genes implicated in mitochondrial functions *(MFN2* and *GDAP1)* lead to CMT. MFN2 interferes with mitochondrial axonal transport by interacting with the adaptor Miro/Milton complex, by which kinesin attaches mitochondria to microtubules. GDAP1 is a mitochondrial fission factor and is probably directly involved in the adaption of the number and size of mitochondria to the requirements of the cell. In addition, mutations in genes involved in protein synthesis and turnover *(GARS, AARS, LRSAM1)*, endocytosis *(RAB7, DNM2)*, calcium homeostasis *(TRPV4)*, myelin assembly *(MPZ)* and gene expression regulation *(MED25)* are also leading to the axonal forms of CMT.

Large axons (with a diameter larger than 1 μm) are wrapped by a myelin sheath, which is a specialized cell membrane with a high lipid-to-protein ratio (lipids account for at least 70% of the dry weight of myelin membranes [Chrast et al., 2011]). The presence of myelin around an axonal segment plays a role as an insulator (by increasing axonal resistance) and participates in the mechanisms leading to the clustering of axonal ion channels at nodes of Ranvier [Barres, 2008]. Myelinating Schwann cells therefore play a critical role in both the architecture and electrical properties of axons, allowing for a substantial increase (up to 100×) in nerve conduction velocity while reducing axonal energy consumption [Hartline and Colman, 2007]. The importance of the appropriate assembly of the myelin membrane is highlighted by the fact that mutations in *PMP22*, *MPZ* and *PRX*, which all participate in the assembly of myelin, lead to CMT (table 2). The observation that changes in myelin protein gene dosage lead to CMT [Suter and Scherer, 2003;

