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An axon regeneration signature in a Charcot-Marie-Tooth Disease Type 2 patient

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Abstract

Charcot-Marie-Tooth Disease (CMT) is one of the most common inherited peripheral neuropathies. The underlying mutations in demyelinating forms tend to affect genes expressed in Schwann cells (CMT Types 1, 3 and 4), while axonal forms of the disease usually have their origins in genes expressed in the affected neurons (CMT Type 2). Repeated rounds of nerve degeneration and regeneration characterize CMT2, but evidence for regeneration has not been demonstrated at a molecular level. Subtractive hybridization was performed on sural nerve biopsies from a patient presenting an axonal form of CMT and an unaffected sibling, which revealed over-expression of genes associated with the regeneration of axons, including PMP22, SPARC/osteonectin, CD9, CD44, EEF1A1 and γ -actin. These results suggest that axonal degeneration elicits a regeneration transcriptional response in the surrounding Schwann cells. This response contrasts with other neurodegenerative diseases in which programmed cell death or an inappropriate immune response are activated. Additionally, Lamin A/C, which is mutated in CMT2B1, was over-expressed in the patient, suggesting that CMT-causing genes may interact in a regulatory network.

Keywords

Nerve degeneration; nerve regeneration; gene expression profiling

INTRODUCTION

Charcot-Marie-Tooth Disease is a genetically heterogeneous group of disorders all leading to degeneration of peripheral nerves. CMT presents a challenge to identify causative mutations as patients may be asymptomatic, and additional symptoms have to be considered to identify affected individuals (Pareyson et al., 2006). Repeated rounds of axonal degeneration and

regeneration characterize the axonal form of the disease (CMT2; Zuchner and Vance, 2006), although this has not been previously correlated with molecular markers. Mutations in ten causative genes have been identified for CMT2, but a recent study found that in 18 families, mutations in the previously identified genes were only responsible for 17% of the cases (Bienfait et al., 2007).

We are in the process of mapping the underlying mutation in a family affected with CMT2. Using the foot deformity *pes cavus*, which arises due to peripheral muscle imbalance, as a surrogate marker, the disease segregates as an autosomal dominant disorder with no anticipation. Three family members have lowered nerve conduction velocities in the demyelinating range, suggesting they are either homozygous for the causative mutation, carry additional modifier loci, or that the condition has a highly variable expressivity. Genetic analysis has excluded the known CMT2 loci (MFN2, RAB7, GARS, NEFL, LMNA, HSPB1, HSPB8, MPZ, GDAP1, KIF1B), as well as additional candidate loci (PMP22, YARS, BSCL2, DNM2, HOXA10, HOXD10) and two loci identified in this study (EFNA5 and SKIL) as responsible (F.C. and A.Q., unpublished results). We performed subtractive hybridization between biopsy material from a patient and an unaffected sibling, with the objective of gaining insight into the disease and identifying molecular markers for future analysis. The observed gene expression differences are strongly suggestive of axonal regeneration occurring in the patient.

MATERIALS AND METHODS

Patient Characteristics

We are studying a large CMT2 family, consisting of fifty family members in four generations. Neurophysiologic evaluation and sural nerve (innervates the lateral portion of the foot) biopsies were performed on individuals who gave their written consent. Three affected individuals have slowed nerve conduction velocities (NCV) and “hammer toe” deformities consistent with a severe axonal neuropathy. Sural nerve biopsies show evidence of axonal degeneration, regeneration and occasional myelin “onion bulbs”, similar to CMT2 subtypes B, E, G, and L. Additional family members without NCV defects display the *pes cavus* foot deformity, a classic CMT symptom. In total, twenty-six family members were examined, thirteen of these had neurophysiological measurements made, and four underwent sural nerve biopsies. All of the observations are consistent with a progressive axonal neuropathy. We selected the patient with the most severe nerve conduction symptoms to investigate. Motor nerve conduction velocity was 34 m/s in the arm; compound muscle action potentials were 10mV in the arm and absent in the leg. Sensory nerve conduction velocity was 34 m/s in the arm; sensory nerve action potentials were 8μV in the arm and absent in the sural nerve. The age of onset of the symptoms was 17 years old, and the patient was 22 years old at the time of the sural nerve biopsy. A sibling of the patient presented with a slight hint of *pes cavus*, but did not display NCV defects. The sibling underwent sural nerve biopsy to provide a definitive diagnosis, allowing management of the disease if positive. The biopsy result was negative presenting an unusual opportunity to compare affected and unaffected tissue. The unaffected sibling was 35 years old at the time of the sural nerve biopsy. All patient-physician interactions followed the guidelines of the Italian National Committee for Bioethics.

