Genetic profiling of the peripheral nervous system

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Chapter 4

N-myc downstream regulated gene I is mutated in hereditary motor and sensory neuropathy - Lom

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Abstract
Hereditary motor and sensory neuropathies, to which Charcot-Marie-Tooth (CMT) disease belongs, are a common cause of disability in adult life. Growing awareness that axonal loss, rather than demyelination per se, is responsible for the neurological deficit in demyelinating CMT disease, has focused research on the mechanisms of early development, cell differentiation and cell-cell interactions in the peripheral nervous system. Autosomal recessive peripheral neuropathies are relatively rare but clinically more severe, and understanding their molecular basis may provide a new perspective on these mechanisms. Here we report the identification of the gene responsible for Hereditary Motor and Sensory Neuropathy-Lom (HMSNL). HMSNL shows features of Schwann cell dysfunction and a concomitant early axonal involvement, suggesting that impaired axon-glia interactions play a major role in its pathogenesis. The gene was previously mapped to 8q24.3, where conserved disease haplotypes suggested genetic homogeneity and a single founder mutation. We have reduced the HMSNL interval to 200 kb and characterised it by large scale genomic sequencing. Sequence analysis of patients and controls for the two genes, which are located in the critical region identified the founder HMSNL mutation: a premature termination codon at position 148 of the N-myc Downstream Regulated Gene I (NDRGI). NDRGI is ubiquitously expressed and has been proposed to play a role in growth arrest and cell differentiation, possibly as a signalling protein shuttling between the cytoplasm and the nucleus. We have studied expression in peripheral nerve and detected particularly high levels in the Schwann cell. Together, these findings point to an important role of NDRGI in the peripheral nervous system, possibly in the Schwann cell signalling necessary for axonal survival.
Hereditary Motor and Sensory Neuropathy Lom (HMSNL) (MIM 601455) is an autosomal recessive form of Charcot-Marie-Tooth disease, which occurs in divergent Romani (Gypsy) groups descended from a small founder population, the Vlax or Danubian Roma. The disorder was first described in affected families from Bulgaria [1] and subsequently diagnosed in Italy [2], Slovenia [3], Germany [4], Spain [5], France and Rumania. HMSNL is an early-onset peripheral neuropathy that progresses to severe disability in adulthood. Clinically, it presents with muscle weakness and wasting, tendon areflexia, skeletal and foot deformities, sensory loss affecting all modalities and severe reduction in nerve conduction velocities [2-4, 6]. Neural deafness develops during the second or third decade of life, with abnormalities in the brain stem auditory evoked potentials suggesting involvement of the entire tract, including the central auditory pathways [3, 6]. The neuropathological observations in HMSNL [3, 4, 6, 7] point to Schwann cell dysfunction, manifested by hypomyelination and demyelination/ remyelination, failure of compaction of the innermost myelin lamellae and poor hypertrophic response (onion bulb formation) to the demyelination process. At the same time, axonal involvement is documented by the early, severe and progressive axonal loss and by the presence of curvilinear intra-axonal inclusions, similar to those seen in the dying-back type of distal axonopathy in experimental vitamin E deficiency.

A number of recent clinical and experimental studies of the common autosomal dominant demyelinating forms of Charcot-Marie-Tooth (CMT) disease have indicated that the neurological deficit in demyelinating neuropathies is related to the axonal loss, rather than to demyelination per se [8-12] features of HMSNL make it impossible to pinpoint the primary defect to either Schwann cells or neurons, and strongly suggest that impairment of SC-axonal interaction is a major component of the pathogenesis of this disease. The molecular basis of HMSNL may thus be of relevance to the general understanding of the pathogenetic mechanisms and causes of disability in demyelinating neuropathies.

