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# Massively Parallel Sequencing Detected a Mutation in the *MFN2* Gene Missed by Sanger Sequencing Due to a Primer Mismatch on an SNP Site

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## Summary

We describe a patient with early onset severe axonal Charcot-Marie-Tooth disease (CMT2) with dominant inheritance, in whom Sanger sequencing failed to detect a mutation in the *mitofusin 2* (*MFN2)* gene because of a single nucleotide polymorphism (rs2236057) under the PCR primer sequence. The severe early onset phenotype and the family history with severely affected mother (died after delivery) was very suggestive of CMT2A and this suspicion was finally confirmed by a *MFN2* mutation. The mutation p.His361Tyr was later detected in the patient by massively parallel sequencing with a gene panel for hereditary neuropathies. According to this information, new primers for amplification and sequencing were designed which bind away from the polymorphic sites of the patient´s DNA. Sanger sequencing with these new primers then confirmed the heterozygous mutation in the *MFN2* gene in this patient. This case report shows that massively parallel sequencing may in some rare cases be more sensitive than Sanger sequencing and highlights the importance of accurate primer design which requires special attention.

Keywords: Massively parallel sequencing (MPS), *mitofusin 2* (*MFN2*), polymerase chain reaction (PCR), primer mismatch, single nucleotide polymorphism (SNP)

## Introduction

Charcot-Marie-Tooth (CMT) disease or hereditary motor and sensory neuropathy (HMSN) is the most common inherited neuromuscular disorder with a prevalence of up to 1/1214 in the general population (Braathen, 2012). According to electrophysiological criteria, CMT is subdivided into two main categories: demyelinating neuropathies with upper limb motor conduction velocities (MCVs) of median or ulnar nerve reduced (< 38 m/s) and axonal forms, which affect mostly axons and are characterized by nearly normal MCVs (> 38 m/s) but reduced amplitude (Harding & Thomas, 1980, Reilly et al., 2011). CMT2A is among the most prevalent type of axonal dominant inherited neuropathy in the Czech Republic and the situation might be similar in other populations (Zuchner & Vance, 2006; Cartoni & Martinou, 2009; Brozkova et al., 2013). CMT2A is caused by mutations in the *mitofusin 2 (MFN2)* gene (Zuchner et al., 2004). Typical clinical symptoms are early onset progressive distal limb muscle weakness and/or atrophy, steppage gait, distal sensory loss, and mobility impairment, which frequently leads to wheelchair dependency (Feely et al., 2011; Stuppia et al., 2015).

We report a patient with an autosomal dominant form of CMT2A, who carries the *MFN2* mutation c.1081C>T (p.His361Tyr) in heterozygous state, initially missed by Sanger sequencing due to a PCR primer mismatch. The patient presented with early onset severe CMT2 with a similarly affected mother in whom CMT2A was also suspected. The causal mutation was initially missed by Sanger sequencing because of a single nucleotide polymorphism under the primer sequence.

Molecular genetic diagnosis of CMT2A (and other CMT forms) is typically achieved by detection of *MFN2* mutations by PCR amplification of 17 coding exons and adjacent

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intronic sequences followed by Sanger sequencing. Polymerase chain reaction is based on repeated cycles of heat denaturation of target DNA and oligonucleotide primer annealing and extension (Mullis et al., 1986). Primer mismatches (e.g., due to variation in the target sequence) can lead to failure of PCR amplification. In diploid genomes a variant in the primer binding site on one allele can result in allele dropout. Other variants on this allele escaping amplification will be missed by sequencing of this PCR product. It is a well-known phenomenon, but there have not yet been many studies published. Before the era of massively parallel sequencing (MPS), only a very limited check existed to detect such errors. There are a few publications (Wong et al., 2001; Lam & Mak, 2006; Rossetti et al., 2012) similar to this article. More recently, new DNA sequencing technologies have emerged and are revolutionizing medical genetic research and clinical testing (Mestan et al., 2011; Rabbani et al., 2014). Technologies such as massively parallel sequencing allow for faster genetic diagnosis at comparatively lower costs (Grada & Weinbrecht, 2013). Although generally considered to be less sensitive and more error-prone than Sanger sequencing, these technologies have occasionally led to the identification of variants in previous Sanger mutation-negative samples (Lam et al., 2012; Ratan et al., 2013).

The project was approved by the Ethics Committee of 2nd Faculty of Medicine, Charles University in Prague and University Hospital Motol.