Subtractive Hybridization

cDNA was amplified from sural nerve biopsies using the SMART system (Clontech). PCR-select cDNA subtraction with mirror orientation selection (Clontech) was used to identify differentially regulated genes. The recovered cDNA clones were sequenced and identified by BLAST analysis. Each clone was dot blotted in duplicate and probed with either patient or unaffected sibling specific subtracted libraries. Densitometric analysis on the signals was

carried out using NIH Image software to give an approximate measure of the differences in expression on all the genes recovered. Thirty-four genes were found to be up regulated, and six down regulated in the patient; for many genes multiple independent clones (“hits”) were recovered (Table 1).

Quantitative PCR

Due to the limitations imposed by the amount of starting material, only seven genes of particular interest were further validated by quantitative PCR using Taqman primer sets (Applied Biosystems). A ratio for the difference between the patient and unaffected sibling was calculated by the comparative C_T method (Table 2). GUSB (beta-glucuronidase) was used as the endogenous control after it was found that RNaseP mRNA levels varied between patient and sibling. Four genes showed robust over-expression, and CD44 showed weak but consistent over-expression. We failed to confirm over-expression of integrin $\beta 1$ and PTP4A2. This was a surprise as PTP4A2 was recovered seven times in the screen; sequence analysis of the recovered clones indicates that the discrepancy does not seem to be due to differential expression of mRNA isoforms.

The studied family shares symptoms with those caused by mutations in lamin A/C (LMNA), so we tested LMNA levels and unexpectedly found significant over-expression in the patient. The absence of LMNA in the genes recovered by subtractive hybridization could be due to the cDNA normalization step in sample preparation (Cao et al., 2004), which may possibly exclude abundant mRNAs from detection. However, we did recover the gene for peripheral myelin protein 22 (PMP22), a major component of PNS myelin, which would be expected to be abundantly expressed in Schwann cells. Mutation or duplication of PMP22 leads to demyelinating CMT (CMT1A; Pareyson et al., 2006). Quantitative PCR revealed that PMP22 is over-expressed 1.8 times in the patient which is higher than the predicted 1.5 times over-expression in CMT patients with a PMP22 duplication (Table 2).

RESULTS AND DISCUSSION

Many of the genes identified as differentially expressed in this study have been previously identified in models of acute injury. Although CMT2 is a chronic condition once established, gene expression data for chronic nerve injury is extremely limited, so we chose to compare to data from acute injury models. Chronic nerve injury models produce Schwann cell responses very similar to those seen in acute injury models (Gupta et al., 2006; Atanasoski et al., 2006), and it seems likely that many of the same genes will be involved in both types of injury. As our biopsy material consisted principally of Schwann cells and axon shafts, we expected to recover genes previously identified as expressed in Schwann cells.

After acute injury, genes that are normally required for the function of the mature nerve are down regulated, and regeneration specific genes are up regulated (Bosse et al., 2006). A subset of genes are up regulated when the regenerating axons re-establish contact with the Schwann cells, leading to subsequent re-myelination. This class is exemplified by PMP22 which was over-expressed in our CMT2 patient. In acute injury models, up-regulation of PMP22 is a sign that axonal regeneration is occurring, and leads to subsequent re-myelination (Maier et al., 2002). We compared the genes identified in this study with one of the most comprehensive studies of gene expression in regenerating nerves. Bosse et al. (2006) examined gene expression of 2361 genes following sciatic nerve crush at six different time points ranging from hours to days after injury. 192 genes were found to differ significantly from uninjured nerves. Twelve of the genes identified in this study were analyzed by Bosse et al. (2006), and six (SPARC, CD9, CD44, PMP22, EEF1A1, H2AFZ) were present in the 192 differentially expressed genes. The probability of this 50% match occurring by chance is extremely low ($p < 0.000001$). For comparison, 36,000 genes were analyzed for changes in expression in mouse models of

CMT1A based on PMP22 and there was no overlap with our dataset, except that UBC is down regulated in the PMP22 knockout mouse, but up regulated in the CMT2 human patient (Giambonini-Brugnoli et al., 2004). In addition, the unfolded protein response (UPR) has been shown to play a critical role in a mouse model of CMT1B (Pennuto et al., 2008), but none of the genes transcriptionally induced by the UPR were recovered in our analysis.

Additional studies of peripheral nerve injury have identified genes recovered in this study: CD9 (Banerjee et al., 1995), SPARC/osteonectin (Gillen et al., 1995; Au et al., 2007), Decorin (Gillen et al., 1995; Braunewell et al., 1995), CD44 (Jones et al., 2000), ACTG1 (Gillen et al., 1995) and TIMP3 (Szpara et al., 2007).

