Open Journal of Genetics, 2014, 4, 426-433
Published Online December 2014 in SciRes. http://www.scirp.org/journal/ojgen
http://dx.doi.org/10.4236/ojgen.2014.46040

Point Mutation Analysis of *PMP*22 in Patients Referred for Hereditary Neuropathy with Liability to Pressure Palsies

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Received 18 August 2014; revised 10 September 2014; accepted 8 October 2014

Abstract

A cohort of 404 patients referred for hereditary neuropathy with liability to pressure palsies was tested initially for the common *PMP*22 whole gene deletion. 94 whole gene deletions were detected, plus three partial gene deletions, and the remaining 307 patients were screened for *PMP*22 point mutations. Nine point mutations were identified (8.5% of all mutations), eight of which were in exon 5, suggesting a point mutation hotspot for individuals with this condition. Sequencing analysis of *PMP*22 exon 5 should therefore be included as a routine diagnostic test for gene deletion-negative patients.

Keywords

Point Mutations, *PMP*22, HNPP

1. Introduction

Hereditary neuropathy with liability to pressure palsies (HNPP) was first described in 1947 [1]. Patients present with transient episodes of weakness and or sensory loss that can present in any limb and sometimes also in the auditory nerve. Motor nerve conduction velocities can be normal or nearly normal with focal areas of slowing at the wrist, around the elbow or knee or distally in the peroneal nerve [2] [3]. Other symptoms can include nerve palsies of the ulna, *pes cavus*, scoliosis, deafness and carpal tunnel syndrome, but due to the episodic nature and transient clinical manifestations, HNPP may often go undiagnosed [4]-[6].

How to cite this paper: Brown, S.B. and Bunyan, D.J. (2014) Point Mutation Analysis of *PMP*22 in Patients Referred for Hereditary Neuropathy with Liability to Pressure Palsies. *Open Journal of Genetics*, **4**, 426-433.
http://dx.doi.org/10.4236/ojgen.2014.46040

Autosomal dominant inheritance of HNPP was first shown in 1954 [4], and the relevant gene was finally identified when two groups showed that a deletion of the entire *PMP*22 gene resulted in the condition [7] [8], due to unequal crossing over between homologous low copy repeats on chromosomes 17 [9]. Although a *PMP*22 whole gene deletion was shown to be the major cause of HNPP [10]-[13], the first point mutation within *PMP*22 was reported in a family with classic HNPP symptoms a few years later [14]. The *PMP*22 gene encodes a 22-kD protein, named peripheral myelin protein 22, that comprises around 5% of peripheral nervous system myelin. It is produced primarily by Schwann cells and expressed in the compact portion of all myelinated fibers in the peripheral nervous system [15].

As *PMP*22 is the only gene that has been linked to HNPP, point mutation analysis of this gene in patients without a known whole or partial gene deletion is therefore likely to reveal additional mutations as mutations that reduce the functional level of *PMP*22 can cause a HNPP phenotype [16]. However, since the first reported *PMP*22 point mutation, very few others have been published. There are only 20 HNPP-causing mutations currently listed on the molgen and HGMD databases (www.molgen.ua.ac.be, www.hgmd.cf.ac.uk) and only a few more present in the literature (see **Table 1**). Almost all of these mutations are from individual reports of a single mutation, so it is difficult to get an idea of the point mutation frequency or gene distribution in HNPP referrals. To this end, we have analysed a cohort of over 400 patients referred with a potential diagnosis of HNPP. Deletions were initially detected using the multiplex ligation-dependent probe amplification (MLPA) technique [42], and the deletion-negative patients were screened for point mutations in the four coding exons of *PMP*22 in order to determine the pick-up rate and distribution of such mutations amongst HNPP referrals.

2. Materials and Methods

2.1. Test Populations

Between 1991 and 2014, a cohort of 404 independently-ascertained patients was collected at our laboratory following referrals from centres within the catchment area of the Wessex Regional Genetics Laboratory. As this is a laboratory-based study, we have no access to clinical data, nor do we have details of the referral criteria used by the clinicians.

This study falls in the realm of routine clinical care and consent for diagnostic testing was obtained from all patients as part of the referral process.