The disease gene was mapped to a 3 cM interval on 8q24.3 where closely related disease haplotypes and strong linkage disequilibrium values suggested a single founder mutation [1]. Similar polymorphic haplotypes were subsequently identified in HMSNL chromosomes across Europe, supporting the assumption of genetic homogeneity and founder effect [13]. We now report the identification of the HMSNL gene and the founder mutation causing the disease.
Subjects and Methods

Physical mapping of the HMSNL region

A contig of genomic clones spanning the HMSNL interval was assembled by screening BAC and PAC libraries for the known STSs in the region and for the end sequences of clones identified in previous rounds of our library screening. The screening was performed by PCR amplification (Research Genetics CITB human BAC library) or filter hybridisation (Pieter de Jonge PAC library #709, RPCI6, obtained through the Resource Centre of the German Human Genome Project). Clone orientation was obtained by STS content mapping and by halo-FISH [14]. Non-overlapping clone ends were used as STSs in the next round of library walking.

Refined genetic mapping

For the identification of new polymorphic microsatellites, BAC and PAC contig clones were digested with frequent cutter restriction endonucleases and shotgun cloned into pBluescript. A replica membrane of the gridded colonies was hybridised with a cocktail of $^{32}$P-ATP end-labelled di/tri/tetranucleotide repeat sequences and positive clones were sequenced. Markers available from the public databases (D8S558, D8S529, D8S378 and D8S256) were PCR-amplified using fluorescently labelled primers (Research Genetics Map Pair Set), length-separated on a PE Biosystems 373 XL DNA analyser and analysed using the PE Biosystems Genotyper software. AFM116y8 and all newly identified microsatellites were analysed through incorporation of $^{32}$P-α-dCTP into the PCR product during amplification. The PCR primers for the newly identified markers were as described [13]. Vertical gel electrophoresis, performed in a Hoeffer Pokerface II apparatus, was followed by autoradiography for 2-12 hours. Allele calling was performed manually. Haplotypes were constructed manually and examined for recent and historical recombinations. The marker positions were determined by the STS content mapping of the contig clones.

A total of 174 individuals were genotyped for 24 markers in the HMSNL region. Informed consent was obtained from all participants in the study.

Sequencing

BAC/PAC DNA isolation and purification with the QIAfilter Plasmid Midi kits followed the manufacturer's protocols (QIAGEN News Issue # 4, 1996). End sequencing was performed using universal primers T7 and SP6. Sequencing analysis of the WISPI and NDRGI genes included all coding regions and at least 100 bp of flanking intronic sequences. PCR amplification was performed using the primers shown in Table 1. The PCR products were purified with the Qiagen QIAquick spin columns. Both strands were sequenced with the same primers as used for the PCR amplification.
Sequencing of end clones and PCR products was performed using PE Biosystems BigDye Terminator reagents. The reactions were run on a PE Biosystems ABI 377 sequencer and analysed using the Sequence Navigator software vs 1.0.1.

For large-scale genomic sequencing, BAC/PAC DNA was isolated using the double acetate method [15]. The closed-circle band was sonicated and 1.5-2 kb fragments were size-selected by agarose electrophoresis and ligated into the Smal site of M13mp18 vector. M13 templates were prepared by the Triton method [16]. Shotgun sequencing was performed using ThermoSequenase (Amersham) and Dye-Terminator chemistry (PE Biosystems). Data were collected using ABI 377 automated sequencers and assembled with the program phrap/cross_match.

Computational analysis

The genomic sequence data were analysed using the RUMMAGE-DP program of the Institute of Molecular Biotechnology, Jena, Germany, which combines more than 25 different programs (references available at the URL of the Jena Institute of Molecular Biotechnology), including five for exon prediction, REPEATMASKER for tagging repetitive sequences, programs for prediction of CpG islands, and homology searches using BLAST version 1.4 and FASTA version 2.0. Recognition of promoter regions and tran-

