NEUROGENETIC ANALYSIS OF HEREDITARY NEUROPATHIES IN THE ERA OF GENOMIC MEDICINE

Ph.D. thesis

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INTRODUCTION

Peripheral neuropathy is a term for a group of conditions in which the peripheral nerves are damaged. Nerve damage can impair the muscle strength, the sensation, and different organ functions. Hereditary neuropathies are chronic conditions affecting symmetric the motor and/or sensory nerves. It is one of the most common inherited neurodegenerative disorders, affecting approximately every one person from 2500. The relative homogenous clinical appearance of the disease is associated with an especially wide genetic background.

Hereditary motor and sensory neuropathy, also called Charcot-Marie-Tooth neuropathy (CMT), affects fundamentally both the motor and sensory nerves. It is characterized by length dependent nerve degeneration which slowly progresses with time leading to worsening of the condition and a gradually developing disability. In addition to classical phenotype, associated features occur frequently due to the complex physiological roles of different CMT genes resulting in atypical signs and symptoms and leading to difficulties in distinguishing CMT from other disorders such as spinal muscular atrophy, hereditary spastic paraplegia, amyotrophic lateral sclerosis ataxias or mitochondrial disorders. Natural histories of patients varies greatly even in families with same mutation which can be explained by the role of modifying factors, such as gene-gene interactions, mutation load, epigenetic modification effects, co-morbidities or disease management.

The four most frequent causative genes are *PMP22*, *GJB1*, *MPZ* and *MFN2* in order and these are responsible for 70-90 percent of all CMT cases. The frequency of major and minor genes varies widely in different cohorts and because of the founder effect, some of the genetic variants are mainly specific for a certain ethnicity or geographical area. In the last decade, numerous study attempt to define an ultimate strategy of genetic testing but these were partly unsuccessful.

The expanding number of clinical data have proved the heterogeneity of the appearance of CMT likely have an impact on its characteristics and leads to a less predictable disease progression. Recently, extensive genotype-phenotype meta-analyses studied the

possible correlation between various symptoms, disease severity and genes concluded that some genes may have associated with non-classical features.

Genetic diagnosis help in the diagnosis of CMT since positive test likely prove hereditary neuropathies especially in uncertain cases.

The result of genetic testing can help to fortify the diagnosis of hereditary neuropathies resuling in relief and acceptance of patients. The multidisciplinary approach of therapy help to improve the fitness of patients, delay the progression, and provide the possibility of aimed therapies. Couples before having kids can get a more precise a risk evaluation during the genetic counselling and can understand the risks. Furthermore, the careful analysis of data gained from research and clinical evaluations may help estimating the prognosis and cope with the disease.

OBJECTIVES

In this study, we aimed to investigate Hungarian CMT patients, unravel the genetic cause of the disease even in still unsolved cases and assess the phenotypical variability and spectrum of different causative genes.

The aims were the followings:

1. To estimate the frequency of most common neuropathy genes – *PMP22, GJB1, MPZ, MFN2, EGR2, CTDP1* and *NDRG1* – in an extensive cohort of Hungarian CMT patients.

2. To assess the disease features and atypical signs and symptoms of CMT in this cohort and detailed descriptions of the phenotype of novel pathogenic and likely pathogenic alterations.

3. To highlight various genotype-phenotype correlations between different CMT subgroups, especially between clinically well-characterized female and male *GJB1* patients.

4. To analyze patient for rare variants with high output genetic methods and identify the causative gene.

PATIENTS AND METHODS

531 Hungarian CMT patients were enrolled. All individuals were born in Hungary, whereof 55 patients were likely of Roma origin. Some of the novel alterations were tested in 350 healthy control individuals as well.

Clinical evaluation and nerve conduction study

Patients underwent neurological examination routinely. Age of onset and family history was taken in all cases by asking about other affected relative and first neuropathy related symptoms, respectively. Nerve conduction studies were performed by standard techniques with superficial registration and stimulation of sensory, motor and mixed nerves. Patients were considered to suffer from CMT if the clinical and electrophysiological signs of motor and sensory neuropathy were present and the family history revealed other affected family members. In sporadic cases, the causes of acquired neuropathy (e.g. metabolic, toxic, inflammatory, infectious and tumor associated polyneuropathies) were excluded using extensive differential diagnostic workup and further diagnostic tests or procedures were performed if required based on the clinical picture. The severity of the disorder was assessed using the CMT examination and/or neuropathy score part of this retrospectively.

Genetic testing of CMT genes

DNA was extracted from whole blood and quantitative changes in the *PMP22* gene was analyzed with multiplex ligation-dependent probe amplification assay. Copy number variation of the *GJB1* gene was screened by real-time PCR methodology with SYBR Green staining. The total coding region of *GJB1*, *MPZ*, *EGR2*, *MFN2*, *PMP22* were analyzed using Sanger sequencing with specific primers and compared to the human reference genome using NCBI's Blast® application. Hotspot mutations in *CTDP1* and *NDRG1* genes were tested with the PCR-RFLP methodology.

