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# Comparison of Single-Strand Conformation Polymorphism and Heteroduplex Analysis for Detection of Mutations in Charcot-Marie-Tooth Type 1 Disease and Related Peripheral Neuropathies

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## Key Words

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## Abstract

To compare the sensitivity of the mutation detection techniques single-strand conformation polymorphism analysis (SSCP) and heteroduplex analysis (HA), we analyzed a cohort of 73 patients with a diagnosis of a demyelinating neuropathy, but without the CMT1A duplication, for mutations in the coding region of the myelin genes PMP22, MPZ and Cx32. In total, 21 samples showed 13 distinct altered migration patterns by one or both methods. Ten altered patterns were detected by both SSCP and HA, two were false negative by HA, and one was false negative by SSCP. Our results suggest that either technique can be useful for mutation detection, but a combination of factors appears to affect the sensitivity of both techniques.

## Introduction

Single-strand conformation polymorphism analysis (SSCP) and heteroduplex analysis (HA) are two techniques that detect single-base alterations, small deletions, or insertions. The SSCP technique is based on the fact that single-stranded DNA samples that differ by as little as a single base can form different secondary structural conformations. On a nondenaturing gel, the mobility of the fragment not only depends on the size of the fragment,

but also on its conformation [1]. The HA technique takes advantage of the formation of heteroduplexes between two different DNA species (i.e. mutant and wildtype) after heating and slowly cooling them together to allow duplexes to form. Heteroduplexes containing single base pair variances can be detected on polyacrylamide gels because they show differential mobility from homoduplexes [2, 3].

Hereditary motor and sensory neuropathy (HMSN) is a heterogeneous group of peripheral neuropathies. The most common subtype is HMSN type I or Charcot-Marie-Tooth type 1 disease (CMT1), which is characterized by progressive weakness and atrophy of the distal limb muscles, reduced or absent deep-tendon reflexes, decreased

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nerve conduction velocities and segmental de- and remyelination [4]. HMSN type III or Déjérine-Sottas syndrome is another HMSN disorder with clinical symptoms similar to those of CMT1, but more severe and of earlier onset [4]. Another peripheral neuropathy, termed congenital hypomyelination, has also been included within the group of HMSN disorders. Congenital hypomyelination is characterized clinically by early onset of hypotonia, areflexia, distal muscle weakness and very slow nerve conduction velocities [5].

CMT1 is genetically heterogeneous with at least five loci and three genes identified to date: the peripheral myelin protein 22 gene (PMP22), the myelin protein zero gene (MPZ) and the gene encoding connexin 32 (Cx32). The major autosomal dominant subtype CMT1A is linked to chromosome 17p11.2–p12 [6]. A minor autosomal dominant subtype, CMT1B, is linked to chromosome 1q22–q23 [7], and an X-linked dominant locus was mapped to chromosome Xq13.1 [8]. In the majority of the CMT1A patients, the disease is associated with a tandem DNA duplication of 1.5 Mb [9–12]. PMP22 was found to be located within the CMT1A duplication region, suggesting that overexpression of this gene causes the CMT1A disease phenotype [e.g. 13, 14]. Identification of point mutations in PMP22 in nonduplicated CMT1A patients confirmed the direct role of this gene in the CMT1A disease process [15, 16]. Dominant PMP22 point mutations have also been found in Déjérine-Sottas syndrome patients [17]. The identification of distinct mutations in MPZ that cosegregate with the disease helped identify MPZ as the CMT1B gene [18, 19]. Point mutations in this gene are also associated with DSS [20]. Mutations in Cx32, the gene encoding connexin 32, were found in CMTX1 patients [21]. Until recently, no mutations had been associated with congenital hypomyelination [22].

The current diagnostic evaluation of a demyelinating neuropathy patient includes molecular analysis for the presence of the CMT1A duplication. In patients that do not have a DNA rearrangement, 3 genes are presently screened for exonic mutations. In this paper, we compare methods for performing the latter analysis and demonstrate the utility and some limitations of these mutational detection methods.

## Materials and Methods

### Patients

A set of 73 unrelated patients were selected based on a diagnosis of demyelinating neuropathy. All patients were tested and negative for the presence of the CMT1A duplication on chromosome

17p11.2–p12 [11]. The clinical diagnoses of the patients with mutations found in PMP22 and MPZ, and of patient 2083-1 are described elsewhere [16, 22–25]. Patients BAB123, BAB523, BAB545, BAB740 and BAB1007 have features consistent with the general criteria for CMT1.