| Table 1. PCR primers for the sequencing analysis of NDRG1 and WISPI |
|-------------------|-------------------|
| **Forward**       | **Reverse**       |
| NDRG1 Exon I      | GACTTCGAGGGTCTGGGAG | CTTAATCCTGGAGTACGC |
| NDRG1 Exon 2      | CTTTCTGCAATGGGACTTG | GCATGCCCATATAAGTGAAAG |
| NDRG1 Exon 3      | GATCGGCTAGAAGGAGAGG | AGAGAAGACGGGGATGAGG |
| NDRG1 Exon 4      | CATCCGATGCTACGCTGCT | GCAATTCTGGCTCTAGGCT |
| NDRG1 Exon 5      | GCCAAAGCCGCCAGACAC | GAGCTACAGAGGCTCTGCTC |
| NDRG1 Exon 6      | GGCAGCAGCAATGGCCAC | TGAGCGGACACACATCTG |
| NDRG1 Exon 7      | GAGCAGAGCTCAGCTAG | CCAAAGCTCAGAGGCTCT |
| NDRG1 Exon 8      | CATCCGCTCCTGCTAAGT | GCTGGATATGCTCAGTCT |
| NDRG1 Exon 9      | GACAGCGACATCTGCTGAT | GCTTGACGCTCAGATCAG |
| NDRG1 Exon 10     | GAGTATCTGACCAGCCTAG | CTTTGGCAGGCTCAGATC |
| NDRG1 Exon 11     | AAGAAGCTGAGCTGAGTG | CTGGATATGCTCAGTCT |
| NDRG1 Exon 12     | CAGGGACCTGGAGTGGAGACC | GAGGACAGGGGCCACCTGG |
| NDRG1 Exon 13     | CAAAGGACACATCTGCTGAT | GCTTGATATGCTCAGTCT |
| NDRG1 Exon 14     | GACAGCGACATCTGCTGAT | GCTTGATATGCTCAGTCT |
| NDRG1 Exon 15     | GAAAGCTGACACACACAC | CATGCCCTCCACACACATA |
| NDRG1 Exon 16     | GTGAGAGCTGAGCTGAGTG | GCTCGAGAACGAGCTCAG |
| WISPI Exon I      | CATATCTGCTGCTGATGG | GTCGACAGGACCCAGTAGAGG |
| WISPI Exon 2      | GACAGTGAGTCATGCGGAGG | GCTGGATCTGCTGAGAC |
| WISPI Exon 3      | GCTGGATCGACGTGGAGC | GCTGGACAGCTGAGTCC |
| WISPI Exon 4      | GTGTGTTGAAAAGTGAGGGTG | GCTTGGTGAAGTGCTGAC |
| WISPI Exon 5      | GTAGGGAGGCTCCTGCCCAC | CAGATCGGGGTAACTGAG |

Subjects and Methods
cription start positions was performed using both Ghosh/Prestridge (TSSG) and Wigender (TSSW) motif databases.

**Screening for the R148X mutation**
Exon 7 of NDRG1 was PCR amplified as a 176 bp product using the following primers 5'AGGCTCCCGTCACTCTG3' (forward) and 5'GTCTCTTCTCATCTTAA AATG3' (reverse). Restriction digests were performed for 4 h at 65°C in a mix containing 1X TaqI buffer, 10 µl PCR product and 10U of TaqI (Promega). The restriction products (104 and 72 bp in length) were separated in 4% agarose gels stained with ethidium bromide and visualised under UV light.

**Expression studies**
SAGE library data were obtained through screening of our own libraries constructed from peripheral nerve, glioblastoma and fetal brain RNA [17] and through searching SAGE public databases. The sequence of the NDRG1 SAGE tag is GGACTTTCCT. Expression levels are given as number of tags/10^6.

Northern blot analysis was conducted on RNA extracted from total peripheral nerve and from cultured non-myelinating Schwann cells and hNT2 cells following standard protocols [18]. RT-PCR of NDRG1 of RNA derived from the same sources as above, was performed using primers AACCCACA CAGTCACCCCT (forward) and GAAGTACTTGAAGGCTC (reverse). The 189 bp products were run on a 1% agarose gel in 1X TBE, blotted and hybridised with the PCR product obtained with the same primers.

