コンレイ比重遠心法で分離してゲノムDNAおよびtotal RNAを抽出し、total RNAからcDNAを作製した。ゲノムDNAを用いて合計77例のHAM患者についてPCR-RFLP法でHTLV-1サブタイプを決定した。各症例のPBMC 1個あたりのHBZ、TaxおよびFoxP3 mRNA発現を定量し、HTLV-1サブタイプ、PVLとの関連を解析した。

一方、TaxまたはHBZの標的配列を持つHTLV-1 LTRプロモーターと、HBZの標的配列を持つFoxP3プロモーターの下流にルシフェラーゼ遺伝子を連結したレポーターベクターを作製し、これらをTax（Tax AおよびTax B）またはHBZ（HBZ-AおよびHBZ-B）の発現ベクターとともに培養細胞株に共導入してルシフェラーゼ活性を比較した。

（倫理面への配慮）

本研究は関連各施設の倫理委員会の承諾を得た後に施行した。十分な説明と同意のもと、書面による研究協力承諾書が得られた被験者から採血した検体のみを用い、完全に匿名化した後に行った。

C. 研究結果

合計77例のHAM患者についてウイルス型を決定した。各症例のPBMC 1個あたりのHBZ、Taxおよび FoxP3 mRNA発現を定量し、ウイルス型、PVLとの関連を解析した結果、TaxA、TaxB 感染HAM患者（TaxA+、TaxB+ HAM）いずれの群においても、PBMC中のHBZ mRNA発現量とPVLに有意な正の相関が認められた。TaxB+ HAMの感染細胞あたりのHBZ mRNA発現量はTaxA+ HAMより有意に高かった。TaxB+ HAMでは、PBMC中のHBZ mRNA発現量とFoxP3 mRNA発現量との間に有意な正の相関関係が認められたが、TaxA+ HAMではこの相関が認められなかった。

各ウイルス型のTaxおよびHBZはHTLV-1 LTR、FoxP3プロモーターのいずれに対しても同等のルシフェラーゼ活性を示し、転写制御因子としての機能に有意な差はなかった。

D. 考察

HAM発見当初、同一のウイルスが慢性炎症性疾患（HAM）と悪性腫瘍（ATL）双方の原因となることから、疾患特異的な株の存在が疑われ検証されたが、否定的な結論が大勢であった。2000年になって、鹿児島県のHAM患者、HTLV-1キャリア、ATL患者の解析から、HTLV-1の転写調節因子Taxの遺伝子配列に2つのウイルス型が存在し、そのうちのTaxAを持つ感染者はTaxBを持つ感染者と比較して、HAM発症のOddsが約2倍高いことが報告された。本研究では、ウイルス型を決定した合計77例のHAM患者について各症例のPBMC 1個あたりのHBZ、Taxおよび FoxP3 mRNA発現を定量し、ウイルス型、HTLV-1 PVLとの関連を解析した。その結果、TaxB+ HAMの感染細胞あたりのHBZ mRNA発現量はTaxA+ HAMより有意に高いこと、TaxB+ HAMでは、PBMC中のHBZ mRNA発現量とFoxP3 mRNA発現量との間に有意な正の相関関係が認められるが、TaxA+ HAMではこの相関が認められないことが明らかになった。これらの結果は、ウイルス型が感染者の生体内におけるウイルス遺伝子、細胞遺伝子の発現制御に影響することで、最終的な疾患感受性にかかわる可能性を示唆している。興味深いことに、TaxAと同じ変異を持つウイルスに感染しているジャマイカにおいては、HAMの発症率が日本の約6倍も高いことが報告されている（Kramer A et al. Am J Epidemiol 142:1212-20, 1995.）。一方、レポーターアッセイにおいて、各ウイルス型のTaxおよびHBZの転写制御因子としての活性に有意な差が認められなかったことから、HTLV-1ウイルス型がウイルス転写制御因子の機能を修飾する以外の方法で宿主遺伝子の発現を制御し、最終的にHAM発症リスクを規定する可能性が考えられる。現時点では、各ウイルス型がどのようなメカニズムで宿主遺伝子の発現を制御しているか不明であり、さらなる解析が必要である。

