# Painful Painlessness

## A novel gene, ENTPD1, and a novel nonsense mutation in NTRK1 (Glu707X) implicated in the phenotype of Congenital Insensitivity to Pain

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## Abbreviations

| | |
|---|---|
| ADP | Adenosine Diphosphate |
| AKT1 | RAC-alpha Serine/Threonine Protein Kinase |
| ALDH18A1 | Aldehyde Dehydrogenase 18 family, member A1 |
| AMP | Adenosine Monophosphate |
| ATL1 | Atlastin1 |
| ATP | Adenosine Triphosphate |
| CCNJ | Cyclin J |
| CD | Cluster of Differentiation |
| CIPA | Congenital Insensitivity to Pain |
| DEPC | Diethyl Pyrocarbonate |
| DNA | Deoxyribo Nucleic Acid |
| DNMT1 | DNA Methyltransferase1 |
| dNTPs | deoxy Nucleoside Triphosphates |
| DRG | Dorsal Root Ganglia |
| EBV | Epstein Barr Virus |
| ENTPD1 | Ectonucleoside Triphosphate Phosphodihydrolase |
| EtBr | Ethidium Bromide |
| FAM | Flourescein Amidite |
| FAM134B | Family with sequence similarity 134, Member B |
| FD | Familial Dysautonomia |
| FRS2 | Fibroblast growth factor Receptor Substrate 2 |
| GAPDH | Glyceraldehyde 3 Phosphate Dehydrogenase |
| Glu | Glutamic Acid |
| GRB2 | Growth factor Receptor Bound Protein 2 |
| HIV | Human Immunodeficiency Virus |
| HSAN | Hereditary Sensory and Autonomic Neuropathy |
| HSN | Hereditary Sensory Neuropathy |
| IKAP | IkB Kinase complex-associated Protein |
| IKBKAP | Inhibitor of kappa light polypeptide gene enhancer in B-cells, Kinase complex-associated Protein |
| KIF1A | Kinesin Family member 1A |

| | |
|---|---|
| MAPK | Mitogen Activated Protein Kinase |
| MGB | Minor Groove Binder |
| NCBI | National Centre for Biotechnology Information |
| NF-K-B | Nuclear Factor kappa B |
| NGFB | Nerve Growth Factor β |
| NTRK1 | Neurotrophic Tyrosine Kinase receptor type 1 |
| PCR | Polymerase Chain Reaction |
| PI3 | Phosphoinositide 3 |
| PLCG1 | Phospholipase C, gamma 1 |
| RAB7 | member RAS oncogene family |
| RNA | Ribo Nucleic Acid |
| ROX | Roxithromycin |
| RPMI | Roswell Park Memorial Institute medium |
| RT | Reverse Transcription |
| SH2B1 | SH2B adaptor protein 1 |
| SH2B2 | SH2B adaptor protein 2 |
| SHC1 | Src Homology 2 domain containing transforming protein 1 |
| SCN9A | voltage gated Sodium Channel Nav1.7 α subunit |
| SPTLC1 | Serine Palmitoyltransferase, Long Chain base subunit-1 |
| SPTLC2 | Serine Palmitoyltransferase, Long Chain base subunit-2 |
| TCTN3 | Tectonic family member 3 |
| WNK1 | Lysine deficient Protein Kinase 1 |
| X | Stop Codon |

## Abstract

Hereditary sensory and autonomic neuropathies (HSANs) are a group of rare congenital disorders, all characterized by widespread sensory and variable autonomic dysfunction due to faulty development of autonomic and sensory neurons. The current classification of the HSAN group of disorders divides the syndromes into five subtypes – HSAN I – V. Hereditary sensory and autonomic neuropathies (HSANs) are a clinically and genetically heterogeneous group of neurological conditions characterized by peripheral nerve degeneration resulting in a loss of sensory perception accompanied by variable degrees of autonomic dysfunction.

There were two patients studied in this respect. Patient 1 has a chromosomal translocation between chromosomes 10 and 22 which has led to a break in the gene ENTPD1 on chromosome 10. Patient 2 has a normal karyotype. Both patients show symptoms of insensitivity to pain and corneal opacity.

This study was undertaken to determine the genetic cause of symptoms of pain insensitivity in the two patients. Known genes were sequenced in both patients to probe the possible involvement of them in the phenotype. Also, as the chromosomal translocation in patient 1 led to the disruption of ENTPD1 gene, the possibility of this gene being involved in the phenotype was also probed.

There are 12 documented genes responsible for different subtypes of HSANs and their specific phenotypes. However, as per the patients' phenotype, the genes tested for possible involvement were NTRK1, NGFB, IKBKAP, WNK1, KIF1A, FAM134B and SCN9A. These genes are autosomal recessive and are responsible for early onset HSAN without motor involvement.

Also, the levels of gene expression of ENTPD1 and genes immediately upstream and downstream to it were studied in patient 1 by Real time PCR. These experiments revealed a significant downregulation of ENTPD1 in the patient.

In patient 2, a novel change was identified in NTRK1 which leads to the conversion of GAG (for Glutamic acid) to a stop codon which might lead to the absence of a significant portion of the kinase domain which is involved in downstream signaling processes.

