Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis

Mutations in the *TRKA*/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis

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Congenital insensitivity to pain with anhidrosis (CIPA; MIM 256800) is an autosomal-recessive disorder characterized by recurrent episodes of unexplained fever, anhidrosis (absence of sweating) and absence of reaction to noxious stimuli, self-mutilating behavior and mental retardation[1-3]. The genetic basis for CIPA is unknown. Nerve growth factor (NGF) induces neurite outgrowth and promotes survival of embryonic sensory and sympathetic neurons[4]. Mice lacking the gene for *TrkA*, a receptor tyrosine kinase for NGF[5,6], share dramatic phenotypic features of CIPA, including loss of responses to painful stimuli, although anhidrosis is not apparent in these animals[7]. We therefore considered the human *TRKA* homologue as a candidate for the CIPA gene. The mRNA and genomic DNA encoding TRKA were analyzed in three unrelated CIPA patients who had consanguineous parents. We detected a deletion-, splice- and a missense-mutation in the tyrosine kinase domain in these three patients. Our findings strongly suggest that defects in TRKA cause CIPA and that NGF-TRKA system has a crucial role in the development and function of the nociceptive reception as well as establishment of thermoregulation via sweating in humans. These results also implicate genes encoding other TRK and neurotrophin family members as candidates for developmental defect(s) of the nervous system.

CIPA is also known as congenital sensory neuropathy with anhidrosis or hereditary sensory and autonomic neuropathy type IV[1-3]. The defects of pain and temperature sensations in CIPA are probably due to an almost complete absence of the first-order afferent system generally considered responsible for pain and temperature sensation[1,8]. A genetically determined defect in differentiation and migration of neural crest elements early in embryogenesis has been postulated to be the cause of this disease[8,9], following the pioneering work by Levi-Montalcini[4]. Electron microscopy studies reveal complete absence of small myelinated and unmyelinated fibers[10]. Sweat glands on skin biopsy in CIPA appeared to be normal, but an ultrastructural study of skin biopsies reveal noninnervation of eccrine sweat glands[11]. In *TrkA*-deficient mice, virtually all dorsal root ganglia (DRG)

neurons associated with nociceptive functions are lost[7]. They also have extensive neuronal cell loss in sympathetic ganglia and a decrease in cholinergic neurons of the basal forebrain.

We examined three candidate genes initially: *NGF*, *p75* neurotrophin receptor (see below), and *TRKA*. To facilitate detection of putative *TRKA* mutations, we selected three unrelated CIPA patients who had consanguineous parents, and in which homozygous abnormalities could be expected (see Methods). Given the lack of information on the whole human *TRKA* gene, we determined part of the gene structure (Fig. 1). Preliminary data shows that *TRKA* is expressed in EB-virus transformed lymphoblastoid cells. Thus, RNA from these cell lines from CIPA-affected individuals could be used to assess this locus as a candidate for CIPA.

A single base C at nucleotide 1726 was deleted in exon C of the first patient (KI-01) (Fig. 2*a*). This deletion occurs in a region encoding the tyrosine kinase domain, causing a frameshift and premature termination codons downstream. The proband and her parents were homozygous and heterozygous for the 1726-C deletion, respectively (Fig. 2*b*; data not shown). RT-PCR analysis revealed a shorter-than-expected band in KI-01, which upon sequencing showed a deletion of 451 nucleotides, from 1421 to 1871 encompassing exons A, B and C (data not shown). We amplified and sequenced all three exons and flanking exon-intron junctions from genomic DNA, but found no abnormalities in these boundaries. The transcript lacking exons A – C was also observed in the two patients described below. Thus, it could be a product of alternative splicing which occurs rarely in lymphoblastoid cells. This alternative transcript in the patient was detected in part because of its shorter size and because of the instability of the normal-size transcript containing the single base deletion. Thus, the 1726-C deletion is apparently the cause of CIPA in this family.

In an Ecuadorian family, two small fragments were amplified from a proband (GM 08382) and his affected brother (GM 08383) by RT-PCR, although a single product was amplified from a control (Fig. 2*d*). The parents yielded three fragments, including two small ones observed for the proband and a normal sized band. Two small cDNA fragments from this patient were subcloned and sequenced. Deletions were found to correspond to an exon (1872-2112) (D in Fig. 1) and part of the same exon (1966-2112) on the other allele, indicating the presence of RNA splicing errors. The partial exon deletion is apparently due to activation of

a cryptic splice donor site. Sequencing of genomic DNA revealed that the 5' splice site of an intron between exons D and E contains a C for A substitution in the third position (Fig. 2c). Similar mutations at the third position of the intron resulted in skipping of the preceding exon[12]. No substitution was found in exon D and the flanking exon-intron junctions. Restriction digestion analysis demonstrated that GM 08382 and GM 08383 were homozygous for the A - C transversion and that parents were heterozygous (Fig. 2e). The mutation was not detected in genomic DNAs from 50 control subjects (data not shown).

