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### A Novel NTRK1 Mutation Associated with Congenital Insensitivity to Pain with Anhidrosis

*To the Editor:*

Among the hereditary sensory and autonomic neuropathies (HSAN), type IV (HSAN IV), also known as congenital insensitivity to pain with anhidrosis (CIPA; MIM 256800), is a rare autosomal recessive disorder clinically characterized by loss of pain sensitivity, self-mutilating behavior, unexplained episodes of fever, anhidrosis, and mental retardation (Rosemberg et al. 1994; Axelrod 1996). Ultrastructural and morphometric studies of the peripheral nerves reveal loss of unmyelinated and small myelinated fibers and no innervation of sweat glands (Langer et al. 1981; Axelrod and Pearson 1984). These features suggest that a defect in the differentiation and migration of neuronal crest elements and a possible deregulation of the NGF/NTRK1 pathway may be responsible for CIPA. It has been reported recently that the NTRK1/NGF receptor gene is implicated in the pathogenesis of CIPA (Kaplan et al. 1991; Klein et al. 1991; Smeyne et al. 1994; Fagan et al. 1996); point mutations within its tyrosine kinase (TK) domain have been detected in four patients with consanguinous parents (Indo et al. 1996). We have recently reported that one of those mutations, a Gly→Arg substitution at codon 571, exerts a loss-of-function effect (A. Greco, R. Villa, L. Fusetti, V. Ranzi, R. Orlandi, and M. Pierotti, unpublished data). We have shown that the NTRK1 TK activity and biological effects are abolished when the Gly571Arg mu-