E. 結論

HTLV-1 ウイルス型の違いにより、感染者の生体内におけるウイルス遺伝子、宿主遺伝子の発現動態が異なり、HAM 発症リスクを規定する可能性を指摘した。

F. 健康危険情報

なし

G. 研究発表

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H. 知的財産権の出願・登録状況(予定含)

1. 特許取得

該当なし

2. 実用新案登録

該当なし

3. その他

該当なし

Ⅲ. 研究成果の刊行に関する一覧表

平成25年度　研究成果の刊行に関する一覧表

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Tanaka Y, Takahashi Y, Tanaka R, Kodama A, Fujii H, Hasegawa A, Kannagi M, Ansari AA, **Saito M.**	Elimination of human T cell leukemia virus type-1-infected cells by neutralizing and antibody-dependent cellular cytotoxicity-inducing antibodies against human T cell leukemia virus type-1 envelope gp46.	AIDS Res Hum Retroviruses.		in press	2014
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Ⅳ. 研究成果の刊行物・別刷

Journal of the Peripheral Nervous System 18:89–93 (2013)

CASE REPORT

Novel mutation in the replication focus targeting sequence domain of *DNMT1* causes hereditary sensory and autonomic neuropathy IE

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Abstract *DNMT1*, encoding DNA methyltransferase 1 (Dnmt1), is a critical enzyme which is mainly responsible for conversion of unmethylated DNA into hemimethylated DNA. To date, two phenotypes produced by *DNMT1* mutations have been reported, including hereditary sensory and autonomic neuropathy (HSAN) type IE with mutations in exon 20, and autosomal dominant cerebellar ataxia, deafness, and narcolepsy caused by mutations in exon 21. We report a sporadic case in a Japanese patient with loss of pain and vibration sense, chronic osteomyelitis, autonomic system dysfunctions, hearing loss, and mild dementia, but without definite cerebellar ataxia. Electrophysiological studies revealed absent sensory nerve action potential with nearly normal motor nerve conduction studies. Brain magnetic resonance imaging revealed mild diffuse cerebral and cerebellar atrophy. Using a next-generation sequencing system, 16 candidate genes were analyzed and a novel missense mutation, c.1706A>G (p.His569Arg), was identified in exon 21 of *DNMT1*. Our findings suggest that mutation in exon 21 of *DNMT1* may also produce a HSAN phenotype. Because all reported mutations of *DNMT1* are concentrated in exons 20 and 21, which encode the replication focus targeting sequence (RFTS) domain of Dnmt1, the RFTS domain could be a mutation hot spot.

Key words: DNA methyltransferase 1, *DNMT1*, hereditary sensory and autonomic neuropathy, missense mutation, next-generation sequencing

Introduction

DNA methyltransferase 1 (Dnmt1), encoded by *DNMT1*, is the principal enzyme responsible for the maintenance of cytosine methylation at cytosine–phosphate–guanine dinucleotides in the mammalian genome *(Feng and Fan, 2009)*, and is also crucial for gene regulation and chromatin stability *(Tohgi et al., 1999; Chen et al., 2003)*. Human Dnmt1 consists of a conserved C-terminal catalytic core and a large N-terminal region harboring multiple globular conserved domains, including the DNA methyltransferase-associated protein 1-binding domain, the proliferating cell nuclear antigen-binding domain, the replication focus targeting sequence (RFTS) domain, the CXXC domain, and two bromo-adjacent homology domains *(Syeda et al., 2011)*.

To date, eight kindreds with *DNMT1* mutations have been reported, half of which were characterized

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by sensory neuropathy, sensorineural hearing loss, and dementia, caused by mutations in exon 20 *(Klein et al., 2011)*; the other half presented with autosomal dominant cerebellar ataxia, deafness, and narcolepsy, and all mutations were located in exon 21 *(Winkelmann et al., 2012)*. It is noteworthy that all peptides coded by exon 20 and 21 belong to the RFTS domain of Dnmt1.

