Table 1 | Clinical characteristics of patients

	(MN–/DPN–)	(MN+/DPN–)	(MN+/DPN+)	(MN–/DPN+)
n	71	25	55	36
Sex (% male)	57.7	32.0	61.8	55.6
Age (years)	52.4 ± 13.4**†	58.4 ± 8.9	61.5 ± 10.5**	59.8 ± 12.3†
Body mass index (kg/m^2)	23.8 ± 4.3	24.2 ± 6.4	23.5 ± 4.4	22.9 ± 5.1
Type 1/type 2	16/55*	3/22	2/53*	2/34
Duration (years)	5.4 ± 6.4**††	5.7 ± 5.6‡§§	13.4 ± 8.4**‡	13.3 ± 8.5††§§
HbA1c (%)	10.1 ± 2.1	9.2 ± 2.2	9.7 ± 2.4	10.0 ± 2.3
Urinary CPR (μg/day)	57.2 ± 37.9	76.0 ± 81.3‡§	40.3 ± 39.8‡	44.5 ± 39.3§
Diet/oral/insulin/none	3/31/6/31*	1/14/1/9	0/21/19/15*	1/21/7/7
Retinopathy (%)	19.7**††	36.0‡§	81.8**‡	75.0††§
PPDR or PDR (%)	1.4**††	12.0‡§§	70.9**‡	61.1††§§
Nephropathy (%)	19.7**††	40.0‡§	80.0**‡	75.0††§

CPR, C-peptide immunoreactivity; PDR, proliferative diabetic retinopathy; PPDR, preproliferative diabetic retinopathy. Data are expressed as mean ± standard deviation. *Patients without median neuropathy at the wrist (MN) or diabetic polyneuropathy (DPN; MN–/DPN–) and patients with MN and DPN (MN+/DPN+), $P < 0.05$; **(MN–/DPN–) and (MN+/DPN+), $P < 0.01$; †(MN–/DPN–) and patients with DPN without MN (MN–/DPN+), $P < 0.05$; ††(MN–/DPN–) and (MN–/DPN+), $P < 0.01$; ‡patients with MN without DPN (MN+/DPN–) and (MN+/DPN+), $P < 0.01$; §(MN+/DPN–) and (MN–/DPN+), $P < 0.05$; §§(MN+/DPN–) and (MN–/DPN+), $P < 0.01$.

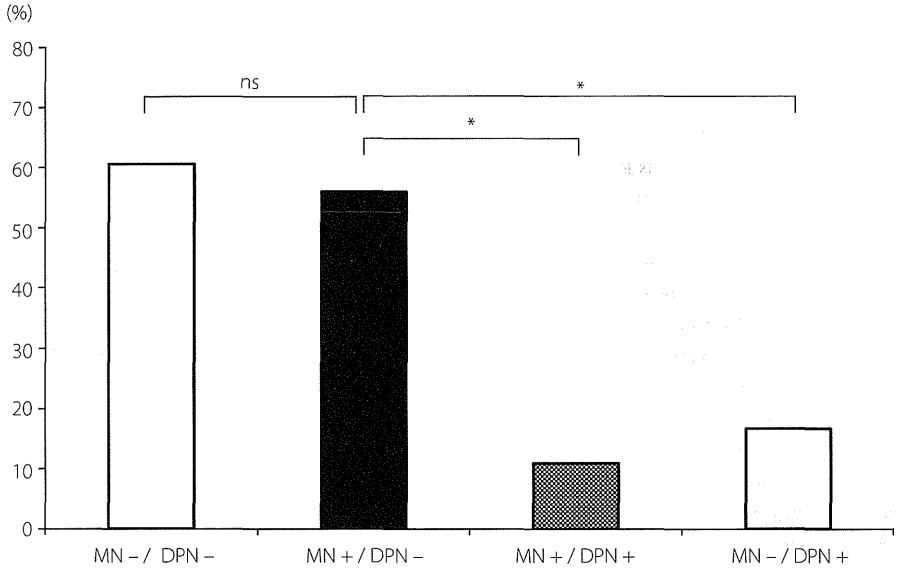

Figure 1 | Percentage of diabetes duration of 5 years or less in the four groups. Data were analyzed by χ^2-test (all significant differences remained after Bonferroni adjustment). *$P < 0.01$. DPN, diabetic polyneuropathy; MN, median neuropathy at the wrist; NS, not significant.

patients with DPN, the MN+/DPN+ group was found to have significantly lower sensory nerve conduction velocities, as well as prolonged median motor latency, sensory palm latency and F-wave latencies than the MN–/DPN+ group. Motor nerve conduction velocities, amplitude of CMAP and SNAPs were not different between the two groups.

DISCUSSION

CTS is reported to occur in 2.6–20% of all patients with diabetes[9]. In the Rochester Diabetic Neuropathy cohort, clinical evidence for MN was found in 9% of patients with type 1 diabetes mellitus and in 4% of patients with type 2 diabetes mellitus. Electrophysiological evidence of asymptomatic MN was found in 22% of patients with type 1 diabetes and 29% of patients with type 2 diabetes[10]. In the current study of patients with poorly managed diabetes, asymptomatic MN was found in 22% of patients with type 1 diabetes and 46% of patients with type 2 diabetes. The prevalence of asymptomatic MN without DPN was 13%, whereas the prevalence of asymptomatic MN with DPN was 29%. This finding is in agreement with previously published data on the prevalence of CTS for this population (14% for CTS without DPN, and 30% for CTS with DPN)[5].

Table 2 | Comparison of median nerve conduction studies of patients

	(MN–/DPN–)	(MN+/DPN–)	(MN+/DPN+)	(MN–/DPN+)
Distal latency (ms)	3.9 ± 0.4**†‡	5.1 ± 0.6**‖	5.4 ± 0.6†¶	4.3 ± 0.4‡‖¶
CMAP (mV)	6.6 ± 1.6**†‡	5.2 ± 1.5**	4.3 ± 1.4†	4.9 ± 1.7‡
MCV (m/s)	53.7 ± 2.7*†‡	51.8 ± 3.0*§§‖	47.1 ± 3.1†§§	48.7 ± 3.4‡‖
F-latency (ms)	26.0 ± 2.0*†‡	27.5 ± 1.8*§§	30.7 ± 3.0†§§¶	29.1 ± 2.8‡¶
SNAP (μV)	18.3 ± 5.5**†‡	10.4 ± 3.9**§§‖	4.1 ± 3.4†§§	5.8 ± 2.6‡‖
SCV (m/s)	54.7 ± 4.4**†‡	42.2 ± 2.9**§‖	39.3 ± 4.8†§¶	49.3 ± 4.7‡‖¶
Palm-latency (ms)	1.5 ± 0.1**†‡	2.1 ± 0.2**‖	2.3 ± 0.3†¶	1.7 ± 0.2‡‖¶

CMAP, compound muscle action potential; MCV, motor nerve conduction velocity; SCV, sensory nerve conduction velocity; SNAP, sensory nerve action potential. Data are expressed as mean ± standard deviation. *Patients without median neuropathy at the wrist (MN) or diabetic polyneuropathy (DPN; MN–/DPN–) and patients with MN without DPN (MN+/DPN–), $P < 0.05$; **(MN–/DPN–) and (MN+/DPN–), $P < 0.01$; †(MN–/DPN–) and patients with MN and DPN (MN+/DPN+), $P < 0.01$; ‡(MN–/DPN–) and patients with DPN without MN (MN–/DPN+), $P < 0.01$; §(MN+/DPN–) and (MN+/DPN+), $P < 0.05$; §§(MN+/DPN–) and (MN+/DPN+), $P < 0.01$; ‖(MN+/DPN–) and (MN–/DPN+), $P < 0.01$; ¶(MN+/DPN+) and (MN–/DPN+), $P < 0.01$.

Interestingly, the frequency of CTS increases after the diagnosis of diabetes in comparison with the rate of CTS before clinical diagnosis. This is because before diagnosis, exposure to hyperglycemia is generally shorter and less severe[11]. On the basis of these reports, we postulate that asymptomatic MN in patients who do not have DPN might be an early manifestation of diabetes. In the current study, compared with the MN and DPN group, the MN without DPN group comprised more patients in the early phase of diabetes (diagnosed within the past 5 years), and fewer patients with diabetic microangiopathy. The present results suggest that MN is found in the early phase of diabetes when DPN has not developed yet.

Although it is not conclusive that diabetes mellitus is a predisposing factor to CTS, the fact known as the "double crush" hypothesis[12], that the median nerve might become more susceptible to pressure effects in the carpal tunnel when underlying diabetic neuropathy is present[9], suggests it. However, CTS without DPN is found in 14% of patients with diabetes worldwide. Additionally, the severity of DPN does not associate with the prevalence of CTS among patients with diabetes[5,6]. Therefore, the double crush hypothesis might not completely account for the mechanisms underlying CTS in patients with diabetes.