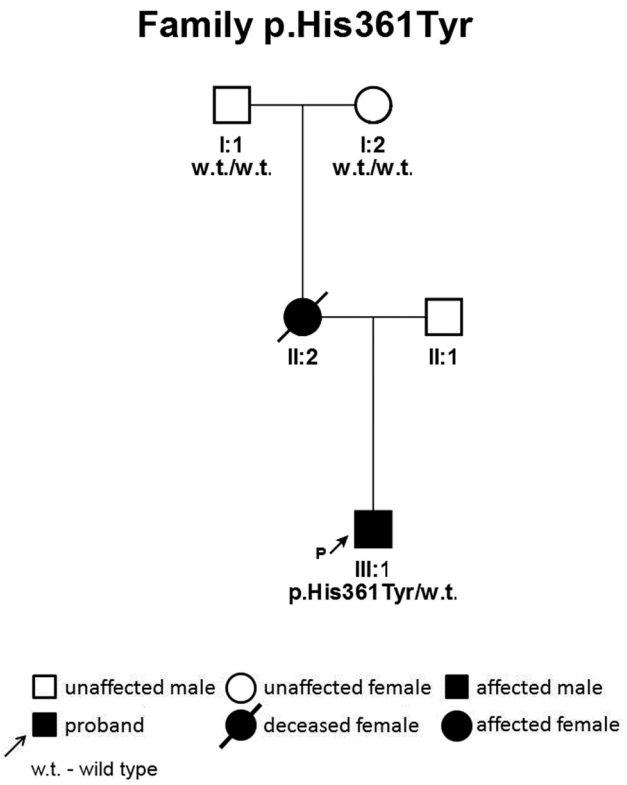


**Figure 1** Pedigree of the patient's family.

## Case Report

We report a patient with an autosomal dominant form of Charcot-Marie-Tooth type 2A (CMT2A). The patient was referred to our clinic at the age of four years. The pedigree is shown in Figure 1. He reached early developmental milestones normally. However, from the age of three, the grandparents noticed he had difficulty in walking, especially downstairs. Upon examination at the age of four, he presented with atrophic calves, bilateral pes cavus and abnormal gait. Muscle tone was reduced at the extremities and reflexes were decreased. Nerve conduction studies (NCS) showed that sensory nerve action potential (SNAP) of the suralis nerve was not recordable. Nerve conduction study results are summarized in Table 1. The mother of the patient is deceased. According to clinical reports she had the same type of CMT2 neuropathy. The disease began at the age of four and her ability to walk just before delivery was limited. The overall clinical presentation and the type of inheritance were suggestive of early onset severe CMT2A due to a dominant mutation in the *MFN2* gene (Feely et al., 2011).

All 17 coding exons of the *MFN2* gene were Sanger sequenced in the first instance. PCR primers were based on the published sequences (Zuchner et al., 2004), but despite the clinical suspicion no pathogenic mutation was found. Analysis of the *MPZ* gene was continued but no causal mutation was identified. The patient DNA was then subjected to MPS – targeted re-sequencing with a panel of genes currently associated with hereditary neuropathy (78 genes).

The data were analyzed with SureCall (Agilent Technologies, Santa Clara, CA, USA). Six hundred and thirty-one variants were called; VCF files were analyzed with Genome Trax (Biobase, MA, USA) and Annovar (http://annovar.openbioinformatics.org).The quality report for the sample showed that the percentage of analyzable target regions that were covered at least 10 times was 97.33 %.

A heterozygous mutation in the *MFN2* gene (NM_014874.3: c.1081C>T, p.His361Tyr) was identified. This mutation had not been detected by Sanger sequencing performed previously. We wanted to understand why the more precise Sanger sequencing method had missed this mutation, while massively parallel sequencing had succeeded. The reason for this phenomenon was found by comparing BAM files and electropherograms. In the BAM file, we found a single nucleotide polymorphism in the primer site of the reverse primer for exon 11 (9th coding) in the *MFN2* gene (dbSNP: rs2236057). The proband was

**Table 1** Neurophysiology in patient III: 1.

| **Motor nerve conduction velocity (MNCV)** | | **Conduction velocity (CV) (m/s)** | **Amplitude (mV)** |
|---|---|---|---|
| | Tibial nerve | 40.0 | 5 |
| | Ulnar nerve | 44.8 | 6 |
| **Sensory nerve conduction velocity (SNCV)** | | **Conduction velocity (CV) (m/s)** | **Amplitude ($\mu$V)** |
| | Sural nerve | not recordable | not recordable |
| | Ulnar nerve | 38.6 | 16 |

