A Novel Point Mutation Affecting the Tyrosine Kinase Domain of the *TRKA* Gene in a Family with Congenital Insensitivity to Pain with Anhidrosis

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A nerve growth factor receptor encoded by the *TRKA* gene plays an important part in the formation of autonomic neurons and small sensory neurons in dorsal root ganglia and in signal transduction through its intracytoplasmic tyrosine kinase domain. Recently, three mutations in the tyrosine kinase domain of *TRKA* have been reported in patients with congenital insensitivity to pain with anhidrosis, which is an autosomal recessive disorder characterized by recurrent fever due to absence of sweating, no reaction to noxious stimuli, self-mutilating behavior, and mental retardation. We examined the *TRKA* gene in five generations of a large Japanese family with many consanguineous marriages who live in a small remote island of the southern part of Japan. We found a novel point mutation at nucleotide 1825 (A→G transition) resulting in Met-581-Val in the tyrosine kinase domain. Two of the three affected patients were homozygous for this mutation; however, the third affected patient was heterozygous. Further analysis revealed that the third patient was a compound heterozygote with the Met-581-Val mutation in one allele and with a single base C deletion mutation at nucleotide 1726 in exon 14 in the other allele, resulting in a frameshift and premature termination codon. ***Key words: nerve growth factor/nerve growth factor receptor/type IV hereditary sensory neuropathy. J Invest Dermatol 112:810–814, 1999***

Congenital insensitivity to pain with anhidrosis (CIPA) (MIM no. 256800) is an autosomal recessive disorder, characterized by the absence of pain and temperature sensation and lack of sweating (Swanson, 1963; McKusick, 1998). Patients have recurrent episodes of unexplained fever, repeated traumatic and thermal injuries, self-mutilating behavior, and occasionally mental retardation.

Nerve growth factor (NGF) and its high affinity receptor, *TRKA*, play an important part in the development and function of sensory and sympathetic neurons (Snider, 1994; Davies, 1994). NGF induces neurite outgrowth and promotes survival of embryonic sensory and sympathetic neurons (Levi-Montalcini, 1987). *TRKA*, a receptor tyrosine kinase originally cloned from a human colon cancer (Martin-Zanca *et al*, 1986) is expressed in the nervous system (Martin-Zanca *et al*, 1990) and is phosphorylated in response to NGF (Kaplan *et al*, 1991; Klein *et al*, 1991). Mice lacking TrkA have severe sensory and sympathetic neuropathies and most die within 1 mo of birth (Smeyne *et al*, 1994). They have extensive neuroneal cell loss in trigeminal, sympathetic, and dorsal root ganglia, as well as a decrease in the cholinergic basal forebrain projections to the hippocampus and cortex (Smeyne *et al*, 1994). As these gene knockout mice including NGF knockout mice (Crowley *et al*, 1994), have phenotypes similar to the human genetic disease, hereditary sensory neuropathies, Indo *et al* (1996) examined these genes and discovered mutations of the *TRKA* gene in patients with hereditary sensory neuropathies type IV, i.e., CIPA. Interestingly, all three mutations found in the *TRKA* gene, a deletion, splice, and mis-sense mutation, were in the intracytoplasmic tyrosine kinase domain, which is thought to be related to intracellular signal transduction. Their findings strongly suggested that defects in *TRKA* cause CIPA and that the NGF-*TRKA* system has a crucial part in the development and function of the nociceptive reception system as well as in the establishment of thermal regulation via sweating in humans. Similar findings that a mutation of a receptor tyrosine kinase for glial cell derived neurotrophic factor (GDNF), RET, causes abnormal enteric neurite outgrowth was reported in Hirschsprung's disease (Romeo *et al*, 1994; Schuchardt *et al*, 1994). These reports suggest that receptor tyrosine-kinase genes function during embryogenesis of the mammalian nervous system.

Here we report the case of an individual who was thought to have suffered from CIPA and whose *TRKA* gene was found to be mutated. Furthermore, we demonstrated that the patient is large family contained two individuals who had a novel mis-sense mutation, which was homozygous and one who was a compound heterozygote.