Metabolic factors should also be considered when investigating entrapment syndromes in patients with diabetes[2]. Interestingly, glycemic control[13] and aldose reductase inhibitor (ARI) treatment[14] result in the improvement of nerve conduction velocities across the carpal tunnel. This suggests that the mechanisms of CTS in patients with diabetes result from the metabolic factors related to hyperglycemia. The major metabolic factors involved in diabetic neuropathy include activation of the polyol pathway and a decrease in Na^+-K^+ adenosine triphosphate (ATP)ase activity[15]. Both impaired Na^+-K^+ pump function by inactivation of Na^+-K^+ ATPase activity, and increased intra-axonal sorbitol concentration would cause intra-axonal Na^+ accumulation, leading to axonal edema[16]. Accumulation of intra-axonal Na^+ would decrease the Na^+ gradient across the axolemma, resulting in reduced Na^+ currents when generating an action potential[17]. ARI treatment increases nodal Na^+ currents and improves the slowing of nerve conduction across the carpal tunnel[18]. ARI treatment could then decrease the pressure of the carpal tunnel; this would be consistent with a lessening of axonal edema[18]. Thus, axonal edema could have a significant impact at common sites of entrapment in patients with diabetes.

DPN results from a complex interaction between functional nerve impairment mediated by metabolic factors directly related to hyperglycemia and structural changes, such as axonal degeneration and demyelination, caused by microangiopathy[15,19,20]. In NCS, diabetic neuropathy is characterized by the coexistence of nerve conduction abnormalities at common sites of entrapment and dying-back degeneration[21]. In the present study, median SNAP was relatively normal, and the degree of axonal dysfunction was milder in patients with MN without DPN than in those with MN and DPN. A slowing of nerve conduction across the carpal tunnel in patients with diabetes without DPN could principally be as a result of an impairment in axonal function. When comparing patients with MN without DPN with those with MN and DPN, the latter are considered dying-back axonal polyneuropathy, whereas the former might partly constitute the pathophysiology of diabetic neuropathy at common sites of entrapment. We speculate that metabolic factors related to hyperglycemia lead to axonal edema, and could contribute to median nerve compression at common sites of entrapment in the early phase of diabetic neuropathy.

Assessment of nerve conduction abnormalities across the carpal tunnel is difficult in patients with DPN, because MN and DPN might affect median nerve conduction in a similar manner. Electrodiagnostic criteria for the diagnosis of MN in patients with an underlying DPN have not been established. Several electrodiagnostic techniques have been proposed to determine MN in patients with DPN[7]. Comparative median-radial sensory nerve studies appear to be the most sensitive electrodiagnostic tests in the detection of MN in diabetic patients[21]. As routine nerve conduction tests were carried out

in the current study, prolongation of the difference in sensory onset latency between the palm to wrist and digit 2 to palm was used in the evaluation of MN. Therefore, many patients were excluded from the analysis to avoid complications caused by the inclusion of other neuropathies. Furthermore, we assessed polyneuropathy as a reduction of SNAPs in the median, ulnar and sural nerves. Patients with more severe symptoms were considered to have polyneuropathy.

At the point of diagnosis, DPN is generally identified by structural changes that are irreversible. Therefore, early detection of neuropathy is important to prevent the progression of DPN. Further investigations using a large sample size and sensitive electrodiagnostic tests will be required to elucidate the mechanisms of functional nerve impairment in patients with diabetes.

In conclusion, abnormalities in nerve conduction across the carpal tunnel are found in the early phase of diabetes at a time when DPN has not developed yet. Asymptomatic MN in patients with diabetes without DPN might be caused by an impairment in axonal function mediated by metabolic factors at common entrapment sites. These findings could be used as guidelines to assist in the identification of early manifestations of diabetic neuropathy.

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原　著

臨床的にCharcot-Marie-Tooth病が疑われた304例のエクソーム解析による網羅的遺伝子診断*

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要旨　原因未同定のCharcot-Marie-Tooth病（CMT）症例304例を対象にエクソーム解析を行い大規模な遺伝子診断を行った。エクソーム解析を行った304例中83例で既知の原因遺伝子に病的変異を確認した。その内訳はCMTが54例、遺伝性運動性ニューロパチー（HMN）が6例、脊髄性筋萎縮症（SMA）が2例、家族性筋萎縮性側索硬化症（FALS）が6例、遠位型ミオパチーが2例、遺伝性痙性対麻痺（HSP）が4例、その他の遺伝性ニューロパチーが8例であった。エクソーム解析により、CMTのみならずCMTと鑑別を要する既知の様々な疾患の原因を特定することができた。同時にCMTとの鑑別を要する疾患は多岐にわたることが分かり、原因の特定の為には、次世代シークエンサーを用いた大規模な遺伝子解析が非常に有用であると思われた。

Key Words：Charcot-Marie-Tooth病，エクソーム解析，次世代シークエンサー

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はじめに

Charcot-Marie-Tooth病（CMT）は遺伝性運動感覚性ニューロパチー（hereditary motor sensory neuropathy：HMSN）とも表現され、遺伝性ニューロパチーの中でもっとも代表的な疾患である。臨床的には四肢遠位筋優位の進行性筋力低下や逆シャンペンボトル様下腿筋萎縮、凹足（pes cavus）、槌状足趾（hammer toe）などを特徴とする。遺伝性ニューロパチーには、CMT以外にも運動神経のみが障害される遺伝性運動性ニューロパチー（hereditary motor neuropathy：HMN）や感覚自律神経が障害される遺伝性感覚性自律神経性ニューロパチー（hereditary sensory and autonomic neuropathy：HSAN）、アミロイドの蓄積がみられる家族性アミロイドニューロパチー（familial amyloid neuropathy：FAP）、圧刺激により誘発される遺伝性圧脆弱性ニューロパチー（hereditary neuropathy with liability to pressure palsies：HNPP）、家族性にneuralgic amyotrophyをきたす家族性神経痛性筋萎縮症（hereditary neuralgic amyotrophy：HNA）など、その他にも様々な病型がある。これまでにCMTは、少なくとも50以上の原因遺伝子が報告されているが、HMNやHSANなどもあわせるとその原因遺伝子の数は65以上にのぼる。また、遺伝性痙性対麻痺（hereditary spastic paraparesis：HSP）や脊髄性筋萎縮症（spinal muscular atrophy：SMA）、遺伝性小脳失調症、家族性筋萎縮性側索硬化症（familial amyotrophic lateral sclerosis：FALS）、遠位型ミオパチー（distal myopathy）などでは、随伴症状としてニューロパチーを呈する病型が多数発見されており、その原因遺伝子も多数同定されている。その他にも、異染性

* Comprehensive mutation analysis of 304 patients with clinically suspected Charcot-Marie-Tooth disease using exome sequencing.

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白質ジストロフィーやKrabbe病、Allgrove症候群などの遺伝性代謝・内分泌異常症でもニューロパチーを随伴することが知られている。これらニューロパチーを呈する疾患をすべてあわせると原因遺伝子の数は優に100を超えており、網羅的に遺伝子解析を行うには従来のサンガー法による直接シークエンスでは労力や費用の面から事実上困難である。しかし、次世代ゲノムシークエンス（Next-Generation Sequencing：NGS）の到来によりそれが可能となった。特に、近年ではエクソーム解析が網羅的遺伝子変異解析や新規原因遺伝子同定の有効なツールとして実用化されている[1-3]。エクソーム解析とは、2万個以上存在すると言われるヒト遺伝子の全エクソン配列（エクソーム）のほぼすべてを解析する手法である。

当教室では、2007年から約5年間で全国から累計500例以上のCMTもしくはCMT疑い症例の遺伝子検査を依頼され、マイクロアレイDNAチップ（28種のCMT原因遺伝子を搭載）を用いた網羅的遺伝子検査を実施してきた[4]。しかしながら、原因を特定できた症例は陽性率が12.4%と低いことが問題となっていた。そこで、今回我々はマイクロアレイ法で病的変異が検出されなかった症例を「原因未同定」とし、その中で臨床的にCMTが強く疑われた症例を対象にエクソーム解析を行い、大規模な遺伝子診断を行った。

対象・方法

2007年4月から2012年4月まで、当院および全国の医療機関から、臨床症状や電気生理学的検査所見、末梢神経組織像などから、CMTと臨床診断された症例もしくはその疑い例、累計544例の遺伝子検査の依頼を受けた。なお、脱髄型CMT症例に関しては、事前にfluorescence *in situ* hybridization（FISH）法によりPMP22の重複・欠失がないことを確認した症例のみ検査の依頼を受け付けた。544例全例にマイクロアレイDNAチップを用い、28個の既知の遺伝性ニューロパチーの原因遺伝子（表1）の変異スクリーニングを行い、68例に病的変異が検出された。残りの原因未同定476例の中から、提供された臨床情報をもとに、臨床的にCMTが強く疑われた症例304例（CMT likely群）を選出・対象にし、エクソーム解析を行った（図1）。CMTの除外目的で検査を依頼された症例や炎症性ニューロパチーが強く疑われた症例、ニューロパチーが軽微で他の神経症候（痙性や固縮、錐体路徴候など）が主症状である症例など計172例（CMT unlikely群）は対象から除外した。

