Mutation and polymorphism analysis of the *TRKA (NTRK1)* gene encoding a high-affinity receptor for nerve growth factor in congenital insensitivity to pain with anhidrosis (CIPA) families

Yuichi Miura[1], Sek Mardy[1], Yutaka Awaya[2, 3], Kenji Nihei[3, 4], Fumio Endo[1], Ichiro Matsuda[1], and Yasuhiro Indo[1]

[1]Department of Pediatrics, Kumamoto University School of Medicine, Kumamoto, Japan;

[2]Department of Pediatrics, Seibo International Catholic Hospital, Tokyo, Japan;

[3]Congenital Insensitivity to Pain with Anhidrosis Association, Japan;

[4]Department of Neurology, National Children's Hospital, Tokyo, Japan

Correspondence should be addressed to Dr. Yasuhiro Indo,

Department of Pediatrics,

Kumamoto University School of Medicine,

Honjo 1-1-1, Kumamoto 860-8556, Japan

Abstract

The human *TRKA* gene encodes a high-affinity tyrosine kinase receptor for nerve growth factor. Congenital insensitivity to pain with anhidrosis (CIPA) is an autosomal recessive genetic disorder reported from various countries and characterized by anhidrosis (inability to sweat), the absence of reaction to noxious stimuli, and mental retardation. We have found that *TRKA* is the gene responsible for CIPA. We have studied *TRKA* in 46 CIPA chromosomes derived from 23 unrelated Japanese CIPA families, including three that have been previously reported, and identified 11 novel mutations. Four (L93P, G516R, R648C, and D668Y) are missense mutations that result in amino acid substitutions at positions conserved in the TRK family, including TRKA, TRKB and TRKC. Three (S131fs, L579fs, and D770fs) are frameshift mutations. Three (E164X, Y359X, and R596X) are nonsense mutations. The other is an intronic branch-site (IVS7-33T→A) mutation, causing aberrant splicing *in vitro*. We also report the characterization of eight intragenic polymorphic sites, including a variable dinucleotide repeat and seven single nucleotide polymorphisms, and describe the haplotypic associations of alleles at these sites in 106 normal chromosomes and 46 CIPA chromosomes. More than 50 % of CIPA chromosomes share the frameshift mutation (R548fs) that we described earlier. This mutation apparently shows linkage disequilibrium with a rare haplotype in normal chromosomes, strongly suggesting that it is a common founder mutation. These findings represent the first extensive analysis of CIPA mutations and associated intragenic polymorphisms; they should facilitate the detection of CIPA mutations and aid in the diagnosis and genetic counseling of this painless but severe genetic disorder with devastating complications.

Introduction

Nerve growth factor (NGF), the first growth factor identified and characterized, supports the survival of sympathetic ganglion neurons and nociceptive sensory neurons in dorsal root ganglia derived from the neural crest and ascending cholinergic neurons of the basal forebrain (Levi-Montalcini 1987; Thoenen and Barde 1980). *TRKA* (also named *NTRK1*) was isolated from a colon carcinoma as a potential new member of the tyrosine kinase gene family (Martin-Zanca et al. 1986) and was later found to be expressed in the nervous system (Martin-Zanca et al. 1990). TRKA protein is a receptor tyrosine kinase that is phosphorylated in response to NGF (Kaplan et al. 1991; Klein et al. 1991). The human *TRKA* gene maps to chromosome 1q (Miozzo et al. 1990; Morris et al. 1991).

Congenital insensitivity to pain with anhidrosis (CIPA: MIM 256800) is a rare autosomal recessive disorder, also known as “congenital sensory neuropathy with anhidrosis”, “hereditary sensory and autonomic neuropathy type IV” or “familial dysautonomia type II”. It is characterized by recurrent episodes of unexplained fever, anhidrosis (inability to sweat), the absence of reaction to noxious stimuli, self-mutilating behavior, and mental retardation (Dyck 1984; McKusick 1994; Swanson 1963). A case similar to CIPA was reported in terms of “generalized anhidrosis” in Japan (Nishida et al. 1951). A pathological feature of CIPA is the absence of small diameter afferent neurons that are activated by tissue-damaging stimuli (Rafel et al. 1980; Swanson et al. 1965). Anhidrosis is probably attributable to a loss of the innervation of eccrine sweat glands by sympathetic neurons (Ismail et al. 1998; Langer et al. 1981).

Mice lacking the orthologous gene of human *TRKA* share dramatic phenotypic features of CIPA, including the loss of responses to painful stimuli (Smeyne et al. 1994; Snider 1994). However, anhidrosis is not apparent in these animals. The deficient mice also have extensive neural cell loss in all dorsal root ganglia neurons associated with nociceptive functions. We have

therefore considered human *TRKA* as a candidate gene for CIPA and identified the genetic basis for this disorder by detecting mutations in the *TRKA* gene in an Ecuadorian and three Japanese families (Indo et al. 1996). We and others have independently determined the structure and organization of human *TRKA* (Greco et al. 1996; Indo et al. 1997). Recently, we have established a method to amplify all coding exons from genomic DNA and analyzed seven CIPA families from five different countries; our results suggest that *TRKA* mutations cause CIPA in various ethnic groups (Mardy et al. 1999).

In the present study, we have investigated the *TRKA* gene for mutations and intragenic polymorphic sites in 106 normal and 46 chromosomes derive from 23 unrelated CIPA families, including three that have been previously reported. We have detected 11 novel mutations and 8 polymorphic sites and characterized the haplotype association. A common founder mutation in the Japanese families seems likely. The analysis of the associations between the CIPA mutations and the *TRKA* polymorphisms provides useful data for population genetic studies and for investigating the history of the mutations.