MATERIALS AND METHODS

Clinical family history We encountered a 57 y old female who was born and grew up on a remote island in the southern part of Japan as shown in **Fig 1**, IV-5. She had episodes of recurrent fever, hypohidrosis, hypalgesia, multiple joint dislocations, bone fractures, and mild mental retardation, although she had normal temperature sensation (**Fig 2*a***). Skin biopsy showed that her eccrine glands were morphologically normal. A careful electron microscopic search revealed no nerve endings in the sweat

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Abbreviation: CIPA, congenital insensitivity to pain with anhidrosis.

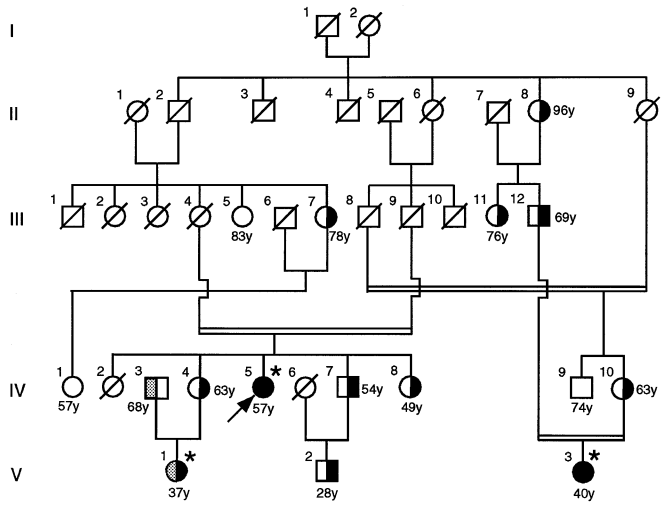

Figure 1. Pedigree and genotype of Met-581-Val mutation and del C^{1726} mutation in the *TRKA* gene of the CIPA family over five generations. Proband (IV-5) is indicated by an arrow. Asterisks represent affected patients. Half-black circles and squares represent heterozygous carriers, black circles represent homozygous patients with the Met-581-Val mutation in the *TRKA* gene. Half-hatched square represents heterozygous carriers, half-hatched and black circle represents a compound heterozygote of the del C^{1726} and Met-581-Val mutations in the *TRKA* gene. Figures with diagonal lines represent deceased individuals. The ages of the family members tested are shown.

glands, as previously reported (Langer *et al*, 1981). Iodine-starch test showed no sweating, and her body temperature rose from 35.6°C to 36.9°C in a 44°C room in 5 min. A lack of physiologic sweat gland response was demonstrated by subcutaneous injection of 1:10,000 acetylcholine. Thus, she was shown to have neurogenic anhidrosis. X-ray examination of bones demonstrated an acetabular dysplasia of the left coxa, absence of a part of the left femoral head (**Fig 2*b***), and a dislocation and/or deformity of the ankle joint. She had a vocabulary IQ of 68. Neurologically, diminished tendon stretch reflexes and pathologic reflex on the left side were noted. Painful stimuli failed to evoke a withdrawal response. The difference between cool (10°C water) and tepid (45°C water) was recognized when tubes containing water at these temperatures were in contact with her extremities and trunk. These suggested a neurologic abnormality similar to CIPA. As shown in **Fig 1**, she had a large family with many consanguineous marriages in which there were at least three patients. The father and mother of the proband (IV-4) are first cousins, while the father of the second patient (V-3) is also a first cousin and her mother the product of a first cousin/aunt marriage. Similarly, the third patient (V-1) is closely related, maternally, but outbred, paternally. IV-4 (**Fig 2*a***, ***b***) and V-3 (**Fig 2*c***) were unmarried and were bedridden and had similar symptoms. V-1, however, who is married and has a daughter, has similar symptoms to the two others except for mental retardation. Sixteen individuals representing four generations were available for DNA analysis (**Fig 1**).