エクソーム解析は、HiSeq2000（Illumina, San Diego, California）でシークエンスを行い、二

表1 マイクロアレイDNAチップによる搭載した既知遺伝子リスト

分類	遺伝子名	疾患名
CMT	AARS	CMT2N
	DHH	46XY partial gonadal dysgenesis, with mini-fascicular neuropathy
	EGR2	CMT1D
	GAN	Giant axonal neuropathy
	GARS	CMT2D
	GDAP1	CMT2K, CMTRIA, CMT4A
	GJB1	CMTX1
	HSPB1	CMT2F
	HSPB8	CMT2L
	KARS	CMTRIB
	LITAF	CMT1C
	LMNA	CMT2B1
	MFN2	CMT2A2
	MPZ	CMT1B
	MTMR2	CMT4B1
	NDRG1	CMT4D
	NEFL	CMT1F, CMT2E
	PMP22	CMT1A, HNPP
	PRX	CMT4F
	RAB7A	CMT2B
	SBF2	CMT4B2
	SH3TC2	CMT4C
	YARS	CMTDIC
	DNM2	CMT2M, CMTDIB
Other hereditary neuropathies	SLC12A6	Agenesis of the corpus callosum with peripheral neuropathy
	APTX	Ataxia, early-onset, with oculomotor apraxia and hypoalbuminemia
	TDP1	SCAN1
	SETX	AOA2, ALS4

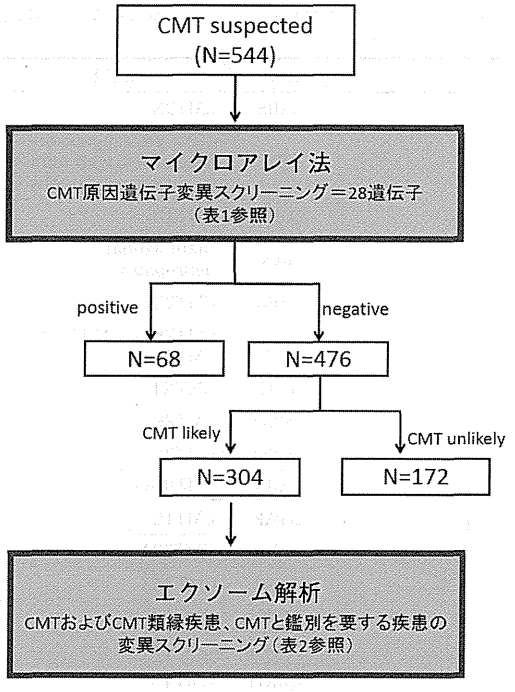

図1　遺伝性ニューロパチーの遺伝子診断ワークフロー
544例を対象にマイクロアレイDNAチップを用いた変異スクリーニングを行い、陰性例304例を対象にエクソーム解析を行った。

次解析ツールにはBWA、Samtoolsを利用した。スクリーニング対象疾患はCMTだけでなく、その近縁疾患であるHMNやHSAN、HSP、FALS、その他CMTと鑑別を要する遺伝性疾患にまで拡げた（表2）。検出された変異リストのなかで、正常多型やコントロール群にも存在する稀な一塩基多型（single nucleotide polymorphisms：SNP）、dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/index.html/) や1000 Genome Project（http://browser.1000genomes.org/）などの公共データベースに照会し、登録されている変異は排除した。登録のない新規変異または既知の病的変異については、Sanger法で変異の再確認を行った。また、病的意義の不明な新規変異に関しては、可能な限り家族メンバーの遺伝子検査を依頼し該当変異の有無を解析（分離解析segregation analysis）し、その病的意義を検証した。確認された変異が本当に疾患の責任変異であるのか、つまり病的意義の確実性については、変異の既報告の有無、表現型の類似性、分離解析の結果により、以下に示すDefinite、Probable、Possible、Less likelyの4つにグレード化した。

（1）確実例（definite）：既知の病的変異と表現型を有する症例。（2）ほぼ確実例（probable）：既知の病的変異であるが表現型が非典型的である症例、もしくは新規変異であるが、表現型が既知の報告に類似しており、分離解析にて該当変異がその家系内に矛盾なく伝わっていることが確認できた症例。（3）疑い例（possible）：新規変異であり、表現型が既知の報告に類似しているが、分離解析が未施行の症例。（4）可能性が低い例（less likely）：新規変異であり、表現型も非典型的で、分離解析も未施行の症例。

表2　エクソーム解析のスクリーニング対象遺伝子リスト

対象疾患	遺伝子数	スクリーニング対象遺伝子
CMT	43	AARS, DHH, DHTKD1, DNM2, DYNC1H1,EGR2, FGD4, FIG4, GAN, GARS, GDAP1, GJB1, GNB4, HARS, HINT1, HK1, HSPB1, HSPB8, INF2, KARS, KIF1A, KIF1B, LITAF, LMNA, LRSAM1, MARS, MED25, MFN2, MPZ, MTMR2, NDRG1, NEFL, PDK3, PMP22, PRPS1, PRX, RAB7A, SBF1, SBF2, SH3TC2, TRIM2, TRPV4, YARS
HMN	6	HSPB3, DCTN1, FBLN5, IGHMBP2, BSCL2, SLC5A7
HSN/HSAN	13	ATL1, CCT5, DNMT1, DST, FAM134B, FLVCR1, IKBKAP, NGF, NTRK1, SCN9A, SPTLC1, SPTLC2, WNK1
Familial ALS	15	ANG, C9orf72, CHMP2B, DAO, FUS, OPTN, PFN1, SIGMAR1, SOD1, TARDBP, UBQLN2, VCP, SETX, VAPB, ALS2
SMA	7	ASAH1, ATP7A, DNAJB2, NAIP, PLEKHG5, SMN1, UBA1
distal myopathies	18	CRYAB, FHL1, FLNC, GNE, HNRNPA2B1, KLHL9, LDB3, MATR3, MYH14, NEB, TIA1, VCP, ANO5, CCDC78, DES, DYSF, MYOT, TTN
HSP	38	ATL1, C12orf65, DDHD1, DDHD2, SACS, SLC16A2, SLC33A1, SPAST, SPG11, SPG14, SPG20, SPG5B, SPG7, SPG9・・・・など
Other hereditary neuropathies	21	ARHGEF10, CTDP1, GJB3, HOXD10, SEPT9, SLC12A6, SOX10, TFG, TTR, AAAS , ABCD1, APTX, CYP27A1, GALC, HSP60, IFRD1, POLG, RNF170, RPIA, SIL1, TDP1

ゲノムDNAはGentra Puregene Blood Kit (Qiagen, Tokyo, Japan) を用いて患者の末梢血から抽出した。患者の臨床情報は、当院の症例については当院の神経内科医が診察、検査を行うことで得た。他の医療機関もしくは研究機関の症例については、主に神経内科医もしくは小児科医が診察を行い、臨床経過や神経学的所見、血液検査や神経伝導検査、神経画像検査をおこなった。本研究は鹿児島大学の倫理委員会で審査を行い承認された。また、すべての患者かつ家族には本研究に参加するためにインフォームドコンセントを行い書面にて同意を得た。

結　果

エクソーム解析の対象となったCMT likely群304例の解析前診断の内訳は、CMT1が77例、CMT2が191例、CMT4が13例、CMTXが11例、HSANが 8 例、HMSN with pyramidal features (HMSN-5) が 2 例、そしてCongenital hypomyelinating neuropathy (CHN) が 2 例であった。

エクソーム解析を行った304例中130例に既知の原因遺伝子になんらかの変異を確認した(図 2)。変異のグレード別の内訳は、Definite：40例、Probable：27例、Possible：16例、Less likely：47例であり、その中で病的意義を有する例もしくはその可能性が高い例 (Definite、Probable、Possible) を合わせると83例であった。この83例の内訳はCMTが54例、HMNが 6 例、SMAが 2 例、家族性ALSが 6 例、遠位型ミオパチーが 2 例、HSPが 4 例、その他の遺伝性ニューロパチーが 8 例であった (図 2)。さらにCMT54例の内訳は、CMT1A (PMP22) が 4 例、CMT1B (MPZ) が 6 例、CMT1D/CMT2E (EGR2) が 1 例、CMT1F (NEFL) が 2 例、CMT2A2 (MFN2) が19例、CMT2C (TRPV4) が 1 例、CMT2D (GARS) が 2 例、CMT2F (HSPB1) が 6 例、CMT2M (DNM2) が 1 例、CMT2N (AARS) が2例、CMT4A/CMT2K (GDAP1) が 2 例、CMT4C (SH3TC2) が 1 例、CMT4H (FGD4) が 1 例、CMT-DIC (YARS) が 1 例、CMT-DIF (GNB4) が 1 例、CMTX1 (GJB1) が 2 例、Giant axonal neuropathy (GAN) が 2 例であった。また、CMT以外の遺伝子変異は29例も見つかり、HMN5Bの原因であるBSCL2変異、HMN7Bの原因であるDCTN1変異、FAPの原因であるTTR変異、ALS1の原因であるSOD1変異、遠位型ミオパチーの原因であるMPD2変異、Charlevoix-Saguenay型痙性失調症 (ARSACS) の原因であるSACS変異、SPG3A の原因であるATL1変異、沖縄型神経原性筋萎縮症の原因であるTFG変異、Allgrove症候群の原因であるAAAS変異、Krabbe病の原因であるGALC変異などの様々な疾患の遺伝子変異を認めた。これら29例のうち、遺伝子診断名を解析前に鑑別として挙げていたのは 9 例のみであった。

