

## Molecular genetics studies in Polish Charcot-Marie-Tooth families

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### Abstract

*Charcot-Marie-Tooth (CMT) disorders are the extremely heterogenous group of diseases of the peripheral nervous system in humans with a prevalence of 1: 2500. Up to date mutations in 30 genes have been reported in various CMT forms. In numerous CMT types only locus is known and some CMT forms were shown not to be linked with any known locus. Genetic studies in CMT disorders cover a wide spectrum of problems ranging from identification of novel mutations through studies of pathogenic nature of mutations to genotype-phenotype correlations. The aim of this study was to present the main directions of genetic analysis performed in Polish families with CMT disease.*

**Key words:** Charcot-Marie-Tooth disease, gene mapping, phenotype-genotype correlations, mutation screening, pathogenic effect of mutations, molecular diagnostics

### Introduction

Hereditary motor and sensory neuropathies (HMSN) in genetic literature called also Charcot-Marie-Tooth disease (CMT) are the most common hereditary group of disorders of the peripheral nervous system in man with prevalence of 1: 2500 newborns [57].

In general, CMT disorders are characterized by slowly progressive atrophy of the distal muscles associated with distal sensory disturbances [16]. In 1980 CMT disease was classified on the basis of the value of the nerve conduction velocity in the motor fibers of the median nerve (MNCV).

The patients with MNCV less than 38 m/sec and demyelinating changes visible on sural nerve biopsy were classified as CMT1. For the patients with the

normal value of MNCV and axonal damage the name of CMT2 was reserved [16].

In the last ten years molecular genetic analysis revealed an extremely high genetic heterogeneity of HMSN. Up to April 2004 thirty genes were reported to be mutated in various forms of HMSN [62]. Both types of genetic heterogeneity i.e. concerning loci and allelic occur in CMT disease. Only in one subtype of CMT, characterized by autosomal dominant trait of inheritance and axonal changes nine loci have been mapped [38]. Similarly to the heterogeneity of loci observed in CMT, the allelic heterogeneity of X-dominant CMTX disease is reflected by the presence of 300 mutations in the gene *Cx32* coding for Connexin 32 [62]. Despite the fact of genetic heterogeneity of HMSN, the distribution of the mutations within the genes is not equal. The number

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of the mutations varies from the single amino-acid substitutions in the *KIF1B* gene detected in one CMT2A family to a common duplication in the 17p11.2-p12 region in CMT1A subtype [46,70].

### Genetic heterogeneity of CMT1 inherited in autosomal dominant trait

Up to date five genes have been reported to be mutated in the CMT1 disease with an autosomal dominant trait of inheritance. The most common mutation, with an estimated frequency at 60% [65] of all CMT cases, is a submicroscopical 17p11.2-p12 duplication encompassing a 1.5 Mb region [43,51].

Although the number of genes in the 17p11.2-p12 region is estimated at 70, only Peripheral Myelin Protein gene (*PMP22*) was shown to play a critical role in CMT1A disease.

Interestingly, the point mutations in the *PMP22* gene have been shown to result also in the CMT1A phenotype [52]. The hereditary neuropathy with liability to pressure palsies (HNPP) was reported to be caused by a 17p11.2-p12 deletion (majority of cases) or point mutations in the *PMP22* gene [10,48].

A minor autosomal dominant CMT1 locus (called CMT1B) has been mapped to chromosome 1q21-q23 [5]. The CMT1D gene maps to chromosome 10 and is associated with mutations in the early growth response gene-2 (*EGR2*), also known as *Krox-20* [63]. The CMT1 affected patients in whom no mutations in the *PMP22*, *MPZ* and *EGR2* genes were detected were designated as CMT1C. Recently the linkage analysis performed in two CMT1C families has revealed the linkage of CMT1C with chromosome 16p. Recently three mutations in the *LITAF/SIMPLE* gene have been identified in three families suggesting that CMT1C may be caused by mutations in the *LITAF/SIMPLE* gene [58,59].

### Autosomal dominant type of CMT2 disease

In the recent years autosomal dominant form of CMT2 disease has been shown to be linked with eleven loci. The first CMT2A locus was mapped on chromosome 1p35-p36 [3]. Although a Q98L in the gene *KIF1B* was identified in the remaining CMT2A families no mutations in the *KIF1B* gene have been detected [69,70].