Materials and methods

Patients

We studied *TRKA* in 46 CIPA chromosomes derived from 23 unrelated Japanese CIPA families, including three that had previously been reported. Family KI-15 had two affected siblings. CIPA was diagnosed in all patients on the basis of clinical findings or by pharmacological and pathological analyses. They had characteristic features of CIPA, viz., recurrent episodes of fever, insensitivity to pain, and anhidrosis. Five families (KI-01, KI-02, KI-03, KI-0, 5 and KI-11) were consanguineous. A study of mutations but not polymorphism for the remaining three families (KI-01, KI-02 and KI-03) has been reported (Indo et al. 1996). Clinical data on the KI-04 patient, including a radiological study, has also been reported (Iwanaga et al. 1996). At least 32 unaffected Japanese individuals were screened in order to verify that mutations found in the CIPA patients were absent in the normal population. DNA samples for all patients, their family members, and normal individuals were obtained from peripheral blood leukocytes using Puregene kits (Gentra Systems). DNA samples from patients and both parents (obligate carriers) were analyzed, except for four families. DNA samples of the patients in two families (KI-05 and KI-16) or of both parents in the other families (KI-07 and KI-14) were not available for testing. Blood was collected from patients with CIPA and from family members referred to the investigators. Informed consent was obtained from all tested subjects.

Polymerase chain reaction amplification of *TRKA* exons and sequencing

TRKA exons were amplified from genomic DNA by using specific primers derived from the 5' and 3' intronic or exonic sequences, and AmpliTaq Gold (PE Applied Biosystems) or the Expand Long PCR system enzyme (Boehringer-Mannheim). Two or three consecutive exons (i.e., 2 and 3; 5 and 6; 9, 10, and 11; and 13 and 14) were amplified simultaneously. The corresponding

polymerase chain reaction (PCR) products were sequenced directly or were subcloned into the plasmid vector. The primers and sequencing of PCR products have been described elsewhere (Mardy et al. 1999). Sequencing of the PCR products was performed by dRhodamine Terminator Cycle Sequencing Kits (PE Applied Biosystems). Sequences were resolved and analyzed on an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). Detected mutations were described according to a nomenclature system for human gene mutations (Antonarakis 1998).

Restriction digestion analysis

For the analysis of some mutations, genomic DNA was amplified with a corresponding set of primers. PCR products were digested with each restriction enzyme and electrophoresed onto a 1.5 % agarose or a 4 % NuSieve agarose gel (FMC BioProducts).

Exon trapping system

An Exon Trapping System (Gibco BRL) was used to confirm a putative branch-site mutation in intron 7; this was heterozygous in five patients. DNA samples from patient KI-19 were used as representatives. From the patient, a 2.6-kb fragment containing exons 7 and 8 and part of the flanking introns was amplified and subcloned into a plasmid vector and sequenced (Mardy et al. 1999). A mutant or a wild- type allele in the exon trapping vector, pSPL3 (Gibco BRL), was transfected into COS-1 cells by Lipofectamin (Gibco BRL) and characterized by reverse transcriptase/PCR (RT-PCR), as described elsewhere (Mardy et al. 1999).

Polymorphism and microsatellite analysis

Single nucleotide polymorphic sites in intron 2 (IVS2+49 and IVS2+84), intron 5 (IVS5+100),

intron 13 (IVS13+118), exon 14 (c.1740), intron 14 (IVS14-4), and exon 15 (c.1953) were characterized by direct sequencing of the PCR products from patients and parents. The primers were same as those used for the amplification of exons 2+3, 5+6, 13+14 and 15 (Mardy et al. 1999).

An analysis of a microsatellite, D1S506 (=AFMa127wh9), in intron 12 was made by using PCR and human genomic DNA. An FAM-labeled forward primer and an unlabeled reverse primer (AFMa127wh9-F and -R, respectively) were used according to the manufacturer's protocol (Research Genetics). The PCR products were resolved on an ABI PRISM 310 Genetic Analyzer, and the results were analyzed by the ABI Genescan Analysis software (PE Applied Biosystems).

A haplotype was established according to the combination of polymorphisms at each site. The disease or normal allele associated with single nucleotide polymorphic site(s) was deduced by inspection of segregating genotypes in the families. First, haplotypes were categorized for the normal alleles derived from both parents in all CIPA families, and then haplotypes in 32 normal controls were deduced by assigning a relatively common haplotype in one allele.

Statistical Analysis

One- and two-sided Fisher's exact tests were used to compare the frequencies of the alleles at polymorphic sites among different groups.

Results

Mutations in *TRKA* in CIPA families

In the present study, 20 unrelated Japanese CIPA families were screened for putative mutation(s) in the *TRKA* gene. This represents a total of 40 CIPA chromosomes. We have screened for a single base deletion (c.1726delC) that causes a frameshift after amino acid Arg548 of *TRKA* (R548fs), since the mutation can be detected by a PCR-restriction enzyme analysis (Indo et al. 1996). If the mutation was found in the heterozygous state or was not found in a sample DNA, we amplified and sequenced all 17 exons of the *TRKA* directly.

We detected *TRKA* mutations in all 40 CIPA chromosomes. Six and eight families were homozygous and heterozygous for the R548fs mutation, respectively, indicating that the mutation was carried by 20 CIPA chromosomes. Eleven novel mutations were identified in the heterozygous state in 14 CIPA families. Four were missense mutations, resulting in the following amino acid substitutions: L93P, G516R, R648C, and D668Y. Three, S131fs, L579fs and D770fs, were frameshift mutations (c.475-476delTC, c.1820delT, and c.2393^2394insT). Three were nonsense mutations (E164X, Y359X, and R596X), and one was the intronic mutation IVS7-33T→A (Table 1, Fig. 1a-k). IVS7-33T→A, D668Y, R596X and R648C were shared by five, four, two and two families, respectively.