In the third patient (KI-02), we found a G - C transversion at nucleotide 1795 in exon C, which causes a Gly-to-Arg substitution at amino acid 571 (Fig. 2f). Following amplification of exon C from parental genomic DNA, we detected both mutant and wild-type alleles (data not shown). Restriction digestion analysis showed that the patient and parents were homozygous and heterozygous for this mutation, respectively (Fig. 2g), whereas the mutation was not detected in genomic DNAs from 50 control subjects (data not shown). Gly 571 is located in the tyrosine kinase domain and is conserved among 14 receptor tyrosine kinases, including human TRKB and TRKC (Fig. 3), suggesting that it is important for enzyme activity. The Gly-to-Arg substitution is likely to be the cause of CIPA in this family.

We have also studied two other potential candidate genes for CIPA, *NGF* itself, and the *p75* neurotrophin receptor, based on the similar phenotypes in the respective knockout mice[13,14]. We did not detect mutation in these genes in an unrelated CIPA patient from non-consanguineous parents.

Anatomical changes in the DRG neurons and spinal cord in *TrkA*-deficient animals are similar to those noted in CIPA, including absence of small neurons in the DRG, lack of small fibers in the dorsal roots, absence of Lissauer's tract and reduction of the spinal tract of the trigeminal nerve[8]. Miotic pupils and slight ptosis associated with loss of neurons in the superior cervical ganglia are the features in these mice, suggesting a defect in sympathetic innervations[7]. These are not common findings in CIPA, but have been reported in some patients[9]. Why CIPA patients with the same apparent gene disruption do not show a deficiency in sympathetic innervations will need to be addressed. This could be due to species differences or alternatively, sympathetic innervations in humans might be maintained

or compensated for by a combination of other neurotrophin and receptor systems. The autonomic ganglia and brain appeared normal in a patient with CIPA[8], but additional pathological studies on CIPA patients are required, including analysis of these ganglia as well as the central nervous system sites of *TRKA* expression in humans[15,16].

A characteristic feature in CIPA is anhidrosis. Sweating is important to maintain body temperature under hot environmental conditions, especially in humans[17]. Eccrine sweat glands are innervated by sympathetic cholinergic fibers originating from the paravertebral ganglia, although the great majority of the post-ganglionic sympathetic fibers are adrenergic[18]. Noninnervation of eccrine sweat glands seems to be main cause of anhidrosis observed in CIPA[11]. Another possibility is that a mechanism controlling body temperature homeostasis does not function properly since input of temperature sensation from the peripheral nervous system is disturbed due to a loss of specific neurons. Eccrine glands are well developed in humans, but are limited to the underside of paws in mice and rats[19]. Rodents probably maintain body temperature by panting or saliva-spreading. Differences in mechanisms of temperature homeostasis might be one reason why unexplained fever is a prominent feature in CIPA. Almost 20% of the reported patients died from hyperpyrexia within the first 3 years of life[20]. Interestingly, about half of the *TrkA* knockout mice died within 20 days of life[7], but their body temperature homeostasis requires further study. Mutations of the *TRKA* gene in CIPA patients suggest that TRKA is essential for innervation of eccrine glands by sympathetic neurons. Thus, the NGF-TRKA system probably plays a crucial role in development and function of the nociceptive reception as well as establishment of thermoregulation via sweating systems in humans. Genetic diseases caused by a loss of function of either neurotrophin and TRK families have not been described previously in humans, although *TRKA* was originally cloned as part of the *TRK* oncogene, a chimaeric transforming gene[21]. Our results implicate genes encoding other member(s) of these families as candidates for developmental defect(s) of the nervous system.

Note added in proof: We have recently discovered that a fourth patient (see Methods) is homozygous for the same single-base deletion in exon C as patient KI-01.

Methods

Subjects.

In the Ecuadorian family, the proband (GM08382) was a male with frequent episodes of unexplained fever, anhidrosis, self-mutilating behavior and mental retardation. Abnormal findings on the histamine test and sural nerve biopsy were reported[22]. He has a similarly affected brother (GM08383) and parents (GM08384 and GM08385A). In the other two families, the probands, KI-01 and KI-02 were females born to Japanese parents with clinical similar symptoms. Lymphoblastoid cell lines from the Ecuadorian family were obtained from the Coriell Cell Repository (Camden, NJ). Establishment of lymphoblastoid cell lines from other patients was described[23].

We also screened two other CIPA patients, from non-consanguineous parents; RT-PCR from both patients revealed abnormally sized bands, and we have preliminary evidence for compound heterozygous *TRKA* mutations in one of them. Complete analysis was hampered by lack of a full-length gene, hence these results are not considered further in this paper.

Exon-intron junctions of *TRKA*.

A human genomic library was screened with human *TRKA* cDNA[24]. One positive genomic clone was isolated. This clone covered the genomic region corresponding to 1/3 of the 3'-end cDNA sequence. The genomic region corresponding to the cDNA sequence 935-1688 was reported[25]. Taken together, exon-intron junctions of the *TRKA* gene encoding the intracellular domain of TRKA were determined.

Primers.