### Molecular Analysis

For SSCP analysis, the coding regions of PMP22, MPZ and Cx32 were amplified using primers published by Roa et al. [16], Nelis et al. [26] and Bergoffen et al. [21] respectively, except for MPZ exon 5. The sequence of this primer set is 5'-GGCCAAACGTACAGCAGTCT-3' (P0ex5-P20) and 5'-TCTCCTTCCCATCTTGCTAGG-5' (P0ex5-M16). Samples (5 µl) of amplified DNA were mixed with 3 µl formamide loading dye and, after denaturation for 2 min and rapid cooling on ice, subjected to electrophoresis on 1 × Hydrolink Mutation Detection Enhancement (MDE) (FMC Bioproducts, Rockland, Me., USA) gels with and without 10% glycerol. Silver staining was carried out as previously described [27]. For HA, the coding regions of PMP22 and Cx32 were amplified using the same primers as above and the conditions as described by Roa et al. [16] and Bergoffen et al. [21]. The MPZ primers were as published by Roa et al. [24]. Five microliters each of PCR products, derived from the individual patients and a nonpatient control, were mixed, denatured at 95 °C for 5 min, and cooled to 35 °C. Loading dye was added (2 µl), and the samples were loaded on a 1 × MDE gel. Fifteen percent urea was added to a few gels to better visualize heteroduplexes by helping to minimize band broadening and the appearance of doublets in the wildtype control. The bands were visualized by ethidium bromide staining. Direct sequence determination was performed as described elsewhere [22]. Restriction digestions were performed to confirm the presence of mutations. Five microliter of PCR products were digested with 5 units of the desired restriction enzyme and incubated for 3 h at the recommended temperature (New England Biolabs).

## Results

Genomic DNA of 73 patients diagnosed with a demyelinating neuropathy who did not have the CMT1A duplication were analyzed for the presence of mutations in the myelin genes PMP22, MPZ and Cx32. All samples were analyzed by both SSCP and HA. All PCR fragments that showed a distinctly altered pattern by SSCP and/or HA were sequenced by direct PCR sequencing, and all showed a sequence variation. The exon 5 and exon 6 polymorphisms in multiple samples were not all sequenced, but instead confirmed by similarity of the pattern on MDE gels or by restriction digestion. The results of the SSCP and HA, direct sequencing and restriction digestions are represented in table 1.

For PMP22, two sequence variations were detected by both SSCP and HA. Sequence analysis revealed two missense mutations [Ser(79)Cys and Thr(118)Met] [16, 23]. In MPZ, 13 sequence variations were detected of which two were detected only by SSCP. Sequence determination

**Table 1.** Overview of the mutations detected by SSCP and/or HA

Gene	Exon	Patient	HA	SSCP	Sequence variation	AA change and position	Restriction site change		Reference
							gain	loss	
PMP22	3	BAB686	+	+	C→G	Ser(79)Cys			16
	4	BAB117	+	+	C→T	Thr(118)Met	<i>NlaIII</i>		23
MPZ	3	BAB511	-	+	C→A	Arg(98)Ser		<b>HhaI</b>	22
	3	BAB1022	+	+	C→T	Arg(98)Cys		<b>HhaI</b>	22
	3	BAB802	+	+	T→C	Ile(135)Thr			24
	3	BAB731	+	+	G→A	Gly(137)Ser			24
	5	BAB136, 808	+	+	G→A	none [Gly(200)]			24
	5	BAB987	-	+	C→T	Gln(215)stop		<b>BsoFI</b>	22
	6	BAB115, 134, 510, 511, 564, 1013	+	+	C→T	none [Ser(228)]	<b>RsaI</b>		24
Cx32	2 (p 1-2) <sup>a</sup>	2083-1	+	-	G→T	Val(13)Leu			25
	2 (p 1-2)	BAB123, 740	+	+	C→T	Arg(15)Trp	<b>BsrI</b>	<b>BsrFI, MspI</b>	
	2 (p 1-2)	BAB545	+	+	C→T + 11-bp deletion	Arg(15)Trp + frameshift	<b>MwoI</b>	<b>BsrFI, MspI, BsmI</b>	
	2 (p 3-5) <sup>a</sup>	BAB523, 1007	+	+	G→A	Val(95)Met		<b>BbrPI, BsaAI, MaeIII, PmlI</b>	

+ = Altered migration pattern; - = no altered migration pattern; restriction enzymes in bold: restriction digestion performed to confirm the presence of the mutation.