## Introduction

Pain is an essential sense that has evolved in all complex organisms to minimize tissue and cellular damage, and hence prolong survival. The onset of pain results in the adoption of behaviours that both remove the organism from a 'dangerous environment' and allow for tissue repair. Pain pathways operate at numerous levels in the nervous system and are under both voluntary and involuntary control. (1)

Whereas individuals with a congenital absence of the sense of vision or of hearing are relatively common, a congenital absence of the sense of pain is very rare. Only a handful of such patients have since been described and are usually categorized as having 'congenital insensitivity to pain' (CIPA). (1)

Hereditary sensory and autonomic neuropathies (HSANs) are a group of rare congenital disorders, all characterized by widespread sensory and variable autonomic dysfunction due to faulty development of autonomic and sensory neurons. Though clinically similar, they are genetically distinct entities resulting from mutations in different genes. The current classification of the HSAN group of disorders divides the syndromes into five subtypes – HSAN I – V. (2) Differential diagnosis between the five main HSAN subtypes is based on their mode of inheritance, pathology, natural history, biochemical, neurophysiologic and autonomic abnormalities. (3)

Hereditary sensory and autonomic neuropathies (HSANs) are a clinically and genetically heterogeneous group of neurological conditions characterized by peripheral nerve degeneration resulting in a loss of sensory perception accompanied by variable degrees of autonomic dysfunction. (4) Peripheral nerve diseases are common, with a prevalence of about 2.5%. Among the most frequent causes are diabetes mellitus, alcohol abuse, toxins, amyloidosis, infections such as HIV or leprosy, and hereditary conditions. (5)

The genes responsible for different subtypes of HSANs and their specific phenotypes are given in Table 1. However, as per the patients' phenotype, the genes tested for possible involvement were NTRK1, NGFB, IKBKAP, WNK1, KIF1A, FAM134B and SCN9A. These genes are autosomal recessive and are responsible for early onset HSAN without motor involvement.

**NTRK1**

Mutations in the Neurotrophic Tyrosine Kinase receptor type 1 (NTRK1) gene is a common cause of HSAN type 4, also termed congenital insensitivity to pain and anhidrosis (CIPA). (6) NTRK1 protein is a receptor tyrosine kinase, which is phosphorylated in response to nerve growth factor (NGF). NGF supports the survival of sympathetic ganglion neurons and nociceptive sensory neurons in the dorsal root ganglia. (7)

**NGFB**

Nerve Growth Factor β (NGFB) belongs to the neurotrophin family of proteins, which regulate neuronal survival, development and function. During development, the interaction of immature sympathetic and sensory neurons with β-NGF derived from peripheral targets is

| Type | Gene | Specific phenotype |
|---|---|---|
| HSAN I | SPTLC1 | Predominantly sensory neuropathy, frequent later motor involvement, neuropathic pain, ulcero-mutilating complications. |
| HSAN I | SPTLC2 | As for SPTLC1 |
| HSAN I | ATL1 | Sensory neuropathy without motor involvement, reflexes may be brisk, ulcero-mutilating complications. Spasticity has been described. |
| HSAN I | DNMT1 | Sensory neuropathy, sensorineural deafness with dementia developing in 4th decade |
| CMT2B | RAB7 | Sensorimotor neuropathy, ulcero-mutilating complications |
| HSAN II | WNK1 | Sensory neuropathy, severe ulcero-mutilating complications, frequent autonomic dysfunction |
| HSAN II | FAM134B | Sensory neuropathy, severe ulcero-mutilating complications, variable autonomic and motor involvement |
| HSAN II | KIF1A | Sensory neuropathy, severe ulceromutilating complications, mild motor involvement |
| HSAN III | IKBKAP | Familial dysautonomia or Riley-Day syndrome, prominent autonomic dysfunction, absent fungiform papillae of the tongue |
| HSAN IV | NTRK1 | Congenital insensitivity to pain with anhydrosis (CIPA), severe sensory neuropathy, anhidrosis, mental retardation, unmyelinated fibers mainly affected |
| HSAN V | NGFB | Congenital insensitivity to pain, minimal autonomic dysfunction, no mental retardation, mainly small myelinated fibers affected |

Table 1: Classification of hereditary sensory and autonomic neuropathies, SPLTC1 - serine palmitoyltransferase, long chain base subunit-1, SPTLC2 - serine palmitoyltransferase, long chain base subunit-2, ATL1- atlastin-1, RAB7 - member RAS oncogene family, DNMT1 - DNA methyltransferase 1, WNK1- nerve specific isoform of WNK lysine deficient protein kinase 1, FAM134B - family with sequence similarity 134, member B, KIF1A - kinesin family member 1A, IKBKAP - inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase complex-associated protein, NTRK1 – neurotrophic tyrosine kinase receptor type 1, NGFB - nerve growth factor beta polypeptide

indispensable for the growth and survival of these cells. In the postnatal period, β-NGF is superfluous for neuronal survival, but mediates inflammatory and immune responses after tissue injury by initiating and maintaining hypersensitivity to noxious stimuli, a phenomenon called peripheral sensitization. (8) Mutations in this gene are responsible for the phenotype of HSAN V.

**IKBKAP**

Inhibitor of κ-Light Polypeptide Gene Enhancer in B Cells, Kinase Complex-Associated Protein (IKBKAP) gene is involved in familial dysautonomia (FD), originally termed the Riley–Day syndrome (9). The IKBKAP gene encodes a protein termed IkB kinase complex-associated protein (IKAP), which is likely a component of the elongator complex and/or is a c-Jun N-terminal kinase-associated protein (7). FD represents HSAN type 3 in the classification. FD is an autosomal-recessive disorder with prominent central and peripheral autonomic perturbances, as well as small-fiber sensory dysfunction. It has been suggested that FD is the most prevalent of the HSAN types and also the most intensively studied. (7)

**WNK1**

The WNK1 gene is involved in an autosomal recessively inherited form of HSAN, corresponding to HSAN type 2. HSAN2 is characterized by early-onset sensory neuropathy in the first two decades. WNK1 stands for lysine-deficient protein kinase 1 gene. (7) WNK1 belongs to the WNK family of serine–threonine kinases—osmotic sensors that regulate sodium, chloride and potassium homeostasis. (8) Its function is unknown, but it is suggested that the protein may play a role in the development or maintenance of peripheral sensory neurons or their supporting cells. (7)