Sequences of forward and reverse primers for *TRKA* cDNA[24] are as follows:

Forward primers:

U-1250 5'-ACCCCATCCCTGACACTAAC-3';

U1291 5'-GAGAAGAAGGACGAAACACC-3';

U-1339 5'-GCCGTCTTTGCCTGCCTCTT-3';

U-1452 5'-GGCCATGTCCCTGCATTTCA-3';

U-1638 5'-CTTCCTTGCTGAGTGCCACA-3';

U-1850 5'-GGGACCTCAACCGCTTCCTC-3';

U-2091 5'-CTACAGCACCGACTATTACC-3';

Int-2 5'-CCAGTCTCCTCTCCCATCAC-3';

Int-3 5'-TCCTGTCCCTGCCGCTTCCA-3'.

Reverse primers:

L-1547 5'-GGCATCACTGAAGTATTGTG-3';

L-1680 5'-CCTTGACAGCCACCAGCATC-3';

L-1705 5'-GCACTCTCGGACGCCTCCTT-3';

L-1895 5'-ATCCTCCCCACCAGCCAGCA-3';

L-2059 5'-ATGCCAAAATCACCAATCTT-3';

L-2170 5'-CTCTCGGTGGTGAACTTACG-3';

L-2475 5'-ATTCCGGCTAACCACTCCCA-3';

L-2578 5'-AGCCCCCACCTGTCCCTTCC-3';

Int-1 5'-CTATGTTCACACATCTGCCT-3'.

Primers, Int-1, Int-2 and Int-3 were designed according to the intronic sequences.

RT-PCR.

Total RNA was extracted from EB-virus transformed lymphoblastoid cells[26]. The first-strand cDNAs were generated from 10 μg of total cellular RNA with 5 pmol of random hexamer, using 200 U of MMLV virus reverse transcriptase (GIBCO BRL) in 20 μl of reaction mixture. PCR was carried out, using 2 μl of the first-strand cDNA as a template and 0.5 μM of each of the primers (U-1250/L-2170 and U-1638/L-2578). Subsequently, products of the first PCR were used as template for a second PCR with U-1291/L-2059 and U-1850/L-2475. The amplification conditions were as follows: 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min.

Amplification of genomic DNA.

Genomic DNAs were prepared from lymphoblastoid cells by standard methods[27].

Amplification of exon-intron junction from genomic DNA was performed by following sets of primers located in consecutive exons (Fig. 1). Approximate size of each product, including an intron, is shown in parenthesis. These are U-1339/L-1547 (750 bp), U-1452/L-1680 (650 bp), U-1638/L-1705 (270 bp), and U-2091/L-2170 (750), generating corresponding exon-intron junction sequences. Sets of primers U-1850/L-Int 1 (210 bp) and Int-2/L-1895 (140 bp), respectively, were used for amplification of two exon-intron junctions between exons C and D.

Cloning and sequencing of PCR products.

Amplified PCR products were cloned into the T-vector[28]. The plasmid vector, pBluescript KS (+) (Stratagene), was digested with *Eco*RV, treated with *Taq* polymerase. Each clone was sequenced by a DNA Sequencer, GENESCAN Model 373A and *Taq* Dye Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc.). Mutations were detected by comparing the cloned sequence with the published cDNA and genomic sequence or normal control sequence and confirmed if multiple clones had the same base change. A primer set of U-2091 / L-2170 was used for sequence analysis of genomic DNA from GM 08382. For sequence analysis of KI-01 and KI-02, Exon C was amplified from genomic DNA with a primer set of Int-3 / Int-1.

Restriction digestion analysis.

For analysis of KI-01, genomic DNA was amplified with the same set of primers as described for KI-02 (see below), digested with *Bsp*1286I and electrophoresed. Conditions for PCR are described above. For analysis of GM 08382, a reverse primer, GM-82, was 5'-CAGGGGAAGGCGTTGGGGACAAAGG<u>CAG</u>CT-3' and complementary to bases +4 - +33 of the intron. Three bases underlined were modified from ACC to CAG to introduce *Pvu*II site into the amplified mutant allele. First, DNA fragments were amplified with a primer set of Int-2/L-2170 or U-2091/L-2170, then were used as template for a second PCR with U-2091/GM-82, generating 55 bp product. Conditions for PCR are described above. The products were incubated with 10 U of *Pvu*II and electrophoresed on 4% Nusieve agarose

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gel (FMC BioProducts). For analysis of KI-02, a forward intronic primer (Int-3) and a reverse primer (Ue-L1796), modified to introduce *Sac*II site in mutated allele, were used to amplify a PCR product from genomic DNA. *Sac*II was added to each of the products, and fragments were subsequently resolved.

 (Ue-L1796: 5'-TGAGCAGGGGGCGGCCCTCGGTGCAG<u>C</u>CGC-3' The underlined base was modified to introduce the *Sac*II site into the mutated allele.)

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Figures.