Recently in the 7 pedigrees with different ethnic background six mutations in the mitochondrial GTPase mitofusin 2 gene (*MFN2*) gene have been

identified [70]. The second CMT2B locus with a phenotype characterized by a severe sensory loss and ulcerations of distal part of the lower limbs was mapped to chromosome 3q13-q22. Recently the CMT2B disease has been shown to be associated with mutation L129F and V162M in the *RAB7* gene coding for a small GTPase late endosomal protein [66]. CMT2C disease was delineated from others CMT2 pedigrees because of its phenotypic hallmarks i.e. progressive muscle weakness and atrophy of limb, diaphragm, vocal cord, and intercostals muscles. CMT2C disease was recently mapped to the 12q23-24 region [27]. Despite its specific phenotype the gene for CMT2C disease has not yet been mapped.

In CMT2D disease characterized by the predominant muscle weakness and atrophy in the hands with an age of onset in the second or third decade four mutations in the *GARS* gene coding for glycyl tRNA synthetase were described [1].

Interestingly the CMT2E subtype mapped on chromosome 8p12 and caused by mutations in the neurofilament light chain gene (*NEFL*) has been shown to be associated both with CMT1 and CMT2 phenotypes [21,45].

In the large multigenerational Russian family a novel CMT2F subtype has been mapped on chromosome 7q11-q21 [20].

The intermediate form of CMT which phenotype shares both axonal and demyelinating features was also shown to be heterogenous. The DI-CMT2A, CMT2B and CMT2C have been mapped to three loci, respectively 10q24.1-25.1, 19p12-13.2 and 1p34-35 [22, 26, 67]. Recently two novel CMT2G and CMT2L subtypes have been mapped respectively on chromosome 12q12-q13.3 and 12q24 [47,60]. Recently a novel early onset CMT2 disease has been shown not to be linked with nine known loci for autosomal dominant type of CMT2 [38].

### Charcot-Marie-Tooth disease linked with X chromosome

Although the first report on X-linked trait of inheritance dates to 1889 this kind of inheritance in CMT disease was viewed with some skepticism [16]. The most frequent CMTX1 form is inherited in a dominant trait, whereas rare X-linked pedigrees (CMTX2 and CMTX3) have been shown to be transmitted in a recessive trait.

In 1985 CMTX was mapped to the proximal long arm of chromosome X [14]. Later on the CMTX1

region was narrowed to Xq13.1 [4]. Finally, in 1993 seven mutations were reported in the *Cx32* gene coding for a Connexin protein of molecular weight of 32 kDa [4].

In the recent 10 years over two hundred fifty mutations in the *Cx32* gene have been reported.

The majority of them have been detected in the patients suffering from "classical" phenotype of peripheral neuropathy. For 15 mutations in the *Cx32* gene additionally the central nervous system involvement was documented (white matter lesions on CT examination and deafness) [62]. It is believed that CMTX1 frequency may be estimated to be around 14% of all CMT types [53]. In a cohort of 153 unrelated patients (141 patients with CMT1, 3 with DSS, 1 with CHN, 1 with hereditary motor and sensory neuropathy V, 1 with HNPP, and 6 with CMT2) eight mutations (7.2%) in the *Cx32* gene have been detected [7]. Apart from the X-dominant type of CMT disease a few pedigrees with X-recessive trait of inheritance have been reported. In one three-generation family five of the seven CMT affected males developed deafness by 5 years of age, and three of these five individuals had also mental retardation. This pedigree has been mapped to chromosome Xq24-q26 [50]. A similar family was identified recently in Poland, but unfortunately, no agreement to genetics analysis was given by the members of the family. The second locus for X-recessive form of CMT disease was mapped to chromosome Xp22.2 in one family. The phenotype was characterized by infantile onset, weakness of lower legs, areflexia, pes cavus, and mental retardation (2 of 5 patients) [19].

To conclude three loci have been identified in X-linked form of CMT disease but only in one X-dominant type the gene (*Cx32*) and mutations have been reported.

### Autosomal recessive CMT disease

The frequency of recessive form of demyelinating Charcot-Marie-Tooth disease (CMT4) is not known, but it seems that CMT4 form is a rare type of CMT disorders. The most common CMT4A form is associated with mutations in the *GDAP-1* gene. Some recessive mutations in the *GDAP-1* gene have been shown to segregate with axonal type of neuropathy whereas others were detected in CMT1 disease [2,11].