Table 2. List of molecular and cellular functions of CMT genes and proteins

Clinical form of CMT	Type of CMT	Gene	Full name	Molecular function	Cellular function	References
Axonal	CMT2A1/2ARCMT2A2	*MFN2*	mitofusin 2	transmembrane GTPase	mitochondrial dynamics	Züchner et al., 2004; Detmer et al., 2008; Cartoni et al., 2010; Misko et al., 2010
	CMT2K/CMT2H	*GDAP1*	ganglioside induced differentiation associated protein 1	glutathione (GHS) metabolism	mitochondrial dynamics	Cuesta et al., 2002; Niemann et al., 2005
	CMT2E	*NEFL*	neurofilament, light polypeptide	intermediate filament protein	neurofilament/mitochondrial dynamics	Mersiyanova et al., 2000; Gentil et al., 2012
	CMT2B1	*LMNA*	lamin A/C	intermediate filament protein	nuclear lamina/neurofilament assembly	De Sandre-Giovannoli et al., 2002
	CMT2A1/2	*KIF1B*	kinesin family member 1B	molecular motor	axonal transport	Zhao et al., 2001
	CMT2O	*DYNC1H1*	dynein, cytoplasmic 1, heavy chain 1	molecular motor	axonal transport	Weedon et al., 2011
	CMT2F	*HSP27 (HSPB1)*	heat shock 27-kDa protein 1	chaperone	cytoskeletal remodeling	Evgrafov et al., 2004; Almeida-Souza et al., 2011; d'Ydewalle et al., 2011
	CMT2L	*HSP22 (HSPB8)*	heat shock 22-kDa protein 8	chaperone	cytoskeletal remodeling	Tang et al., 2005; Irobi et al., 2010
	CMT2D	*GARS*	glycyl-tRNA synthetase	tRNA synthetase	protein synthesis	Antonellis et al., 2003; Seburn et al., 2006; Motley et al., 2011
	CMT2N	*AARS*	alanyl-tRNA synthetase	tRNA synthetase	protein synthesis	Latour et al., 2010
	CMT2P	*LRSAM1*	leucine rich repeat and sterile alpha motif containing 1	E3-ubiquitin ligase	protein degradation	Guernsey et al., 2010; Weterman et al., 2012
	CMT2B	*RAB7*	RAS-associated protein RAB7	GTPase	endocytosis	Verhoeven et al., 2003a; Spinosa et al., 2008
	CMT2M	*DNM2*	dynamin 2	GTPase	endocytosis/cytoskeletal remodeling	Fabrizi et al., 2007
	CMT2C	*TRPV4*	transient receptor potential cation channel, subfamily V, member 4	calcium channel	calcium homeosthasis	Klein et al., 2011; Landouré et al., 2010
	CMT2I/2J	*MPZ*	myelin protein zero	structural myelin protein	myelin assembly	Marrosu et al., 1998; Chapon et al., 1999; De Jonghe et al., 1999
	CMT2B2	*MED25*	mediator complex subunit 25	transcriptional coactivator	gene expression regulation	Leal et al., 2009
Demyelinating	CMT1A/CMT1E	*PMP22*	peripheral myelin protein 22	structural myelin protein	myelin assembly	Lupski et al., 1991; Adlkofer et al., 1995; Sereda et al., 1996
	CMT1B/CMT4E	*MPZ*	myelin protein zero	structural myelin protein	myelin assembly	Hayasaka et al., 1993; Kulkens et al., 1993; Martini et al., 1995; Pennuto et al., 2008
	CMT4F	*PRX*	periaxin	signaling	myelin assembly	Gillespie et al., 2000; Guilbot et al., 2001; Court et al., 2004
	CMT4B1	*MTMR2*	myotubularin-related protein 2	lipid phosphatase	membrane trafficking	Bolino et al., 2000, 2004; Vaccari et al., 2011
	CMT4B2	*MTMR13 (SBF2)*	myotubularin-related protein 13	pseudophosphatase	membrane trafficking	Azzedine et al., 2003; Senderek et al., 2003a; Tersar et al., 2007
	CMT4J	*FIG4*	FIG4 homolog, SAC1 lipid phosphatase domain containing	lipid phosphatase	membrane trafficking	Chow et al., 2007; Vaccari et al., 2011
	CMT4D (HMSNL)	*NDRG1*	N-myc downstream regulated 1	signaling	membrane trafficking	Kalaydjieva et al., 2000; Okuda et al., 2004
	CMT4A	*GDAP1*	ganglioside induced differentiation associated protein 1	glutathione (GHS) metabolism	mitochondrial dynamics	Baxter et al., 2002; Niemann et al., 2005; Noack et al., 2012
	CMT1F	*NEFL*	neurofilament, light polypeptide	intermediate filament protein	neurofilament/mitochondrial dynamics	Jordanova et al., 2003; Gentil et al., 2012
	CMT4C	*SH3TC2*	SH3 domain and tetratricopeptide repeats 2	Rab11 effector	endosomal recycling	Senderek et al., 2003b; Arnaud et al., 2009; Lupo et al., 2009; Roberts et al., 2010; Stendel et al., 2010
	CMT1C	*LITAF*	lipopolysaccharide-induced TNF factor	?	protein sorting	Street et al., 2003; Somandin et al., 2012
	CMT1D/CMT4E	*EGR2*	early growth response 2	transcription factor	gene expression regulation	Warner et al., 1998; Nagarajan et al., 2001
	CMT4G (HMSNR)	*HK1*	hexokinase 1	converts glucose into glucose-6-phosphate	glucose metabolism	Hantke et al., 2009
	CMT4H	*FGD4/Frabin*	FYVE, RhoGEF and PH domain containing 4	guanine nucleotide exchange factor (GEF)	cytoskeletal remodeling	Delague et al., 2007; Stendel et al., 2007
Intermediate	DI-CMTE	*INF2*	inverted formin, FH2 and WH2 domain containing	regulator of actin polymerization/ depolymerization	cytoskeletal remodeling	Boyer et al., 2011
	Slow SNV	*ARHGEF10*	Rho guanine nucleotide exchange factor (GEF) 10	guanine nucleotide exchange factor (GEF)	cytoskeletal remodeling	Verhoeven et al., 2003b
	DI-CMTB	*DNM2*	dynamin 2	GTPase	endocytosis/cytoskeletal remodeling	Züchner et al., 2005; Sidiropoulos et al., 2012
	DI-CMTC	*YARS*	tyrosyl-tRNA synthetase	tRNA synthetase	protein synthesis	Jordanova et al., 2006; Storkebaum et al., 2009
	DI-CMTD	*MPZ*	myelin protein zero	structural myelin protein	myelin assembly	Mastaglia et al., 1999
	I-CMT/CMTX1	*GJB1/CX32*	fap junction protein, beta 1, 32 kDa	gap junction channel component	myelin assembly/intra-myelin transport	Bergoffen et al., 1993; Anzini et al., 1997; Abrams et al., 2003
	CMTX5	*PRPS1*	phosphoribosyl pyrophosphate synthetase 1	phosphoribosylpyrophosphate synthetase	nucleotide synthesis	Kim et al., 2007