Using a next-generation sequencing (NGS) system, we screened a panel of candidate genes in a Japanese patient with sensory neuropathy, autonomic nervous system dysfunctions, sensorineural hearing loss, and slight dementia. This screen identified a novel missense mutation in exon 21 of *DNMT1*. We also reviewed all reported cases with *DNMT1* mutation and investigated the pathogenesis of various *DNMT1*-related phenotypes.

Case Report

The patient was a 41-year-old Japanese male from a non-consanguineous family (Fig. 1A). No neurological disorders were found in other family members. Pain perception began to decrease in his distal lower limbs after high school, and this condition progressed slowly. At the age of 30 and 32, after local infection, he had osteomyelitis in his first right toe and fifth left toe, respectively, and amputations were performed. Meanwhile, he began to experience hearing loss, and a hearing aid was used in the right ear. After his gait became unsteady he was referred to a department of neurology. Physical examination revealed foot ulcers and mutilations (Fig. 1B). His muscle strength was normal; however, a marked decrease was observed in his sense of pain and touch (1/10) in the lower limbs. Vibration perception was present in the fingers but absent in the lower limbs, and Romberg test was positive. Mild mental retardation was noted. Examination of the cranial nerves was normal except for bilateral hearing loss. Cerebellar function examination showed no abnormalities on finger-to-nose or heel-to-knee testing, or rapidly alternating pronation and supination of hands. Muscle tone was normal, without speech abnormality. The deep tendon reflexes of the lower limbs were absent. The pure tone audiometry test suggested moderate to severe bilateral sensorineural hearing loss. A 24-h Holter monitor indicated sinus bradycardia (43/min on average). Plain radiographs showed an amputation stump of the right hallux and left little toe, accompanied by bone destruction, cortical bone thickness or sclerosis, and an irregular articular surface (Figs. 1C and 1D). Brain magnetic resonance imaging (MRI) revealed mild diffuse cerebral and cerebellar atrophy (Fig. 1E). Clinical and radiological examinations revealed several saprodontia, bronchiectasis in the middle and lower lobe of the right lung, and accessory sinusitis.

In the electrophysiological study, except for the slight slowing of motor nerve conduction velocity in the right tibial nerve, motor nerve conduction studies were almost normal in the median, ulnar, and posterior tibial nerves. However, the sensory nerve action potential could not be evoked in the right median, ulnar, and sural nerves.

The protocol of the studies described below was reviewed and approved by the Institutional Review Board of Kagoshima University. The patient provided his written informed consent to participate in this study.

Methods and Results

Sixteen candidate genes, including 11 genes related to hereditary sensory and autonomic neuropathies (HSAN) and another 5 genes (Table 1) associated with sensory and autonomic dysfunctions were screened on the MiSeq sequencing system Illumina, San Diego, CA, USA).

After one run for 28 h, 400,122 (150 × 2) reads were generated for this patient on the NGS; 93.7% of the reads could be mapped to the reference genome and 98.1% of the target regions were covered at least 10 times. In 27 high-confidence variants, 24 known single nucleotide polymorphisms (SNPs) were coincident with the dbSNP (http://www.ncbi.nlm.nih.gov/snp/) or 1000 Genomes data (http://browser.1000genomes.org). Of the remaining three non-synonymous variants, c.3248A>C in *KIF1A* and c.3448T>C in *SCN9A* were also found in the normal control, and were thus considered SNPs. Besides, a heterozygous missense mutation, c.1706A>G (p.His569Arg) in exon 21 of the *DNMT1* gene (NM_001130823.1, NP_001124295.1) remained and was confirmed by Sanger sequencing (Figs. 2A and 2B). This mutation is located in a highly conserved domain among different species (Fig. 2C). Using the web-based programs, this His569Arg alteration was predicted to be pathogenic in POLYPHEN2 (0.982) and SIFT (0.00).