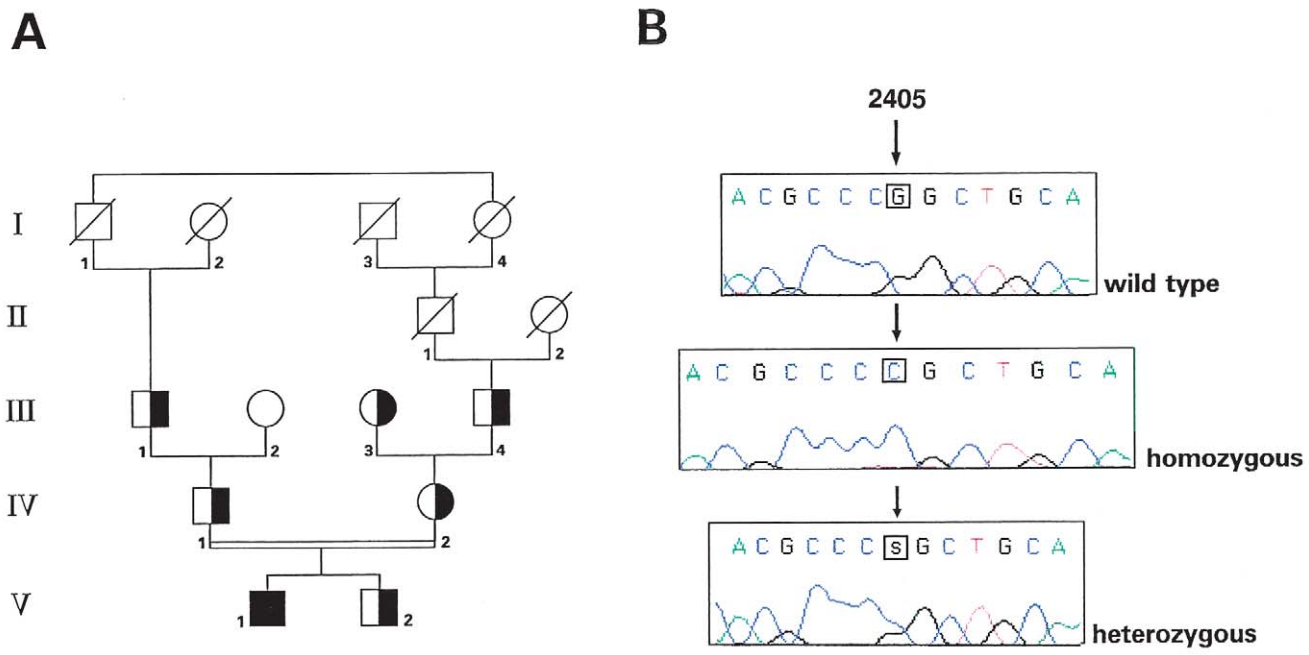


**Figure 1** Detection of NTRK1 Arg774Pro mutation in an HSAN IV patient. *A*, Pedigree of the family analyzed in this work. Blackened symbols denote patients with HSAN IV homozygous for the 774 mutation (proband); half-blackened symbols denote unaffected individuals heterozygous for the 774 mutation; unblackened symbols denote unaffected individuals homozygous for the wild-type NTRK1 sequence; slashed symbols denote unexamined individuals. Individual II-1 is reported to have insensitivity to pain. *B*, Sequence analysis of NTRK1 exon 17 in the proband family. Exon 17 was amplified from genomic DNA by PCR with primers 17F (5′-GGACTGGCCTCACTCTCTTG-3′) and 17R (5′-GTCATCCCAATAACTGGCAAG-3′). The amplified fragments were subjected to nucleotide sequence with an ABI Prism 377 DNA sequencer (Perkin-Elmer). The proband (V-1) shows a G→C transversion at nucleotide 2405 (codon 774); individual III-2 displays the wild-type sequence; in all other family members a mix of C and G (S, according to the International Union of Biochemistry code) is detected at position 2405.