In the CMT4B1 type characterized by focally folded myelin sheaths at the sural nerve biopsy mutations have been shown in the *MTMR-2* gene coding for phosphatidylinositol phosphatase [8,9].

Interestingly the other CMT4 neuropathy also associated with phenotype of focally folded myelin and early onset glaucoma was shown to be associated with mutations in the *MTMR-13* gene belonging to the family of phosphatidylinositol phosphatases [55].

CMT4C mapped to region 5q32 is characterized by early onset, severe clinical course with severe scoliosis requiring surgery and extended Schwann cells processes. CMT4C neuropathy was up to date reported in the Algerian, Dutch and Turkish consanguineous families [13,41]. Recently mutations in the gene encoding for a novel SH3/5PR domain have been shown to be associated with CMT4C neuropathy [56]. The recessive form of Dejerine-Sottas syndrome has been reported to be associated with mutations in the periaxin gene (*PRX*) encoding L- and S-periaxins, proteins required for maintenance of peripheral nerve myelin [6,15]. Two axonal forms of recessive neuropathy (ARCMT2) have been reported. The ARCM2A disease is caused by mutations in the *LMNA* gene, the ARCM2B is linked to the 19q13.3 locus [12]. There are also two forms of recessive CMT occurring only in Romy (Gypsy) families. The hereditary motor and sensory neuropathy identified in Russe (HMSNR) was mapped to the 10q22-q23 region, and the second HMSN initially described in Gypsy from Bulgarian city LOM which was shown to be caused by the R184X mutation in the N-myc Downstream Regulated gene 1 (*NDRG1*) [24,25,61].

### Mutations in the Myelin Protein Zero gene are associated with spectrum of Charcot-Marie-Tooth phenotypes

For the first time in 1982, a demyelinating form of Charcot-Marie-Tooth disease was mapped to the chromosome 1q21-q23, to the Duffy locus [5]. Since the genetic linkage to the 1q21-q23 region was shown only in some CMT1 families the minor locus was called CMT1B. Later on CMT1B was shown to be caused by mutations in the Myelin Protein Zero gene (*MPZ*) coding for a most abundant protein occurring in the myelin of the peripheral nervous system [17]. Due to the initially described phenotype-genotype

correlation, the *MPZ* gene has been analyzed for years only in CMT1 affected patients. For the first time, in 1996 a set of mutations was reported in patients suffering from DSS disease and congenital hypomyelinating neuropathy (CHN) [64]. Recently the *MPZ* gene mutations have been shown to segregate also with axonal form of CMT disease (CMT2).

The spectrum of phenotypes associated with mutations in the *MPZ* gene was also observed in Polish CMT affected patients. A heterozygous T124K mutation in the *MPZ* gene was identified in the patient suffering from CHN. The detection of the first amino-acid substitution in the *MPZ* gene associated with CHN phenotype suggests a dominant-negative effect of this mutation [30]. The phenotype of an early onset demyelinating neuropathy may be located between CHN and CMT1B phenotypes. A novel L190fs mutation was identified in the *MPZ* gene in a patient with early onset CMT1 disease [31]. The phenotype of the so-called focally folded myelin (FFM) is a particular form of CMT1B disease caused by mutations in the *MPZ* gene. Within four mutations in the *MPZ* gene detected so far in patients with FFM two were identified in Polish CMT affected patients [29,32]. Finally, also in Polish patients the CMT2 family with mutations in the *MPZ* gene was reported. A novel E56K mutation in the *MPZ* gene was found in four members of CMT2 affected family. CMT2 phenotype in this family was characterized by late onset and slowly progressive course [33,49].

In conclusion the patients suffering from CHN, CMT1, CMT2 with focally folded myelin and CMT2 affected patients should be screened for point mutations in the *MPZ* gene [34].

### **Mild early onset axonal Charcot-Marie-Tooth disease in Polish family not linked to other Charcot-Marie-Tooth loci**

The family was identified in the northern part of Poland. Neurological examination was performed in twelve members of the family. The age of onset ranged between 6 and 14 years. The atrophy of the forearm muscles and the thenar eminence muscles was present in four patients. In all patients except one deep tendon reflexes in the upper limbs were preserved. In the lower limbs distal muscle atrophy was observed in all patients. The knee reflexes were

preserved in all patients, whereas Achilles tendon reflexes were retained in the youngest 12 year – old patient. All sensory modalities were impaired distally in both the upper and lower limbs in all examined patients.