None of the eleven novel mutations was observed in a sample of 64 normal control chromosomes or in a sample of 42 parental chromosomes that were not transferred to the CIPA patients, suggesting that these changes do not represent frequent polymorphisms. Mendelian inheritance of the mutations was confirmed in 16 families for which both patient and parent samples were available for testing (data not shown).

Exon trap analysis of intronic mutation

We characterized the IVS7-33T→A mutation by using exon trap analysis, since mRNA preparations from the patients were not available for testing. We wanted to know whether intron 7 was excised from a primary transcript and whether exons 7 and 8 were incorporated into an mRNA. RT-PCR analysis demonstrated that the corresponding exons were incorporated in the wild type allele but not in the mutant allele (Fig. 2a). A large DNA fragment was observed in the mutant sample. Sequence analysis of this fragment revealed an intronic sequence from IVS7-137 to IVS7-1, including the T-A substitution at IVS7-33 (Fig. 2b).

Polymorphic sites within the human *TRKA* gene

We analyzed microsatellite, D1S506, since a (GT)n repeat located in intron 12 was noted during inspection of the 23-kb-long sequence of the human *TRKA* gene (Indo et al. 1997). Sequence analysis of the *TRKA* gene during the search for mutations in CIPA patients revealed nucleotide alterations that each could be regarded as a single nucleotide polymorphism (SNP). We report the identification and analysis of seven SNPs at the *TRKA* locus. The seven SNPs correspond to IVS2+49G/T, IVS2+84G/A, IVS5+100C/T, IVS13+118T/C, c.1740G/A, IVS14-4A/delA, and c.1953C/T (Fig. 3). The c.1740G/A and c.1953C/T mutations are silent and are located in exons 14 and 15, respectively. IVS2+49G/T, IVS2+84G/A, IVS5+100C/T, IVS13+118T/C, and IVS14-4A/delA are located in introns 2, 2, 5, 13 and 14, respectively. We determined the allele frequency at eight sites in two groups: one was a sample of 64 chromosomes from 32 disease-free Japanese controls, whereas the other was a sample of 42 parental chromosomes not transferred to the affected patients. The latter “internal control” was recommended for use in association studies (Lander and Schork 1994). We combined these two control groups, since no significant difference in distribution was observed in each polymorphic site, when compared by the Fisher’s exact test (data not shown). The allele frequencies at these polymorphic sites in a total of 106 normal

Japanese chromosomes are shown in Table 2.

Haplotype analysis of polymorphic sites within the *TRKA* gene

Haplotype associations between alleles at eight polymorphic sites within the *TRKA* gene were established for the total of 106 normal and 46 CIPA chromosomes. A large number of different *TRKA* haplotypes was generated when the polymorphic microsatellite repeat D1S506 was incorporated into categorization of haplotypes. To eliminate excessive variability in the analysis and to obtain useful information on the genetic structure of the Japanese population, with regard to the *TRKA* locus, we did not consider variation at this site. For this reason, we studied the haplotype association at seven polymorphic sites: IVS2+49G/T, IVS2+84G/A, IVS5+100C/T, IVS13+118T/C, c.1740G/A, IVS14-4A/delA and c.1953C/T. We detected six representative haplotypes that were shared by at least 1 % of normal controls (Table 3). We termed these haplotypes A, B, C, D, E, and F. The most frequent haplotype was A (~44%), followed by B (~37%), C (~4%), D and E (each ~3%), and F (~2%). We also detected eight rare haplotypes with less than 1 % frequency in normal controls. As indicated above, each of these haplotypes showed considerable variability at the D1S506 site. However, as indicated in Table 3, particular D1S506 alleles were more frequently associated with each of these haplotypes.

In contrast, we detected five representative haplotypes in the 46 CIPA chromosomes, three of whose haplotypes (CIPA1, CIPA2, and CIPA3) are shown in Table 3. CIPA1 but not CIPA2 and CIPA3 was detected in one of 106 normal chromosomes. The other two haplotypes were A and B, which were commonly found in normal chromosomes.

Association study of the *TRKA* mutations and haplotypes

To establish the haplotypic association between the *TRKA* polymorphisms and the *TRKA*

mutations in the CIPA patients, all eight polymorphic markers were characterized. A complete description of the *TRKA* haplotypes for 46 CIPA chromosomes from 23 CIPA families is presented in Table 4, including three families reported elsewhere (Indo et al. 1996). The frequency of the R548fs mutations was 52 % in a total of 46 CIPA chromosomes. Eighteen R548fs mutations were associated with a rare haplotype, CIPA1. We were not able to phase three CIPA patients carrying this mutation because either patient's DNA (KI-05) or parent's DNA (KI-14) was not available for testing in two families and the patient and both parents showed heterozygosity at two polymorphic sites (KI-19). Thus, the haplotype of four chromosomes carrying this mutation could not be considered definite. However, this is most likely to be CIPA1, as determined by an inspection of the distribution and segregation pattern of the genotypes. If we count the haplotype of these four chromosomes as a CIPA1 haplotype, more than 90 % of the R548fs mutations are associated with the unique haplotype and over 80 % of the R548fs chromosomes share a 137 allele at the D1S506 locus. Two R548fs chromosomes derive from a homozygous patient (KI-01) have a C instead of a T at the polymorphic site, viz., IVS5+100C/T.

IVS7-33T→A was detected in five chromosomes, four of which were associated with the haplotype CIPA2. A substitution from the T to a G at the site IVS2+49 was observed in the other IVS7-33T→A chromosome. All IVS7-33T chromosomes share the same allele at the D1S506 locus.