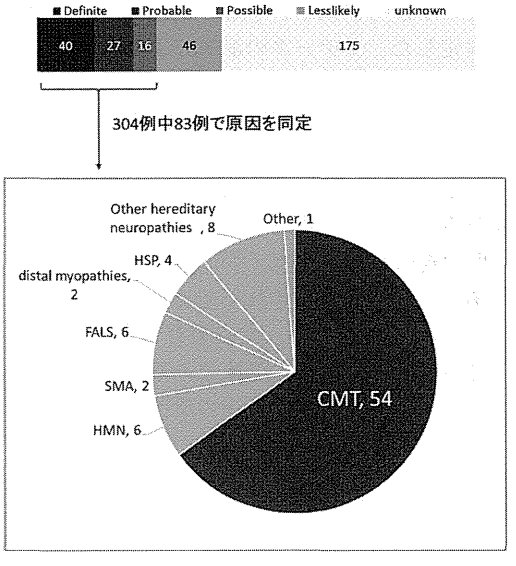

図 2 304例のエクソーム解析で検出された変異の内訳
304例中83例で原因を同定し、その内訳はCMTが54例、その他が29例であった。

考　察

当教室では累計544例のCMTもしくはCMT疑い症例を対象にマイクロアレイDNAチップを用いて網羅的遺伝子解析を実施してきたが、原因を同定できた症例は68例で同定率が12.4%と低いことが問題となっていた。今回、原因未同定例を対象にエクソーム解析を行い83例で原因を同定することができ、マイクロアレイ法の同定率を合わせると、全体の同定率は27.6%(544例中151例) であり (図 3)、脱髄型CMT

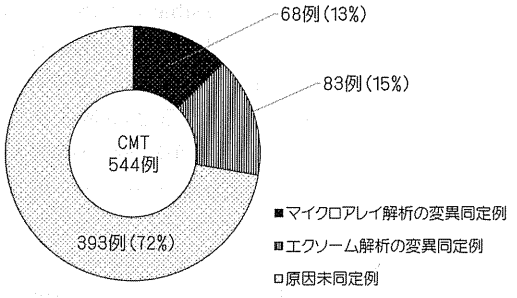

図3 マイクロアレイ解析およびエクソーム解析による変異同定率
マイクロアレイ解析およびエクソーム解析にてそれぞれ68例、83例に病的変異を同定し、全体の同定率は28%（544例中151例）であった。

と軸索型CMTの同定率はほぼ同等であった。また、病的変異の同定率を過去の報告と比較してみると、Saportaら[5]およびMurphyら[6]による網羅的なCMT遺伝子解析では、原因同定率がそれぞれ67%（787例中527例）、44.3%（1607例中712例）であるが、PMP22重複・欠失によるCMT1AおよびHNPPを除外した症例を対象に同定率を計算し直すと、それぞれ42%、18.5%であり、同定率は本研究と同等であった。また、83例中55例はマイクロアレイDNAチップのスクリーニング対象遺伝子の変異であったことも判明し，エクソーム解析は網羅性だけでなく検出感度の面からも有用であると思われた。

エクソーム解析を用いた遺伝子変異スクリーニングにはメリットとデメリットがある。一番のメリットはメンデル遺伝性疾患の原因の約85%が存在するといわれているエクソン領域を網羅的かつ迅速に解析できるため、スクリーニングという点ではきわめて有効な手法となる。特に、CMTに限らず、先天性難聴、網膜色素変性症、遺伝性心筋症のように遺伝的に多様な疾患のスクリーニングには、効率やコストの面から非常に有効な手段となる。また、多検体を同時に解析できる点や臨床的に鑑別に挙げられなかった疾患を偶然発見できることもメリットの一つである。実際、今回の研究で原因の同定に至った症例のなかで、FAPや遠位型ミオパチー、Krabbe病、Allgrove症候群などのいくつかの症例は検査依頼時には臨床的には鑑別に挙げられなかった症例であった。一方、エクソーム解析にはデメリットもある。全エクソンの約10%の塩基は正確に解読できないと言われており、GC含有率が高い部位は解読できないこともある。また、検出された変異のなかには疑陽性と偽陰性の両方存在するため、エクソーム解析はあくまでスクリーニングとして利用し、最終的にはサンガー法でその変異を確認する作業が必要である。また、エクソーム解析では予想しない疾患の遺伝子変異が見つかる可能性もある。また特に若年者を対象にしたエクソーム解析では発症前診断をしてしまうリスクも生じる。

エクソーム解析はヒト遺伝子の全エクソン配列のほぼすべてを解析することができる一方、検出される変異の数も膨大になる。エクソーム解析で1症例あたりに検出される変異数は非同義変異（アミノ酸置換を引き起こす塩基置換）だけでも10,000～12,000個にのぼるため、その中から疾患の原因となる病的変異を同定することは決して安易ではない。また、疾患の既知の原因遺伝子に変異が確認されても、すぐさまその変異が責任変異であると決めつけてはいけない。その部位や種類によってはタンパク質レベルでその機能に影響を及ぼさないものも多く存在するからである。我々はこれらの膨大な数の変異の中から、以下の手順で疾患の遺伝子変異を同定した。①データの品質によるフィルタリング（クオリティ値やread depthの低い変異は除外）、②同義変異（アミノ酸置換が起こらない変異）や正常多型、公共データベースに登録されている稀なSNPの除外、③スクリーニング対象遺伝子（表2）の変異の抽出、④病的変異の既報告の有無の確認、⑤segregation analysisである。特に、確認された変異が病的変異かどうかの判断には、④、⑤の過程が重要である。確認された変異が過去に疾患の原因として報告されていれば、それが疾患の原因であることに疑いようはないが、新規変異の場合はその病的意義については慎重に判断すべきである。例えば、CMT2Cの原因遺伝子TRPV4にヘテロ接合性ミスセンス変異（c.2344C>T, p.R782C）が確認された症例で、この変異は過去に報告のない新規変異であったが、segregation analysisを実施したところ、健常である母親にも同変異が

確認されたことから、病的意義を証明することができず、稀なSNPであると判断した。このような稀なSNPが疾患の原因と誤って判断されないように注意しなければならない。

以上、本研究では大規模なCMT症例を対象にしたアレイ解析およびエクソーム解析による網羅的遺伝子診断について概説した。近年、NGSによるゲノム解読技術はますます向上しており、一般的な研究室においても安価で迅速に遺伝子診断をできるようになってきている。近い将来、エクソーム解析が疾患遺伝子変異スクリーニングのスタンダートなると思われる。個々の症例の遺伝子診断は、遺伝性疾患解明の第一歩であり、今回我々が行ったエクソーム解析をもちいた網羅的遺伝子診断の結果は、今後の遺伝子診断の方向性や治療対策などを検討する基礎データになるであろう。一方で、遺伝的原因を同定できていない症例もかなり多く、現在、エクソーム解析で原因未同定の症例を収集しCMTの新規の原因遺伝子の探索にも取り組んでいる。多くの遺伝的原因が解明されれば、CMT の分子メカニズムの全体像が明らかとなり、そのことは末梢神経疾患のみならず、神経変性疾患の全体の理解を深めるものと思われる。

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Comprehensive mutation analysis of 304 patients with clinically suspected Charcot-Marie-Tooth disease using exome sequencing.

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Charcot-Marie-Tooth (CMT) disease comprises a group of clinically and genetically heterogeneous inherited peripheral neuropathies. Genetic studies have revealed at least 50 disease-causing genes in patients with CMT. Concomitant with the increase in the number of genes, the labor and reagent costs for molecular genetic testing have significantly increased. We performed whole exome sequencing in 304 patients with suspected CMT that was refractory to molecular diagnosis, using a custom microarray containing DNA of 28 known CMT disease-related genes. We found apparent causative mutations in 83/304 (27.3%) patients. These pathogenic mutations affected not only CMT-related genes but also the causative genes of neuromuscular diseases mimicking CMT including those in 54 patients with CMT-related genes, 6 patients with hereditary motor neuropathy related genes, 2 patients with spinal muscular atrophy-related genes, 6 patients with amyotrophic lateral sclerosis-related genes, 2 patients with distal myopathy-related genes, 4 patients with hereditary spastic paraparesis-related genes, and 8 patients with other hereditary neuropathy-related genes. Therefore, this study demonstrates that exome sequencing is an accurate and efficient molecular diagnostic tool for patients with inherited peripheral neuropathy.

Key Words: Charcot-Marie-Tooth disease, exome analysis, next-generation sequencing

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RESEARCH REPORT

Neurofilament light mutation causes hereditary motor and sensory neuropathy with pyramidal signs

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Abstract To identify novel mutations causing hereditary motor and sensory neuropathy (HMSN) with pyramidal signs, a variant of Charcot-Marie-Tooth disease (CMT), we screened 28 CMT and related genes in four members of an affected Japanese family. Clinical features included weakness of distal lower limb muscles, foot deformity, and mild sensory loss, then late onset of progressive spasticity. Electrophysiological studies revealed widespread neuropathy. Electron microscopic analysis showed abnormal mitochondria and mitochondrial accumulation in the neurons and Schwann cells. Brain magnetic resonance imaging (MRI) revealed an abnormally thin corpus callosum. In all four, microarrays detected a novel heterozygous missense mutation c.1166A>G (p.Y389C) in the gene encoding the light-chain neurofilament protein (NEFL), indicating that *NEFL* mutations can result in a HMSN with pyramidal signs phenotype.