Although the underlying mutation for the CMT2 family we are studying has not been identified, electrophysiological measurements are completely consistent with an axonal pathology. It is therefore interesting to see up-regulation of genes typically expressed by Schwann cells in normal and injured tissue, suggesting that the Schwann cells in the patient are attempting to compensate for a genetic deficit in the axons. The dramatic up-regulation of SPARC, suggests that it could be a potential biomarker for the severity of the disease, especially if the protein enters the bloodstream. Finally, the observed up-regulation of PMP22 and LMNA, genes that underlie other CMT subtypes suggests that CMT causing genes may participate in an interacting regulatory network.

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Genes identified as differentially expressed between the CMT2 patient and an unaffected sibling by the subtractive hybridization screen.

Up-regulated Genes	Accession	Symbol	Name	Hits	Ratio
<u>Growth Factors</u> <u>Extracellular Matrix</u>	NM_021146	ANGPTL7	Angiopoietin-like 7	1	6.6
	NM_003118	SPARC	Osteonectin	5	3.5
	NM_001920	DCN	Decorin	2	2.2
<u>Membrane Proteins</u>	NM_000362	TIMP3	Tissue inhibitor of metalloprotease 3	2	3.1
	NM_001769	CD9	CD9 antigen (p24)	1	2.5
	NM_001001392	CD44	CD44 antigen	1	7.2
	NM_001025080	CD47	Integrin associated protein	1	5.8
	NM_002211	ITGB1	Integrin beta 1	1	3.3
	NM_021999	ITM2B	Integral membrane protein 1	1	2.2
<u>Myelin</u>	NM_000304	PMP22	Peripheral myelin protein 22	2	1.9
	NM_003953	MPZL1	Myelin protein zero-like 1	1	7.3
<u>Signal Transduction</u>	NM_006908	RAC1	Small GTP binding protein	1	3.9
	NM_001386	DPYSL2	Dihydropyrimidase-like 2 variant protein	1	2.8
	NM_145117	NAV2	Neuron navigator 2	1	1.4
<u>Cytoskeleton</u> <u>Phosphatases</u>	NM_001614	ACTG1	Actin gamma 1	1	3.5
	NM_003479	PTP4A2	Protein tyrosine phosphatase type IVA	7	6.1
	NM_000944	PPP3CA	Calcineurin A alpha	2	4.0
<u>Ion Transport</u>	XM_167044	SLC35F1	Nucleotide sugar transporter	2	1.7
	NM_016929	CLIC5	Chloride intracellular channel 5	2	4.0
	NM_000702	ATP1A2	ATPase, Na+/K+ transporting alpha 2	1	6.8
<u>Translation</u>	NM_001402	EEF1A1	Translation elongation factor 1 alpha 1	5	3.4
	NM_001005	RPS3	Ribosomal protein S3	1	1.4
	NM_001010	RPS6	Ribosomal protein S6	1	1.9
<u>Nuclear</u>	AK124855	CNOT6L	Putative mRNA deadenylase		
	NM_003107	SOX4	SRY (sex determining region Y) box 4	1	2.3
	AL110141	GAS5	Growth arrest specific 5	1	1.5
	NM_012412	H2AFV	H2A histone family member 5	1	4.9
	NM_002106	H2AFZ	H2A histone family member Z	1	1.3
	NM_006275	SFRS6	Splicing factor, arg/ser rich	1	5.7

Up-regulated Genes	Accession	Symbol	Name	Hits	Ratio
<u>Protein Degradation</u>	NM_021009	UBC	Ubiquitin C	1	1.3
	AL080234	MARCH-VI	Membrane associated ring finger	1	1.2
<u>Other</u>	NM_019852	METTL3	Methyltransferase like 3	2	2.3
	AK096118	FLJ38799	Clorf107	2	3.7
Down-regulated Genes	AY713999	-	Mitochondrial NADH dehydrogenase 4	2	1.5
	NM_001962	EFNA5	Ephrin A5	1	0.264
	NM_005414	SKIL	SKL-like	1	0.546
	NM_020948	MI-ER1	N1-beta	1	0.310
	AK125888	FLJ43900	Similar to F-box only protein 32	1	0.222
	AK022236	FLJ12174	Novel	1	0.529
	AK000176	FLJ20169	Novel	1	0.277

Table 2

Validation of selected genes by quantitative PCR.

Gene		Ratio
SPARC	osteonectin	4.9
CD9	CD9 antigen (p24)	1.5
CD44	CD44 antigen	1.1
ITGB1	integrin beta 1	0.7
PMP22	Peripheral myelin protein 22	1.8
ACTG1	actin gamma 1	1.8
PTP4A2	protein tyrosine phosphatase type IVA	0.9
LMNA	Lamin A/C	4.6