This mutation was not observed in 100 Japanese control samples, nor did we find it on the 1000 Genomes web site, which catalogs human genetic variations using 2,500 samples, including 500 East Asian (100 Japanese) samples.

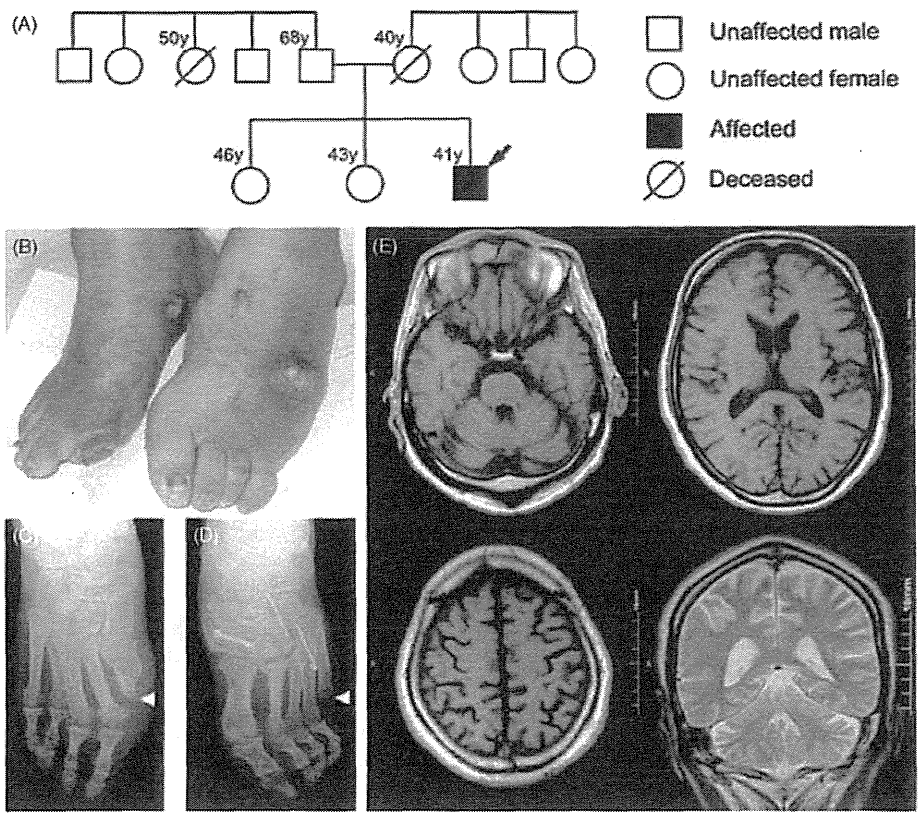

Figure 1. Pedigree and clinical photographs. (A) Pedigree of the patient. The arrow (→) indicates the index case. (B) Foot ulcers and mutilations. (C, D) Radiographs showing the amputation stump of the right hallux and left little toe (◻) accompanied by chronic osteomyelitis. (E) Brain magnetic resonance imaging showing mild and diffuse cerebral and cerebellar atrophy.

Table 1. Candidate genes screened using Miseq sequencing system.

Gene symbol	Locus	Coding exons	Reference sequences
SPTLC1	9q22.31	15	ENST00000262554
SPTLC2	14q24.3	12	ENST00000216484
ATL1	14q11	14	ENST00000441560
DNMT1	19p13.2	41	ENST00000359526
WNK1	12p13.33	28	ENST00000537687
FAM134B	5p15.1	9	ENST00000306320
KIF1A	2q37.3	49	ENST00000498729
IKBKAP	9q31.3	37	ENST00000374647
NTRK1	1q23.1	16	ENST00000368196
NGF	1p13.2	3	ENST00000369512
DST	6p12.1	84	ENST00000244364
SCN9A	2q24.3	27	ENST00000303354
CCT5	5p15.2	11	ENST00000280326
PRNP	20p13	2	ENST00000379440
FLVCR1	1q32.3	10	ENST00000366971
RNF170	8p11.21	7	ENST00000527424

Discussion

We report a Japanese patient with suspected HSAN. Using a high-throughput NGS system, we established a diagnostic procedure involving screening of 16 candidate genes in one run, and identified a novel missense mutation in exon 21 of *DNMT1*.