**KIF1A**

Mutations in the kinesin family member 1A gene, KIF1A, have been identified with a typical HSAN-II phenotype but without autonomic disturbances. KIF1A is a molecular motor protein with a role in the anterograde axonal transport of synaptic vesicle precursors. Interestingly, KIF1A interacts with the domain of WNK1 encoded by the HSN2 exon. The functional effect of this interaction is still unknown. (8)

**FAM134B**

Mutations in FAM134B have been described in families with HSAN-II. The function of FAM134B is poorly understood, although it is known to be a component of the *cis*-Golgi matrix, and has a role in shaping and tethering the membrane stacks of this structure. FAM134B is predominantly expressed in sensory and autonomic ganglia; this preferential expression in peripheral nerves might provide a basis for the PNS-restricted phenotype in patients with HSAN-II. (8)

**SCN9A**

SCN9A encodes Nav1.7, the a-subunit of a tetrodotoxin-sensitive voltage-gated sodium channel that is expressed at high levels in peripheral sensory neurons, most notably in nociceptive small-diameter dorsal root ganglia (DRG) neurons. Voltage-gated sodium channels underlie the depolarizing phase of action potentials in excitable cells and tissue-

specific expression of the various family members help to shape the excitability and repetitive firing properties of different neurons. (1)

Apart from the possible involvement of the above known genes, one of the patients is seen to have a chromosomal translocation between chromosomes 10 and 22 which leads to a breakpoint in the gene ENTPD1 on chromosome 10 (described in detail later). ENTPD1, also known as CD39, is an ectonucleotidase that hydrolyzes ATP and ADP to AMP, initiating an enzymatic cascade that leads to the generation of adenosine receptors. By regulating nucleotide levels, ENTPD1 controls the activity of two purinergic receptors (P2X and P2Y) and downstream adenosine (P1) receptors. ENTPD1 is widely expressed, particularly in blood vessels and on cells of the immune system, glomeruli, afferent arterioles, and larger vessels of the kidney. (10)

We have explored the possibility of the involvement of ENTPD1 in the phenotype of the patient, as a consequence of its downregulation due to its translocation from chromosome 10 to 22, by excluding the possible involvement of documented genes, and by demonstrating the levels of ENTPD1 in the patient as compared to controls. At the same time, we have also tested the expression of genes upstream and downstream ENTPD1 to study the extent of the effect of the translocation on either side on the chromosome. As a result, we have studied the expression of ALDH18A1 (Aldehyde Dehydrogenase 18 family, member A1) and TCTN3 (Tectonic family member 3) upstream ENTPD1 and CCNJ (Cyclin J) downstream ENTPD1.

## Materials and methods

## Case report

Patient 1: The child is 6 years old and shows symptoms of pain insensitivity and corneal opacity. The child has normal intelligence. Karyotype analysis revealed a heterozygous translocation between chromosomes 10 and 22 as shown in Figure 1. The breakpoint is in the gene ENTPD1 on chromosome 10. The parents of the child have a consanguineous marriage, the father being married to his sister's daughter. The parents have a normal karyotype and are asymptomatic. The patient has a 7 month old sister who also has a normal karyotype and is asymptomatic.

Patient 2: The child has similar symptoms as patient 1 i.e. insensitivity to pain, corneal opacity and normal intelligence. The parents are asymptomatic and the patient has a normal karyotype.

## PCR and Sequencing

Genomic DNA was extracted from blood samples of both the patients and parents of patient 1. Primers were designed for all exons and exon-intron boundaries of the genes NTRK1, NGFB, FAM134B, KIF1A, WNK1, IKBKAP and SCN9A using the Primer3.0 software (11) and were ordered from Sigma-Aldrich®. The PCR conditions were standardized for each primer set and PCR was done using the thermal cycler. The PCR reaction mix contained 1X Taq Polymerase Buffer, 0.8 mM dNTPs, 1.5 mM $MgCl_2$, 0.25 µM of each primer, 0.05 U/µl Taq Polymerase (NEB) and 5 ng/µl DNA in a 20 µl reaction. The PCR products were

electrophoresed on 1.5% agarose gel containing 2 µl EtBr (2 µg/ml), and purified using a membrane filter plate. The sequencing reactions were performed using 0.8 µl of BigDye® Terminator v3.1 Cycle Sequencing reaction mix, 1X sequencing buffer, 0.25 µM primer and 2 µl of purified PCR product in a 20 µl reaction mix. The following cycling conditions were used: initial denaturation at 95 °C for 1 minute, followed by 25 cycles of denaturation at 96 °C for 10 seconds, annealing at 50 °C for 5 seconds and extension at 60 °C for 4 minutes, and a final hold at 4 °C. Sequencing was done in an automated DNA sequencer. Variations were identified by comparison with reference sequences obtained from NCBI and sequence analysis software (SeqMan™ II). Variants seen in the sequence were confirmed by sequencing with the second primer. The variants found were checked in NCBI (12), 1000genomes (13) and Exome Variant Server (14) to determine whether they were reported previously.