All four D668Y chromosomes were associated with haplotype B, which was relatively common in normal controls. Both G571R and R596X mutations were noted in two chromosomes and were associated with haplotype A , which was most common in normal controls. Each of two R648C chromosomes was associated with haplotype A or B. Each of the remaining seven novel mutations (L93P, S131fs, E164X, Y359X, G516R, L579fs and D770fs) was carried on one chromosome. All these chromosomes, except for L579fs, were associated with either common

haplotype A or B. The L579fs chromosome was associated with haplotype CIPA3 , which was not found in normal controls.

Discussion

Twenty-four of the 46 CIPA chromosomes carried the same R548fs mutation, suggesting that it is a common mutation in Japanese. We have also identified 11 novel mutations, including four missense mutations, three frameshift mutations, three nonsense mutations, and one intronic branch-site mutation, all being detected in the heterozygous state. All missense mutations altered amino acid residues conserved among the human TRK families, including TRKA, TRKB, and TRKC (Nakagawara et al. 1995). In addition, G516 and R648 are conserved among at least 14 receptor tyrosine kinases (Martin-Zanca et al. 1989), which means that they are probably important for the activity of the protein. The effects of all these missense mutations on the functions of the TRKA protein are currently unknown, and experiments are in progress to address this question. The frameshift and nonsense mutations are self-evident for CIPA.

We detected the intronic mutation IVS7-33T→A in five CIPA patients; this causes aberrant splicing *in vitro*. The mutation is involved in a branch-site that is critical for intron excision. The site usually resides anywhere from 9 nucleotides to 59 nucleotides upstream of the 3' splice site and has the RNA consensus sequence YNYURAC (where A is the nucleotide of branch formation; Maquat 1996). The "invariant A"is critical for the formation of the lariat structure, an intermediate of normal eukaryotic splicing (Reed and Maniatis 1988; Ruskin and Green 1985). CCCTGAC (IVS7-36 to IVS7-30) of intron 7 in *TRKA* is the only consensus sequence detected upstream of the 3' splice site in intron 7. The mutation IVS7-33T→A substitutes A for a conserved T (U in the transcript) at the fourth position of the branch-site and causes aberrant splicing of intron 7 *in vitro*. Alternative splicing observed in the mutant allele resulting in an insertion of a 137-bp segment is probably attributable to utilization of an upstream cryptic splice acceptor site. *In vitro* splicing studies demonstrate that altering the U to A at the fourth position of the branch-site severely reduces splicing efficiency (Reed and Maniatis 1988). In addition,

similar mutations at the fourth position of the branch-site have been noted in genetic disorders (Burrows et al. 1998; Kuivenhoven et al. 1996). Thus, the IVS7-33T→A mutation is probably responsible for CIPA.

Recently, two missense mutations, Met581Val and Arg774Pro, have been noted in the tyrosine kinase domain of the *TRKA*, one from Japan (Yotsumoto et al. 1999) and the other from Italy (Greco et al. 1999). The former was detected in three affected patients in a large family with many consanguineous marriages; all these patients live in a small remote island of the southern part of Japan. Interestingly, two patients were homozygous for the Met581Val mutation, but the other was a compound-heterozygote having R548fs in a separate allele, probably derived from outbred marriage. The Italian Arg774Pro mutation is interesting, since the residue is not conserved among the TRK families but does show a loss-of-function effect (Greco et al. 1999). In our two previous consecutive studies, we detected three (Indo et al. 1996) and eleven (Mardy et al. 1999) novel mutations in various regions of *TRKA*. Thus, we have detected a total of 25 mutations, 13 of which have been detected in the Japanese and do not overlap with those in other countries, including UAE, Kuwait, Spain, Italy, and Canada (Mardy et al. 1999).

We have characterized seven single nucleotide polymorphic sites: IVS2+49G/T, IVS2+84G/A, IVS5+100C/T, IVS13+118T/C, c.1740G/A, IVS14-4A/delA, and c.1953C/T in the *TRKA* gene. A comparison of our sequence data with the published cDNA sequence shows two differences at c.1833 and c.1884. The nucleotide of these two sites is a T instead of a C in all DNAs that we have analyzed. Four SNPs (two in exon 14, one in exon 15, and one in intron 15) have been noted in a study of prostate tumors from a mixed race population, viz., white, black, oriental, and unknown (George et al. 1998). These correspond to c.1740G/A, c.1833C/T, c.1953C/T, and IVS15-16T/C according to our nomenclature system. Polymorphism at the two sites c.1740G/A and c.1953C/T has also been observed in the present study, which suggests that these are

polymorphic in various ethnic groups. The study of prostate tumors also showed a T at position c.1833 instead of a C, but it did not refer to the site c.1884. In contrast, we could not detect polymorphism at the site IVS15-16 in any DNA sample that we analyzed. A site that is heterozygous in one population may be monomorphic in others; hence, an SNP marker map must be developed for multiple populations.

The IVS14-4A/delA polymorphic site is interesting, since it is located at the 3' splice site in intron 14. The potential splice scores for IVS14-4A and IVS14-4delA are 94.3 and 95.9, respectively (Shapiro and Senapathy 1987). The deletion probably does not affect the score, because the nucleotide at this position is usually an N (any nucleotide). IVS14-4delA has been detected in the heterozygous state in 5 of 64 normal control chromosomes. It has also been detected in the maternal mutant allele in a CIPA family (KI-10). This allele carries a frameshift mutation, L579fs, responsible for CIPA in this family. Thus, we consider that IVS14-4delA is a polymorphism, not a mutation.

We found a microsatellite, D1S506(AFM127wh9), in intron 12 of the *TRKA* gene, when we characterized the entire structure (Indo et al. 1997). In the present study, we have shown that heterozygosity and the size range at this locus are 71 % and 129-139, respectively, in 106 normal Japanese controls. Heterozygosity and size range at the D1S506 (AFM127wh9) are 57 % and 123-141, respectively, in Caucasians (Dib et al. 1996). We have observed that allele sizes and each frequency in Japanese differ from those in Caucasians. Recently, such differences of allelic distributions at many of the marker loci have been noted in these two racial groups (Yamane-Tanaka et al. 1998).