In 2011, *DNMT1*-related dementia, deafness, and sensory neuropathy was demonstrated in four kindreds from America, Europe, and Japan and was designated HSAN IE. It is an autosomal dominant degenerative disorder of the central and peripheral nervous systems characterized by sensory impairment, sudomotor dysfunction (loss of sweating), dementia, and sensorineural hearing loss. Affected individuals are normal in their youth but begin to manifest progressive sensorineural deafness and sensory neuropathy by the age of 20–35 *(Klein et al., 2011)*. In 2012, another four kindreds from Europe were found to have early onset (18–44 years) of a narcolepsy/cataplexy syndrome followed by ataxia, deafness, sensory neuropathy, and memory loss, which was reported to be associated with *DNMT1* mutations *(Winkelmann et al., 2012)*.

The present patient was normal until graduation from senior high school, but began to manifest progressive inability to perceive pain and experienced painless osteomyelitis. Deafness, as the second symptom, started after the age of 30, and an examination

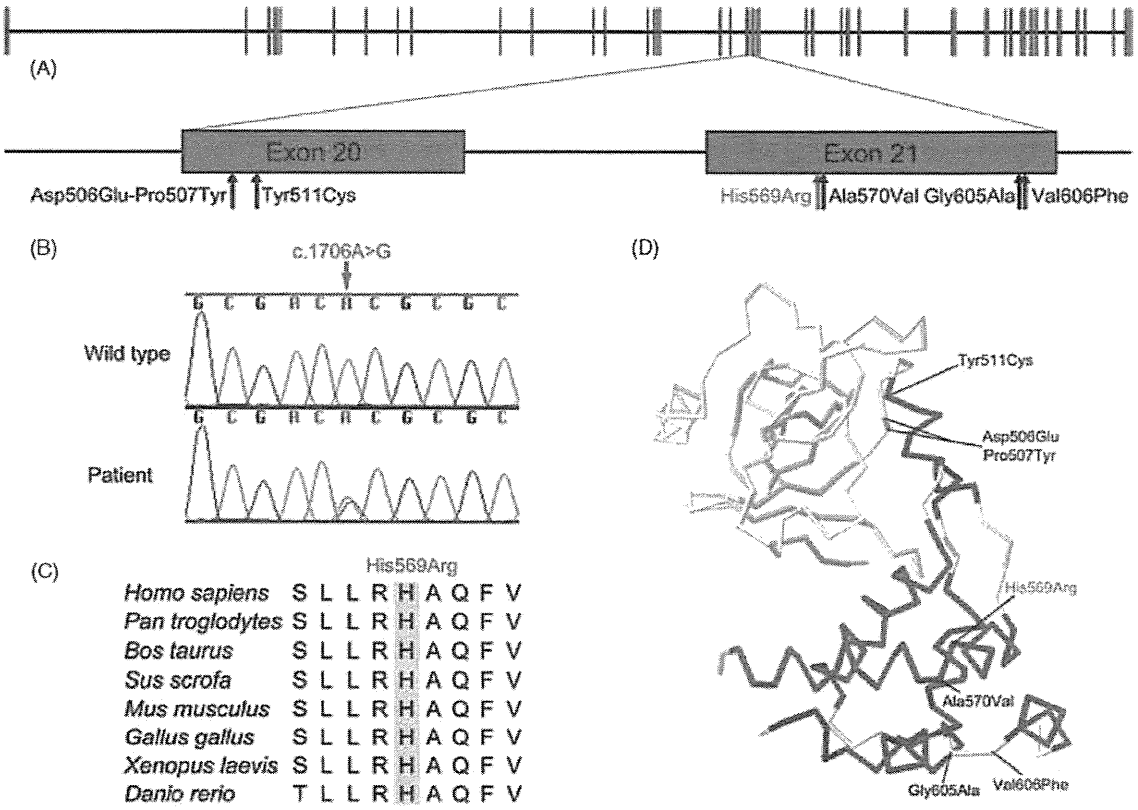

Figure 2. Genetic studies and mutations review. (A) Schematic overview and all mutations in exons 20 and 21 of *DNMT1*. (B) Sequencing chromatogram of the c.1706A>G mutation. Red arrow indicates the mutation site in present patient. (C) Amino acid sequence at the mutation site in homologs of DNA methyltransferase 1 aligned by CLUSTALW. A yellow bar indicates the highly conserved histidine at position 569. (D) Location of mutated residues in the crystal structure of the replication focus targeting sequence domain (Protein Data Bank accession number 3EPZ, showing residues 367–616 in NP_001124295.1).

revealed bilateral sensorineural hearing loss. His gait became ataxic and there was no vibration perception in the lower limbs. Although cerebellar atrophy was revealed by the brain MRI, no definite cerebellar dysfunction was identified. After examination, his ataxia was considered mainly due to loss of deep sensation. The electrophysiological studies revealed sensory dominant axonal polyneuropathy. In addition, mental retardation was observed by the neurologist and diffuse cerebral and cerebellar atrophy was noted in the brain MRI. All these findings were consistent with HSAN IE. The patient's sinus bradycardia and other dysfunctions of the respiratory system might have resulted from the autonomic nervous system dysfunction, but no reliable test was performed to check his autonomic nervous function.