2.2. MLPA Analysis

MLPA was carried out according to the manufacturer's instructions with the current *CMT* probe mix at the time of referral (MRC-Holland, The Netherlands) either kit P033 or kit P405. Any patients referred prior to 2002 were retroactively tested with the P033 kit. MLPA PCR products (0.5 µl) were added to 0.2 µl of GeneScan™-500 ROX™ Size Standard (Applied Biosystems, USA) and 9 µl of Hi-Di Formamide (Applied Biosystems, USA) and separated on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, USA) using a 36 cm array and 3130 POP-7™ array polymer (Applied Biosystems, USA). The run conditions were as follows: injection voltage = 1.2 kVolts, injection time = 5 seconds, oven temperature = 60˚C, run voltage = 15 kVolts, run time = 20 minutes. Subsequent data was analysed using the MLPA analysis function of the Gene Marker (version 1.85) software (SoftGenetics, USA).

2.3. *PMP*22 Sequencing Analysis

Mutation analysis of the four coding exons of the *PMP*22 gene (exons 2-5 Ensembl transcript ENST-00000395938, gene ENSG00000109099) was carried out by direct sequencing analysis. PCR products were first generated using a standard PCR reaction with a 25 µl volume and a 60˚C annealing temperature using the relevant exon primers; 2F-ctcctcgcaggcagaaact, 2R-ctgaaccagcaggagcacg, 3F-tccccttttccttcactcct, 3R-ccaataagcgtttccagctc, 4F-catggccagctctcctaac, 4R-actaatcattccgcagacttg, 5F-ccgctctgccatggactc and 5R-ttccctatgtacgctcagag. These products were then sequenced using the standard protocol of the Big-Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, USA) and separated on an ABI 3130*xl* Genetic Analyzer (Applied Biosystems, USA). Subsequent data was analysed using the Mutation Surveyor (version 3.1) software (SoftGenetics, USA).

Any missense mutations were assessed using the Alamut mutation interpretation software (version 1.5-

Table 1. A summary of HNPP-causing *PMP*22 mutations.

Location	Base change	Protein change	Mutation	Reference
Exon 2	c.11delT	p.(Leu4fs)	Frameshift	[17]
Exon 2	c.19_20delAG	p.(Leu7fs)	Frameshift	[18]
Exon 2	c.31_39delCTCCACGTC	p.(Leu11fs)	Frameshift	[19]
Exon 2	c.65C>T	p.(Ser22Phe)	Missense	[14]
Intron 2	c.78+1G>T		Splicing	[20]
Intron 2	c.78+5G>A		Splicing	[21]
Intron 2	c.79-13T>A	? p.(Gln27fs)	Splicing/Frameshift	This study
Exon 3	c.88G>A	p.(Val30Met)	Missense	[22]
Intron 3	c.178+1G>C		Splicing	[23]
Intron 3	c.179-1G>C		Splicing	[24]
Exon 4	c.183G>A	p.(Trp61Ter)	Termination	[25]
Exon 4	c.183G>A	p.(Trp61Ter)	Termination	[26]
Exon 4	c.199G>A	p.(Ala67Thr)	Missense	[27]
Exon 4	c.227delG	p.(Ser76fs)	Frameshift	[28]
Exon 4	c.281_282insG	p.(Gly94fs)	Frameshift	[29]
Exon 4	c.281_282insG	p.(Gly94fs)	Frameshift	[30]
Exon 4	c.289delT	p.(Tyr97fs)	Frameshift	[31]
Exon 4	c.297delT	p.(Gly100fs)	Frameshift	[32]
Intron 4	c.320-1G>C		Splicing	[21]
Intron 4/ Exon 5	c.320-1_320delGGinsTA		Splicing	This study
Exon 5	c.320G>T	p.(Gly107Val)	Missense	[33]
Exon 5	c.328G>A	p.(Val110Met)	Missense	This study
Exon 5	c.353C>T	p.(Thr118Met)	Missense	[34]
Exon 5	c.353C>T	p.(Thr118Met)	Missense	[35]
Exon 5	c.353C>T	p.(Thr118Met)	Missense	This study
Exon 5	c.353C>T	p.(Thr118Met)	Missense	This study
Exon 5	c.364_365delCC	p.(Pro122fs)	Frameshift	[36]
Exon 5	c.372G>A	p.(Trp124Ter)	Termination	[37]
Exon 5	c.372G>A	p.(Trp124Ter)	Termination	[38]
Exon 5	c.392C>G	p.(Ser131Cys)	Missense	This study
Exon 5	c.395A>G	p.(Tyr132Cys)	Missense	This study
Exon 5	c.419G>A	p.(Trp140Ter)	Termination	[21]
Exon 5	c.433_434insC	p.(Leu145fs)	Frameshift	[39]
Exon 5	c.434delT	p.(Leu145fs)	Frameshift	This study
Exon 5	c.434delT	p.(Leu145fs)	Frameshift	[40]
Exon 5	c.469C>T	p.(Arg157Trp)	Missense	This study
Exon 5	c.475C>T	p.(Arg159Cys)	Missense	[41]