Key words: Charcot-Marie-Tooth disease, gene chip array, hereditary motor and sensory neuropathy with pyramidal signs, light-chain neurofilament protein (NEFL), mitochondrial accumulation

Introduction

Hereditary motor and sensory neuropathy (HMSN) with pyramidal signs includes a genetically and clinically heterogeneous group of neuropathies affecting motor and sensory nerves and the spinal cord. The following three subtypes are identified according to the hereditary pattern: autosomal dominant, autosomal recessive, and X-linked (*Borhoumi et al., 2001; Goto et al., 2003*).

Despite clinical heterogeneity, mutations in only one gene, *mitofusin 2* (*MFN2*), have been linked to HMSN with pyramidal signs *(Zhu et al., 2005)*. In contrast, more than 30 types of hereditary spastic paraplegia (HSP) are caused by mutations in separate genes *(Patel et al., 2002; Irobi et al., 2004; Klebe et al., 2006; Rainier et al., 2008)*. Moreover, these etiologically distinct HMSNs have many overlapping features. Some patients with distal hereditary motor neuropathy 5 (HMN5), resulting from a *BSCL2* mutation, were expressed as a different phenotype with pyramidal signs and slight sensory loss *(Windpassinger et al., 2004; Luigetti et al., 2010)*.

Light-chain neurofilament protein (NEFL) gene encodes the light chain neurofilament protein. Mutations in *NEFL* are associated with demyelinating Charcot-Marie-Tooth disease (CMT) (CMT type 1F), axonal CMT (CMT type 2E), and unspecified CMT *(Mersiyanova et al., 2000; De Jonghe et al., 2001; Georgiou et al., 2002; Yoshihara et al., 2002; Jordanova et al., 2003; Choi et al., 2004; Leung et al., 2006; Miltenberger-Miltenyi et al., 2007; Yum et al., 2009)*. Here we report five generations of a Japanese family with autosomal dominant

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HMSN with pyramidal signs caused by a novel *NEFL* mutation.

Materials and Methods

We investigated five generations of a Japanese family including 10 individuals with suspected HMSN with pyramidal signs. To confirm the diagnosis and elucidate the underlying genetic cause, four affected members of this family were examined (Fig. 1). The study protocol was reviewed and approved by the Institutional Review Board of Kagoshima University. All family members provided written informed consent.

Standard nerve conduction studies were performed in patient 1 at the age of 57 years and in patient 2 at the age of 65 years. Skin temperature was maintained above 30°C.

A left sural nerve biopsy obtained from patient 2 at the age of 65 years was analyzed for morphometric changes using light and electron microscopes.

Genomic DNA was extracted from the peripheral blood. The custom MyGeneChip® CustomSeq® Resequencing Array (Affymetrix, Inc., Santa Clara, CA, USA) was designed to screen CMT and related diseases such as ataxia with oculomotor apraxia types 1 and 2, spinocerebellar ataxia with axonal neuropathy, and distal hereditary motor neuropathy. We designed 363 primer sets to include the entire coding regions and flanking sequences of the following 28 disease-causing genes: *early growth response 2 (EGR2), peripheral myelin protein 22 (PMP22), myelin protein zero (MPZ), gap junction protein beta 1 (GJB1), periaxin (PRX), lipopolysaccharide-induced TNF-α factor (LITAF), neurofilament light chain polypeptide (NEFL), ganglioside-induced differentiation-associated protein 1 (GDAP1), myotubularin-related protein 2 (MTMR2), SH3 domain and tetratricopeptide repeats 2 (SH3TC2), SET-binding factor 2 (SBF2), N-myc downstream regulated 1 (NDRG1), mitofusin 2 (MFN2), Ras-related GTPase 7 (RAB7), glycyl-tRNA synthetase (GARS), heat shock protein 1 (HSPB1), HSPB8, lamin A/C (LMNA), dynamin 2 (DNM2), tyrosyl-ARS (YARS), alanyl-ARS (AARS), lysyl-ARS (KARS), aprataxin (APTX), senataxin (SETX), tyrosyl-DNA phosphodiesterase 1 (TDP1), desert hedgehog (DHH), gigaxonin 1 (GAN1),* and *K-Cl cotransporter family 3 (KCC3)*. In addition, primer sets were designed to include the entire coding regions and flanking sequences of following nine candidate genes: *ankyrin 3 (ANK3), contactin 1 (CNTN1), CNTN2, cysteinyl-ARS (CARS), glutamyl-prolyl-ARS (EPRS), hystidyl-ARS (HARS), methionyl-ARS (MARS), seryl-ARS (SARS),* and *sodium channel, voltage gated, type VIII, alpha subunit (SCN8A)*. The details of gene chip analysis have been previously described *(Zhao et al., 2012)*; therefore direct sequencing was performed to confirm the mutations revealed by gene chip analysis.

Results

Patient 1

Patient 1 (IV-6, Fig. 1), a 61-year-old male, developed gradually progressive gait disturbance beginning at age 50. He had a spastic and ataxic gait with mild distal atrophy and diffuse weakness (4/5) in the lower limbs. He had lower limb spasticity, brisk patellar tendon reflexes, and positive Babinski signs. Bilateral *pes cavus* was noted. Light touch and proprioception were decreased in all limbs, whereas vibration was markedly decreased at the ankles. The ankle and upper limb deep tendon reflexes were absent. There was no evidence of extrapyramidal involvement. CMT neuropathy score was 12 *(Murphy et al., 2011)*.

Patients 2 and 3

Patients 2 (IV-3, Fig. 1) and 3 (IV-2, Fig. 1) were 66- and 67-year-old brothers of patient 1. They both experienced gait dysfunction and weakness in the lower limbs that started in their mid-50s. Examination revealed *pes cavus*, mild weakness in the lower limbs, absent Achilles tendon reflexes, brisk patellar tendon reflexes, and positive Babinski signs. Vibration was moderately decreased at the ankles. CMT neuropathy score of patient 2 was 9, but patient 3 could not be calculated because of lack of nerve conduction studies.

Patient 4

Patient 4 (III-4, Fig. 1) was the 93-year-old bedridden mother of patients 1, 2, and 3. She suffered from contracture, *pes cavus*, atrophy in the lower limbs, and severe spasticity. She had brisk patellar tendon reflexes and positive Babinski signs but absent Achilles tendon reflexes. CMT neuropathy score could not be calculated.

The thickness of genu (G), middle trunk (MT), and splenium (S) of corpus callosum was measured in the sagittal magnetic resonance imaging (MRI) of patient 1 (T2-weighted) and patient 2 (T1-weighted). In patients 1 and 2, the thickness of G (normal range: 12.6 ± 1.5 mm), MT (normal range: 7.0 ± 0.8 mm), and S (normal range: 12.6 ± 1.6 mm) were 7.1 and 7.9 mm, 3.6 and 3.5, 7.5 and 7.7 mm, respectively *(Okamoto et al., 1990)*. These results revealed thinning of the corpus callosum in both patients (Fig. 2A and 2B). The MRI of the cervical and thoracic spinal cord was normal.

In patient 1, motor nerve conduction velocities (MCVs) of tibial (normal: >41.7 m/s) and peroneal (normal: >41.8 m/s) nerves were 33 and 36 m/s, respectively. Compound muscle action potential (CMAP) of

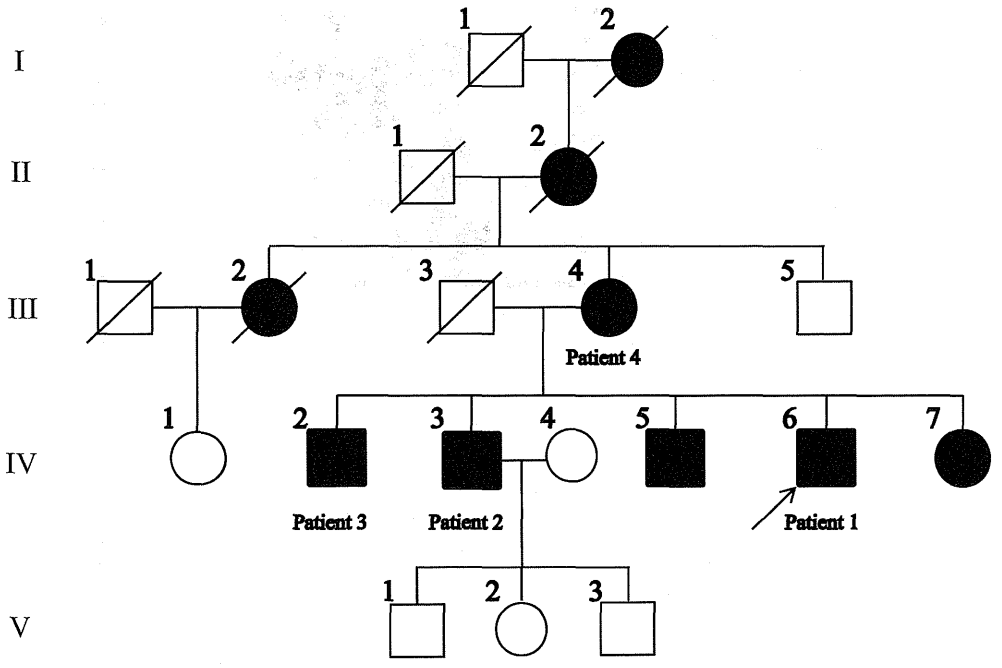

Figure 1. Pedigree of the hereditary motor and sensory neuropathy-V (HMSN-V) family. The arrow indicates the proband. The affected individuals are represented by solid black symbols, and healthy individuals by open symbols.