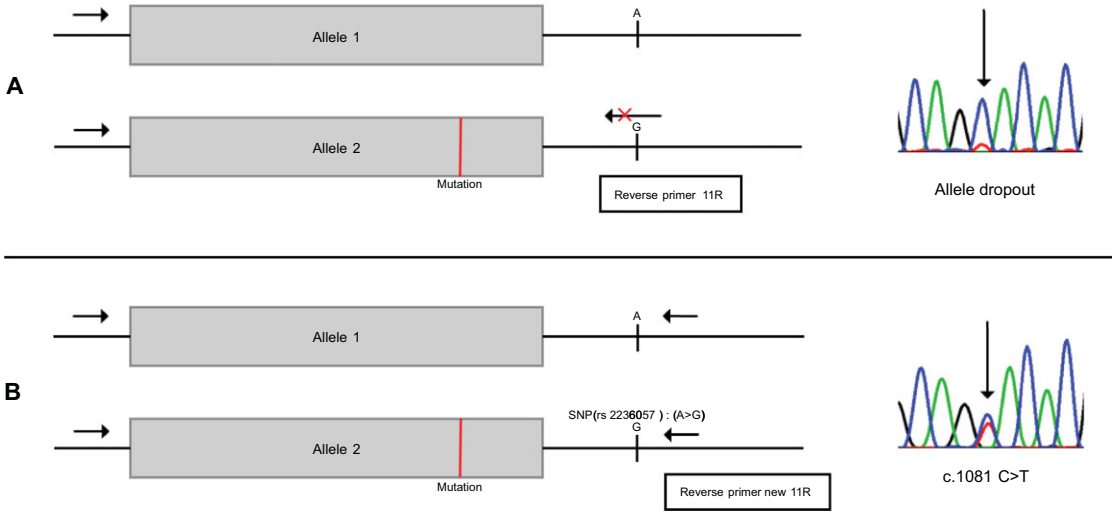


**Figure 2** PCR amplification for exon 11 was initially carried out with previously designed primers. Due to the G allele for rs2236057 on the mutated chromosome, we detected only the wild-type allele. A mutant allele was not amplified and only a tiny red dropout peak was visible (A). For new re-designed primers we moved the sequence further away from the polymorphic site to avoid the SNP (rs2236057). New analysis was performed. The alleles were therefore equally amplified and the mutation c.1081C>T (p.His361Tyr) was clearly visible (B).

heterozygous for the A (reference) and the G (alternative) alleles (according to GRCh37/hg19). The G allele is the more frequent allele at this locus (frequency 66.15%-http://exac.broadinstitute.org/), despite the A allele being the reference allele. This SNP caused inadequate annealing and failed amplification of the mutant allele (Fig. 2A).

The original PCR primer (Reverse primer 11R) had been derived from the A allele (reference sequence), suggesting that the missed mutation resided on the G allele which could not be amplified (Fig. 2A). Based on the BAM file we were able to design new primers (Reverse primer new 11R) in such a way, that the new primer would anneal to a part of the DNA sequence, where this patient is not carrying any SNPs (Fig. 2B). PCR was performed again and the mutation c.1081C>T (p.His361Tyr) was confirmed in the heterozygous state. The sequences obtained were compared to reference sequence NM_014874.3. We retrospectively checked the Sanger sequencing chromatograms and found that a small peak is visible at the mutation site and there is minimal decrease of the signal of the wild type peak. Such a picture may have been caused by mosaicism, but the family history was contradictory and further results showed another explanation. This variant has already been reported as pathogenic (Verhoeven et al., 2006). The c.1081C>T (p.His361Tyr) variant was predicted to be deleterious by SIFT (http://sift.jcvi.org/) and to be disease causing by MutationTaster (http://www.mutationtaster.org/). This position is highly conserved. There are no data for minor allele frequency in the Exome Variant Server-EVS (http://evs.gs.washington.edu/EVS/) or the Exome Aggregation Consortium - ExAC (http://exac.broadinstitute.org/).

Segregation of the mutation with the disease in the family was further tested. The unaffected father was not tested as he was not available for DNA testing. The patient´s mother, who was similarly affected, is deceased and her DNA was not available. However, we tested the maternal grandparents, who

 

are without any neurological symptoms, and the mutation was not detected. Therefore, we assume that the mutation arose *de novo* in the affected mother; however, we were unable to prove this by DNA analysis.

## Discussion

Allele dropout is a well-known cause of missed mutations due to unequal amplification of heterozygote alleles. The present case illustrates that MPS can enable detection of allele dropout in previous Sanger sequencing mutation-negative samples. This case further highlights the importance of designing primers for successful and correct amplification. As we saw in this case, and as has been described in only a few publications, a single nucleotide polymorphism under the primer binding site may lead to the failure of PCR by allele dropout. Due to allele dropout, the mutation is not visible and misdiagnosis may occur (Mullins et al., 2007).

As reported in the literature, primers should ideally be completely SNP free. However, this standard is in reality very difficult to achieve, because there may be rare new SNPs in individual patients. It seems advisable to compare primer sequences against previously published SNPs, as well as against databases such as EVS, ExAC and dbSNP. Moreover, primers for exon 11 published in the original paper (Zuchner et al., 2004) should not be used for diagnostic testing of the *MFN2* gene in CMT patients since there is a substantial risk of missing the mutation in this exon due to allele dropout of the mutated allele. For CMT patients, we successfully use DNA testing depending on the clinical presentation, type of neuropathy, mode of inheritance and age of onset for the most common causes: CMT1A/HNPP by the MLPA method and Sanger sequencing of genes *GJB1, MPZ* and *MFN2* in the first instance. If these tests do not reveal the cause of the disease, we continue with MPS with a disease-specific gene panel. MPS is a valuable tool for rare, unusual or atypical cases and types of CMT. In contrast to ordinary Sanger sequencing technology, MPS detects SNPs by default. Special care must be taken when selecting PCR primers for clinical testing.

## Acknowledgements

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## Web Resources

http://annovar.openbioinformatics.org
http://www.ncbi.nlm.nih.gov/snp/
http://evs.gs.washington.edu/EVS/
http://exac.broadinstitute.org/
http://sift.jcvi.org/
http://www.mutationtaster.org/

## Conflict of Interest

Authors declare no conflict of interest.

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