Maeda et al., 2012] is in agreement with the evidence that mutations in *EGR2*, a transcription factor that regulates myelin gene expression, also lead to a CMT phenotype. The estimated 6,500-fold increase in membrane surface occurring during myelination [Webster, 1971] and the highly polarized structure of Schwann cells suggest that membrane assembly, transport and maintenance are critical for proper Schwann cell function. The identification of mutations in *MTMR2, MTMR13/SBF2, FIG4* and *NDRG1* genes (encoding proteins involved in membrane trafficking), in *SH3TC2* (involved in endosomal recycling), in *LITAF* (involved in protein sorting) and in *FGD4* (involved in cytoskeletal remodeling) supports this hypothesis and provides molecular insight into the various mechanisms involved in myelin membrane homeostasis.

Intermediate forms of CMT are expected to affect both glial and neuronal functions and highlight the close relationship between these 2 cell types. Mutations affecting genes encoding proteins involved in cytoskeletal remodeling *(INF2* and *DNM2)*, protein synthesis *(YARS)*, myelin assembly *(MPZ* and *GJB1)* and nucleotide synthesis *(PRPS1)* lead to defects in Schwann cells and in axons.

The diversity of cellular and molecular functions of proteins that are associated with different forms of CMTs demonstrates that the identification of disease genes is a crucial step to obtain insights into both the pathophysiology of the disease and the biology of the peripheral nervous system. In terms of future gene identification efforts, the plethora of genes and gene products involved in CMTs (table 2) precludes simple predictions of additional CMT genes based solely on functional data. This aspect is further highlighted by the ubiquitous expression/function of many CMT genes identified so far (e.g. *HSP27, HSP22, GARS, AARS, YARS, MTMR2, MTMR13/SBF2*). Therefore, while data obtained through characterization of various CMT forms have generated a list of possible candidate pathways that may be selectively compromised in patients, one cannot exclude that other biological structures or biochemical reactions can be important in the molecular pathology of CMT.

From Positional Cloning to Highly Parallel Genetic Technologies

The majority of CMT genes have been identified by positional cloning, usually involving large families with multiple affected individuals to allow fine-mapping of the culprit mutation to a small region containing only few genes. In more recent times, gene hunting in CMTs (and other heterogeneous conditions) has been forced to adapt to less favorable conditions, classically represented by patients from small families yielding extended or even several regions of potential linkage. Scientists have therefore become more inventive, designing or integrating various techniques to overcome technical limitations. For example, research combining systematic screens with linkage results (e.g. blind mutational screenings of all genes present in a given linkage region) have allowed the discovery of mutations in genes that a priori were not considered as prime candidates (e.g. [Chakarova et al., 2007]). As mentioned above, strategies to prioritize candidate genes based on gene function (if known) and on similarity with or functional relation to genes involved in the same disease [Dryja, 1990, 1997] may not always be successful as structurally and functionally diverse gene products might cause the same or a similar phenotype. Another attractive strategy is to characterize the expression profile of candidate genes in the organ or tissue targeted by the disease. However, for hereditary neuropathies, this approach has been hampered so far by the scarcity of transcriptional data from the affected tissues, i.e. spinal cord and dorsal root ganglion neurons or Schwann cells. Recently, with the availability of high throughput transcriptional analysis techniques, we and others have generated large-scale expression data characterizing peripheral nerve development, degeneration and regeneration, which are now available for gene prioritization [Verheijen et al., 2003; Giambonini-Brugnoli et al., 2005; Verdier et al., 2012].

Regardless of the technique used to pre-select possible culprit sequences, detection of mutations has relied so far on the use of mid- to large-scale systems, i.e. Sanger sequencing [Sanger and Coulson, 1975], sometimes preceded by screening techniques such as single-strand conformation polymorphisms, denaturing high performance liquid chromatography or denaturing gradient gel electrophoresis. More recently, the discovery and the recent commercialization of next-generation sequencing (NGS) instruments have represented a real technological revolution in the field of molecular genetics.

In NGS-driven genomic and exomic approaches, the bulk of the analytical procedures consists in data filtering, since comprehensive sequencing of the human genome (or of its coding sequences) obviously generates much more information than what is required for the identification of the cause of a monogenic disease. In the context of single-gene hunting, only 1 or 2 of the ~4 million variants detected genome-wide (or ~20,000 for a typical exome) represent the 'signal', to be distinguished

from the overwhelming noise represented by all other DNA changes.