We have also analyzed the association between haplotypes and CIPA mutations. Some CIPA mutations are strongly associated with rare *TRKA* haplotypes, although a few variations can be detected at some polymorphic sites. Chromosomes carrying the most common R548fs mutation

were found to share the unique haplotype that was detected in less than 1 % of normal chromosomes. This strongly suggests that the mutation was derived from a single ancestral mutation that occurred in the Japanese population. We consider that the R548fs mutation shows linkage disequilibrium with this rare haplotype. The association has not been significantly eroded during the population's history, but we predict at least one recombination between intron 5 and intron 12 and two mutations at the D1S506 locus that might have occurred separately. Some variations at markers in these CIPA chromosomes suggest that the R548fs mutation is not a recent event. It is tempting to speculate that the mutation originated in an isolated region of Japan before the people were allowed to move freely about 130 years ago in this country. Perhaps the mutation recently spread into various regions in Japan. However, one cannot completely rule out the possibility that the R548fs mutation originated in ancient Japanese populations and spread through Japan. In either case, the R548fs mutation is a common founder mutation in Japanese CIPA families.

The IVS7-33T→A mutation is also associated with a unique haplotype. Variation in one chromosome seems to be derived from recombination. Again, this argues in favor of a common origin for the CIPA chromosomes carrying IVS7-33T→A, albeit the frequency is low compared with that of the R548fs. In contrast, the D668Y mutation is associated with a common haplotype in normal controls.

The major mutations, R548fs, IVS7-33T→A, and D668Y, account for 72 % of 46 chromosomes from 23 unrelated Japanese CIPA families. The present findings should facilitate the detection of CIPA mutations and aid in the diagnosis and genetic counseling of this painless but severe genetic disorder with devastating complications. The data in this article also represent the first extensive analysis of CIPA mutations and associated intragenic polymorphisms and provide a general understanding of the variability at the *TRKA* locus in both CIPA and normal

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Figures

Fig. 1a-k. Eleven novel mutations of *TRKA* detected in 20 Japanese CIPA families.