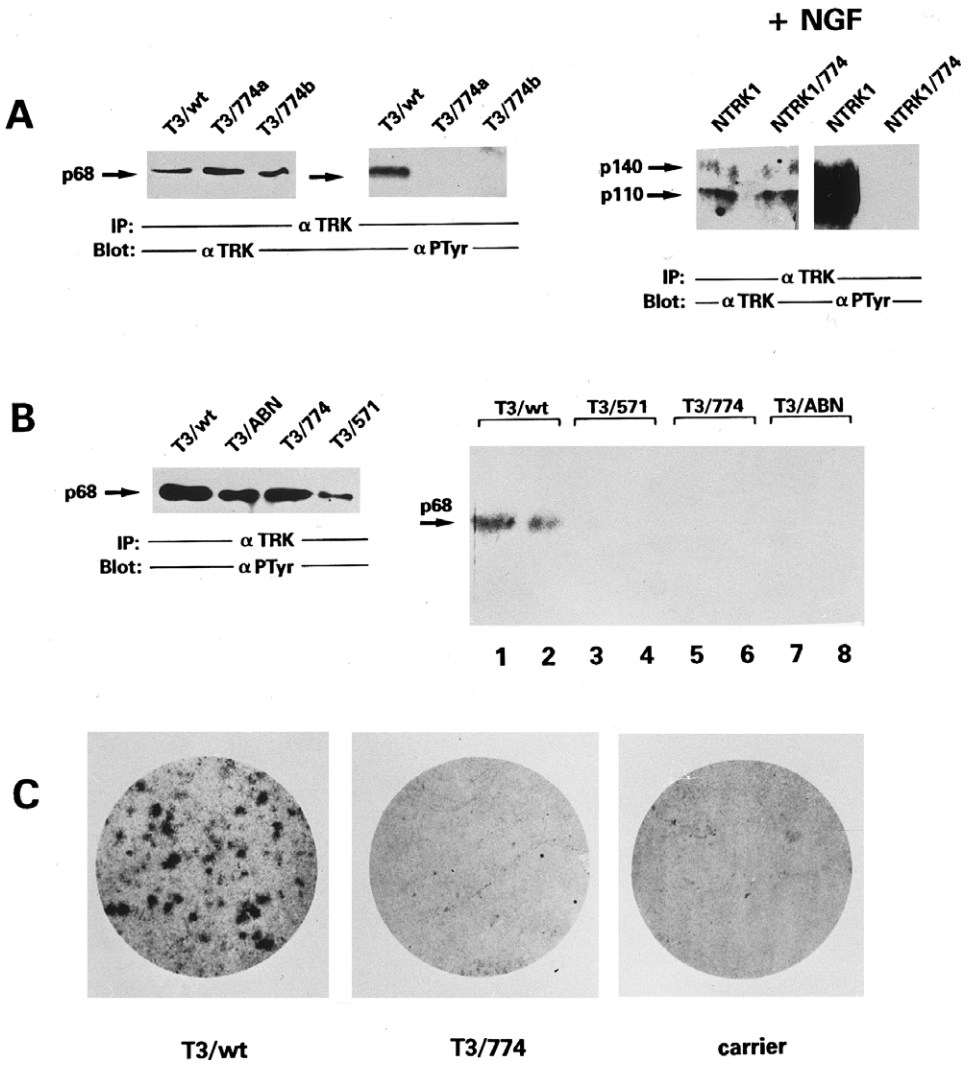


**Figure 2** Biochemical and biological analysis of TRK-T3 and NTRK1 proteins carrying the Arg774Pro mutation. *A,* Expression and phosphorylation of wild-type (wt) and mutated proteins. wt and mutated TRK-T3 and NTRK1 cDNAs were cloned into the pRC/CMV expression vector. COS1 cell transfection and western blot analysis were performed as described elsewhere (Greco et al. 1998). Two different TRK-T3 mutated clones are shown (T3/774 *a* and *b*). Cells transfected with the NTRK1 constructs were treated with NGF (50 ng/ml) for 10 min before extraction. *B,* In vitro immunocomplex kinase assay. wt, ATP-binding negative (ABN; Greco et al. 1998), 774, and 571 (A. Greco, R.Villa, L. Fusetti, V. Ranzi, R. Orlandi, and M. Pierotti, unpublished data) mutant TRK-T3 proteins transiently expressed into COS1 cells were immunoprecipitated with anti-TRK antibody and blotted with the same antibody (*left*) or subjected to immunocomplex-kinase assay (*right*). In the latter, two replicates for each sample are shown. *C,* Transforming activity of T3/774 protein. NIH3T3 cells were transfected with 500 ng of the indicated plasmid DNA by the calcium phosphate precipitation method, using 30 μg HMW NIH3T3 DNA as carrier. Transfected cells were selected in a G418-containing medium or in a 5% serum medium. G418-resistant colonies and transformed foci were fixed and stained after selection for 2 weeks. Both constructs produced an equivalent number of G418-resistant colonies.

tation is introduced into both the NTRK1 receptor and the constitutively active TRK-T3 oncogene (Greco et al. 1995).

Here we report the detection of a novel NTRK1 mutation associated with CIPA and demonstrate its inactivating effect. An Italian CIPA patient and his family (fig. 1) were screened for NTRK1 mutations. All the NTRK1 exons were amplified by PCR from peripheral blood lymphocite–DNA and sequenced by an ABI Prism 377 DNA sequencer (Perkin-Elmer). As a control, Hela DNA was amplified and sequenced simultaneously. A homozygous G→C transversion at nucleotide 2405 (exon 17, Arg→Pro substitution at amino acid 774) was detected in the proband. All the other members of the family, including a younger, unaffected brother, are heterozygous, except for the paternal grandmother, who is homozygous for the wild-type sequence. Interestingly, the Arg774Pro mutation was present in both maternal

grandparents, who had no documented consanguinity but were both from the same village.

To ascertain that the Arg774Pro mutation, rather than a rare polymorphism, was the cause of CIPA in the family, we performed functional studies, as reported for the Gly571Arg mutation (A. Greco, R. Villa, L. Fusetti, V. Ranzi, R. Orlandi, and M. Pierotti, unpublished data), in an attempt to unveil a loss-of-function effect. The Arg774Pro mutation was introduced into the constitutively active TRK-T3 oncogene and the wild-type NTRK1 receptor cDNAs (Greco et al. 1995) by site-directed mutagenesis. The mutated constructs (T3/774 and NTRK1/774) express protein of the expected molecular weight, as deduced by western blot analysis with anti-TRK antibodies (fig. 2*A*). Immunoblotting with antiphosphotirosine antibodies showed that the Arg774Pro mutation abrogates the constitutive phosphorylation of the TRK-T3 oncogene and the NGF-mediated phosphorylation of the wild-type NTRK1 receptor (fig. 2*A*). We analyzed the in vitro TK activity of mutant T3/774 with an immunocomplex autokinase assay. Two kinase-defective mutants (T3/ABN [Greco et al. 1998] and T3/571 [A. Greco, R. Villa, L. Fusetti, V. Ranzi, R. Orlandi, and M. Pierotti, unpublished data]) and the wild-type construct were used as negative and positive controls, respectively. As shown in figure 2*B*, the amounts of all the proteins were similar; the T3/774 and two kinase-defective mutants display an undetectable level of phosphorylation activity. In the NIH3T3 transfection/focus formation assay, no transformed foci arose from NIH3T3 cells transfected with T3/774 (fig. 2*C*), indicating that the introduced mutation abrogates the TRK-T3 transforming activity. All these findings demonstrate the loss-of-function effect of the Arg774Pro mutation, analogous to what was reported for the Gly571Arg mutation (A. Greco, R.Villa, L. Fusetti, V. Ranzi, R. Orlandi, and M. Pierotti, unpublished data).

In conclusion, we have detected the following novel NTRK1 mutation associated with CIPA: a G→C transversion at nucleotide 2405 causing an Arg→Pro substitution at codon 774. Biological and biochemical studies are consistent with a loss-of-function effect, demonstrating the pathogenic role of this mutation. Arg 774 is located at the C terminus of the NTRK1 TK domain; it is surrounded by residues conserved among the different members of TRK receptor family, but it is present only in NTRK1. Its role in receptor activation is unknown. However, our data outline the importance of such residues for NTRK1 activity. It is interesting to note that all reported mutations associated with HSAN IV occur within the NTRK1 TK domain. Other point mutations occurring in the extracellular portion of NTRK1 or mutations of other genes encoding neurotrophin receptors might cause other variants of HSAN.