An in-house designed CMT gene panel was used for target sequencing using MiSeq platform. Exome Capture was performed in Miami University, Hussman Institute. The procedure was executed according to the manufacturer's protocol.

In silico and statistical analysis

In silico analyses were performed with PolyPhen2, MutationTaster and SIFT softwares. The significance of detected alterations was checked with HGMD (www.hgmd.cf.ac.uk), dbSNP (www.ncbi.nlm.nih.gov/SNP/), ClinVar (www.ncbi.nlm.nih.gov/clinvar/) and CMT database (http://www.molgen.ua.ac.be). The nature of novel alterations was assessed based on the ACMG guideline.

The group comparisons were performed with independent sample t-test and Mann Whitney U test regarding means. Percentages were compared with Chi square test. p values of <0.05 was considered statistically significant. Spearman test and linear regression model was used for estimating the relationship among variables. Odds ratio in case control studies and the 95% confidence intervals for proportions and means were calculated using standard formulas.

RESULTS

Clinical and electrophysiological characteristics of CMT patients

From the 531 studied CMT patients, 409 (77%) were classified as CMT1 and 122 (23%) as CMT2. Family history was positive in 148 cases (51%) while 142 patients (49%) were sporadic. The inheritance pattern was autosomal dominant in 123 cases, and X-linked dominant and autosomal recessive inheritance were present in and 12 (P/F: 1.8) cases, respectively.

CMTES and CMT related additional features could be assessed in 309 cases (58.2%). The mean CMT examination score was 8.9±4.3, with a minimum of 0 and a maximum of 22. Symptoms began before the age of 30 in 69.3% of CMT cases; however, the age of onset ranged between the first and seventh decade of life. Additional features were found in a total of 22.3% of patients as follows: CNS involvement in 7.8% (24), facial, glossopharyngeal and recurrent laryngeal nerve palsy in 5.2% (9, 4 and 3 respectively), bilateral sensorineural hearing impairment in 4.9% (15), immune dysfunction in 2.9% (9), autonomic nervous system (ANS) involvement in 1.6% (5), cataract in 1.3% (4) and optic atrophy in 0.7% (2) of the cases.

Result of genetic testing of frequent genes

Within the studied cohort, genetic testing confirmed the causative gene in 276 CMT1 and in 42 CMT2 patients. Altogether 318 CMT patients (59.9%) received a genetic diagnosis, while in 213 individuals we could not detect any pathogenic alterations within the genes studied.Regarding the entire CMT cohort, the most frequent causative gene alteration occurred in *PMP22* (40.1%) followed by *GJB1* (9.6%), *MPZ* (4.5%), *MFN2* (2.4%) *NDRG1* (1.5%), *EGR2* (0.8%) and *CTDP1* genes (0.8%). Homozygous founder mutations in *NDRG1* and *CTDP1* genes were present in 21.8% of investigated Roma patients, with eight and four cases respectively.

Results of statistical analysis of CMT genetic subgroups

Age, age of onset and disease duration did not significantly differ between the studied subtypes of CMT.

The Spearman test and linear regression analysis were performed in CMT, *PMP22*, *GJB1*, *MPZ* and *MFN2* cohorts. There was a positive correlation between CMTES and age as well as between CMTES and disease duration in each group. No potential association was apparent with linear regression analysis other than a moderately weak correlation between CMTES and disease duration.

The frequency of atypical CMT features were also statistical analyzed. Dysimmune mechanisms were more frequently associated with *PMP22* (6,5%) duplication while hearing impairment was dominant in *PMP22* duplication (6,5%) and *NDRG1* (75%) founder mutations The frequency of CNS involvement was higher in *MFN2* (50%, N=5) and male *GJB1* patients (37.5%, N=6). These two genes were responsible for half of the cases with CNS symptoms.

Clinical features and disease burden of *GJB1* mutated males and females a broad range depending on gender. Age at onset was earlier in males than in females. The disease duration did not differ significantly between genders, however, the disease severity based on certain CMT neuoropathy subscores, differed significantly and the disease burden of males was more pronounced than of females.