For recessive Mendelian diseases, one of the most successful approaches for noise reduction is filtering for regions of the genome for which patients carry identical (homozygous or autozygous) alleles inherited from a common ancestor of both parents. In autosomal recessive CMT this is particularly valuable, as in geographic regions and communities with a high prevalence of consanguineous marriages (e.g. Arab countries, Turkey, Iran, Pakistan, Roma populations) autosomal recessive inheritance accounts for 30–50% of all CMT cases [Patzko and Shy, 2011]. Detection of autozygosity is usually performed by using polymorphic markers (commonly SNPs) and the identification of DNA stretches for which homozygosity of alleles is observed over several kilobases. Using whole-genome SNP homozygosity mapping and subsequent DNA sequencing, Fischer et al. [2012] were able to provide molecular diagnosis to 15 (63%) out of 24 index cases with sporadic or autosomal recessive CMT. Information on SNPs can be obtained prior to exome or genome sequencing, or directly extracted from the genome or exome information. The difference between these 2 approaches relies mostly on costs: in large families, microarray-based genotyping for several members followed by the analysis of a single exome/genome is (currently) cheaper, while in small families or in single cases direct sequencing is to be preferred. Another advantage of homozygosity mapping over classical linkage is that in principle even small families can be informative enough to lead to the successful identification of the disease gene.

An alternative strategy for gene hunting for autosomal recessive CMT makes use of compound heterozygosity observed in the offspring of non-consanguineous parents with children with a recessive disorder: in this scenario, sequencing data will be filtered for genes in which 2 non-polymorphic variants (putative mutations) are simultaneously present. This process allows reducing the number of candidate genes by up to 3 orders of magnitude, compared to the genes containing only a single variant. Finally, selection of DNA changes that are shared among affected members of the same pedigree can further reduce the list of putative disease genes to the point only the true mutation is left.

In autosomal dominant or X-linked CMTs, noise reduction can be achieved by a similar pipeline, such as filtering for variants that are shared among multiple patients from the same families [Weedon et al., 2011].

Highly Parallel Genetic Technologies and CMT: Recent Discoveries

While still in its infancy, various highly parallel genetic technology approaches have already lead to a successful identification of the causative mutations in some families affected with peripheral neuropathies.

In their landmark study, Lupski et al. [2010] described the complete genome sequencing of an individual affected with CMT1 performed with the use of one of these sequencing platforms. Comparison of the proband's sequence with the human genome reference sequence led to identification of 1,165,204 intragenic SNPs of which 9,069 were non-redundant SNPs predicted to lead to non-synonymous codon changes. The authors then concentrated on 54 coding SNPs (out of 3,148 putative SNPs) present in 40 genes known to be involved in neuropathy-like conditions. Of these, two were located in the *SH3TC2* gene (1 previously unreported missense mutation, p.Y169H, and 1 known disease-causing nonsense mutation, p.R954X) indicating compound heterozygous mutations in this gene as a cause of CMT1.

An exome sequencing approach was used by Weedon et al. [2011] to study a large, 4-generation family affected by CMT2, in which various mutations in genes commonly associated with CMT2 *(MFN2, MPZ, NEFL* and *LMNA)* had been previously excluded by Sanger sequencing. Whole-exome sequencing was performed in 3 affected individuals and, based on an assumed autosomal dominant model of inheritance, the authors concentrated on novel heterozygous variants. Out of 177, 192, and 199 missense, nonsense, frameshift or splice site variants present in individual patients, only 1 variant was shared by all 3 affected individuals. The detected missense change (c.917A>G, p.H306R) in cytoplasmic dynein heavy chain 1 gene *(DYNC1H1)* affects a highly conserved residue within its homodimerization domain.

A similar approach was used by Landouré et al. [2012] to study a family with a CMT2C phenotype (axonal CMT with vocal cord paralysis). One affected individual was analyzed by exome sequencing leading to identification of a novel sequence variant (c.557G>A, p.R186Q) in the transient receptor potential vanilloid 4 gene *(TRPV4)* that was already known to cause CMT2C. Sanger sequencing confirmed the segregation of the mutation with the phenotype in the family. In addition, authors confirmed the pathogenicity of this variant in a cellular system.