Using the MiSeq sequencing system, a heterozygous missense mutation, c.1706A>G (p.His569Arg), was identified in exon 21 of *DNMT1*. As the present patient showed a definite HSAN phenotype, our findings indicated that the variable *DNMT1*-related phenotype was unlikely to have been determined by the location of the mutation. Although no narcolepsy/cataplexy was noted either in our case or the original four kindreds, the mechanism underlying the varied phenotypes requires further investigation.

It is noteworthy that all reported mutations of *DNMT1* were located in exon 20 (*Klein et al., 2011*: p.Asp506Glu-Pro507Tyr, p.Tyr511Cys; NP_001124295.1) and exon 21 (*Winkelmann et al., 2012*: p.Ala570Val, p.Gly605Ala, p.Val606Phe; NP_001124295.1), and that the original presentations were sensory neuropathy and narcolepsy/cataplexy syndrome, respectively. In Dnmt1, all the peptides encoded by exons 20 and 21 belong to the RFTS domain (Fig. 2D). Previous research indicated that this RFTS domain, inserted deeply into the DNA-binding pocket *(Takeshita et al., 2011)*, contributes to the inhibition of Dnmt1 binding to naked DNA oligonucleotides and native polynucleosomes *(Syeda et al., 2011)*. The RFTS domain also contains a binding site for Uhrf1 *(Achour et al., 2008)*, which recognizes and binds to the hemimethylation sites of DNA and recruits Dnmt1 *(Bostick et al., 2007; Arita et al., 2008;*

Avvakumov et al., 2008). Mutations in exon 20 and 21 of *DNMT1* would transform the structure of the RFTS domain and affect the recognition and binding procedure of hemimethylated DNA, creating abnormal methylation and gene silencing. On the basis of our findings and previous studies, we surmise that the RFTS domain is a mutation hot spot compared with the other Dnmt1 domains. The other possibility is that mutation in other functional domains might cause global genome demethylation and embryonic lethality.

In conclusion, using a MiSeq sequencing system we identified a novel missense mutation in exon 21 of *DNMT1* in a Japanese patient with the typical HSAN IE phenotype. We also reviewed all eight of the kindreds with *DNMT1* mutations in previous reports, and excluded the presumption that varied phenotypes were generated by mutations in different exons. However, further research is required to elucidate the mechanisms of alterations in the RFTS domain and their influence on the DNA methylation procedure.