Interactive Biosoftware, France). Alamut uses the programs SIFT (sift.jcvi.org) and Polyphen (genetics. bwh. harvard.edu/pph2/) to predict causality. SIFT predicts whether an amino acid substitution in a protein will have a phenotypic effect, based on the degree of conservation of amino acid residues in sequence alignments derived from closely related sequences, collected through PSI-BLAST (blast.ncbi.nlm.nih.gov/Blast.cgi). The amino acid substitution is given a score from 0 to 1 and is predicted to be damaging if the score is ≤0.05, and tolerated

if the score is >0.05 [43]. Polyphen classifies amino acid substitutions as "benign", "possibly damaging", or "probably damaging" based on data compiled from all damaging alleles with known effects on the molecular function causing human Mendelian diseases present in the UniProtKB database (uniprot.org), together with common human non-synonymous single nucleotide polymorphisms without annotated involvement in disease [44].

Potential splice site mutations were analysed using the Fruitfly splice predictor program within Alamut (http://www.fruitfly.org/seq_tools/splice.html).

3. Results

3.1. MLPA Analysis

Dosage analysis of the *PMP*22 gene showed that 94 of the 404 referrals had the standard whole gene deletion, while three had a partial gene deletion—the first had a deletion of exons 1-3, the second had a deletion of exons 4 and 5 and the third had a deletion of exons 2 and 3. The remaining 307 patients were screened for point mutations within *PMP*22.

3.2. *PMP*22 Sequencing Analysis

A single sequence change was found in nine of the 307 patients (see **Table 2**). All mutation nomenclature is based on Ensembl transcript ID ENST00000395938 where base 1 is the first base of the translation initiation codon. Eight of these changes were within exon 5 of *PMP*22 with one destroying the exon 5 splice acceptor site and one frameshift mutation. The remaining six caused an amino acid substitution, and Alamut mutation interpretation software predicts that all six are likely to be pathogenic. The ninth change was in intron 2 where the substitution c.79-13T>A was predicted by the Fruitfly splice predictor program to create a new splice site at position c.79-25_-24 which has a higher likelihood of usage than the original one at c.79-2_-1. This would result in a 23 base pair insertion if the alternative splice site is used (c.78_79insGATATCTATCTGATTCTCTCTAG).

4. Discussion

This study confirms that deletions are the most common HNPP-causing mutation but also demonstrates that point mutation analysis extends the diagnostic yield for HNPP referrals. Nine sequence changes were detected in patients referred with a diagnosis of HNPP. The nine changes comprised of eight different mutations, so no point mutation founder effect was evident. Two of these mutations (c.353C>T p.(Thr118Met) and c.469C>T p.(Arg157Trp)) are listed as deleterious by Clin Var (ncbi.nlm.nih.gov/clinvar/), UniProt (uniprot.org) and dbSNP (ncbi.nlm.nih.gov/SNP/) while the other six were novel and unclassified. The p.(Thr118Met) mutation was originally published in the literature as a possible polymorphism [45], but subsequent studies showed that it did cause HNPP [34] [35], and although heterozygosity for p.(Arg157Trp) had not been reported as causing HNPP, it had been shown to cause Dejerine-Sottas disease in homozygous form [46]. *In silico* analysis suggests

Table 2. The *in silico* predictions of the nine *PMP*22 sequence changes found in the study.