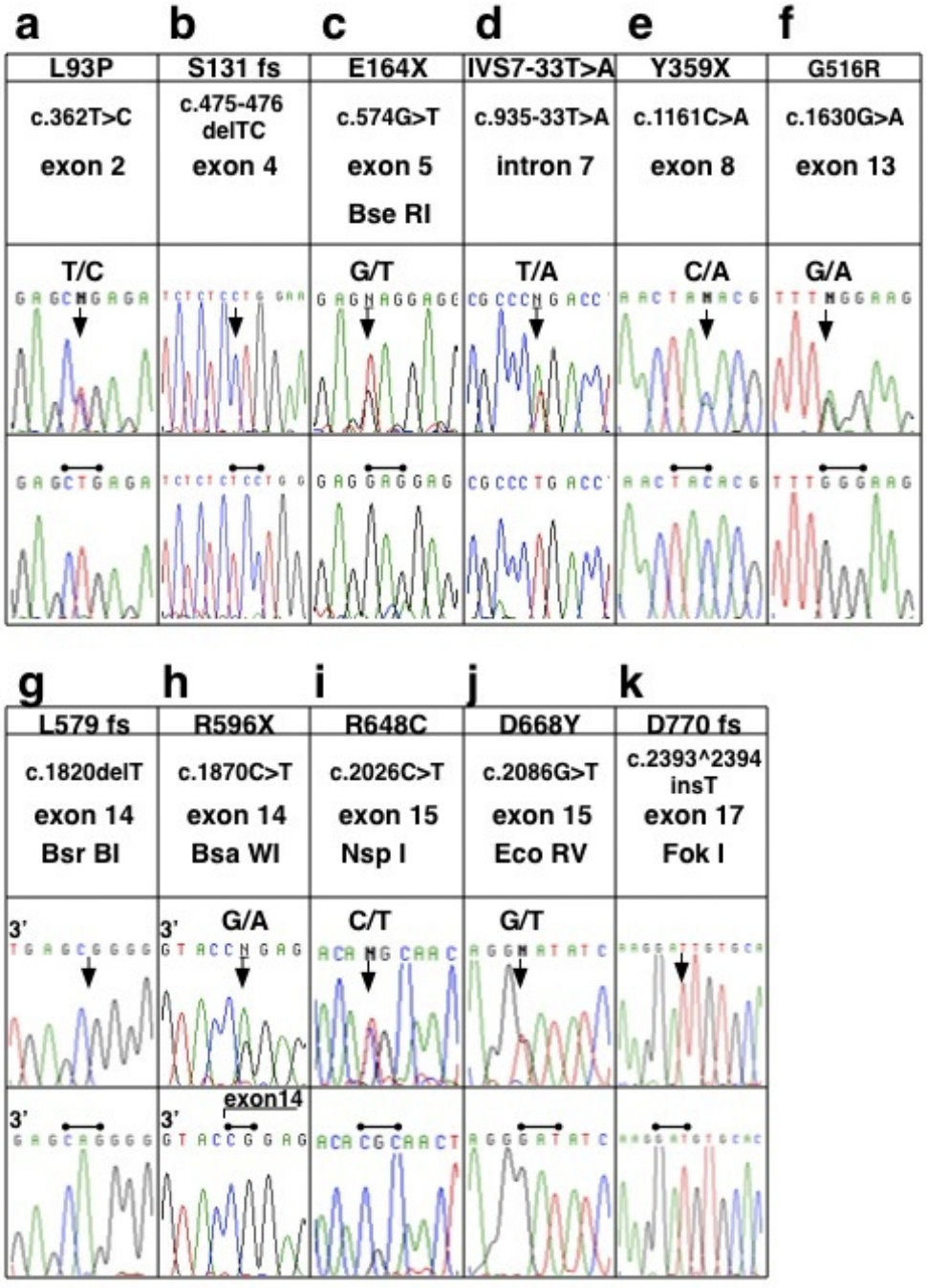


Fig. 1a-k. Eleven novel mutations of *TRKA* detected in 20 Japanese CIPA families. A summary of each mutation is shown in the *upper box*, including the name of the restriction enzyme, if a mutation destroys or creates such a site. Sequences of a representative mutation and a normal control are shown in the *middle* and *lower boxes*, respectively. All mutations in the patients were detected in the heterozygous state. Results of direct sequencing are shown, except for three frameshift mutations in which mutant alleles were subcloned and sequenced. The *arrow* in the *middle box* and a *bar* with bilateral *closed circles* indicate the position of the mutation and an affected codon, respectively.

a A T→C transition at nucleotide 362 in exon 2 causes a Leu→Pro substitution at amino acid 93. The patient and father carry this mutation in family KI-17.

b A 2-base deletion at nucleotides 475-476 in exon 4 causes a frameshift after amino acid Ser131. The patient and father carry this mutation in family KI-13.

c A G→T transversion at nucleotide 574 in exon 5 causes a Glu→Stop substitution at amino acid 164. The patient carries this mutation in family KI-07. DNA samples of parents were not available for testing.

d A T→A transversion at nucleotide –33 in a putative branch-site for splicing in intron 7. The mutation was detected in five families: KI-08, KI-13, KI-18, KI-19, and KI-23. The patients and fathers (in KI-08 and KI-18) or mothers (in KI-13, KI-19, and KI-23) carry it.

e A C→A transversion at nucleotide 1161 in exon 8 causes a Tyr→Stop substitution at amino acid 359. The patient and mother carry this mutation in family KI-04.

f A G→A transition at nucleotide 1630 in exon 13 causes a Gly→Arg substitution at amino acid 516. The patient and mother carry this mutation in family KI-21.

g A single base deletion at nucleotide 1820 in exon 14 causes a frameshift after amino acid Leu 579. Reverse sequences are shown. The patient and mother carry this mutation in family KI-10.

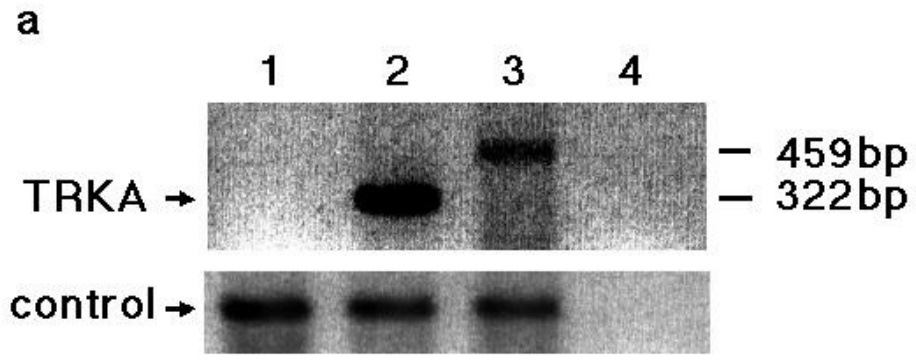
h A C→T transition at nucleotide 1870 in exon 14 causes an Arg→Stop substitution at amino acid 596. Reverse sequences are shown. The patient and father carry this mutation in family KI-04. The patient in family KI-14 also carries it, but DNA samples of parents were not available for testing.

i A C→T transition at nucleotide 2026 in exon 15 causes an Arg→Cys substitution at amino acid 648. Two affected siblings and the patient carry this mutation in families KI-15 and KI-16, respectively. Their father is heterozygous for this mutation.

j A G→T transversion at nucleotide 2086 in exon 15 causes an Asp→Tyr substitution at amino acid 668. The mutation was detected in four families: KI-07, KI-12, KI-15, and KI-18. The patient and father carry this mutation in family KI-12, whereas affected patients and mothers carry it in the other families.

k A single base insertion at nucleotides 2393-2394 in exon 17 causes a frameshift after amino acid Asp770. The patient and mother carry this mutation.

Fig. 2a, b Exon trap analysis of the branch-site mutation in *TRKA*.



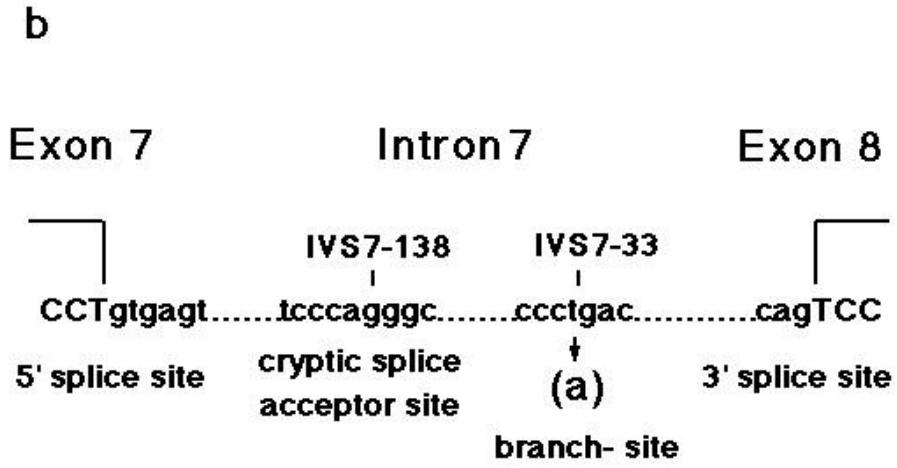


Fig. 2a-b Exon trap analysis of the branch-site mutation in *TRKA*.

a Characterization of the IVS7-33T→A mutation.