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## Electronic-Database Information

Accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for CIPA [MIM 256800])

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## Mutations in the *RP2* Gene Cause Disease in 10% of Families with Familial X-Linked Retinitis Pigmentosa Assessed in This Study

*To the Editor:*

X-linked retinitis pigmentosa (XLRP; MIM 312600) is a severe form of retinal degeneration that typically presents as loss of the peripheral visual field and night blindness in affected males within the 1st or 2d decade of life, owing to primary degeneration of rod photoreceptor cells and bone spicule pigmentary deposits (Bird 1975). The disease is progressive: loss of central vision occurs later in life, and complete functional blindness often occurs by 40–50 years of age. *RP2* was the first genetically mapped retinitis pigmentosa (RP) locus (Bhattacharya et al. 1984) and showed disease segregation with the RFLP detected by the probe L1.28 (DXS7). Subsequent genetic-mapping studies have shown exceptional genetic heterogeneity for autosomal RP and XLRP, which is illustrated on the short arm of the X chromosome and on *RP2, RP3, RP15,* and *RP23* (Ott et al. 1990; McGuire et al. 1995; Thiselton et al. 1996; A. J. Hardcastle, D. L. Thiselton, T. S. Mah, M. B. Gorin, and S. S. Bhattacharya, unpublished data). Extensive homogeneity and heterogeneity analyses of worldwide XLRP family collections suggested that *RP3* (frequency 70%–75%) and *RP2* (frequency 20%–25%) are the predominant forms (Ott et al. 1990; Teague et al. 1994). The *RP3* gene was positionally cloned, facilitated by the identification of submicroscopic deletions in affected patients (Meindl et al. 1996; Roepman et al. 1996). The causative gene, a putative guanine-nucleotide-exchange factor (*RPGR*), appears to be mutated in only 15%–20% of patients with XLRP (Meindl et al. 1996; Roepman et al. 1996; Buraczynska et al. 1997), not the 70%–75% expected from the results of genetic-mapping studies (Teague et al. 1994). Investigation of splice variants, promoter/enhancer mutations, and as yet unidentified exons may account for the low mutation rate detected in this gene; however, a more likely explanation for these findings is microheterogeneity. In comparison, genetic mapping suggested that *RP2* is rare (15%–20% of patients with XLRP), and the close proximity to *RP3,* together with a lack of disease-associated deletions, has hampered the search for the *RP2* gene for many years. We reported haplotype analysis for two pedigrees, which defined both proximal and distal boundaries of the *RP2* critical interval to a region of 5 cM between *DXS8083* (Xp11.3) and *DXS6616* (Xp11.23) (Thiselton et al. 1996) and which excluded the candidate gene *TIMP1* (Hardcastle et al. 1997*b*). The *RP2* gene recently has been positionally cloned by targeting this interval by use of the YAC representation hybridization technique (Schwahn et al. 1998). *RP2* consists of five exons, encodes a polypeptide of 350 amino acids, and is ubiquitously expressed. The predicted amino acid sequence has homology to cofactor C, over 151 amino acids (30.4%), which is involved in $\beta$-tubulin folding (Schwahn et al. 1998). Currently, this provides the only functional clue for the *RP2* gene; hence, the overall function and specific role in the retina are unknown at present. We have screened our panel of patients with XLRP for mutations in the *RP2* gene, to define the spectrum of mutations causing disease. Of particular interest were the two definitive genetically defined families with *RP2* that we had described previously (families F72 and NRP; Thiselton et al. 1996).

Appropriate informed consent was obtained from patients and relatives. Of a total of 59 families with XLRP assessed in this study, 26 families were excluded from *RP2* gene mutation screening, because haplotype and linkage analyses excluded the *RP2* interval or because *RPGR* mutations had already been identified. The remaining 33 families with XLRP were assessed for *RP2* mutations.

DNA from affected male patients from each of the 33 pedigrees (and two normal male controls) was amplified with primer pairs described by Schwahn et al. (1998), by use of 150 ng of initial template DNA and with minor modifications to the suggested annealing temperatures (details available on request from the authors). Purified PCR products (8 $\mu$l of product incubated with 1 U shrimp alkaline phosphatase [SAP; Amersham Life Science] and 1 U Exonuclease I [United States Biochemical] in SAP buffer, at 37°C for 30 min followed by 80°C for 15 min) were aliquoted (5 $\mu$l) for cycle sequencing in the forward and reverse directions, by use of the ABI Prism Ready Reaction Dye Terminator cycle sequencing kit (fluorescent sequencing kit, Perkin-Elmer), in accordance with the manufacturer's instructions. Reactions then were electrophoresed on an ABI 373A automated sequencer.

Segregation of the mutation, with the disease, was