the tibial nerve (normal: >4.4 mV) was 4.1 mV. The F-wave latencies of the median (normal: <28.2 ms), ulnar (normal: <29.7 ms), and tibial (normal: <51.7 ms) nerves were 33, 30, and 56 ms, respectively. Sensory nerve action potentials (SNAPs) from the median, ulnar, and sural nerves were undetectable. Similarly, in patient 2, MCVs of the tibial and peroneal nerves were 39 and 32 m/s, respectively. CMAPs of the ulnar (normal: > 6.0 mV), tibial, and peroneal (normal: > 2.2 mV) nerves were 3.8, 2.0, and 0.2 mV, respectively. SNAPs from the median, ulnar, and sural nerves were undetectable. The F-wave latency of the tibial nerve was 53 ms. Nerve conduction studies on patients 1 and 2 suggested an axonal type of motor and sensory neuropathy (Table 1). Needle electromyogram was not performed in all patients.

A sural nerve biopsy from patient 2 exhibited slight decrease in large-diameter myelinated fiber densities in all fascicles. Although giant axons and onion bulb formation were not detected, fibers with relatively thin myelin were frequently observed together with occasional small fiber clusters (Fig. 2C). The proportion of myelinated fibers with diameters >6.0 μm was 18%, and the myelinated fiber density was 7,686/mm^2 (>8,000/mm^2). The histogram of myelinated fibers shows unimodal distribution (Fig. 2D). Electron microscopic analysis showed abnormal mitochondria and mitochondrial accumulation in about 10% of neurons and Schwann cells (Fig. 2E and 2F). The accumulation of intermediate filament was not observed.

Using the custom gene chip, we identified a missense heterozygous mutation, c.1166A>G (designated p.Y389C), in exon 2 of *NEFL* in patient 2. Subsequently, the other three affected family members showed the same mutation (Fig. 2G). In healthy family members, no gene testing was done. A sequence homology search was performed to align protein sequences from multiple species using the HomoloGene (http://ncbi.nlm.nih.gov/homologene) system. Tyrosine 389 was conserved among all species analyzed (Fig. 2H). This mutation was neither found in 453 controls with inherited neuropathy nor in the 1,000 Genomes websites listing human genetic variations in 2,500 samples (including 500 East Asian samples). Furthermore, we could computationally predict the effect of the p.Y389C mutation on protein function using the MUpro (http://mupro.proteomics.ics.uci.edu) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) algorithms. The MUpro score of −0.737 is indicative of a decrease in protein stability (scores <0 indicate a decrease in protein stability) and a PolyPhen-2 score of 1.00 indicates a significant probability of pathogenesis. Using a sequence homology search, we showed that p.Y389 is a completely conserved amino acid residue, suggesting that it may have a potential functional impact on NF-L. In order to exclude diagnosis of HMN5 with pyramidal sign and sensory loss by the *BSCL2* gene mutation, we analyzed that gene by Sanger method resequencing, we did not find any mutation.

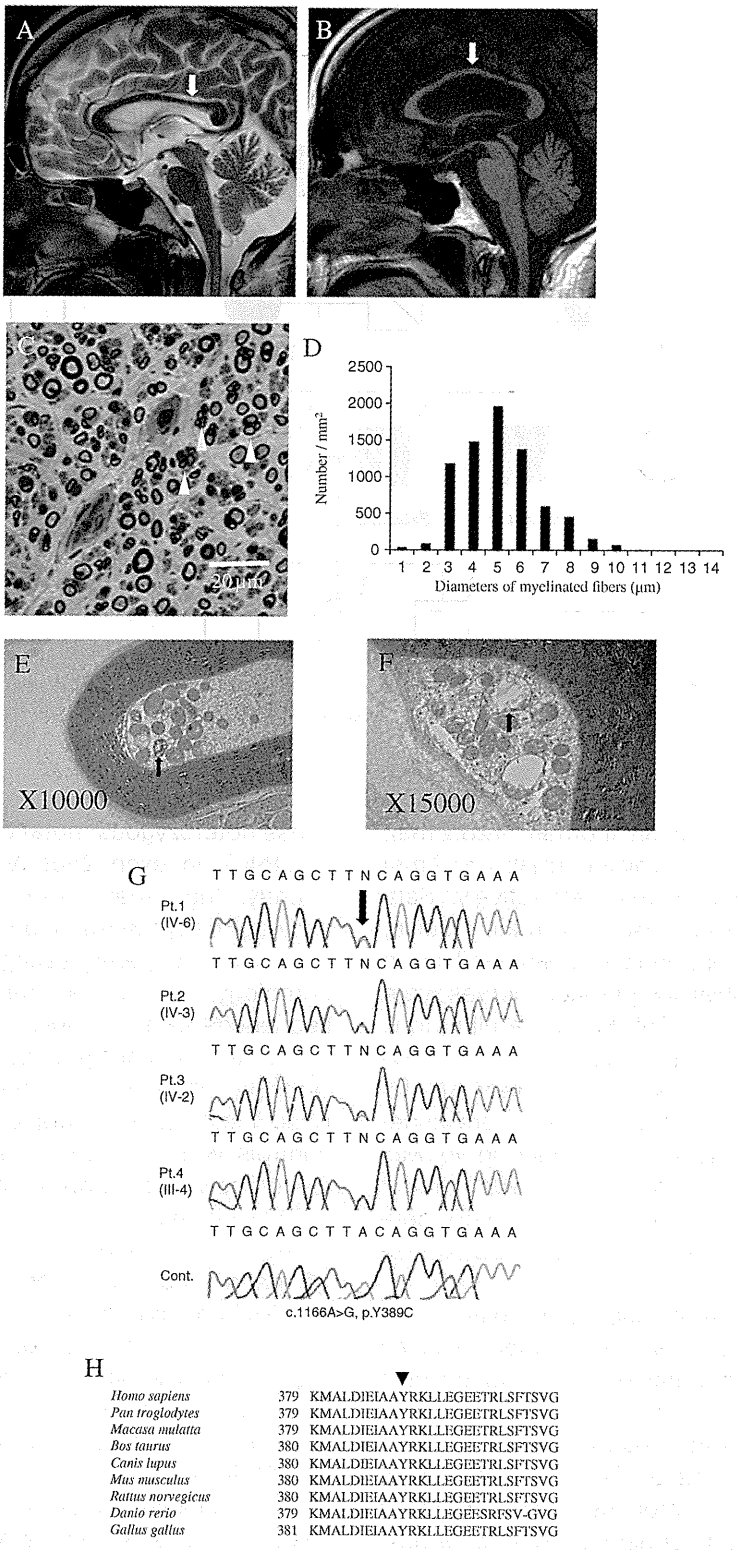

Figure 2. Radiological, pathological and genetic findings of the hereditary motor and sensory neuropathy-V (HMSN-V) family. (A) Brain magnetic resonance imaging (MRI) of patient 1 and (B) patient 2 showing a thin corpus callosum (arrows). (C) Toluidine blue and safranin staining of left sural nerve from patient 2 shows occasional small fiber clusters (arrowheads). (D) Histogram of myelinated fibers. (E, F) Electron microscope images show mitochondrial accumulation and abnormal mitochondrial structure (arrow) in neuron (E) and Schwann cell (F). (G) Chromatogram of the heterozygous c.1166A>G (Y389C) mutation in exon 2 of NEFL: upper, four affected members; bottom, control. (H) Comparison of aligned NEFL amino acid sequences between species. Arrow head (▼) indicates the mutated amino acid.

Table 1. Nerve conduction studies in patients 1 and 2.

	Median				Ulnar				Tibial		Peroneal		Sural	
Nerve	MCV (>49.6)	CMAP (>3.1)	SCV (>47.2)	SNAP (>7.0)	MCV (>50.1)	CMAP (>6.0)	SCV (>46.9)	SNAP (>6.9)	MCV (>41.7)	CMAP (>4.4)	MCV (>41.8)	CMAP (>6.7)	SCV (>40.8)	SNAP (>5.0)
Pt. 1	48.7	9.2	N.R.	N.R.	50	8.8	N.R.	N.R.	33.3	4.1	36.4	2.9	N.R.	N.R.
Pt. 2	48.2	7.5	41.6	7.4	52.3	3.8	N.R.	N.R.	38.8	2	32.1	0.2	N.R.	N.R.