A fragment containing exons 7 and 8, and parts of flanking introns, was subcloned into the exon trap vector, pSPL3, and transfected into COS-1 cells. RT-PCR analysis was performed on the mRNA from such cells transfected with each vector construct. A vector alone (*lane 1*), a vector with a normal *TRKA* gene (*lane 2*), a vector with a mutant *TRKA* gene (*lane 3*), and a PCR without a product by reverse transcription (*lane 4*) are shown. Sequencing of the PCR product from a normal sample revealed the presence of consecutive exons 7+8, whereas sequencing of the product from mutant sample showed the presence of a 137- base intronic sequence from IVS7-137 to IVS7-1, including a T→A transversion at IVS7-33. A housekeeping gene was analyzed, as a control for the integrity of the mRNA and of the RT-PCR.

b Schematic representation of the IVS7-33T→A mutation.

The 5' splice site, 3' splice sites, a putative branch-site, and a cryptic splice acceptor site in intron 7 are shown. *Dots* are interrupted sequences. An *arrow* indicates the substituted A for a conserved T (U in the transcript) at the fourth position of the branch-site. This mutation activates an upstream cryptic splice acceptor site at IVS7-138, resulting in an aberrant splicing. Thus, a 137-base fragment is incorporated into the mRNA between exons 7 and 8.

Fig. 3 Localization of mutations and polymorphic markers in *TRKA*.

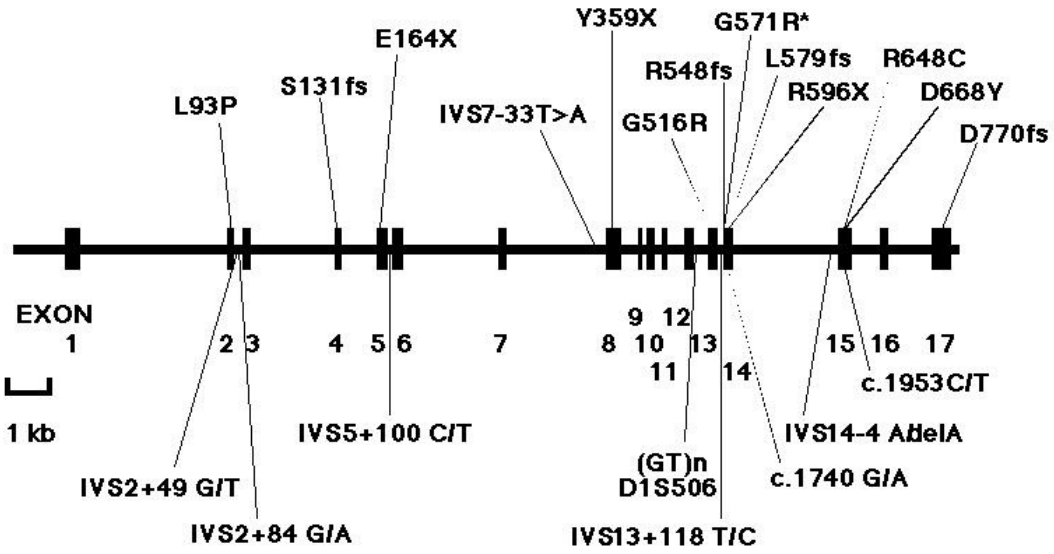


Fig. 3 Localization of mutations and polymorphic markers in *TRKA.*

Amino acid numbering of the TRKA protein and the structure of the *TRKA* gene are according to Martin-Zanca et al. (1989) and Indo et al. (1997), respectively. Mutations reported in this study are listed *above* the *TRKA* gene diagram. *Asterisk* indicates mutation previously reported but not detected in 40 chromosomes in this study. Polymorphic markers are listed *below* the TRKA diagram. IVS2+49G/T, IVS2+84G/A, IVS5+100C/T, IVS13+118T/C, c.1740G/A, IVS14-4A/delA, and c.1953C/T are single nucleotide polymorphisms. D1S506 is a dinucleotide repeat.

Table 1 *TRKA* mutations identified in Japanese CIPA patients

Mutation	Type	Nucleotide change	Amino acid change /predicted consequence	No. of chromosomes	Reference
L93P	Missense	c.362T→C	Leu93Pro	1	Present report
S131 fs	Frameshift	c.475-476delTC	Truncation after Ser131	1	Present report
E164X	Nonsense	c.574G→T	Truncation on Glu164	1	Present report
IVS7-33T →A	Branch site mutation	c.935-33T→A	Aberrant splicing	5	Present report
Y359X	Nonsense	c.1161C→A	Truncation on Tyr359	1	Present report
G516R	Missense	c.1630G→A	Gly516Arg	1	Present report
R548 fs	Frameshift	c.1726delC	Truncation after Arg548	24	Indo et al. (1996)
G571R	Missense	c.1795G→C	Gly571Arg	2	Indo et al. (1996)
L579 fs	Frameshift	c.1820delT	Truncation after Leu579	1	Present report
R596X	Nonsense	c.1870C→T	Truncation on Arg596	2	Present report
R648C	Missense	c.2026C→T	Arg648Cys	2	Present report
D668Y	Missense	c.2086G→T	Asp668Tyr	4	Present report
D770 fs	Frameshift	c.2393^2394insT	Truncation after Asp770	1	Present report

Positions of nucleotide change are from the transcription start site, as described (Martin-Zanca et al. 1989).
The ATG initiation codon is located at nucleotide position c.85. The structure and organization of human *TRKA* are as described by Indo et al. (1997).