PCR and DNA sequencing for mutation detection After informed consent was obtained, blood samples were collected and genomic DNA was extracted from the buffy coats (QIAGEN, Hilden, Germany). As a control, genomic DNA from 51 normal healthy Japanese people were used. Specific primers (Int-3; 5′-TCCTGTCCCTGCCGCTTCCA-3′ and Int-1; 5′-CTATGTTCACACATCTGCCT-3′) for amplification of exon 14 used in this study are those which had been reported (Indo *et al*, 1996). Amplification conditions were 95°C for 1 min, followed by 35 cycles of 94°C for 40 s, 60°C for 40 s, and 72°C for 1 min, and then final extension at 72°C for 3 min. PCR products (377 bp) were cloned into TA vector (Invitrogen, San Diego, CA) and sequenced with the Dye Terminator Cycle Sequencing Ready Reaction (Perkin Elmer, Warrington, U.K.) and an ABI 310 autosequencer.

Restriction enzyme digestion for DNA diagnosis The Met-581-Val mutation abolished one of the two *Nla*III restriction enzyme sites in the 377 bp fragment, which enabled us to distinguish a mutant allele (289 and 88 bp fragments) from the wild-type allele (226, 88, and 63 bp fragments). The single base C deletion at nucleotide 1726 (del C^{1726}) results in a loss of one of the three restriction enzyme sites for *Bsp*1286I in the 377 or 366 bp fragment, which allowed us to distinguish a mutant allele (241, 96, and 39 bp fragments) from the normal allele (194, 96, 48, and 39 bp fragments). Digestion with these restriction enzymes was done according to the manufacturer's recommendations (New England Biolabs, Beverly, MA). The digested samples were examined by electrophoresis on 3% agarose gels.

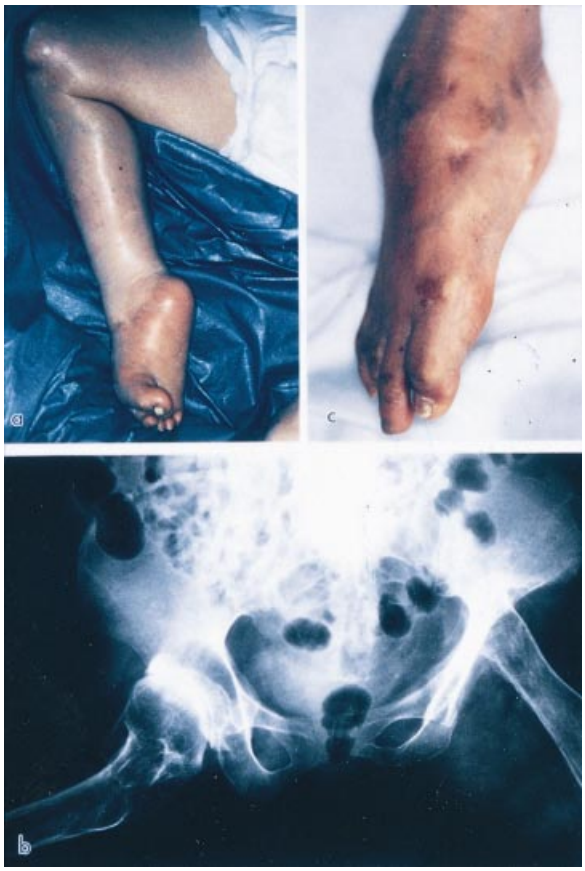

Figure 2. Clinical appearance and X-ray finding of patients with CIPA. The proband (IV-5) (*a*, *b*) and the patient (V-3) (*c*) show that the joint destruction caused by the failure to react to painful stimuli was the most prominent feature.

RESULTS

Identification of a novel point mutation in exon 14 of the intracytoplasmic tyrosine kinase domain The entire exon 14 with flanking segments of the *TRKA* gene from the genomic DNA of patients IV-5 and V-3 and normal healthy controls was amplified, cloned into the vector, and sequenced. Results of the sequence in both patients (IV-5 and V-3) revealed a novel point mutation at nucleotide 1825 (A→G transition) resulting in Met-581-Val in the tyrosine kinase domain in contrast with that of normal controls (**Fig 3*a***). The Met-581-Val mutation abolished one of the two *Nla*III restriction enzyme sites in the 377 bp PCR products, which enabled us to distinguish a mutant allele (289 and 88 bp fragments) from the wild-type allele (226, 88, and 63 bp fragments) (**Fig 3*b***). *Nla*III digestion of the 377 bp PCR product (**Fig 3*c***) revealed whether or not individual family members had this mutation (**Figs 1** and **3*c***). The proband (IV-5) was homozygous for the mutation (data not shown). Patient V-3 with consanguineous parents was homozygous for this mutation, while her father (III-12), mother (IV-10), and aunt (III-11) were heterozygous, and her uncle (IV-9) and others did not carry the mutation (**Fig 3*c***). This mutation was not detected in genomic DNA from 51 control subjects.