In 6 examined patients median nerve conduction velocities and motor action potentials were normal, whereas in the lower limbs the amplitudes of the compound motor action potential were reduced except one patient.

Molecular genetic analysis was performed in 13 members of CMT2 family. All the patients gave their informed consent. DNA was extracted from white blood cells. The *Cx32* gene was sequenced and no abnormalities were found. Also the *MPZ* gene locus was excluded by linkage analysis. The genetic linkage analysis was performed for nine known CMT2 loci. A negative score (<-2) was obtained for at least one marker at each locus. These results indicate that CMT2 disease reported in the Polish family is not linked to any known locus of autosomal dominant CMT2 disease.

At the clinical level CMT2 disease reported in the Polish family is characterized by slowly progressive motor and sensory neuropathy without any additional features observed in other CMT2 types. The disproportionate distribution of the motor and sensory nerve involvement between the upper and lower limbs seems to be an electrophysiological hallmark of CMT2 disease reported in the Polish family [38].

### **Mutations in the *Cx32*, *NEFL* and *PMP22* genes – how pathogenic are they?**

In contrast to the well known mutations in genetic disorders, vast majority of the amino-acid substitutions identified in CMT disorders were detected in single patients. Thus, the detection of an association between a certain amino-acid substitution and particular phenotype of CMT is not equal with the identification of mutation that causes CMT disease.

Up to date over 200 mutations in the *Cx32* gene have been identified in the CMTX disease [62]. Only 10% mutations in the *Cx32* were proved to segregate with CMTX disease. The pathogenic status of 9% of *Cx32* mutations is unknown, since those mutations do not have any phenotypic characteristics. Functional analysis was performed only for a few mutations in the *Cx32* gene [44]. In Poland, a first



mutation in the *Cx32* gene (E208G) was described in 2001 [28]. Interestingly the E208G mutation in the *Cx32* gene has not been detected so far in other CMT families. Nevertheless, the codon 208 of the *Cx32* gene was shown to be mutated (E208K) mutation in several CMTX families. Up to date six mutations in the *Cx32* gene have been reported in Polish CMTX families. Although these mutations were not detected in the control group, their pathogenic status differs from well documented E208K substitution to novel G110D, V152D and K167E substitutions detected in CMT affected patients [35]. Interestingly the E208K mutation in the *Cx32* gene was identified in a mosaic 79 years old patient [36].

Mutations in the Peripheral Myelin Protein 22 gene (*PMP22*) were reported in CMT1 affected patients, Dejerine-Sottas disease and congenital hypomyelination. The most common point mutation of the *PMP22* gene, S72L mutation was detected in patients with severe CMT1 disease, DSS disease and congenital hypomyelination [62]. It seems important to note that S72L mutation is associated with a severe phenotype of CMT disease also in two Polish unrelated patients with phenotype of severe CMT1 disease [37]. The similarity between CMT phenotypes associated with the same S72L mutation in the *PMP22* gene indicates that this amino-acid substitution is pathogenic.

The *NEFL* gene coding for Neurofilaments with light molecular mass was shown to be mutated both in CMT1 and CMT2 affected patients [21,45]. The Glu528del mutation in the *NEFL* gene was initially identified in CMT affected patient. According to the authors the Glu528del segregated with CMT phenotype, however, this observation was not confirmed by molecular genetic analysis in the CMT2E family. The Glu528del was not detected in 65 healthy controls (130 chromosomes) [21]. The studies of the *NEFL* gene performed in the group consisted of 248 healthy individuals (496 chromosomes) from Japan revealed the presence of the Glu528del polymorphism with a frequency of 0.018 [68]. Recently a novel I214M amino-acid substitution in the *NEFL* gene was identified in two unrelated patients with a severe and mild CMT phenotype. The results of functional analysis of the I214M mutation in the *NEFL* gene did not confirm the pathogenic nature of this amino-acid substitution [23]. The question whether a particular amino-acid substitution is a benign polymorphism

or pathogenic mutation is not limited to the genes of CMT disease but seems to concern numerous genetic disorders [42].