Acknowledgements

The authors thank Ms. A. Nishibeppu of our department for her excellent technical assistance. The authors would like to thank Enago (www.enago.jp) for the English language review. The authors thank the Joint Research Laboratory, Kagoshima University Graduate School of Medical and Dental Sciences for the use of their facilities. The study was funded by the research on the Nervous and Mental Disorders and Research Committee for Charcot-Marie-Tooth Disease, Neuropathy, Ataxic Disease and Applying Health and Technology of Ministry of Health, Welfare and Labour, Japan.

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Hereditary sensory and autonomic neuropathy type IID caused by an *SCN9A* mutation

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ABSTRACT

Objective: To identify the clinical features of Japanese patients with suspected hereditary sensory and autonomic neuropathy (HSAN) on the basis of genetic diagnoses.

Methods: On the basis of clinical, in vivo electrophysiologic, and pathologic findings, 9 Japanese patients with sensory and autonomic nervous dysfunctions were selected. Eleven known HSAN disease-causing genes and 5 related genes were screened using a next-generation sequencer.

Results: A homozygous mutation, c.3993delGinsTT, was identified in exon 22 of *SCN9A* from 2 patients/families. The clinical phenotype was characterized by adolescent or congenital onset with loss of pain and temperature sensation, autonomic nervous dysfunctions, hearing loss, and hyposmia. Subsequently, this mutation was discovered in one of patient 1's sisters, who also exhibited sensory and autonomic nervous system dysfunctions, with recurrent fractures being the most predominant feature. Nerve conduction studies revealed definite asymmetric sensory nerve involvement in patient 1. In addition, sural nerve pathologic findings showed loss of large myelinated fibers in patient 1, whereas the younger patient showed normal sural nerve pathology.

Conclusions: We identified a novel homozygous mutation in *SCN9A* from 2 Japanese families with autosomal recessive HSAN. This loss-of-function *SCN9A* mutation results in disturbances in the sensory, olfactory, and autonomic nervous systems. We propose that *SCN9A* mutation results in the new entity of HSAN type IID, with additional symptoms including hyposmia, hearing loss, bone dysplasia, and hypogeusia. ***Neurology*® 2013;80:1641–1649**

GLOSSARY

CIP = channelopathy-associated insensitivity to pain; **CMAP** = compound muscle action potentials; **HSAN** = hereditary sensory and autonomic neuropathy; **$Na_V1.7$** = voltage-gated sodium channel; **dbSNP** = single nucleotide polymorphism database; **SCV** = sensory nerve conduction velocity; ***SCN9A*** = sodium channel, voltage-gated, type 9, α; **SNAP** = sensory nerve action potentials.

Hereditary sensory and autonomic neuropathy (HSAN) is a clinically and genetically heterogeneous group of disorders. Until now, HSAN has been classified into 6 main groups on the basis of their mode of inheritance and clinical features, and 11 HSAN disease–causing genes have been identified (table 1[1–17]).

SCN9A encodes the voltage-gated sodium channel ($Na_V1.7$), and the gain-of-function mutations result in several painful disorders, including inherited erythromelalgia,[18] paroxysmal extreme pain disorder,[19] and small nerve fiber neuropathy.[20] Loss-of-function *SCN9A* mutations have been linked to channelopathy-associated insensitivity to pain (CIP), which is characterized by congenital insensitivity to pain perception and anosmia; however, the autonomic dysfunction has been regarded as exclusionary criteria for the diagnosis of CIP.[21] It is noteworthy that no definite abnormalities have been recorded using either nerve conduction studies or sural nerve pathologic examinations in all of the previous cases with homozygous loss-of-function *SCN9A* mutations and part with compound heterozygous mutations.[22–26] However, a slight reduction in sensory nerve action potentials (SNAP) was recorded in two cases with compound heterozygous *SCN9A* mutations.[25,27]

Supplemental data at www.neurology.org

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