CMAP, compound muscle action potential; MCV, motor conduction velocity; N.R., not recordable; Pt, patient; SCV, sensory conduction velocity; SNAP, sensory nerve action potential.

Discussion

All four patients showed a gradually progressing spastic gait, which along with findings from physical examinations and nerve conductions, confirmed the involvement of upper motor neurons and sensory and motor peripheral nerves. MRI of patients 1 and 2 revealed unremarkable changes other than a markedly thin corpus callosum, a structural abnormality not previously reported in patients with NEFL mutations, but common in some forms of SPG. To the best of our knowledge, no pyramidal tract disorder has been noted in any patients with NEFL mutations.

NEFL encodes the neurofilament light polypeptide (NF-L), the smallest of the three neurofilament isoforms, and a cytoskeletal protein almost universally expressed in the central nervous system (CNS), and peripheral nervous system (PNS). At the subcellular level, neurofilaments are observed in the somata, axons, and dendrites, where they control developmental and plastic changes in the neuronal morphology.

NF-L is divided into the following three domains: head, rod, and tail. Several mutations that cause CMT type 1F or 2E are distributed in the head or rod domain, whereas the p.Y389C mutation is located in the Coil2B section of the rod domain. The following three additional mutations in the Coil2B domain were reported in patients with CMT: p.Q333P, p.L334P, and p.E397K *(Mersiyanova et al., 2000; Choi et al., 2004)*. Including these, all NEFL mutations, autosomal dominant p.P8R, p.P8Q, p.P8L, p.T21fs, p.P22T, p.P22S, p.E89K, p.L93P, p.N97S, p.A148V, p.Q333P, p.L334P, E397K, and autosomal recessive p.E210X, do not cause pyramidal signs *(Mersiyanova et al., 2000; De Jonghe et al., 2001; Georgiou et al., 2002; Yoshihara et al., 2002; Jordanova et al., 2003; Choi et al., 2004; Leung et al., 2006; Miltenberger-Miltenyi et al., 2007; Yum et al., 2009)*. However, some patients with autosomal dominant p.L93P mutation had cerebellar ataxia and schizophrenia *(Miltenberger-Miltenyi et al., 2007)*, and a patient with autosomal dominant p.N97S mutation had learning disorder and nystagmus *(Jordanova et al., 2003)*. Furthermore some patients with autosomal recessive p.E210X mutation had learning disorder and prolonged visual-evoked responses *(Yum et al., 2009)*. These findings suggest the potential to cause CNS disorders by *NEFL* mutation.

Another NF-L mutation altered the intracellular distribution of mitochondria in cultured neurons *(Perez-Olle et al., 2004)*. Mutations in *MFN2*, a gene associated with both CMT and HMSN with pyramidal signs, may cause axonal damage by mitochondrial fusion disorder. Similarly, the abnormal mitochondria and mitochondrial accumulation in the neurons and Schwann cells in our patient, NEFL mutation may also result in axonal damage by altered mitochondrial distribution both in PNS and CNS because of the broad pattern of expression.

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INTERNAL MEDICINE

□ CASE REPORT □

A Family with Distal Hereditary Motor Neuropathy and a K141Q Mutation of Small Heat Shock Protein *HSPB1*

Kengo Maeda[1], Ryo Idehara[1], Akihiro Hashiguchi[2] and Hiroshi Takashima[2]

Abstract

We herein describe a Japanese family with distal hereditary motor neuropathy carrying a K141Q mutation of small heat shock protein *HSPB1*. Two patients among them had late onset disease (older than 50 years). The muscles of the distal legs were weak and atrophic. Sensory and autonomic dysfunction were not seen. Even eight years after onset, one patient could still walk without support. A nerve conduction study revealed axonal degeneration of the motor nerves of the legs. A heterozygous K141Q mutation was detected in the affected patients. The late onset and mild clinical phenotype might reflect the mild biochemical alteration of HSP27 induced by the K141Q mutation.

Key words: Charcot-Marie-Tooth disease, distal hereditary motor neuropathy, heat shock protein

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Introduction

Mutations of several genes are known to cause distal hereditary motor neuropathy (dHMN). There is often an overlap with Charcot-Marie-Tooth disease (CMT2) and the juvenile form of amyotrophic lateral sclerosis. Heat shock protein (HSP) 27 is one of the causative proteins resulting in dHMN or CMT2F. Its clinical phenotypes differ based on the *HSPB1* mutations (1). In Japan, only three families with dHMN and *HSPB1* mutations have been reported so far (2-4). We herein report another family with dHMN with a mutation in this gene.

Case Reports

Case 1 (the proband)

A 69-year-old man presented with gait disturbance which had appeared one year earlier. He did not have numbness in his feet. Before the onset, he had been exercising at a gym near his house. He had not been exposed to any toxic organic solvent or heavy metals. There were some individuals showing similar symptoms in his family.

During the initial examination, the patient was alert, and there were no abnormal finding for his cranial nerves. Muscular atrophy was obvious in his legs, and pes cavus was observed (Fig. 1A). The muscle strength was decreased to 4/4 in the anterior tibial muscles. The strength of the other muscles was normal. Sensations of light touch, pain, temperature, vibration, and position were normal. The tendon reflexes were symmetrical and decreased in all four limbs. No pathological reflex was evoked. The patient's coordination was normal. He could not stand on his heels. He did not have any autonomic symptoms, such as orthostatic hypotension or urinary incontinence. The complete blood cell count was normal. The erythrocyte sedimentation ratio was 13/33 mm (one hour/two hours). The parameters of liver function and renal function were within the normal limits. The serum level of creatine kinase was mildly elevated, to 488 IU/L. The levels of fasting blood glucose, electrolytes, and lipids were normal. Anti-nuclear antibody, proteinase 3-anti-neutrophil cytoplasmic antibody (PR3-ANCA), and myeloperoxidase (MPO)-ANCA, and the levels of vitamin B 1, B12 and folic acid were normal.

The cell count of the cerebrospinal fluid (CSF) was 2/3 mm^3. The levels of protein and glucose in the CSF were 46 mg/dL and 64 mg/dL, respectively. A nerve conduction

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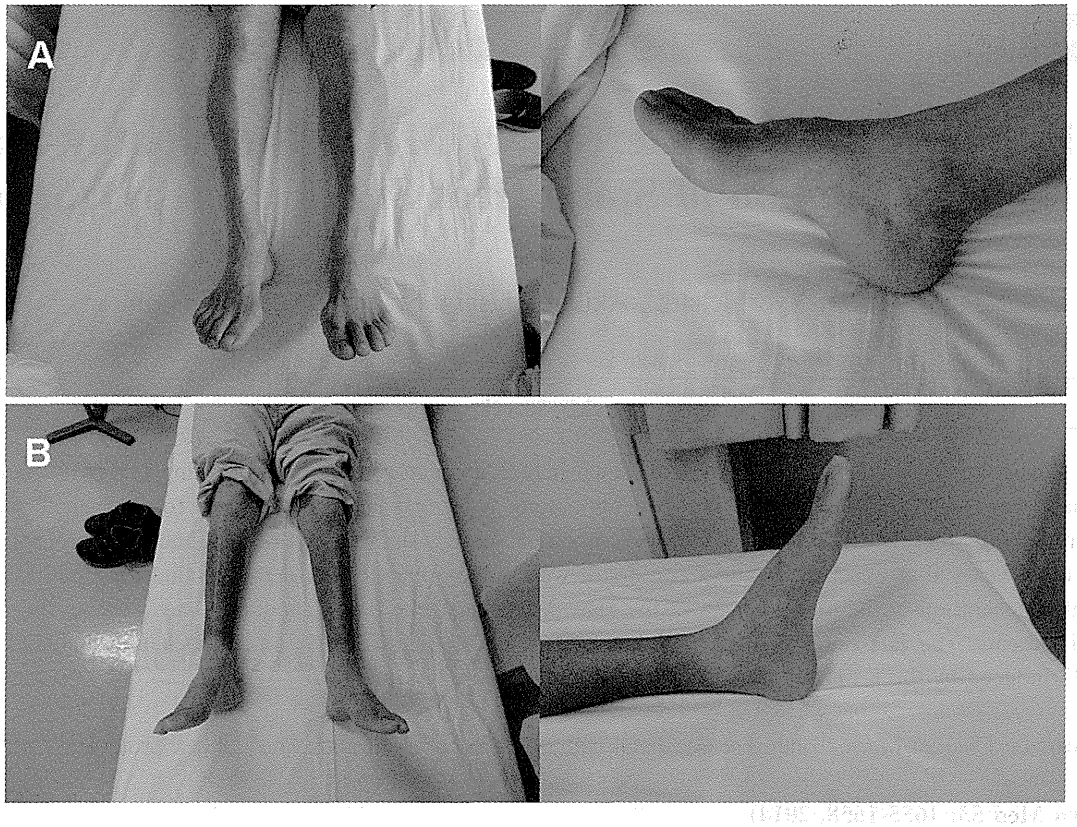

Figure 1. Photographs of the patients' legs (left) and feet (right). A: Case 1, B: Case 2

Table. The Results of the Nerve Conduction Study of Case 1

Nerve	Motor						Sensory					
	DL (ms)		CV (m/s)		CMAP(mV)		DL (ms)		CV (m/s)		SNAP (μV)	
	R	L	R	L	R	L	R	L	R	L	R	L
Median	5.5	4.8	53	52	7.3	6.7	3.3	2.9	60	62	24	33
Ulnar	2.7	2.5	61	61	4.7	7.3	2.3	2.2	66	78	25	26
Tibial	6.9	6.4	40	34	0.4	1.1						
Sural							3.3	3.0	42	47	4.2	14

DL: distal latency, CV: conduction velocity, CMAP: compound muscle action potential, SNAP: sensory nerve action potential, R: right, L: left

study (Table) revealed a decreased amplitude of the compound muscle action potentials in the tibial nerves. The amplitudes of the other motor nerves were relatively spared. The amplitude of the sensory nerve action potential was not decreased, except for the right sural nerve. The conduction velocities were not decreased in either the motor or sensory nerves. He refused to undergo a sural nerve biopsy.