### Mutations in the *LITAF* gene and the phenotype of CMT1C disease

After identification of mutations in the *PMP22* (CMT1A), *MPZ* (CMT1B) and *EGR2* (CMT1D) genes it became clear that some CMT1 patients do not have mutations in these genes. This observation suggested a further genetic heterogeneity of CMT1. In 2002 the other form of CMT1 was linked to the 16p13.3-p12 locus [58]. One year later, three mutations in the *LITAF* gene were reported in CMT1 disease linked to the 16p13.3-p12 locus and this form was called CMT1C [59]. The clinical characteristics of CMT1C disease is limited to a few pedigrees, nevertheless, CMT1C phenotype seems to be indistinguishable from the other CMT1 forms. Recently in the multicenter study of *LITAF* gene encompassing CMT1 affected patients with different ethnic background four novel mutations in the *LITAF* gene were reported. One of the *LITAF* mutations was identified in a Polish CMT1 affected patient. The Leu122Val mutation was found in a patient with a mild form of CMT1 disease, characterized by the presence of typical demyelinating changes in sural nerve biopsy. Although the Leu122Val mutation was not detected in the large group of healthy controls, its pathogenic nature is not completely understood [54]. In contrast to the numerous genes mutated in CMT disease, the biological function of the *LITAF* protein remains unknown. In this context the understanding of the pathogenic nature of the mutations in the *LITAF* gene seems to be extremely difficult. The further studies both of CMT1 affected patients and transgenic animals are needed for better understanding of CMT1C pathogenesis.

### Molecular diagnostics and genetic counseling in Charcot-Marie-Tooth disorders

Similarly to other genetic disorders molecular diagnostics in CMT in the majority of countries is limited to the most common forms of CMT i.e. CMT1A disease caused by the 17p11.2-p12 duplication and HNPP disease caused by the 17p11.2-p12 deletion. Although the average

frequency of 17p11.2-p12 duplication within CMT1 affected patients in Europe was estimated to 70.7%, its frequency varies from 34.3% in Spain through 37.5% in Sweden to 69.7% in Belgium [18,46]. It seems possible that the low frequency of 17p11.2-p12 duplication in Swedish CMT patients results from a higher percentage of recessive forms. Similarly to the 17p11.2-p12 duplication the differences of mutation distribution in other CMT genes may be expected.

In the large cohort of CMT patients consisting from 153 unrelated CMT patients the 17p11.2-p12 duplication was observed in 51.5% individuals. The *Cx32* gene mutations were found in 7%, mutations in the *MPZ* and *PMP22* genes were found in 6.6%. Only in 1.95% of CMT patients mutations in the *EGR2*, *PRX* and *NEFL* genes were identified. Interestingly, no mutations in 8 analyzed genes were found in 32.7% CMT patients [7]. The estimation of distribution of mutations in CMT genes in a certain region or country is crucial for a specific molecular diagnostics. Although the frequency of rare disorders with autosomal recessive trait of inheritance in Poland is not high, five mutations in *GDAP1* gene were detected in Polish patients, whereas only 20 mutations in the *GDAP1* gene were identified in the other countries.

It seems possible that in the Polish patients with early onset CMT2 disease molecular analysis of the *GDAP1* gene should be performed. Molecular genetic diagnostics in CMT disorders is crucial for genetic counseling for patients. Due to the high rate of sporadic cases, the analysis of pedigree is not informative for a genetic counselor. A limited penetrance of 17p11.2-p12 deletion, mutations in the *Cx32* gene or even in *MPZ* gene may result in a false detection of de novo mutations. In congenital hypomyelinating neuropathy the diagnosis based on the sural nerve biopsy findings is not sufficient for genetic counseling due to the autosomal recessive and dominant trait of inheritance in CHN.

### Genetic studies in CMT disorders in future

In the recent ten years the number of CMT genes rose from 4 to 30. The genetic heterogeneity of CMT disorders is not reflected by molecular genetic diagnostics which is still limited to the CMT1A and HNPP diseases. Thus, for the effective genetic counseling in CMT disorders a wider spectrum of

CMT genes should be screened in future. For numerous not yet mapped CMT types the detection of novel genes may be expected. Although a number of CMT genes still rises, the biological function of them remains unclear. The understanding of the molecular pathophysiology of CMT disorders will require the complete characteristics of CMT genes at the cellular level. Every month novel mutations in CMT affected patients are detected, however, for numerous mutations their pathogenic status remains unknown. It is possible that fine phenotype-genotype correlations in CMT will result in better diagnostics of this group of disorders. Finally, hopefully, after DNA „era” in CMT diagnostics some experiments of gene therapy may be developed in gene therapy of CMT disorders.

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