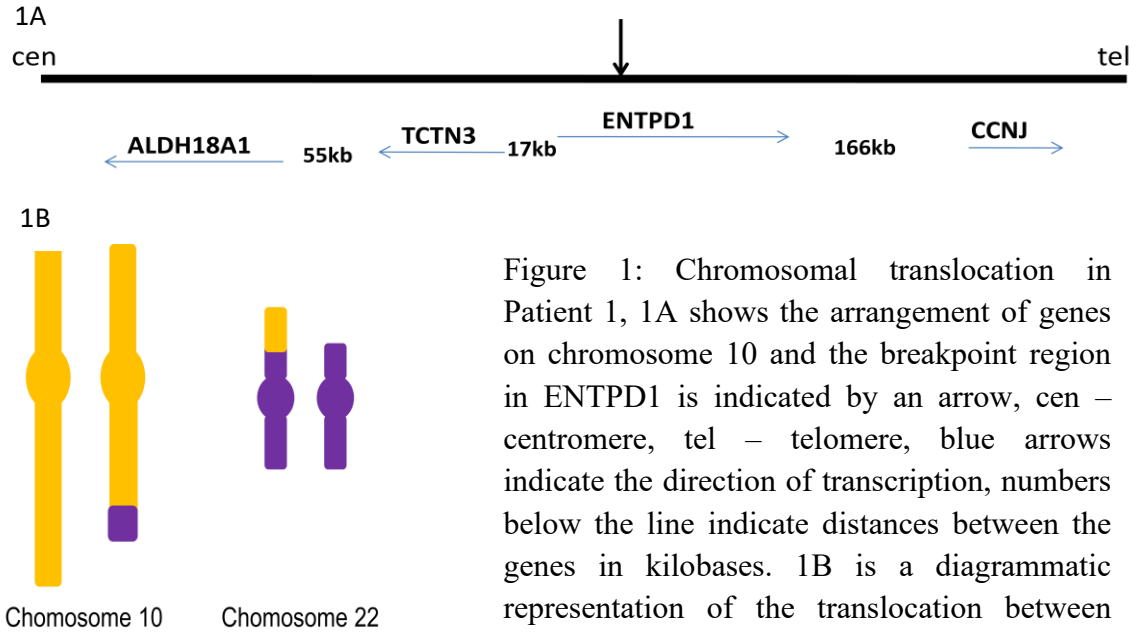



Figure 1: Chromosomal translocation in Patient 1, 1A shows the arrangement of genes on chromosome 10 and the breakpoint region in ENTPD1 is indicated by an arrow, cen – centromere, tel – telomere, blue arrows indicate the direction of transcription, numbers below the line indicate distances between the genes in kilobases. 1B is a diagrammatic representation of the translocation between chromosomes 10 and 22.

## RNA extraction and reverse transcription

The DNA of patient, parents and control were transfected in EBV cell lines and cultured in RPMI media for 14 days. RNA of patient, parents and control was extracted from EBV transformed cell lines as follows. The cells were centrifuged at 1000 rpm for 10 minutes at room temperature and the pellets were washed with 3 ml RPMI. The pellets were resuspended in TRIZOL® Reagent (Gibco BRL) (1 ml per $cm^2$ of the culture dish). The tubes were incubated at room temperature for 5 minutes and centrifuged at 1000 rpm for 10 minutes at room temperature. The supernatant was collected in fresh tubes and chloroform (0.2 ml per ml of TRIZOL® Reagent used) was added and the contents were mixed by inverting the tubes. The tubes were incubated at room temperature for 3 minutes and

centrifuged at 12,000 rpm for 15 minutes at 4 °C. The upper aqueous layer was transferred to fresh tubes and Isopropyl alcohol (0.5 ml per ml of TRIZOL® Reagent used) was added and the contents were mixed gently by inverting the tubes. The tubes were incubated at room temperature for 10 minutes and centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was discarded and the pellets were washed twice with 1 ml 75% ethanol by inverting the tubes to mix the contents followed by centrifugation at 7500 rpm for 5 minutes at 4 °C. The RNA pellets were air dried and dissolved in 50 µl of DEPC-treated water. The samples were electrophoresed on a 1% agarose gel (Figure 2) and their concentrations were determined using Nanodrop spectrophotometer.

The RNA samples were treated with DNase I in a reaction mix containing 1X DNase I Reaction Buffer (NEB), 2 U DNase I enzyme and 10 µg RNA in a 100 µl reaction mix. The tubes were incubated at 37 °C for 30 minutes and then EDTA was added to a final concentration of 5 mM followed by a further incubation at 55 °C for 10 minutes.

Reverse transcription reaction was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The 2X RT reaction mix was made and 2 µg RNA was added to it and placed in a thermal cycler at the following conditions: 25 °C for 10 minutes, 37 °C for 120 minutes, 85 °C for 5 minutes followed by a final hold at 4 °C. This cDNA was directly used for quantitative PCR.

## Real time PCR

The TaqMan® gene expression assays were ordered for the genes ENTPD1, TCTN3, CCNJ, ALDH18A1 and endogeneous control GAPDH. Their details are given in Table 2. The real time PCR master mix contained 1 X TaqMan® Universal Master Mix, 1 X primer-probe mix (MGB probe labeled with 6-FAM) and 2 µl of the cDNA in a 10 µl reaction. The passive reference dye used here was ROX™. The amplification was performed in StepOnePlus™ Real Time PCR System in a 96-well plate using the following conditions: 50 °C for 2 minutes, 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. A negative control comprising of the reaction mix without cDNA was included in each reaction. The entire experiment was performed 3 times using different batches of EBV transformed cell lines in each setup. Standard curves were constructed for each primer-probe set to determine the efficiencies of the reactions before analyzing the data by comparative $\Delta\Delta C_T$ method.

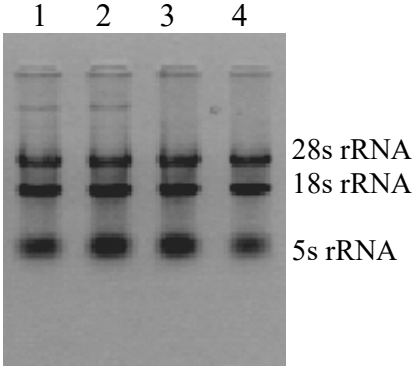



Figure 2: 1% agarose gel showing RNA extracted from EBV transformed cells, Lane 1 – RNA from an unrelated control, Lane 2 – RNA from father, Lane 3 – RNA from mother, Lane 4 – RNA from patient

| Gene | Assay ID | Location of probe |
|---|---|---|
| ALDH18A1 | Hs00913261_m1 | Exon boundary 17 – 18 |
| TCTN3 | Hs00383917_m1 | Exon boundary 8 – 9 |
| ENTPD1 | Hs00969559_m1 | Exon boundary 9 – 10 |
| CCNJ | Hs00908190_g1 | Exon boundary 5 – 6 |
| GAPDH | Hs99999905_m1 | Exon 3 |

Table 2: List of genes for which Taqman® gene expression assays were ordered, their assay IDs and location of the probes within the cDNA.