Analysis for tissue-specific transcripts was performed by 5'-RACE and by RT-PCR of two fragments spanning the entire coding region of NDRG1. 5'-RACE (Boehringer Mannheim 5'/3' RACE kit) was performed on total RNA from human fetal brain, adult peripheral nerve and lymphocytes, using the following NDRG1-specific primers: NDRG1-R1 5'ACACAGCGTGACGTGAACAG3' (for the reverse transcription step), and NDRG1-R2 5'CAGAGCCATGTAAAGTCTCG3' and NDRG1-R3 5'ATGTCTCTGCTCCTGGACATC3' for the 5'-RACE reactions. The products were tested on agarose gels and sequenced with primer NDRG1-R3. One-step RT-PCR was performed on the same sources of RNA as the 5'-RACE, using the following two primer pairs: NDRG1 5'UTR-F 5'GAAGCTCCTGTCAGTCGACC3' and NDRG1 Exon4-R 5'GTGATCTCCTGATGCTCCTC3'; NDRG1 Ex4-F 5'GAGGA CATGCAGGAGATCAC3' and NDRG1 Ex15-R 5'CCAGAGGCTGTCGACC3'.

**Radiation hybrid mapping**
The chromosomal location of NDRG2 was determined by radiation hybrid (RH) mapping. PCR screening of the GeneBridge RH panel was performed using primers selected from the unique 3'UTR sequence of KIAA1248, showing no homology to NDRG1.
Microsatellite mutation
Recombinant haplotypes

Figure 1 (a) Diagram of the BAC, PAC and cosmID (HCT) clone contig of the HMSNL region. Known genes are represented by arrows pointing in the direction of their transcription. Also indicated are the positions of polymorphic microsatellites used to construct haplotypes on disease chromosomes (b). The HMSNL critical interval is flanked by markers pJ10 and 369CA3 and contains the WISPI and NDRG1 genes.
Figure 2. Scale diagram of the genomic and cDNA organisation of NDRG1 with an illustration of the HMSNL mutation. (a) Genomic organisation: The NDRG1 gene consists of 16 exons spanning 60kb of genomic sequence. A CpG island overlaps with the first exon and the 5' end of intron 1. A potential promoter with a TATA box, a GC-box similar to the N-myc binding region of mouse Ndr1 and a TATA-less enhancer were located 39bp, 65bp and 81bp upstream respectively of the first exon. The 5'UTR is split between the first two exons. (b) cDNA structure: translation starts from nt 123. (c) HMSNL mutation: C → T substitution at base 564 results in a stop signal (TGA) at codon 148 in exon 7 (R148X). All nt positions are given relative to the published sequence (GenBank Accession #D87953).
or NDRG3. The primer sequences were as follows: NDRG2RH-F2 5'-CTGGGGGCTC-CATTCAACAAAGC and NDRG2RH-R2 5'-AGCCCGAGCCCAAGCTTAGCTC. The results were submitted for calculation to the RH server of the Whitehead Institute/MIT Center for Genome Research.

Results

Physical and refined genetic mapping

We have assembled a 1 Mb contig of genomic BAC, PAC and cosmid clones, with a minimum tiling path shown in Figure 1. The contig spans the entire HMSNL region as defined by the recombinations identified in the initial study [1]. The contig was anchored to the four known markers in this region on 8q24, following the order provided by public databases (cen-D8S529/D8S378-AFM116yh8-D8S256-tel). Our subsequent findings have shown the correct orientation to be cen-AFM116yh8-D8S378-D8S529-D8S256-tel. The contig clones were used for the physical mapping of ESTs roughly positioned in this region and for the identification of new polymorphic markers. The refined genetic mapping included 174 individuals (60 patients and 114 unaffected relatives) from seven European countries, genotyped for 24 polymorphic microsatellites, of which 19 were identified in our study [13]. Ten recombinant haplotypes, whose distribution differed between disease chromosomes originating from the diverse Romani groups, helped to narrow down the HMSNL gene region (Figure 1b). In 5 of the 7 centromeric recombinations (bottom of Figure 1b) the breakpoints mapped to the same 90 kb interval between markers pJ10 and 458b14, thus placing the centromeric boundary of the region at pJ10. Haplotype analysis of the telomeric recombinants placed the distal boundary at marker 369CA3 (right hand side of Figure 1b). Within the pJ10-369CA3 interval, all HMSNL chromosomes shared an identical haplotype for markers 458a13-458b57-369a89. This haplotype was not found in any of the 88 normal chromosomes studied. Marker 458b14 presented with three different alleles in the disease chromosomes, however on the basis of the conserved flanking haplotypes this variation was assumed to result from microsatellite mutations (similar to those observed in 339CA2, 189CA17 and especially D8S378; green boxes in Figure 1b).