Montenegro et al. [2011] also used exome sequencing to study a family with an axonal or intermediate form of CMT. Sequencing was performed in 2 affected individu-

als leading to the identification of 86 unique single-nucleotide variants in 82 genes and 146 insertions/deletions (66 in coding exons) segregating in both individuals. One of these variants (c.283G>A, V95M) affected the previously identified CMT gene *GJB1*.

A recent study by Auer-Grumbach et al. [2011] described the successful use of a combination of linkage analysis and NGS to identify missense variants in fibulin-5 that lead to a dominant form of CMT1. A genetic linkage study was performed in 2 families leading to the identification of a critical region on chromosome 14q32. After the exclusion of a few candidate genes *(CALM1, GALC, PTPN21, KCNK13)*, the critical region was captured using a custom sequence capture array (Roche NimbleGen) and sequenced (454 GS-FLX, Roche). Sequence analysis revealed 12 non-synonymous sequence variants, eleven of which were known SNPs. The only new variant that was identified was in the *FBLN5* gene (c.1117 C>T, p.R373C). Two additional fibulin-5 variants (c.268 G>A; p.G90S and c.376 G>A; p.V126M) were identified in 6 additional CMT patients.

A very similar approach was used by Weterman et al. [2012] to identify a frameshift mutation in *LRSAM1*. The linkage analysis run on a large 3-generation family led to the identification of a 5-Mb region on 9q33q34 with a LOD score of 5.12. The authors then used a custom sequence capture array covering the identified linkage region, to enrich, by sequence capture, the DNA from 1 affected individual which was subsequently sequenced. Sequence analysis revealed 5 heterozygous changes compatible with an autosomal dominant mode of inheritance. Subsequent segregation analysis and bioinformatic analyses revealed that the p.Leu708Argfx28 frameshift mutation in *LRSAM1* is underlying this dominant type of CMT.

NGS and CMT: Perspectives

Data from recent studies in large cohorts of CMT patients indicated that molecular diagnosis could be established in ~50–70% of them [Baets et al., 2011; Saporta et al., 2011]. These results strongly indicate that an important number of additional CMT genes remains to be discovered. As discussed above, NGS technologies have the potential to help in achieving this goal.

In addition to their potential as a gene discovery tool, NGS technologies should be considered also as a diagnostic screening method. This is especially true in cases of CMT with substantial locus heterogeneity and/or where multiple large genes, making conventional screening of each patient time- and money-consuming, are implicated. Recent work by Choi et al. [2012] has already demonstrated the successful use of exome sequencing as a molecular diagnostic tool in CMT. In this study, 25 CMT patients who were negative for mutations in major CMT genes were analyzed by exome sequencing, leading to the identification of mutations in 32% of them. While this detection rate is relatively high, especially considering that these samples were pre-tested for the most common CMT genes, it also indicates the current limitations of this approach [Coppola and Geschwind, 2012].

In exome sequencing, the most prominent of these limitations is the selection of DNA to be analyzed. Since only exons and their flanking regions are interrogated, mutations in promoter areas, untranslated regions or any other non-exonic areas, as well as copy number variations, will be missed. Most important, in some instances capture kits allowing isolation of exonic regions are inefficient in isolating specific areas of the genome. As a consequence, the sequence coverage of CMT-related genes varies substantially (from 0 to more than 100 reads per base position) and some neuropathy-related genes including *NEFL, WNK1* and *SEPT9* cannot be analyzed at all [Montenegro et al., 2011]. However, this issue should be resolved, or at least substantially improved, with the commercialization of the next generation of exon capture kits, which should provide more comprehensive exome coverage.

The second and probably more challenging limitation is the issue of adequate exome data analysis. Each sequencing experiment leads to the identification of thousands of variants requiring subsequent filtering of previously identified benign variants. Various databases can be used to achieve this (1,000 Genomes Project: http://www.1000genomes.org; dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/, etc.). However, often even a very restrictive filtering step will lead to the identification of tens of candidates that are genetically and functionally potentially compatible with the disease. This highlights the importance of clinically well-characterized cohorts for data pooling and comparison or extended pedigrees allowing selection of meaningful variants through segregation analysis.

Although large-scale genetic approaches already provide useful tools to characterize patients affected by CMT disease, from the examples mentioned above it is clear that both NGS technologies and their analytical pipelines still need some improvement. The anticipated technological development leading to better sequence representa-

tion, optimized filtering procedures and the establishment of a repository of coding variants identified in CMT patients will contribute to an improved success rate of NGS as a tool for both new gene discovery and diagnosis of CMT patients.

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