## Results

## Patient 1

## PCR and sequencing

All exons and exon-intron boundaries of NTRK1, NGFB, FAM134B, KIF1A, WNK1, IKBKAP and SCN9A were amplified and sequenced. The list of variations found in the patient sample is given in Table 3 and the sequence traces can be found in Figure 3. The variations were not found to be of clinical significance.

## Real time PCR

Real time PCR was done using cDNA from Patient 1, both parents and an unrelated control. The graph showing the levels of relative gene expression in patient compared to the controls is shown in Figure 5. As is evident from the graph, ENTPD1 shows a significant decrease in gene expression in the patient as compared to gene expression in controls. It was observed that the levels of gene expression in the father were much higher in comparison with the corresponding levels in the mother. Also, there was a lot of variability in the levels of gene expression in the father and the patient, although the levels were found to be comparatively stable in the mother.

## Patient 2

## PCR and sequencing

As for patient 1, all exons and exon-intron boundaries of NTRK1, NGFB, FAM134B, KIF1A, WNK1, IKBKAP and SCN9A were amplified and sequenced. The list of variations found in the patient sample is given in Table 4 and the sequence traces can be found in Figure 5. It was seen that one of the variations (not mentioned in table 4)(Glu707X)(Figure 6) in NTRK1 arising from a homozygous change from G>T leads to the conversion of GAG coding for glutamic acid to TAG which is a stop codon. This change is in the second last

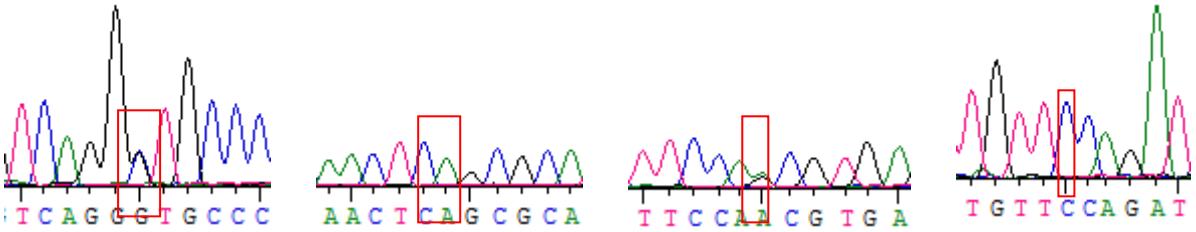
exon (Exon 16) of the gene which could possibly lead to the loss of its kinase domain which is important for its downstream signaling.

As we do not have the patient's parents' DNA samples, it was not possible to study the inheritance of this change.

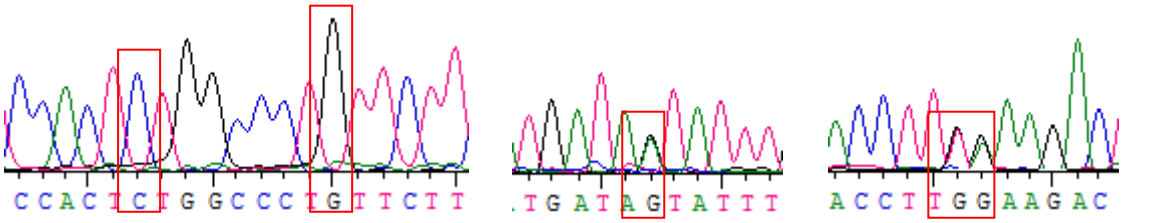
| Gene | Variation found | rs ID |
|---|---|---|
| NGFB | g.85799476C>G | Unreported |
| NTRK1 | g.8274259G>A | rs1800601 |
| | g.8334875G>A | rs6334 |
| | g.8337552delA | rs1799770 |
| | g.8337746G>C | rs2274499 |
| | g.8338754T>G | rs2274500 |
| IKBKAP | g.40835239T>C | rs838824 |
| SCN9A | g.17372791C>A | rs4443015 |
| | g.17372790T>C | rs58349687 |
| | g.17372461T>C | rs9646771 |
| | g.17371695G>A | rs4429487 |
| | g.17308576T>C | rs6746030 |
| | g.17294542A>C | rs10199799 |
| | g.17294560_17294561delTT | rs58519838 |
| | g.17292396T>C | rs7595255 |
| | g.17270402_17270405delTTTG | rs59145121 |
| | g.17269867A>T | rs10180721 |
| WNK1 | g.906208G>A | rs2240284 |
| | g.914404T>C | rs7300829 |
| | g.927482G>C | rs1012729 |

Table 3: Variations found in patient 1, their locations and rs IDs

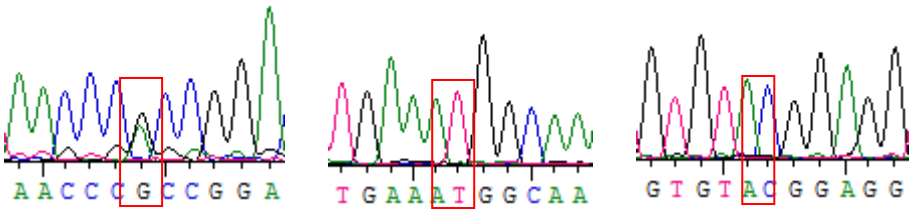
| | | |
|---|---|---|
| | g.930912A>C | rs956868 |
| | g.931009C>T | rs7953912 |
| | g.933930C>T | rs7300444 |
| | g.934487G>C | rs7955371 |
| | g.938365G>T | rs12828016 |
| | g.943837C>T | rs2301880 |