Case 2 (the son of the proband's cousin)

The second patient was sixty years old at the time of our examination. He had experienced difficulty walking for eight years. His grandmother had also experienced difficulty walking. The patient's calf was atrophic and pes cavus was also found (Fig. 1B). The muscle strength was 1/1 at the anterior tibial muscles and 2/2 at the gastrocnemius muscles. The strength of the other muscles was normal. He did not have any sensory symptoms, such as numbness. The sensations of light touch and temperature were normal, but the vibration sensation was slightly decreased. All tendon reflexes were decreased. No pathological reflex was evoked. He could walk by himself without any support.

Other family members (Fig. 2A)

Three cousins of the proband had similar symptoms. One of them had previously been examined at another hospital. He had undergone sural nerve biopsy and was diagnosed with axonal type CMT, but had not undergone a genetic diagnosis. Although the surviving patients had some level of walking disability, their activity of daily life was relatively preserved. The disease onset was after age fifty in all of the patients. Although we asked the family members to undergo re-examination of their neurological condition, they rejected our proposal.

Gene analysis of the proband and his family (Fig. 2B)

Genomic DNA was extracted from the peripheral blood leukocytes of the patients using the Gentra Puregene Blood

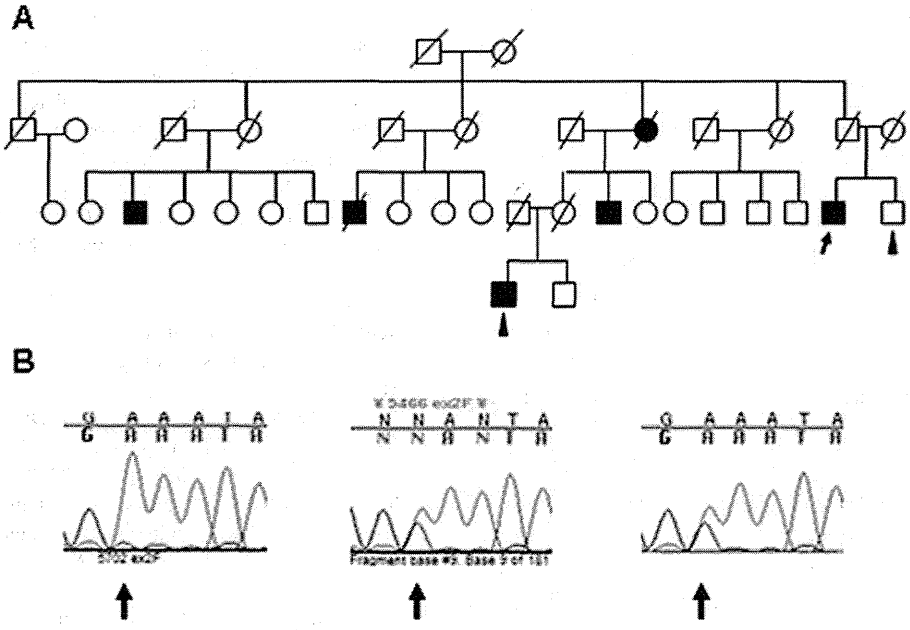

Figure 2. The pedigree of the family with the heat shock protein 27 (HSP27) c.412A>C mutation (A). The arrow indicates Case 1 (the proband). The arrowheads indicate subjects who underwent a genetic analysis. Open circles (women) and squares (men) denote unaffected individuals, and filled symbols denote affected family members. Symbols with a strike through them indicate deceased family members. The electropherograms for the brother of Case 1 (left), Case 1 (middle), and Case 2 (right) (B). The arrows indicate nucleotide 421 in *HSPB1*.

Kit (Qiagen, Duesseldorf, Germany). A panel of sixty genes, including 40 known CMT disease-causing genes and 20 candidate genes were screened (AARS, ANKG, APTX, ARHGEF10, CARS, CNTF, CNTN2/TAG1, DARS, DHH, DNM2, EGR2, EPRS, FARSA, FARSB, FGD4, FIG4, GAN, GARS, GDAP1, GJB1, HARS, HK1, HOXD10, HSPB1, HSPB8, IARS, KARS, KCC3, LARS, LITAF, LMNA, MARS, MED25, MFN2, MPZ, MTMR2, NARS, NDRG1, NEFL, PEPD, PMP22, PRPS1, PRX, QARS, RAB7, RARS, SARS, SBF2, SCN8A, SETX, SH3TC2, SOX10, TARS, TDP1, TRPV4, TTR, VARS, WARS and, YARS). Using the Primer 3 program, we designed 861 oligonucleotide primers covering the entire coding exons and exon-intron junctions, with an amplicon length of 350-500 base pairs. Briefly, all fragments were amplified by multiplex polymerase chain reaction (PCR) (Qiagen Multiplex PCR Kit; Qiagen) and then were mixed to build the amplicon DNA library. As an initial input, 50 ng of the DNA library was fragmented and tagged simultaneously with the Nextera transposome, then multiple index 1 (i7) and index 2 (i5), as well as common adapters (P5 and P7, respectively) were ligated. After small DNA fragments (shorter than 300 bp) were removed using the AMPure PCR purification system (Agencourt Bioscience, Beverly, USA), the library was adjusted to a working concentration of 2 nM. The target resequence analysis was performed using a next-generation sequencer (MiSeq®, Illumina, San Diego, USA). After cluster generation through a bridge PCR, paired-end sequencing (150×2) was performed on a flow cells; clusters were imaged using light emitting diode (LED) and filter combinations specific to each of the four fluorescently-labeled dideoxynucleotides. After base-calling, filtering, and quality scoring, fastq files were generated. Using the CLC Genomics Workbench 6 software program (CLC bio, Aarhus, Denmark), the output reads were aligned with the reference sequence, and thereafter the variants were called and annotated for the analysis.

To confirm the mutation revealed by next-generation sequencer, the proband and two members of the family underwent a genetic analysis by the Sanger method for direct sequencing. In the two affected individuals, we detected a heterozygous c.421A>C (p.K141Q) missense mutation in the *HSPB1* gene. The proband's younger brother, who was neurologically normal, did not have this mutation.

Discussion

This family is the fourth reported Japanese family with autosomal dominant dHMN with a *HSPB1* mutation. The K141Q mutation was first reported by Ikeda et al. (4). The ages of onset of their two cases were 47 years and in the fifties. Compared with these patients, the onset in our Case 1 occurred at an older age. In the first reported family with this mutation, severe dysfunction of the autonomic nervous system was reported. Unlike that family, our patients did not complain of orthostatic hypotension or neurogenic bladder. However, the dysautonomia in the first reported family could have been due to complicated diabetes mellitus. The sensory involvement was minimal or subclinical in our cases, as well as in the previously reported cases.

Since the first report of *HSPB1* mutation (5), 16 different autosomal dominant mutations and one autosomal recessive mutation have been reported in families with CMT2 and dHMN (6-12). HSP27 is one of a stress-induced chaperone protein and forms oligomers to maintain a misfolded protein in a refolding-competent state. The upregulation of HSP27 has been reported to be required for the survival of motor and sensory neurons injured by apoptotic stress (13). In fact, higher levels of serum HSP27 have been reported in diabetic patients with better nerve function (14). Individuals with mutations in the C-terminal domain of HSP27 show a more severe phenotype, with ages at onset as young as four and seven years (2).

The K141Q substitution is located in the α-crystallin domain of HSP27. The K141Q mutation does not dramatically affect the quaternary structure of HSP27. The chaperone-like activity associated with the K141Q mutation is only a little less than that of the wild-type protein. However, oligomers formed by proteins with the K141Q mutation are slightly larger and less stable than those formed of the wild type (15). The effects of the K141Q mutation on the aggregation of neurofilament light polypeptide or incorporation of neurofilament medium polypeptide into the cytoskeletal network remain to be clarified. Unlike patients with mutations in the C-terminal domain, patients with the K141Q mutation show late onset and a mild clinical phenotype, reflecting the minimal biochemical changes associated with this mutation (15).

The authors state that they have no Conflict of Interest (COI).

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