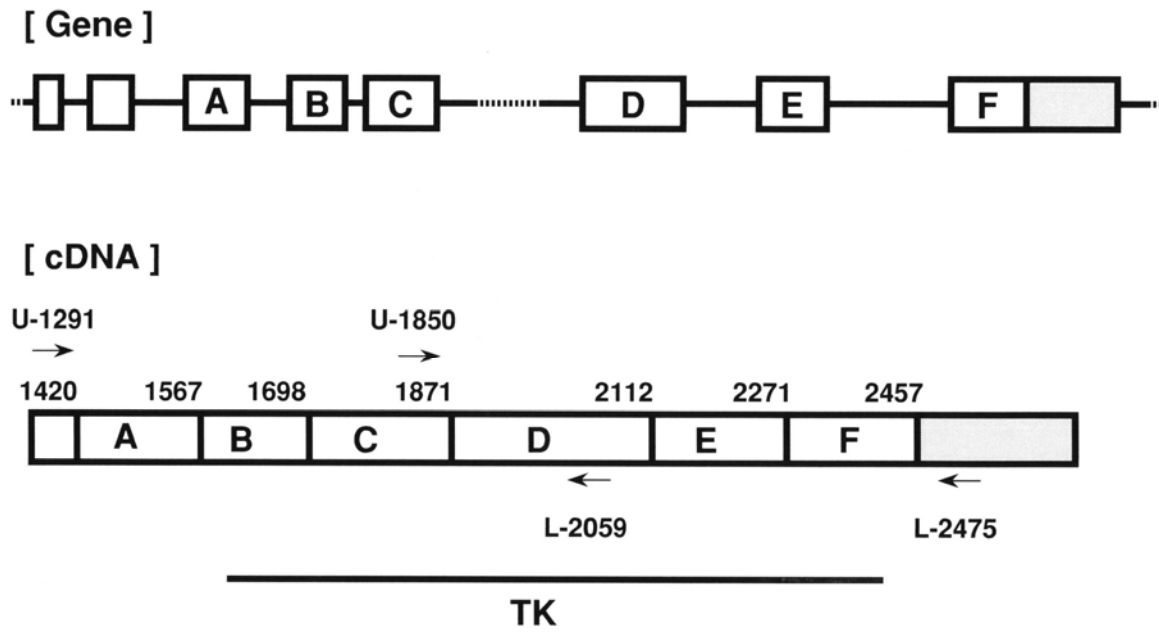**Fig. 1 Human gene and cDNA encoding intracellular domain of TRKA.**

Fig. 1 Human gene and cDNA encoding intracellular domain of TRKA.

The open boxes (A-F) represent 6 exons encoding the intracellular domain. The shaded box shows the 3'-noncoding region. The dotted line is an interrupted intron. Numbers above the cDNA boxes indicate the 3'-end of each exon, according to the published sequence[24].

Arrows indicate forward and reverse primers used for PCR. The region corresponding to the tyrosine kinase domain (TK) is shown below the cDNA.

Fig. 2 Detection of *TRKA* mutations in congenital insensitivity to pain with anhidrosis.

Fig. 2a

Sequence analysis of exon C amplified from genomic DNA of KI-01.
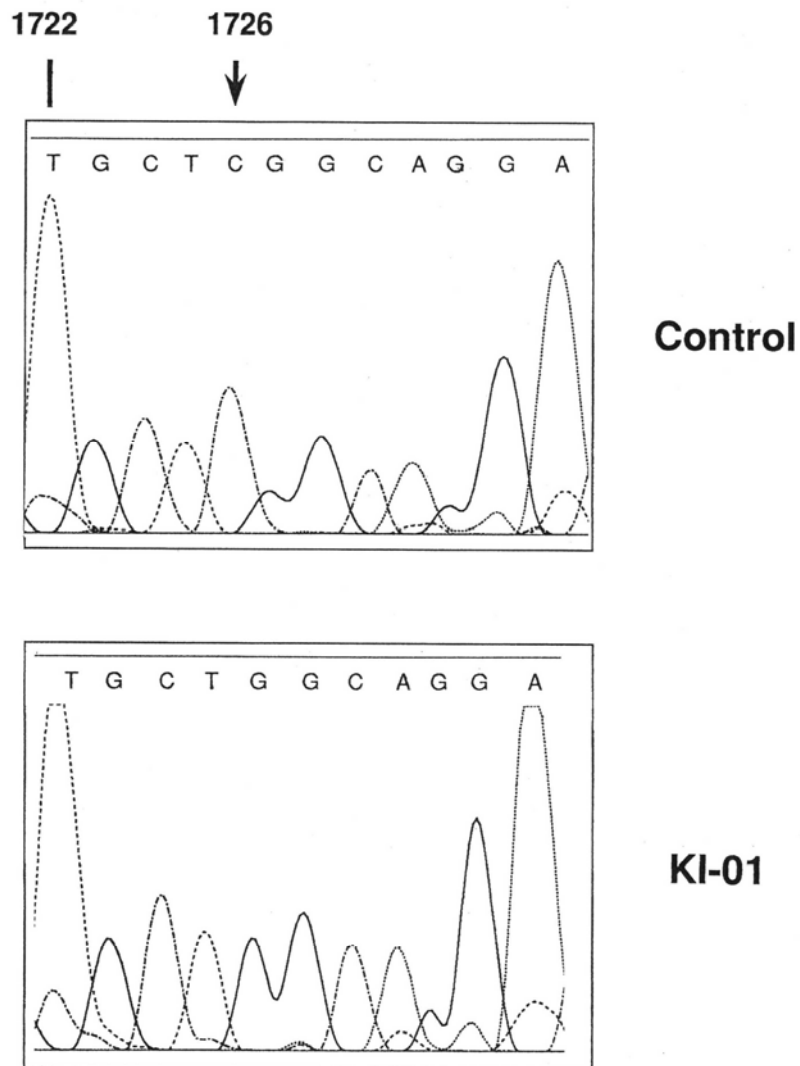
Fig. 2 Detection of *TRKA* mutations in congenital insensitivity to pain with anhidrosis.