The critical HMSNL gene interval was thus defined on the basis of recombination and homozygosity mapping to be located between newly identified markers pJ10 and 369CA3. The entire region was contained in 3 overlapping genomic clones, PAC 709A2498Q2 and BACs 458A3 and 369M3 (Figure 1). Large-scale sequencing of these clones identified the final exons of thyroglobulin in PAC 709A2498Q2 and the full length of two known genes: Wnt1-inducible signalling protein 1, WISPI [19] in BAC 458A3 and N-myc downstream regulated gene 1, NDRG1, aliases RTP, NDR1, DRG1, CAP43 [20-24] in BACs 458A3 and 369M3 (Figure 1a). WISPI and NDRG1 are located tail
to tail, in opposite orientations and separated by a small distance of about 3 kb. The WISP1 gene spans about 38 kb of genomic DNA, with coding regions split into 5 exons. NDRG1 is spread over 60 kb of genomic DNA and consists of 16 exons, including an untranslated first exon (Figure 2).

The HMSNL mutation
The search for the mutation was conducted by sequencing the untranslated and promoter regions, all exons and at least 100 nucleotides of the flanking introns of WISP1 and NDRG1 in a panel of DNA samples from HMSNL patients and unaffected controls from the same population.

This analysis revealed a total of 13 single nucleotide polymorphisms (SNPs) in the two genes (Table 2), of which only one was in WISP1. The difference is due to the fact that sequence variation in NDRG1 was investigated more extensively in individuals of diverse ethnic background whereas WISP1 was analysed only in the Roma. Our results so far show that SNPs in the NDRG1 gene occur with a frequency of at least 1 per 423 nucleotides. In HMSNL patients, the WISP1 gene sequence was identical to the published wild-type sequence.

The analysis of NDRG1 in the HMSNL affected individuals identified a C→T transition in exon 7, at nucleotide position 564 (following the numbering of the RTP sequence published under accession # D87953). This substitution results in the replacement of arginine by a translation termination signal at codon position 148 (Figure 2). The RI48X mutation was found in the homozygous state in all 60 HMSNL patients included in our study.

The C→T substitution abolishes a TaqI site and a restriction assay was designed as a screening test for the RI48X mutation. In the HMSNL families, the mutation segregated in 100% agreement with the carrier status predicted by haplotype analysis. The analysis of 69 additional unaffected members of the extended kindred where the disease was first described (the Lom pedigree), detected 24 carriers.

The RI48X mutation screening also included 10 Romani families with unspecified autosomal recessive peripheral neuropathies from Rumania. The RI48X mutation was found in 6 of these families, where it co-segregated with the disease phenotype and occurred in the homozygous state in the affected patients.

We did not find the RI48X mutation among 101 unrelated unaffected control individuals, including 68 non-Romani Bulgarians and 33 Roma who originate from the same groups as the HMSNL patients but belong to kindreds with other genetic disorders.