Result of analysis with new generation sequencing

From the studied CMT2 cohort, 15 well-characterized CMT patients have been analyzed – 6 with whole exome sequencing and 9 dHMN-HMSN overlapping phenotype with target sequencing. These patients do not harboured mutations of any of the screened genes . Based on our results, pathogenic or likely pathogenic alterations were found in 5 patients. We identified two pathogenic mutations, the heterozygous *TRPV4* p.R269H alteration in one and the homozygous *HINT1* p.R37P in three young patients. POLG and MME variants with a likely pathogenic nature was found in the

TRPV4 c.806G>A pathogenic alteration

The c.806G>A, p.R269H known pathogenic mutation of *TRPV4* was indentified with whole exome sequencing . The alteration was found in a two and half years old girl whose symptoms started at age of 8 months with clumsiness and delayed motor

development. Her status indicated distally prominent and moderate paresis and spared tactile and vibratory sensation of all limbs. Electrophysiological investigation revealed a severe reduction of CMAP amplitude in peroneal, tibial ulnar and median nerves. SAP amplitude reduction of sensory nerves were minimal. NCV was spared of all nerves. Both parents have not shown any clinical or electrophysiological signs of neuropathy and genetic analysis proved that they do not carry the *TRPV4* alteration.

Co-occurance of *POLG* and *MME* alterations

Exome sequencing confirmed the presence of the POLG c.3244 G>A and the MME c.1946 T>C missense nucleotide substitution in a 35 years old patient. Based on ACMG guideline, the POLG variant is likely pathogenic alteration while the MME mutation was declared as variant with unknown significance. Deletion of mitochondrial DNA (mtDNA) was also tested and found a 95% heteroplasmy rate of common deletion. At the age of 35 had moderate distal muscle weakness which was more pronounced in lower than in upper limbs. Electrophysiological evaluation revealed axonopathy of motor, sensory and mixed nerves with spared nerve conduction velocities.

HINT1 founder mutation with axonopathy and neuromytonia

Three patients had delayed muscle relaxation, two CNS involvement (cerebellar ataxia and pyramidal sign, respectively), and one patient suffered from prominent scapular weakness. All the patients genetic data were carefully checked for pathogenic and likely pathogenic alterations using ClinVar database, PubMed and ACMG guideline. The same homozygous pathogenic mutation was found in three patients (*HINT1*, c.110 G>C, p.R37P). Patients, carrying *HINT1* mutation, presented progressively decreasing muscle strength, delayed muscle relaxation, elevated CK levels, impaired mental performance, and mild sensory disturbance. Age of onset ranged between 9-14 years. Interestingly, beside axonal neuropathy, EMG registered neuromyotonia which was characterized by prolonged muscle relaxation after voluntary muscle contractions, causing by the hyperexcitability of lower motor nerves.

CONCLUSION

This study is the first genetic epidemiology study among Hungarian CMT patients. In conclusion, our findings can be summarized as follows:

1. Analysis of the most common CMT genes in Hungarian patients has revealed the genetic etiology almost in 60% of cases which is a satisfactory result compared to international findings. Our data also highlight that data screening of certain alterations should precede other gene tests in selected cases based on clinical data and ethnicity.
2. We identified nine novel pathogenic or likely pathogenic variants of *GJB1*, *EGR2* and *MPZ* genes in 531 Hungarian CMT patients which further broadens the spectrum of pathogenic alterations.
3. Detailed clinical characteristics of patients provided valuable data in prediction and prognosis. Our findings indicate that genetic diagnosis is a strong predictor of disesase progression but numerous genetic and environmental modification have to be taken into consideration which measurement and identification are not solved yet.
4. Current research first reported the frequency of associated features of neuropathy. Different symptomps were more frequently associated with certain genes. Based on that observations, certain features can help in determining the most likely genes.
5. New generation sequence platforms can be a robust diagnostic tool to unwrap sporadic cases and study overlapping syndromes. Cases, where traditional genetic analyses have not confirmed the pathogenic variant, new approach have lead to a genetic diagnosis in multiple cases.
6. We also determined a possibly frequent *HINT1* pathogenic alteration which analysis should be inserted into the CMT diagnostic workflow in Hungary, especially in neurmyotonia specific cases. All these observations can facilitate the diagnostic algorithm of CMT along with the family history and accelerate the cost-effective diagnosis. of patients.

PUBLICATIONS

Relevant for the PhD thesis:

Milley GM, Varga ET, Grosz Z et al. Genotypic ad phenotypic spectrum of the most common causative genes of Charcot-Marie-Tooth disease in Hungarian patients. Neurmuscul Disord. 2017;28(1):38-43 (2.612)

Milley GM, Varga ET, Grosz Z et al. Three nove lmutations and genetic epiemiology analysis of the Gap Junction Bet 1 (GJB1) gene among Hungarian Charcot-Marie-Tooth disease patients. Neurmuscul Disord. 2016;26(10):706-711 (IF: 2.969)

Further publications:

Pentelenyi K, Remenyi V, Gal A, **Milley GM,** Csosz A, Mende BG, Molnar MJ: Asian-specific mitochondrial genome polymorphism (9bp deletion) in Hungarian patients with mitochondrial diesease. Mitochondrial DNA. 2014 Sep 22:1-4

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