Fig. 2*a*, Sequence analysis of exon C amplified from genomic DNA of KI-01.

A single base C at nucleotide 1726 is deleted, causing a frameshift and premature termination codon downstream.

Fig. 2b

Restriction digestion analysis of the genomic region around the mutation in family members of KI-01.
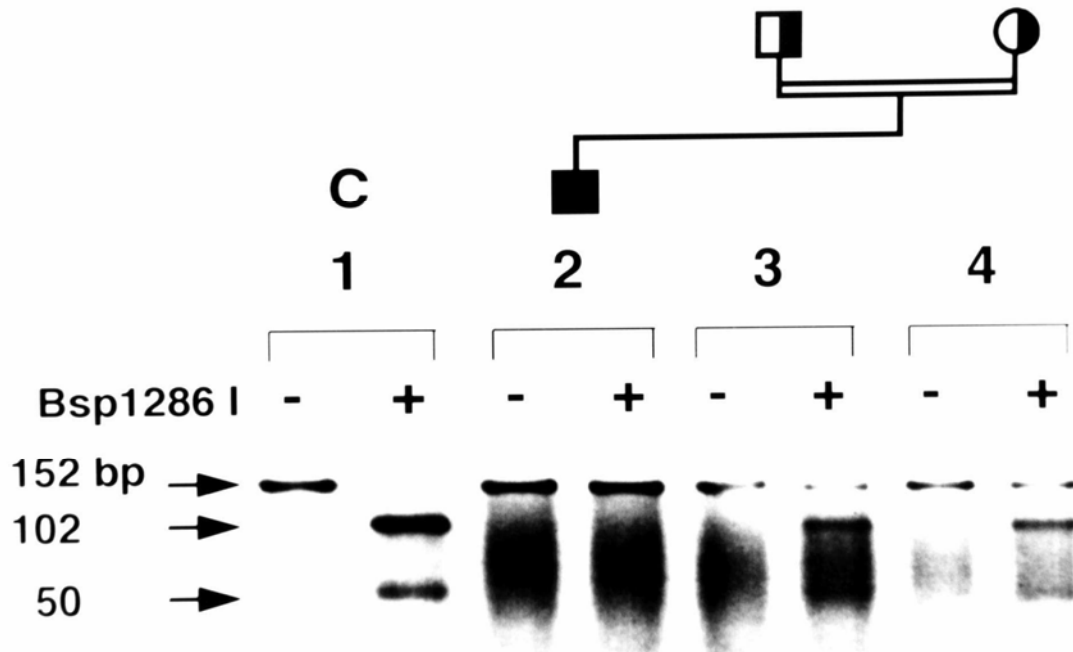
Fig. 2*b*, Restriction digestion analysis of the genomic region around the mutation in family members of KI-01.

PCR products of 152bp were digested with *Bsp*1286I and electrophoresed on a 4% agarose gel. The symbols (+) and (-) denote presence and absence of the enzyme for incubation, respectively. There is a *Bsp*1286I site in the normal control sequence, providing fragments of 102 and 50 bp (lane 1). The single base deletion at nucleotide 1726 disrupts this site, preventing digestion. Analysis of the PCR product for each individual is shown below the corresponding symbols in the pedigree.