The SNP positions are designated as proposed by Antonarkis et al. [25], with positive IVS (intronic) numbers starting from the G of the donor site invariant GT, and the negative IVS numbers starting from the G of the acceptor site invariant AG.
The N-myc downstream regulated gene family

NDRG1 is a known gene that has been identified previously by several independent in-vitro studies of human cell lines [20-22, 24]. The encoded protein is highly conserved in evolution [26-28]. The genomic organisation of NDRG1 revealed in our study (Figure 2), is also conserved and closely related to that of the mouse gene [26]. Previous experiments suggested that NDRG1 is a unique gene [20, 29], however a recent study has demonstrated the existence of a Ndr gene family in the mouse [30]. Since the existence of homologous genes in humans could affect the specificity and hence reliability of expression studies, we have used the novel mouse sequences to search the human genome databases. This search has confirmed the existence of related human genes, which we will refer to as NDRG2 and NDRG3 respectively, for the genes homologous to mouse Ndr2 and Ndr3.

NDRG2 was found to be represented by 147 ESTs and two cDNA sequences. To determine its chromosomal localisation, we have performed radiation hybrid mapping using the GeneBridge panel. NDRG2 was localised to chromosome 14q11.2, at 6.72 cR from D14S264, with a lod score of 15.0.

The NDRG3 gene was represented by 86 ESTs and a genomic clone from chromosome 20q11.21-q11.23. This provisional chromosomal localisation was confirmed by electronic PCR. This search identified four STSs in the same genomic clone (three flanking NDRG3 and one located in its 3'UTR) that have also been independently localised to chromosome 20 by radiation hybrid mapping.

The BLAST comparison showed considerable homology between the three human NDR genes, with greater divergence in the terminal parts of the sequences. At the protein level, the identity (similarity) is 54% (70%) between NDRG1 and NDRG2, 67% (81%) between NDRG1 and NDRG3 and 58% (71%) between NDRG2 and NDRG3. These values are very similar to the percent homology reported for the members of the mouse Ndr family [27]. Both mouse [27] and human NDRG2 and 3 lack the highly hydrophilic amino acid sequence motif (GTRSRSHTE) typical of NDRG1 and repeated three times at its C-terminus.

Expression analysis of NDRG1

The ubiquitous expression of NDRG1 is documented by 343 entries in UniGene Cluster Hs.75789 and by the previous studies using various experimental systems [20-24, 29]. No information has been published to date on the peripheral nervous system. To obtain a quantitative comparison of the levels of NDRG1 expression in different tissues, we have performed SAGE library screening and database searches. The following results, presented as tags/10^6, were obtained: peripheral nerve 400; colorectal cancer (HCT116) 213; glioblastoma multiformae libraries 210 and 99; brain 146; normal colon and some primary colon tumours 81-105; and prostate cancer 139,158. The above values indicate that NDRG1 is abundantly expressed in peripheral nerve, where the levels of expression are significantly in excess of those in the other tissues examined.
Table 2. Single Nucleotide Polymorphisms (SNPs) Identified in NDRG1 and WISP1

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Nucleotide Position</th>
<th>Ethnic background</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDRG1</td>
<td>T/G</td>
<td>5’UTR; nt15(^a)</td>
<td>Afro-American</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>5’UTR, nt3(^b)</td>
<td>Dutch; Roma</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>Intronic; IVS1+48</td>
<td>Dutch; Roma</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>Intronic; IVS2-5</td>
<td>Afro-American</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>Intronic; IVS6-33</td>
<td>Afro-American; Dutch; Roma</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>Intronic; IVS10+83</td>
<td>Dutch</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>Intronic; IVS10-50</td>
<td>Afro-American; Dutch; Roma</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>Intronic; IVS11-7</td>
<td>Roma</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>Intronic; IVS13+147</td>
<td>Afro-American; Dutch; Roma</td>
</tr>
<tr>
<td></td>
<td>A/G (293Pro→Pro)</td>
<td>Exon 14; 989(^b)</td>
<td>Afro-American</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>Intronic; IVS14-124</td>
<td>Afro-American</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>3’UTR 1395(^b)</td>
<td>Afro-American</td>
</tr>
<tr>
<td>WISP1</td>
<td>C/T</td>
<td>(307Asn→Asn) Exon 5; 1009(^c)</td>
<td>Roma</td>
</tr>
</tbody>
</table>