Table 2 Polymorphic markers within the *TRKA* gene
(*HET* heterozygosity. *PIC* polymorphism information content, *FRQ* frequency)

Marker	Type	HET	PIC	Allele	FRQ
IVS2+49G/T	Single base change	0.090	0.086	G	0.953
				T	0.047
IVS2+84G/A	Single base change	0.037	0.036	G	0.981
				A	0.019
IVS5+100C/T	Single base change	0.155	0.143	C	0.915
				T	0.085
D1S506	(GT)n	0.707	0.658	129	0.358
				131	0.085
				133	0.028
				135	0.038
				137	0.377
				139	0.113
IVS13+118T/C	Single base change	0.499	0.375	T	0.519
				C	0.481
c.1740G/A	Single base change	0.478	0.364	G	0.604
				A	0.396
IVS14-4A/delA	Single base deletion in splice acceptor site	0.090	0.086	A	0.953
				delA	0.047
c.1953C/T	Single base change	0.073	0.070	C	0.962
				T	0.038

Table 3 *TRKA* haplotypes in normal controls and CIPA patients

Name	Frequency[a]	IVS2 +49G/T	IVS2 +84G/A	IVS5 +100C/T	D1S506[b]	IVS13 +118T/C	c.1740 G/A	IVS14-4 A/delA	c.1953 C/T
A	0.443	G	G	C	129 133 137 139	T	G	A	C
B	0.368	G	G	C	129 131 133	C	A	A	C
C	0.038	T	G	C	137	T	G	A	C
D	0.028	G	G	C	129	C	G	delA	C
E	0.028	G	G	T	135	C	G	A	T
F	0.019	G	G	T	129	C	A	A	C
CIPA1	0.009	G	G	T	137/139	T	G	A	C
CIPA2[c]	0.000	T	G	C	129	C	A	A	C
CIPA3[c]	0.000	G	A	C	129	C	G	delA	C

[a] The frequency of each haplotype was calculated for 106 normal Japanese chromosomes.

[b] D1S506 alleles that are found predominantly associated with each of the *TRKA* haplotypes.

[c] Haplotypes CIPA2 and CIPA3 were observed in CIPA patients but not in normal chromosomes.

Table 4 *TRKA* haplotypes associated with CIPA alleles (*P* paternal allele, *M* maternal allele, numbers 1 or 2 at the *end* indicate each allele in the corresponding CIPA family)

Mutation	Fre-quency (n=46)	Associated polymorphism								
		IVS2+ 49G/T	IVS2+ 84G/A	IVS5+ 100 C/T	D1S 506	IVS13+ 118T/C	c. 1740 G/A	IVS14-4 A/delA	c. 1953 C/T	Allele
R548 fs	24	G	G	T	137	T	G	A	C	KI-06-P
		G	G	T	137	T	G	A	C	KI-06-M
		G	G	T	137	T	G	A	C	KI-08-M
		G	G	T	137	T	G	A	C	KI-09-P
		G	G	T	137	T	G	A	C	KI-09-M
		G	G	T	137	T	G	A	C	KI-10-P
		G	G	T	137	T	G	A	C	KI-11-P
		G	G	T	137	T	G	A	C	KI-11-M
		G	G	T	137	T	G	A	C	KI-16-M
		G	G	T	137	T	G	A	C	KI-17-M
		G	G	T	137	T	G	A	C	KI-20-P
		G	G	T	137	T	G	A	C	KI-20-M
		G	G	T	137	T	G	A	C	KI-21-P
		G	G	T	137	T	G	A	C	KI-22-P
		G	G	T	137	T	G	A	C	KI-22-M
		G	G	T/C	137	T	G	A	C	KI-05-1
		G	G	T/C	137	T	G	A	C	KI-05-2
		G/T	G	T/C	137	T	G	A	C	KI-19-P
		G	G	T	139	T	G	A	C	KI-03-P
		G	G	T	139	T	G	A	C	KI-03-M
		G	G	C	137	T	G	A	C	KI-01-P
		G	G	C	137	T	G	A	C	KI-01-M
		G	G	T	125	T	G	A	C	KI-23-P
		G	G	T/C	125/ 137	T	G	A	C	KI-14-1
IVS7-33 T→A	5	T	G	C	129	C	A	A	C	KI-08-P
		T	G	C	129	C	A	A	C	KI-23-M
		T	G	C	129	C/T	A/G	A	C	KI-13-M
		T/G	G	C/T	129	C	A	A	C	KI-19-M
		G	G	C	129	C	A	A	C	KI-18-P
D668Y	4	G	G	C	129	C	A	A	C	KI-07-1
		G	G	C	129	C	A	A	C	KI-12-P
		G	G	C	129	C	A	A	C	KI-15-M

		G	G	C	129	C	A	A	C	KI-18-M
G571R	2	G	G	C	139	T	G	A	C	KI-02-P
		G	G	C	139	T	G	A	C	KI-02-M
R596X	2	G	G	C	137	T	G	A	C	KI-04-P
		G	G	C/T	137/125	T	G	A	C	KI-14-2
R648C	2	G	G	C	129	C	A	A	C	KI-15-P
		G	G	C	137	T	G	A	C	KI-16-P
L93P	1	G	G	C	131	T	G	A	C	KI-17-P
S131 fs	1	G	G	C	133	T/C	G/A	A	C	KI-13-P
E164X	1	G	G	C	129	C	A	A	C	KI-07-2
Y359X	1	G	G	C	133	T	G	A	C	KI-04-M
G516R	1	G	G	C	129	C	A	A	C	KI-21-M
L579 fs	1	G	A	C	129	C	G	delA	C	KI-10-M
D770 fs	1	G	G	C	137	T	G	A	C	KI-12-M