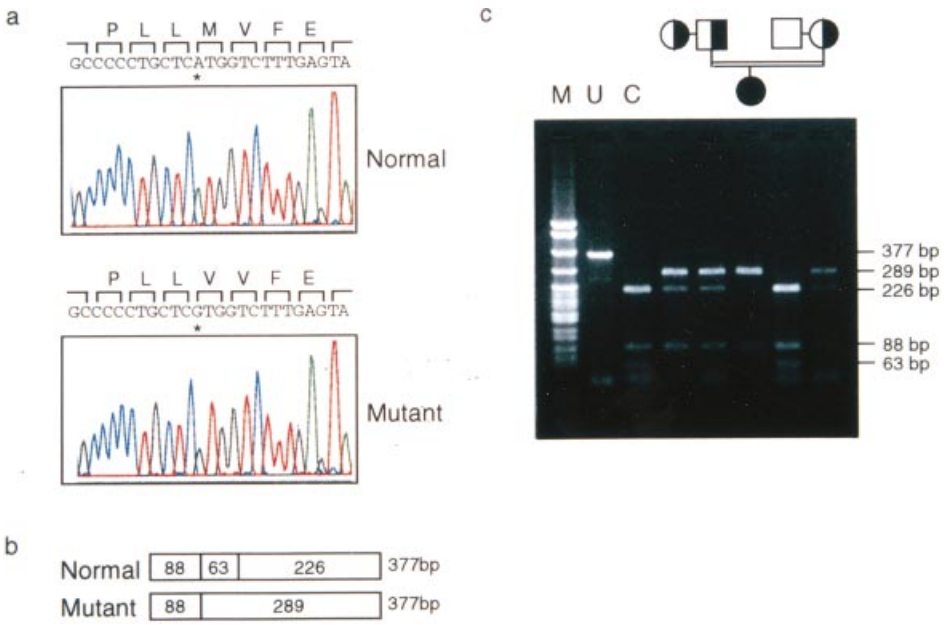

Figure 3. The proband and the patient V-3 were homozygous for the Met-581-Val mutation. (*a*) Sequence analysis of exon 14 and the flanking intronic sequence of the *TRKA* gene from genomic DNA reveals a homozygous A-to-G transition (*asterisks*) at position 1825 in the mutant allele (*lower panel*) as compared with the normal control sequence (*upper panel*). The mutation changes codon ATG to GTG (Met-581-Val). (*b*) Scheme of the fragment pattern of the PCR products formed by restriction enzyme *Nla*III digestion. The nucleotide substitution abolishes a *Nla*III restriction enzyme site. In a normal allele, the 377 bp PCR product is digested to 226, 88, and 63 bp fragments, whereas in a mutant allele, 289 and 88 bp fragments appear. (*c*) Restriction enzyme digestion analysis of the genomic region around the mutation in family members III-11, III-12, IV-9, IV-10, and V-3 as shown in Fig 1. The 377 bp PCR products were digested with *Nla*III and electrophoresed on a 3% agarose gel. Analysis of the PCR product for each individual is shown below the corresponding symbols in the pedigree. M, molecular marker; U, undigested PCR product; C, normal control individual.