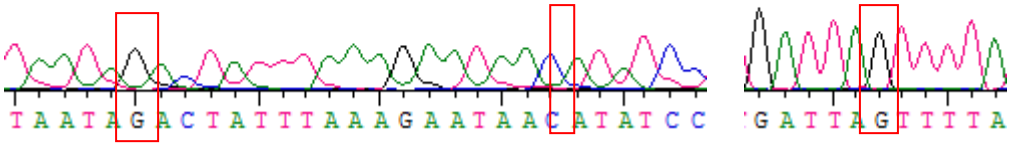
NGFB-g.85799476C>G rs1800601 rs6334 rs1799770



rs2274499; rs2274500 rs838824 rs4443015; rs58349687



rs9646771 rs4429487 rs6746030



rs10199799; rs58519838 rs7595255

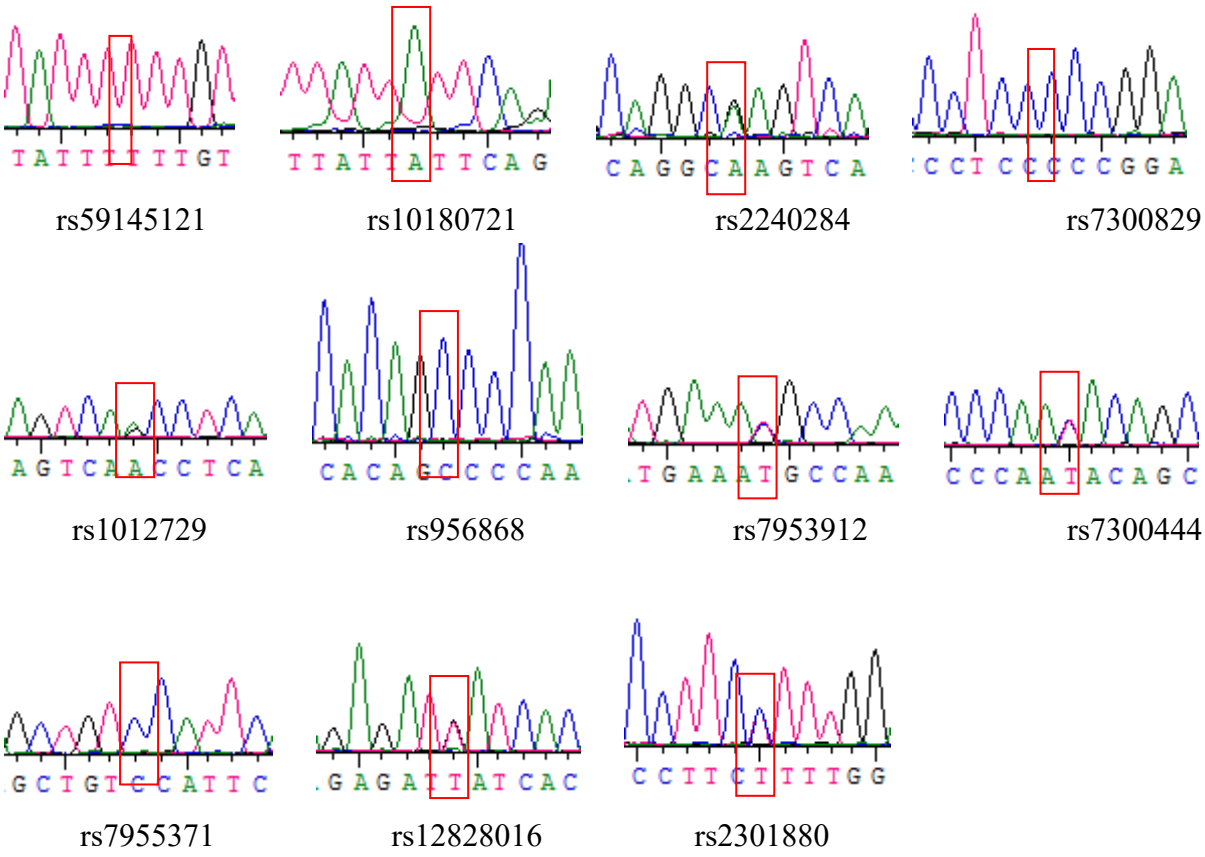



Figure 3: Sequence traces of variations found in patient 1. The peak corresponding to the variation is indicated in each case.

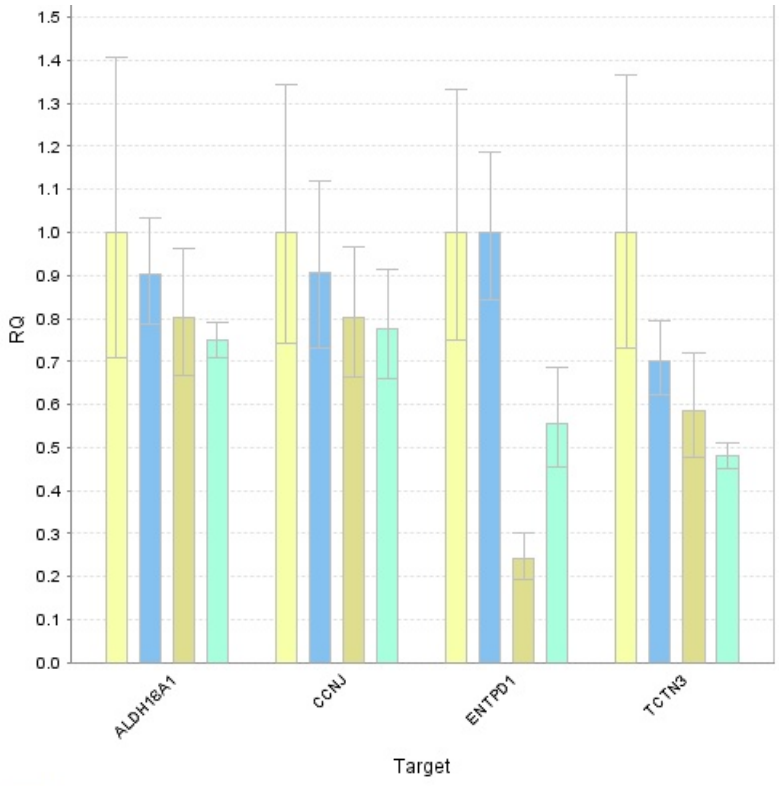
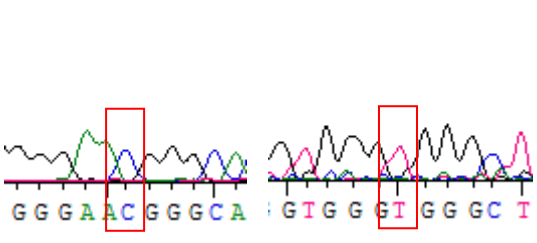