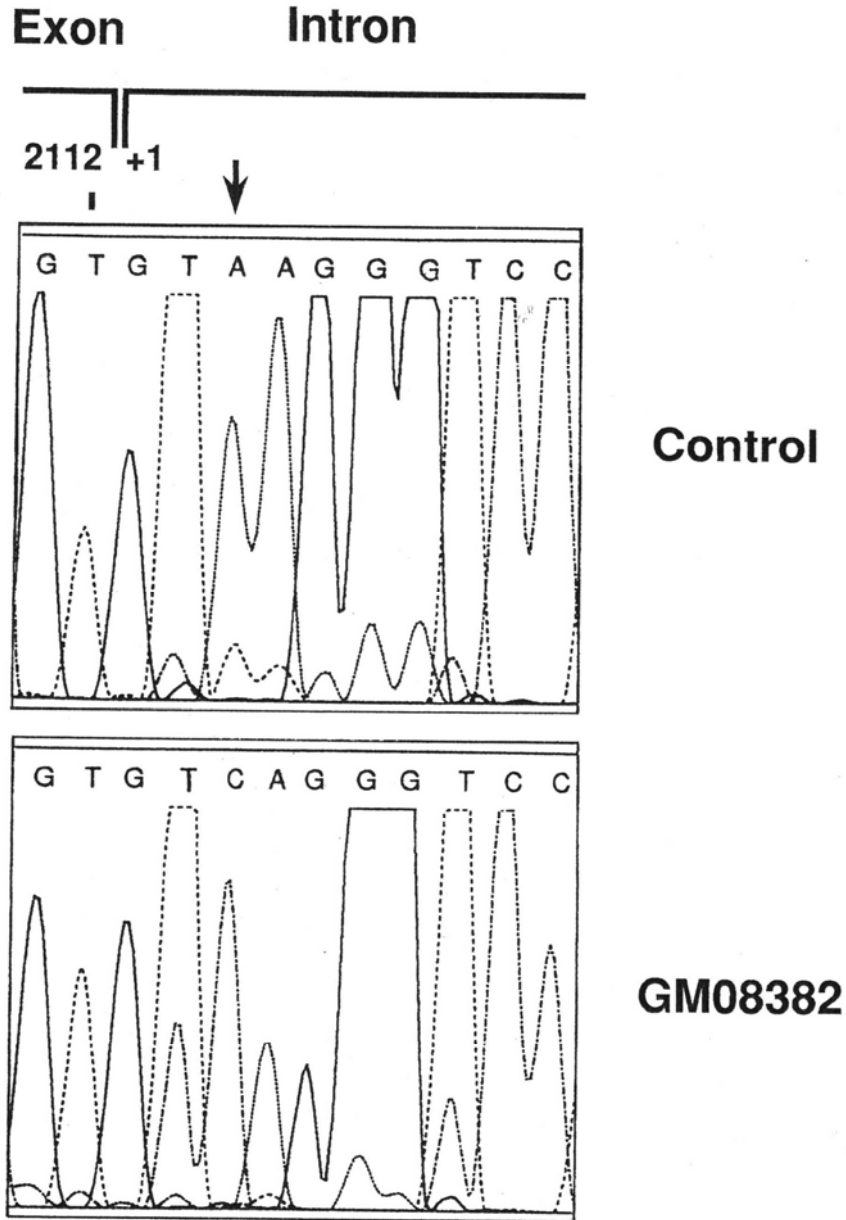**Fig. 2c A - C mutation in *TRKA* from Ecuadorian patient GM08382.**

Fig. 2*c*, A - C mutation in *TRKA* from Ecuadorian patient GM08382.

The sequence of the 5' splice site of an intron between exon D and E contains a point

mutation substituting a C for A in the third position.

Fig. 2d

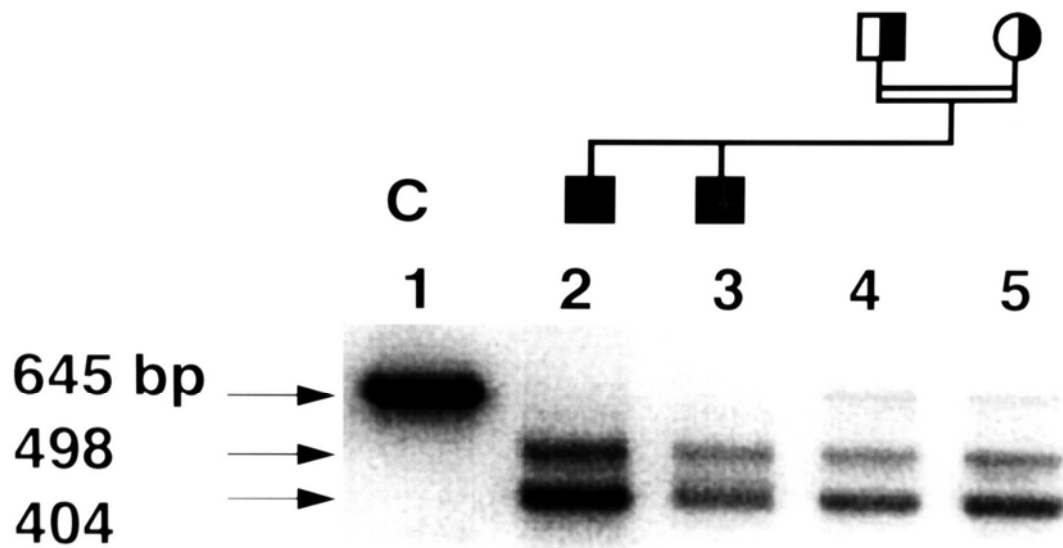**Analysis of RT-PCR products from the Ecuadorian family.**

Fig. 2*d*, Analysis of RT-PCR products from the Ecuadorian family.

A set of primers was used to amplify a product extending from part of the tyrosine kinase

domain to the C terminus. A single RT-PCR product of 645 bp was amplified from a control

(lane 1). In contrast, two small fragments with 498 and 404 bp were amplified from the

proband, GM08382 (lane 2) and an affected brother, GM08383 (lane 3). Analysis of

consanguineous parents, GM08384 (lane 4) and GM08385A (lane 5), show all three

fragments.