Identification of a compound heterozygote in the same family The third 37 y old female patient (V-1) in the same family was heterozygous for the Met-581-Val mutation (**Fig 1**). As we expected to detect another mutation in this patient (V-1) as a compound heterozygote, PCR products with the same primers (Int-3 and Int-1) from the genomic DNA of the patient (V-1) and her father (IV-3) were cloned into the TA vector and sequenced. The sequences revealed a single base C deletion mutation at nucleotide 1726 in exon 14 (**Fig 4*a***), resulting in a frameshift and premature termination codon. This del C^{1726} mutation was seen in five of seven clones from the patient (V-1) and in six of eight clones from her father (IV-3). This mutation was not detected in the healthy controls (**Fig 4*a***) as also reported elsewhere (Indo *et al*, 1996). The del C^{1726} mutation results in a loss of one of the three restriction enzyme sites for *Bsp*1286I in the 377 or 366 bp fragment, which allowed us to distinguish a mutant allele (241, 96, and 39 bp fragments) from a normal allele (194, 96, 48, and 39 bp fragments) (**Fig 4*b***). The patient (V-1) and her father (IV-3) were heterozygous for the del C^{1726} mutation, while her mother (IV-4) did not carry it (**Fig 4*c***, *lower panel*). On the other hand, the patient (V-1) and her mother (IV-4) were heterozygous for the Met-581-Val mutation, while her father (IV-3) did not carry it (**Fig 4*c***, *upper panel*). Therefore, we concluded that this patient (V-1) was a compound heterozygote with a combination of the Met-581-Val mutation and the del C^{1726} mutation.

DISCUSSION

CIPA is a rare clinical disorder, only 32 cases have been reported in the literature most of which were children, in contrast to our adult cases. In addition to the three mutations previously reported (Indo *et al*, 1996), we found a novel point mutation and the first case of a compound heterozygote in the same family (**Table I**). All three mutations in Japanese cases were in exon 14, while a point mutation resulting exon 15 skipping in Ecuadorian cases was in the splice-donor site in intron 15. Because the affected patients had characteristic clinical symptoms and features, all the family members know the symptoms of the disease well. The affected patients could not feel pain, especially from bone fractures. Therefore, they easily developed bone deformities from suppurative arthritis, osteomyelitis and joint dislocation of hands, elbows, knees, and ankles. Sensory neurons which innervate periosteum and joint capsules seem to be affected as are those in skin and muscle. As the homozygous patients with the Met-581-Val mutation have relatively long survival and have normal temperature sensation, the substitution from methionine to valine at position 581 in *TRKA* in this family might cause milder clinical symptoms as compared with others (Vardy *et al*, 1979; Ishii *et al*, 1988; Indo *et al*, 1996). The characteristic clinical features in our three Japanese patients are: (i) they are adult cases; (ii) familial incidence is present, which is rare in Japanese cases reported (Ishii *et al*, 1988); (iii) they lacked impairment of temperature sense; and (iv) the third patient (V-1) obviously lacked mental retardation.

Both dorsal root and sympathetic ganglia arise from the neural crest (Brown and Podosin, 1966). In the chick embryo, tactile exteroceptive cells appear at 2.5–6 d, nociceptive and proprioceptive cells at 10 d over the early differentiation of neural crest cells (Hamberger and Levi-Montalcini, 1949). The timing of the onset of the disorder may critically determine the degree of system involvement (Brown and Podosin, 1966). A congenital defect in the neural crest, causing defective development of pain and temperature afferent neurons and efferent sympathetic neurons to sweat glands, might explain the syndrome in these patients (Swanson, 1963; Swanson *et al*, 1965). With light microscopy eccrine sweat glands appeared to be morphologically normal; however, we could not find the nerve fiber which should reach the secretary portion of eccrine sweat glands by electron microscope (data not shown). These data suggest that the NGF-*TRKA* system is related to the formation or extension of neural fibers during development in the early embryonal period.