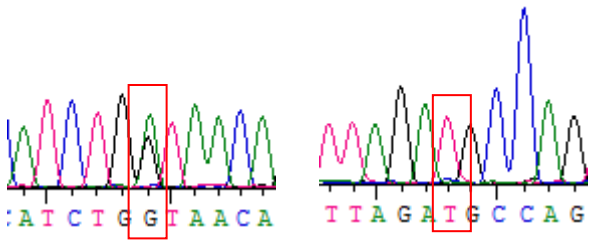

Figure 4: Graph showing relative gene expression of ALDH18A1, CCNJ, ENTPD1 and TCTN3 in an unrelated control, father, mother and patient. Significant decrease in the levels of ENTPD1 is seen in the patient as compared to controls, RQ – Relative Quantity, Target – indicated the genes that were probed for checking expression.

| Gene | Variation found | rs ID |
|---|---|---|
| NTRK1 | g.8326783T>C | rs1800879 |
| | g.8334762C>T | rs1800880 |
| SCN9A | g.14157551A>C | rs7588362 |
| | g.17346538C>T | rs4525717 |
| | g.17308576T>C | rs6746030 |
| | g.17294542A>C | rs10199799 |
| | g.17294560_17294561delTT | rs58519838 |
| | g.17292396T>C | rs7595255 |
| | g.17270402_17270405delTTTG | rs59145121 |
| | g.17269867A>T | rs10180721 |
| WNK1 | g.914404T>C | rs7300829 |
| | g.927482G>C | rs1012729 |
| | g.930912A>C | rs956868 |
| | g.931009C>T | rs7953912 |
| | g.933930C>T | rs7300444 |

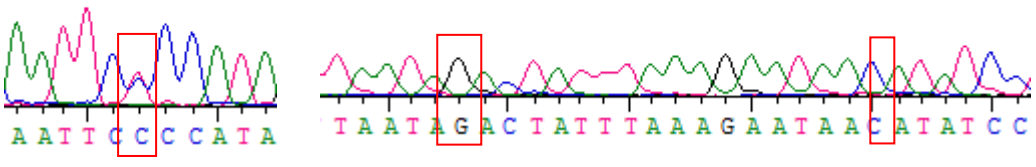
Table 4: Variations found in patient 2, their locations and rs IDs



rs1800879 rs1800880



rs7588362 rs4525717



rs6746030 rs10199799; rs58519838

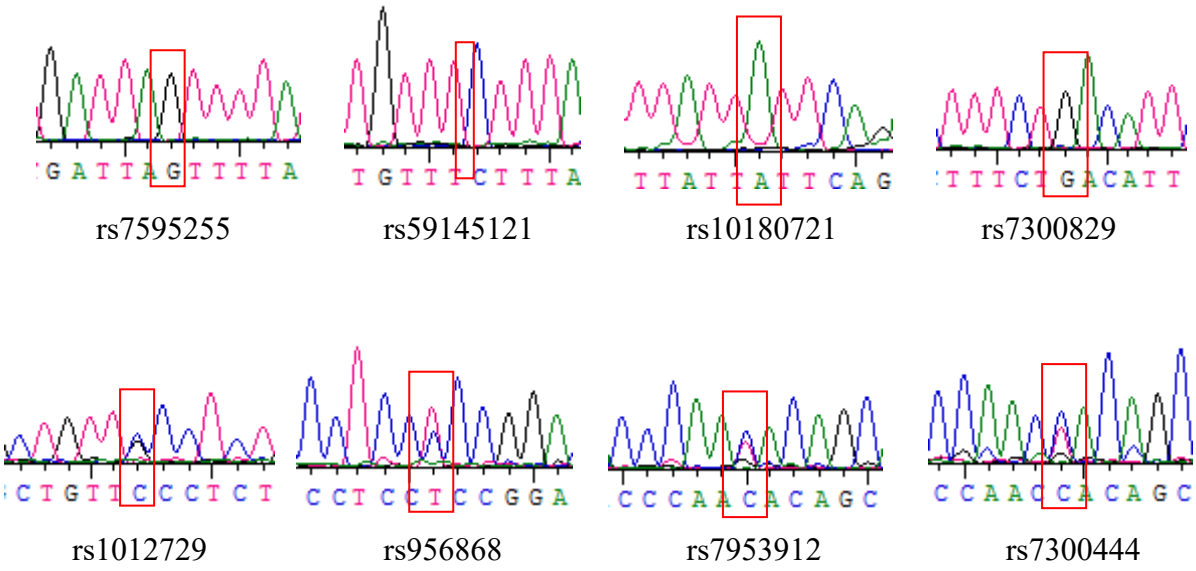



Figure 5: Sequence traces of variations found in patient 2. The peak corresponding to the variation is indicated in each case.

