The M581V Mutation, Associated with a Mild Form of Congenital Insensitivity to Pain with Anhidrosis, Causes Partial Inactivation of the NTRK1 Receptor

To the Editor:

Congenital insensitivity to pain with anhidrosis (CIPA; MIM 256800), also known as hereditary sensory and autonomic neuropathy type IV, is a rare autosomal recessive disorder associated with consanguinity (Axelrod et al, 1984; Axelrod, 1996) and is characterized by the absence of pain and temperature sensation and the lack of sweating. CIPA patients are generally children; they have recurrent episodes of unexplained fever, self-mutilation behavior, and occasionally mental retardation (Rosenberg et al, 1994). CIPA is the consequence of a genetic defect in the differentiation and migration of neural crest elements involved in nociceptive reception as well as in thermal regulation. Recently, mutations of the NTRK1 gene have been implicated in the pathogenesis of this disease. So far 37 mutations of NTRK1 have been detected in patients from different ethnic groups by several laboratories, including ours. Mutation types include frameshift, non-sense, splice site, and missense (Indo, 2001).

The NTRK1 gene encodes one of the receptors of the nerve growth factor (NGF). NTRK1 protein is a tyrosine kinase receptor comprising an extracellular portion, involved in NGF binding, a single transmembrane region, a juxtamembrane domain, a tyrosine kinase domain, and a C-terminal tail. Most of CIPA mutations occur within the NTRK1 tyrosine kinase domain and a few within the extracellular domain. Recently, we analyzed the biologic effect of several NTRK1 germline missense mutations and discovered at least three different pathogenic mechanisms responsible for the disease. Most of the mutations inactivate the NTRK1 receptor by completely abrogating its catalytic activity. The L213P mutation, in the extracellular domain, interferes with the receptor processing, causing its retention in the endoplasmic reticulum. The third mechanism, described for mutation D668Y, involves a reduction of the NTRK1 receptor activity, which is unlikely to be sufficient for the proper neuronal differentiation (Greco et al, 1999, 2000; Miranda et al, 2002).

Recently the M581V mutation, occurring within subdomain V (β5 strand) of the NTRK1 tyrosine kinase domain, has been detected in a large Japanese CIPA family. The three affected individuals were adults and displayed milder clinical symptoms compared with other CIPA patients, including normal temperature sensation and a relatively long survival. Two patients were homozygous for the M581V mutation, whereas the third one was a compound heterozygous with a combination of M581V mutation and C1726 deletion (Yotsumoto et al, 1999).

We investigated the effect of the M581V mutation on NTRK1 receptor activity. The mutation was introduced into the NTRK1 cDNA subcloned into the pRC/CMV mammalian expression vector, which carries the G418-resistance gene as a selectable marker. The NTRK1/M581V and NTRK1/wt constructs were transiently transfected into COS1 cells. Transfected cells were treated with or without NGF (50 ng per ml) for 10 min or 16 h, and cell extracts were immunoprecipitated with the MGR12 antibodies, which are specific for the receptor extracellular portion. Western blot analysis with anti-TRK antibodies (reacting with the intracellular NTRK1 portion) showed that the NTRK1/M581V receptor produced the 110 and 140 kDa forms indicating its proper expression (Fig 1A, upper panel). After western blot analysis with anti-phosphotyrosine antibodies, we detected a basal autophosphorylation of the wild-type receptor (due to the high expression level), which increased after NGF treatment. With respect to the NTRK1/M581V mutant, no phosphorylation was observed in untreated cells. NGF treatment induced a weak phosphorylation, highly reduced with respect to wild type, at 10 min and 16 h (Fig 1A, lower panel). The low tyrosine phosphorylation level achieved by NTRK1/M581V mutant after a long NGF treatment suggests a defect in the intrinsic kinase activity, rather than in the autophosphorylation kinetics. To investigate whether a deregulated phosphatase activity might account for the reduced phosphorylation, phosphorylation assays were performed on untreated and NGF-stimulated NTRK1-transfected cells. The M581V mutation caused a delayed, but almost complete, autophosphorylation with respect to the wild-type receptor. The effect of the M581V mutation on kinase activity was confirmed by in vitro kinase activity assay. 

Figure 1. Biochemical analysis of NTRK1 receptor carrying the M581V mutation. The M581V mutation was introduced by site-directed mutagenesis (Gene Editor, Promega) into the NTRK1 cDNA inserted into the pRC/CMV expression vector. The sequence of the oligonucleotide is the following: 5′-CCCTGTCGTGTCCTCG3′ (the mutated nucleotide is underlined). Mutated clones were identified by nucleotide sequence of a PCR fragment spanning the mutation. A selected mutant clone was subjected to a complete nucleotide sequence to exclude possible additional mutations that accidentally occurred during the mutagenesis reaction. One microgram of the indicated plasmid DNA was transfected in combination with 19 μg of carrier pRC/CMV DNA into COS1 cells as previously described (Miranda et al, 2002). Two days later, cells were serum starved overnight then subjected to the indicated treatments. (A) Expression (top) and phosphorylation (bottom) of wild-type and mutated proteins. Cells were treated with 50 ng NGF per ml for the indicated times, lysed with PLCBL buffer and subjected to immunoprecipitation using MGR12 antibodies directed towards the extracellular portion of NTRK1 (Tagliabue et al, 1999). Immunoprecipitates were resolved in 6.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis then subjected to western blotting with anti-TRK (Santa Cruz Biotechnology, Inc.) or anti-phosphotyrosine (Upstate Biotechnology, Inc.) antibodies and revealed by enhanced chemiluminescence (Amersham Biosciences). (B) Effect of sodium orthovanadate (Na3VO4) on NTRK1/M581V tyrosine phosphorylation. Transfected COS1 cells were treated with 5 mM Na3VO4 for 2.5 h then subjected to western blot analysis as described above.
presence of both 1 and 2 ng per ml of NGF, in agreement with previous data (Miranda et al, 2002). The M581V receptor produced foci only in the presence of 2 ng per ml of NGF, with strongly reduced efficiency with respect to the wild type. The transforming activity of D668Y mutant, used as a control, was significantly reduced with respect to the wild type, as previously reported (Miranda et al, 2002), but slightly higher than M581V.

We have shown that the M581V mutation causes a reduction of activity of the NTRK1 receptor and therefore we have identified an important residue for receptor activity. The molecular structure of NTRK1 receptor remains unsolved; however, a model of its inactive and active forms can be produced by analogy of the insulin receptor kinase. In this model methionine 581 is totally buried in a hydrophobic environment. Valine, despite being equally hydrophobic, is definitely smaller and could cause destabilization of this part of the molecule leading to a reduction in receptor responsiveness but not to complete inactivation. Thus, the residual activity may be sufficient for some of the NGF effects, and would explain the mild phenotype of the CIPA patients carrying the M581V mutation.

In conclusion, our data indicate that a reduction in NTRK1 receptor activity may be a common mechanism in CIPA disease, being ascribed to two different mutations, the D668Y and the M581V, so far reported.

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