\(^a\)Position relative to NDRG1 5’UTR novel sequence (GenBank Accession # AF230380)
\(^b\)Position relative to mRNA for RTP (GenBank Accession number D87953)
\(^c\)Position relative to mRNA WISP1 (GenBank Accession number AF100779)

Northern blot analysis, comparing total adult peripheral nerve RNA, cultured non-myelinating Schwann cells and hNT2 cells, which can be induced to neuronal differentiation showed strong signal in total peripheral nerve and Schwann cells; expression was lower in undifferentiated hNT2 cells; no signal was obtained in differentiated hNT2. In view of the high sequence homology between the genes of the NDRG family and the possibility of cross-hybridisation, these results were verified and confirmed by RT-PCR using NDRG1-specific primers (Figure 3). Our preliminary immunocytochemistry findings in peripheral nerve point to NDRG1 localisation in the Schwann cell cytoplasm, with no evidence of axonal expression (not shown).

We have used 5’-RACE and RT-PCR to check for the presence of tissue-specific NDRG1 transcripts in peripheral nerve, fetal brain and lymphocytes. 5’-RACE did not provide evidence of different transcription start sites; these experiments identified a short (15 nt) novel additional sequence immediately upstream of the 5’UTR of longest published NDRG1 sequence [21], which however was common to all three transcripts. RT-PCR, followed by sequencing, of the entire coding region of NDRG1 in peripheral nerve, fetal brain and lymphocytes revealed a single transcript, identical to the published cDNA sequence, with no evidence of tissue-specific alternatively spliced forms.
Discussion

The heterogeneous category of hereditary motor and sensory neuropathies consists of a large number of clinically and genetically distinct conditions (recently reviewed in [31, 32], including autosomal recessive forms, some of which have been placed on the human genetic map [33-37]. Relative to autosomal dominant CMT disease, these conditions are rare. However, they are clinically more severe [38] and less likely to result from mutations in structural myelin proteins, therefore understanding their genetic basis may provide an insight into hitherto unknown molecular mechanisms of peripheral nervous system development and axon-glia interactions. The genetic heterogeneity of autosomal recessive peripheral neuropathies and the limited number and size of families affected by any single disorder, have presented a major obstacle to molecular research and gene identification. In the case of HMSNL, positional cloning was facilitated by the substantial number of patients identified over a short period of time after the initial description of the disease, as well as by the history of the disease-causing mutation. HMSNL occurs in an ethnic group that is marginalised by most health care systems, therefore ascertainment can be predicted to be limited. The number of affected individuals already diagnosed suggests that the disease is relatively common and may be the prevalent form of peripheral neuropathy among the Roma. On the other hand, the origin of the HMSNL mutation has been estimated to pre-date the arrival of the proto-Roma in Europe [1], indicating that the mutation was present in the ancestral population before it split into numerous small groups separated by geographic dispersal, social pressures and rules of endogamy. The independent evolution and diversification of disease haplotypes in the different Romani groups across Europe has provided a powerful tool for the refined mapping of the HMSNL gene.

The molecular defect shared by all affected individuals was found to be a truncating mutation in N-myc downstream regulated gene 1. This gene encodes a highly conserved protein with a high degree of homology to the proteins in other species. The
amino acid similarity is 44% to the Drosophila protein, 48% to sunflower [27], 75% to rat Bdm1 [28] and 96% to mouse Ndrl [26]. These proteins show no homology to known motifs, except for a putative phosphopantetheine-binding site [20, 21, 29] and a 46% similarity to the ligand-binding domain of the inositol 1,4,5-triphosphate receptor [27].