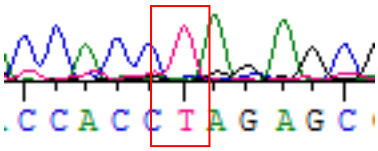


Figure 6: Sequence trace of a change in NTRK1 from G>T leading to change from Glutamic acid (GAG) to stop codon (TAG) in patient 2.

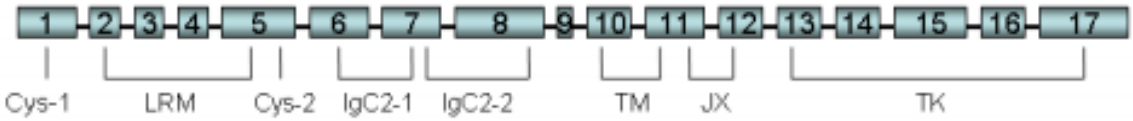



Figure 7: Domain structure of NTRK1. Numbers inside the boxes indicate exons. Extracellular part of the protein consists of: two cysteine clusters (Cys); three leucine-rich motifs (LRM) and two immunoglobulin-like C2 domains (Ig-C2). It is followed by a short transmembrane portion (TM), an intracellular juxtamembrane domain (JM) and tyrosine kinase domain (TK). (18)

## Discussion

## Patient 1

As noted earlier, none of the variations found in the known genes seem to be of clinical significance. All the variations are frequent in the population indicating that the symptoms of patient 1 are independent of these variations. This brings our attention to the chromosomal translocation between chromosomes 10 and 22.

The translocation has resulted in a breakpoint in the gene ENTPD1 leading to a part of the gene on chromosome 10 and another part on chromosome 22. The real time PCR experiments

show a significant decrease in the expression of ENTPD1 in patient 1 compared to gene expression in parents and an unrelated control. This leads us to a possibility that the decrease in levels of ENTPD1 might be responsible for the symptoms of pain insensitivity, although this point needs to be further validated.

The chromosomal aberration has also resulted in the translocation of genes downstream to ENTPD1, including CCNJ from chromosome 10 to chromosome 22. However, as per the real time PCR data, the expression of genes upstream and downstream to ENTPD1 does not seem to be affected. Thus, the translocation has only affected the expression of ENTPD1 and all other genes are normally expressed.

Another phenomenon that is apparent from the real time PCR results is the relative over expression of all genes in the father, patient 1 and a male control compared to mother. The levels of expression in all genes can be seen to be higher than that of the female parent although the levels are inconsistent across experimental setups. This observation can be attributed to position effect variegation.

Position effect variegation was first studied in *Drosophila melanogaster*. It refers to the inactivation of genes contained in a euchromatic region when translocated to a heterochromatic region. (15) This results in gene expression in some somatic cells and not in others. (16) As one of the breakpoints lies in the heterochromatin region, the euchromatin region juxtaposed next to it may be taking on some of the characteristics of the heterochromatic state. The heterochromatin-driven cis-inactivation occurs only in a proportion of cells during development. So, essentially the affected individual is a mosaic of mutant and wild type phenotypes. It has been proposed that the adjacent heterochromatin may cause some modification to the variegating gene which could result in the inhibition of its normal expression. (17) This could explain the inconsistency in the levels of gene expression across different experimental setups in males.

## Patient 2

The NTRK1 gene is composed of 17 exons and to date different reports have demonstrated various mutations in different ethnic groups along the entire length of the gene, both in the extracellular, NGF-binding domain, and the intracellular, tyrosine kinase domain. (2)

Here, we report the presence of a novel homozygous change from G>T in the DNA sequence leading to a change from GAG (Glutamic acid) to TAG (stop codon) in the protein sequence. This change is found at position 2119 of the transcript and 707 of the corresponding polypeptide. This transcript is 2391 nucleotides long and codes for a 796 residue polypeptide. Amino acid residues 440 to 796 form a potential cytoplasmic domain and amino acids 510 to 781 form a tyrosine kinase domain which is important for downstream signaling. The NTRK1 domain structure is shown in Figure 7.

NTRK1 is a receptor tyrosine kinase involved in the development and the maturation of the central and peripheral nervous systems through regulation of proliferation, differentiation and survival of sympathetic and nervous neurons. Its primary ligand is NGFB and it recruits, phosphorylates and/or activates several downstream effectors including SHC1, FRS2, SH2B1, SH2B2 and PLCG1 that regulate distinct overlapping signaling cascades driving cell

survival and differentiation. Through SHC1 and FRS2, it activates a GRB2-Ras-MAPK cascade that regulates cell differentiation and survival. Through PLCG1, it controls NF-K-B activation and the transcription of genes involved in cell survival. Through SHC1 and SH2B1, it controls a Ras-PI3 kinase-AKT1 signaling cascade that is also involved in regulating survival.

The change observed might lead to the absence of 89 residues at the C-terminus leading in turn to the absence of a major portion of its kinase domain. This might also lead to the loss of a tyrosine residue at position 791 which is autophosphorylated (19) and is involved in interactions with PLCG1, thus affecting the signalling pathway associated with it.

## Conclusion

This study was undertaken to identify the genetic cause of symptoms of pain insensitivity in two patients. One of these patients was found to have a chromosomal translocation between chromosomes 10 and 22 leading to the disruption of ENTPD1 gene. Sequencing of known genes in this patient did not reveal clinically significant changes in the sequence. However, when we checked ENTPD1 at the RNA level in the patient, we found a significant decrease of the gene as compared to normal controls. We contemplate a possible involvement of ENTPD1 in the patient's phenotype, and further validation is under way.

The second patient has a normal karyotype and sequencing of known genes revealed a homozygous change – Glu707X – in NTRK1, leading to a possible truncated protein. NTRK1 is a receptor tyrosine kinase, and the loss of the cytoplasmic kinase domain is implicated, which might disrupt downstream signalling, and lead to the phenotype.

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