Patient	Location	Base change	Protein change	Polyphen prediction	SIFT prediction
1	Intron 2	c.79-13T>A	Potential frameshift (c.78_79ins23)	Not applicable	Not applicable
2	Exon 5	c.320-1_320delGGinsTA	Destruction of normal exon 4/exon 5 splicing	Not applicable	Not applicable
3	Exon 5	c.328G>A	p.(Val110Met)	Probably damaging	Deleterious
4	Exon 5	c.353C>T	p.(Thr118Met)	Probably damaging	Deleterious
5	Exon 5	c.353C>T	p.(Thr118Met)	Probably damaging	Deleterious
6	Exon 5	c.392C>G	p.(Ser131Cys)	Possibly damaging	Deleterious
7	Exon 5	c.395A>G	p.(Tyr132Cys)	Possibly damaging	Deleterious
8	Exon 5	c.434delT	Frameshift	Not applicable	Not applicable
9	Exon 5	c.469C>T	p.(Arg157Trp)	Probably damaging	Deleterious

causality (loss of *PMP*22 function) for the three unclassified amino acid substitutions (c.328G>A p.(Val110Met), c.392C>G p.(Ser131Cys) and c.395A>G p.(Tyr132Cys)), while the c.320-1_320delGGinsTA (destruction of splice site) and c.434delT (protein reading frame shift) mutations are clearly deleterious. The remaining base change, c.79-13T>A, is potentially deleterious based on the *in silico* data, but RNA analysis would be required to prove causality, and at present we have been unable to obtain an RNA sample from this patient.

The interesting finding from this study is the distribution of the point mutations, with eight out of nine changes clustering within a single coding exon, namely exon 5. There is a paucity of reported *PMP*22 mutations in the literature, but our results are supported by the Inherited Peripheral Neuropathies Mutation Database (IPNMD; molgen.vib-ua.be) and a literature search which garnered 28 other HNPP-causing *PMP*22 mutations (see **Table 1**), eleven of which are in exon five. Combining the mutation database figures with those from our study, a total of 51% of all point mutations (19 out of 37), and 77% of amino acid substitutions (10 out of 13), are within the last coding exon of *PMP*22.

Mutations in *PMP*22 can also cause Charcot-Marie-Tooth type 1A (CMT1A) [47] but, in contrast to HNPP, the IPNMD shows a clustering of *PMP*22 point mutations in CMT1A referrals within exon 4 of the gene (28 out of 40; 70%). It seems clear from this that genotype-phenotype correlations exist in *PMP*22, with the development of HNPP or CMT1A symptoms dependant upon the location of the point mutation. Exons 4 and 5 each account for approximately one-third of the coding sequence of *PMP*22, so a slight increase in mutations identified in these exons would be expected, but the distribution frequencies in CMT1A referrals (exon 4; $\chi^2 = 12.8$, $p < 0.01$) and HNPP referrals (exon 5; $\chi^2 = 6.0$, $p < 0.01$) are clearly skewed. *PMP*22 has four transmembrane domains involving amino acid numbers 5-26, 65-84, 96-117 and 137-156 [48]. Of the ten different amino acid substitutions listed in **Table 1**, six of these lie adjacent to these transmembrane domains at positions 30, 118, 131, 132, 157 and 159, while four are within the transmembrane regions at positions 22, 67, 107 and 110, so presumably these amino acid substitutions cause a HNPP phenotype by disrupting the transmembrane domains.

The majority of mutation positive cases from our cohort (n = 94) had the standard whole gene deletion, while three had a partial gene deletion. These 97 deletion cases were all detected by MLPA analysis, but the identification of nine point mutations, accounting for 8.5% of all positive cases, highlighted the importance of *PMP*22 sequence analysis in patients with HNPP. As eight of the nine mutations were within exon 5 of the gene (as are ~51% of all reported HNPP-causing point mutations), we would recommend that, as a minimum, sequencing of this exon is performed on all HNPP gene deletion-negative cases.

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