The evolutionary conservation of NDRG1-related proteins points to an important biological role. The previously proposed functions of human NDRG1 are based on studies of non-neural tissues. NDRG1 has been shown to be repressed in cell transformation [20, 24] and upregulated in growth-arrested differentiating cells [20, 24, 29] and under conditions of cellular stress [21-23]. Inducing agents include p53 [24], increased intracellular Ca$^{2+}$ and forskolin [22], retinoic acid and vitamin D [29]. NDRG1 expression has been shown to cycle with cell division [24] and studies of the intracellular localisation of the protein suggest translocation between the cytoplasm and the nucleus [20, 24, 29]. A role as a developmental gene has been documented for Ndrl, which, in the mouse embryo, is repressed, by N-myc and upregulated in cells committed to terminal differentiation [26]. The accumulated data suggest involvement in growth arrest and cell differentiation during development, and in the maintenance of the differentiated state in the adult, possibly as a signalling protein shuttling between the cytoplasm and the nucleus.

In terms of patterns of expression and proposed general functions, NDRG1 clearly resembles PMP22/gas3. PMP22 is also widely expressed in embryonic and adult tissues [39, 40] and believed to be involved in growth arrest and cell differentiation [41, 42]. The highest levels of expression are found in the myelinating Schwann cell where PMP22 is a component of compact myelin [43]. PMP22 is now known to be responsible for CMT disease type IA, hereditary neuropathy with liability to pressure palsies and some forms of Dejerine-Sottas syndrome in humans [39, 44-47] and for naturally occurring models of peripheral neuropathy in the mouse [48, 49]. A number of studies of affected humans, as well as of natural and transgenic rodent models, have pointed to the complex pathogenesis of these disorders where altered myelin stability and demyelination are only one aspect. The observed significant phenotypic changes in both Schwann cells and axons [9-12, 50] have suggested that, in addition to its function as a myelin protein, PMP22 plays a role in early PNS development and differentiation and in Schwann cell-axonal interactions (reviewed in [51]).

Axons and glia in the peripheral nervous system are involved in a most complex system of communication, whose integrity is essential for the differentiation, survival and normal function of both types of cell [52-54]. The involvement of NDRG1 in these mechanisms, and a possible functional link to PMP22, remain to be investigated in functional studies, as well as through the identification of NDRG1 mutations in other peripheral neuropathies. The high levels of NDRG1 expression in peripheral nerve and specifically in the Schwann cell, together with the characteristics of the HMSNL phe-
notype point to a possible involvement of NDRG1 in the Schwann cell differentiation and the signalling necessary for axonal survival. The role of NDRG1 in growth arrest and cell differentiation, proposed for other tissues, may thus be conserved in the PNS and related to the complex developmental transitions marking the stages of differentiation of the Schwann cell lineage and Schwann cell-axonal interactions [53, 54]. At the same time, the abundant expression in adult peripheral nerve, and the putative phosphopantetheine-binding domain present in the NDRG1 protein, point to a possible dual role and additional involvement in the lipid biosynthetic pathways operating in the myelinating Schwann cell.

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Electronic database information

The URLs and accession numbers for data in this article are as follows:
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for HMSNL [MIM 601455])
http://www.genome.washington.edu/UWGC/ analysistools/phrap.htm
http://genome.imb-jena.de/~schattev/
http://dot.imagen.bcm.tmc.edu/9331/gene-finder/gf.html/
http://www.genome.wi.mit.edu/
GenBank, http://www.ncbi.nlm.nih.gov/Genbank (for BAC369M3 [AF186190]; BAC 458A3 [AF192304]; NDRG1 (RTP) mRNA [D87953]; NDRG1 5'UTR novel sequence [AF230380]; NDRG1 UniGene cluster [Hs 75789]; NDRG1 LocusLink [ID 10397]; sunflower SF21 [AF189148]; D.melanogaster BcDNA.GH02439 [AF145594]; Rattus norvegicus Bdm1 [AF045564]; mouse Ndr1 [U60593]; Ndr2 [AB033921]; Ndr3 [AB033922]; sequences representing human NDRG2 [AF159092 and AB033074] and NDRG3 [AL031662]
References


36. Casaubon, L., et al., The gene responsible for a severe form of peripheral neuropathy and agenesis