Of interest is that the homozygote of the Met-581-Val mutation had mental retardation but the heterozygote for the Met-581-Val and del C^{1726} mutations did not. More than 90% of CIPA

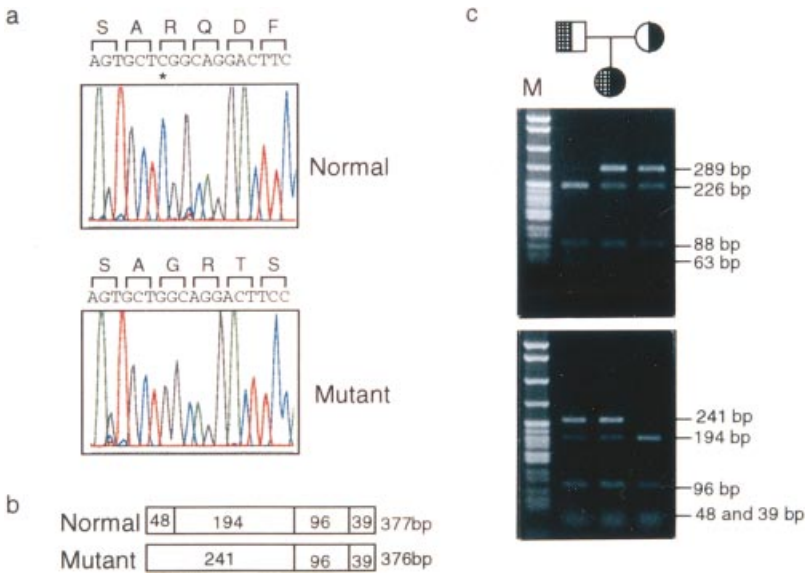

Figure 4. The third patient V-1 is a compound heterozygote for the Met-581-Val mutation and the del C^{1726} mutation in exon 14. (*a*) Sequence analysis of exon 14 and the flanking intronic sequence of the *TRKA* gene from genomic DNA reveals a deletion of a single base C at nucleotide 1726 (*asterisks*) in the mutant allele (*lower panel*) as compared with the normal control sequence (*upper panel*), causing a frameshift and premature termination codon downstream. (*b*) Scheme of the fragment pattern of the PCR products formed by digestion with restriction enzyme *Bsp*1286I. The nucleotide deletion abolishes a *Bsp*1286I restriction enzyme site. In a normal allele, the 377 bp PCR product is digested to 194, 96, 48, and 39 bp fragments, whereas in the mutant allele, 241, 96, and 39 bp fragments appear after *Bsp*1286I digestion. (*c*) Restriction enzyme digestion analysis of the genomic region around the mutation in family members IV-3, IV-4, and V-1 as shown in **Fig 1**. PCR products of 377 and 366 bp were digested with *Nla*III (*upper panel*), and with *Bsp*1286I (*lower panel*), and electrophoresed on a 3% agarose gel, respectively. Analysis of the PCR product for each individual is shown below the corresponding symbols in the pedigree. Solid halves represent the maternally inherited Met-581-Val mutation; striped halves represent the paternally inherited del C^{1726} mutation. M, molecular marker.

Table I. TRKA gene mutations found in CIPA patients

Origin of family	Nucleotide change	Predicted effect on protein product	Families	Reference
Japan	del C^{1726}	Frameshift at residue 548	KI-01 homozygous	Indo *et al*., 1996
Ecuador	$IVS15^{+3}$, a→c	exon 15 skipping	GM08382 homozygous	Indo *et al*., 1996
Japan	C^{1795}→G	G571R	KI-02 homozygous	Indo *et al*., 1996
Japan	A^{1825}→G	M581V	homozygous	this study
	A^{1825}→G/del C^{1726}	M581V/Frameshift at residue 548	heterozygous	this study

cases reported have mental retardation in spite of existence of consanguinity. There were no other retards in the extended, consanguineous family. It had been reported that the relationship between forebrain contribution and mental retardation in neural crest syndrome was not clear (Brown and Podosin, 1966). In adult TrkA-deficient mice, cholinergic projections of the basal forebrain to the hippocampus and cerebral cortex are severely decreased (Smeyne *et al*, 1994). The third case (V-1) without mental retardation suggest there is a possibility that the heterozygous change of the *TRKA* in CNS may not affect the fiber outgrowth or maintenance of the cholinergic phenotype. Although it is difficult to explain the different phenotypic expression of the same gene mutation, further investigation to detect the mutations of the *TRKA* gene in patients with CIPA might reveal a correlation between the pattern of the mutation and the phenotypic involvement of the CNS.

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