Fig. 2e

Restriction digestion analysis in the genomic region around the mutation in GM08382.
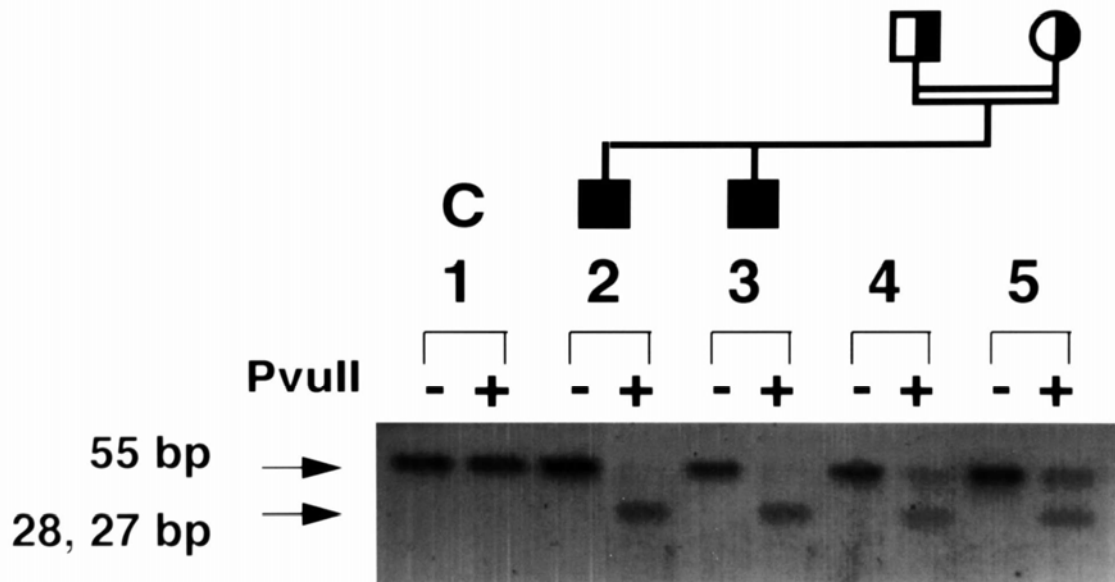

Fig. 2*e*, Restriction digestion analysis in the genomic region around the mutation in GM08382.

PCR products of 55 bp were digested with *Pvu*II. There is no *Pvu*II site in the normal control sequence (lane 1). However, the A-C transversion mutation creates a *Pvu*II site, producing cleavage of fragments of 28 and 27 bp. (Resolution of these two bands was not apparent in the 4% agarose gel.)

Fig. 2f

Sequence of the region around *TRKA* nucleotide 1795 of KI-02, demonstrating a G - C transversion (arrow) at this nucleotide.
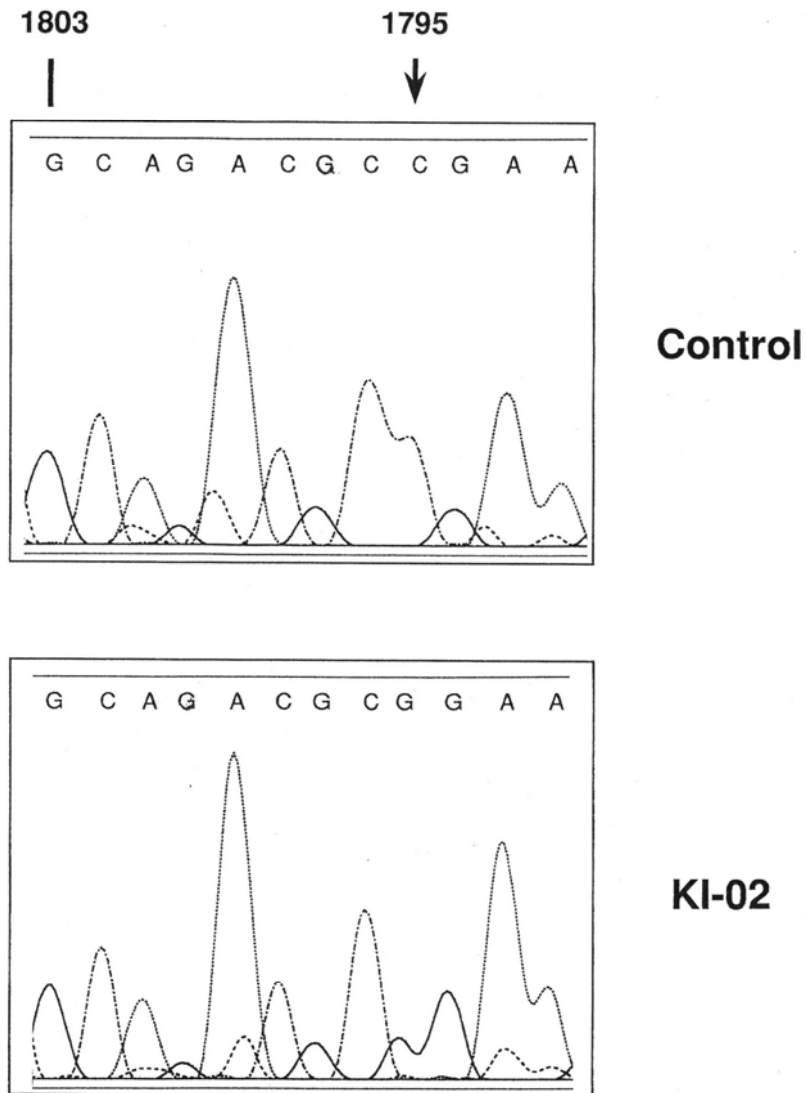

Fig. 2*f*, Sequence of the region around *TRKA* nucleotide 1795 of KI-02, demonstrating a G - C transversion (arrow) at this nucleotide.