<sup>a</sup> Primer sets for amplification of Cx32 exon 2 [27].

showed four different missense mutations [Arg(98)Ser, Arg(98)Cys, Ile(135)Thr and Gly(137)Ser], one nonsense mutation [Gln(215)stop] and two different silent mutations [Gly(200) and Ser(228)] [22, 24]. Five sequence variations were observed in Cx32 by both SSCP and HA, and an additional one was detected only by HA. The visualization of the heteroduplex in patients BAB523 and BAB1007 required the addition of 15% urea to the MDE gels. Sequence analysis revealed three distinct missense mutations [Val(13)Leu, Arg(15)Trp and Val(95)Met] [25], and in one patient the missense mutation was combined with an 11-bp deletion [Arg(15)Trp + deletion and frameshift].

## Discussion

To compare the sensitivity of the mutation detection techniques SSCP and HA, we analyzed a set of 73 patients without the CMT1A duplication for mutations in the coding region of the myelin genes PMP22, MPZ and Cx32. The main advantage of both SSCP and HA is their simplicity and relative sensitivity, as well as their significant

time and cost advantages over direct sequencing when searching for relatively rare mutations in a large number of patients. Sequencing is required only in the few patients in whom SSCP and/or HA detects an altered migration pattern. SSCP analysis detects 79% of the mutations in PCR products of 212 bp or less [28]. The sensitivity of the method decreases with an increase in size of the PCR product. Recent studies indicate a level of sensitivity for HA similar to SSCP analysis (80–90%) in small DNA fragments (<300 bp) [2].

In total, 13 distinct altered migration patterns were detected; ten altered patterns were detected by both SSCP and HA, two were false negative by HA and one was false negative by SSCP (table 1). Nevertheless, we cannot exclude the possibility that some samples are false negative by both techniques. It is not known why some samples give false negatives when using HA or SSCP. Several factors have been suggested to affect the sensitivity of HA. They include the length of the PCR fragment analyzed, the type of mismatch (i.e. specific base mismatch or point mutation versus deletion), and the position of the base change within the PCR fragment [3]. These effects are evident in this study. The effect of size can be seen when

comparing the detection of a mismatch in the 210-bp MPZ exon 6 fragment with the 430-bp Cx32 fragment between primers 3 and 5. The detection of the mismatch in the Cx32 fragment required the addition of 15% urea, while the mutation in the MPZ fragment was easily seen without urea. The consequence of the type of mismatch on HA sensitivity can be seen by comparing patients BAB511 and BAB1022. These two patients have different base substitutions at the identical position in MPZ exon 3. However, patient BAB1022, who has a C-A/G-T mismatch, is detected by HA, while patient BAB511, who has a C-T/A-G mismatch, is not detected. Moreover, the importance of the placement of the mismatch within the PCR fragment is shown by the fact that the mismatch in patient BAB987, that is only 46 bp away from the 3'-end of the PCR product, is not detected by HA. However, these factors alone cannot account for the false negatives listed above because the mismatch detected by HA in patient 2083-1 is found only 46 bp away from the 5'-end of the PCR product, and is the same mismatch pair as that found in BAB511. Therefore, it is thought that a combination of the above-mentioned factors, as well as the degree of hydrogen bonding of adjacent base pairs and base-stacking interactions, determines the ability of a particular single-base mismatch to be detected by HA [3].

All the factors listed above for HA could also theoretically apply to SSCP since they would all be expected to influence secondary structure. Size is a crucial factor in the sensitivity of SSCP with a significant decrease in sensitivity observed for large molecules (>400 bp), as well as for extremely small fragments (<130 bp), which is likely due to constraints on the ability to form stable secondary structures [28]. Sheffield et al. [28] have also demonstrated that the base position of a substitution is more important than the type of substitution (i.e. transversion versus transition), and that modification of the flanking sequences also affects the sensitivity of the assay.

In conclusion, it can be said that similar factors affect both SSCP and HA, but in distinct ways because they identify alleles on the basis of different principles. The results of this study demonstrate that both SSCP and HA are good methods to screen for mutations in a large set of samples because both have a comparable sensitivity. In our study, 13 (18%) out of 73 patients have an apparent disease-associated mutation. There are several possible reasons why no mutation was detected in the other 60 patients. These include: (1) mutations were missed by both techniques; (2) patients may have mutations in the regulatory sequences of PMP22, MPZ or Cx32 or, (3) patients may have mutations in as yet unidentified genes. Since the majority of the patients analyzed were single cases or cases belonging to nuclear families, linkage analyses could not be performed to see whether they mapped to any of the known CMT1 loci on chromosomes 1, 17 and X. However, to date no CMT1-linked families have been described that had no mutation in the respective CMT1 genes MPZ, PMP22 or Cx32 [12]. Because of the high level of sensitivity for both methods (80–90%) and the known heterogeneity of these diseases (at least 5 known loci), it is more likely that the majority of the mutations in these 60 patients are in as yet unidentified genes or the regulatory regions.

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