Exon C was amplified from genomic DNA and complementary sequences of control and KI-02 are shown. The mutation changes codon GGC to CGC (Gly571Arg).

Fig. 2g

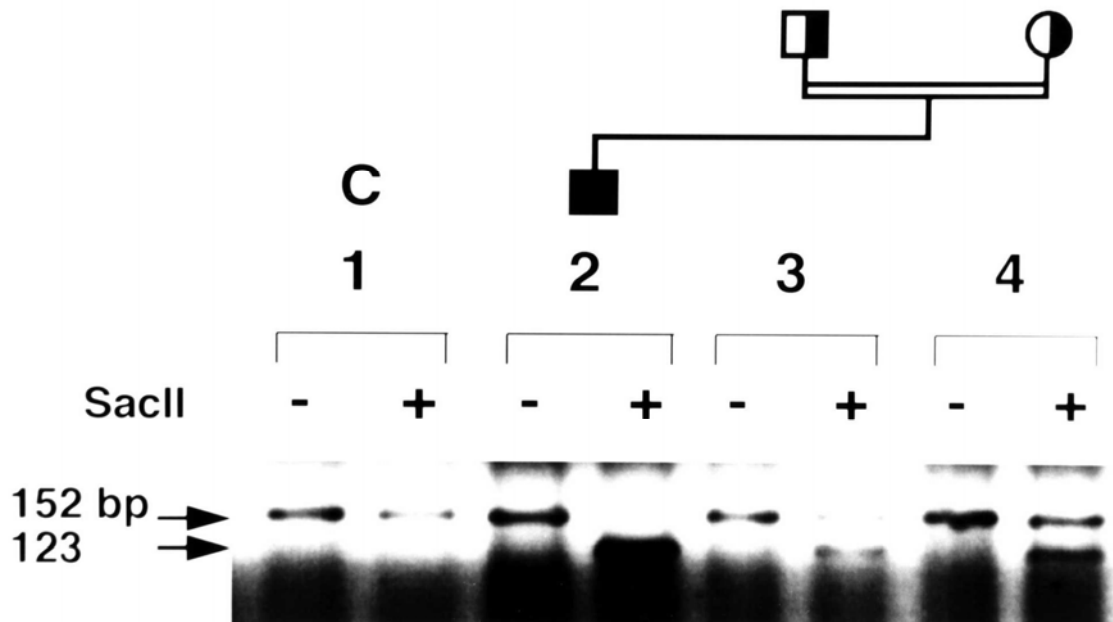**Restriction digestion analysis of the genomic region around the mutation of KI-02.**

Fig. 2*g*, Restriction digestion analysis of the genomic region around the mutation of KI-02. PCR products of 152bp were digested with *Sac*II. A *Sac*II site, absent in the normal control sequence (lane 1), is created by the G-C transversion at nucleotide 1795 results in fragments of 123 and 29 bp. Only the 123bp-fragments are shown.

Fig. 3

Conservation of a mutated residue in *TRKA* in various growth factor receptors.


```
                565              571    573
                O O            O   O  ●
TrkA    HIVRFFGVC
TrkB    HIVKFYGVC
TrkC    HIVKFYGVC
IR      HVVRLLGVv
IGF-IR  HVVRLLGVv
c-ros   NILKILGVC
met     NVLsLLGIC
eph     HILHLeGVv
EGFR    HVcRLLGIC
erbB2   yVsRLLGIC
ret     HVIKLYGaC
CSF-IR  NIVnLLGaC
PDGFR   NVVnLLGaC
c-kit   NIVnLLGaC
```

Fig. 3 Conservation of a mutated residue in *TRKA* in various growth factor receptors. Shown is the region of the tyrosine kinase domain flanking Gly571, mutated in KI-02 (alignment based on refs 24, 29). Uppercase indicates either identical or conserved residues. Lowercase indicates unrelated residues. Closed and open circles show residues shared by all receptors listed and conserved residues shared by at least 13 of the 14 receptors, respectively. Amino acid 565 to 573 were aligned with those corresponding to the homologous regions of the human cell surface receptor for TrkB, TrkC, insulin (IR), IGF-1 (IGF-IR), EGF, and CSF-1, the mouse PDGF receptor, products of the human homologue of the retroviral v-*ros* (c-*ros*) and v-*kit* (c-*kit*) oncogenes, those of the human *ret* and *met* proto-oncogene, the *erbB2* and the *eph* gene.