Homozygous mutations in *NTRK1* gene underlie congenital insensitivity to pain with anhidrosis in Pakistani families

[1]Humaira Aziz Sawal, [2]Muhammad Ikram Ullah, [3]Arsalan Ahmad, [2]Abdul Nasir, [4]Ali Amar, [3]Ejaz A. Khan, [3]Mamoon Rashid, [4]Saqib Mahmood, [1]Peter John, [2]Wasim Ahmad, [5]Christian A. Hübner, [1]Muhammad Jawad Hassan

[1]*Department of Healthcare Biotechnology, Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences & Technology (NUST), Islamabad;* [2]*Department of Biochemistry, Faculty of Biological Sciences, Quaid i Azam University, Islamabad;* [3]*Sections of Neurology, Pediatrics & Plastic Surgery, Shifa International Hospital, Shifa Tameer e Millat University, Islamabad;* [4]*Department of Human Genetics and Molecular Biology, University of Health Sciences, Lahore; Pakistan;* [5]*Universitätsklinikum Jena, Institut für Humangenetik, Postfach, Jena, Germany*

Abstract

Congenital insensitivity to pain with anhidrosis is a rare autosomal recessive disorder presenting with loss of pain sensation, thermal sensation defects, and self-mutilating behavior. In the present study, we recruited two consanguineous pedigree showing pain insensitivity symptoms from Pakistan for clinical and molecular investigations. In family A, one female patient displayed classical CIPA symptoms along with microcephaly and severe intellectual disability. During course of the disease, her right foot was amputated and had remarkable dental degeneration and teeth shedding. In family B, one boy presented with classical symptoms of congenital insensitivity to pain with anhidrosis. Blood was collected from both families for molecular studies. Sequencing with the Ilumina Trusight One Sequencing Panel covering 4813 OMIM genes revealed a known homozygous mutation c.2084C>T; p.P695L of *NTRK1* in family A and a novel truncated mutation c.2025C>G; p.Y681X in family B. Protein modeling analysis of both mutations (p.P695L and p.Y681X) predicted loss of the rigidity in tyrosine kinase domain of NTRK1 that led to conformational changes as well as deleterious effect on protein function. The known mutation was reported more than a decade ago in a family from Northern Israel and other non-sense mutation is newly identified. It is interested that most of *NTRK1* mutations are associated with this domain. This is first ever report of *NTRK1* variants in congenital insensitivity to pain with anhidrosis patients from Pakistan.

INTRODUCTION

Congenital insensitivity to pain with anhidrosis (CIPA; OMIM #256800), also designated as hereditary sensory and autonomic neuropathy IV (HSAN IV), is a rare autosomal recessive disorder which manifests during the first month of life.[1] Fundamental characteristics includes loss of pain sensation, mainly in extremities and tongue, thermal sensation defects, self-mutilating behavior and intellectual disability.[2] The self-mutilating behavior is mainly pertaining to oro-facial tissues and manifests as premature loss of teeth, numerous other dental anomalies, various ulcers of oral tissues, tongue injuries, and scare formation.[3]

Insensitivity to pain results from the degeneration/absence of primary afferent fibers; while loss of sweating (anhidrosis) is due to loss of sympathetic postganglionic neurons.[4] Anhidrosis often presents as recurrent attacks of unexplained fever and can have serious impacts. Sweat glands in CIPA patients were morphologically intact in number and structure, but were devoid of innervating nerve fibers.[3,5]

CIPA is caused by mutations of the *NTRK1* gene (OMIM # 191315), or called as TRKA[3, 6-10] which maps to chromosome 1 (1q21-q22). It comprises of 17 coding exons (Ensembl gene identifier-ENSG00000198400] and responsible to encode the highly active enzyme tyrosine

Address correspondence to: Muhammad Jawad Hassan PhD, Assistant Professor Healthcare Biotechnology, Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences & Technology (NUST), Sector H-12, Islamabad, 44000, Pakistan. Tel: +92 51 9085-6135, Email: mjh@asab.nust.edu.pk

kinase receptor I for Neurotrophic Growth Factor (NGF) domain.[11] NGF is essential for the precise differentiation and maintenance of sympathetic ganglia and nociceptive sensory neurons.[12] To date, more than 73 mutations are documented including 31 missense and 12 non-sense mutations (HMGD professional 2015.2) in different ethnic groups worldwide being more predominant and frequent in Asian population.

The present study describes one novel (p.Y681X) and one previously described (p.P695L) mutation of NTRK1 gene in two Pakistani consanguineous families with CIPA. The p.P695L mutation was earlier documented in a Northern Israeli Bedouin family.[13] *In silico* studies predicted the damaging effect of these mutations on protein structure and function. This is the first report of clinical and genetic evaluation of CIPA families from the Pakistani population.

METHODS

Approval for the study was obtained from respective Institutional Review Boards (IRBs) of Shifa College of Medicine-Shifa Tameer-e-Millat University, Islamabad, Pakistan and Atta ur Rahman School of Applied Biosciences (ASAB), National University of Sciences & technology (NUST), Islamabad, Pakistan. Written informed consent was obtained from all participants. Parents gave consent for their children.

Recruitment of consanguineous family with CIPA and clinical evaluation

Family A (Janjua Rajput tribe of Kashmiri language group) belonged to District Kotli, Azad Jammu Kashmir (AJK) region of Pakistan and Family B (Awan Tribe of Saraiki language group) originated from a village near Dera Ismail Khan, KPK, Pakistan. The affected individuals of both families presented with insensitivity to pain, episodic fever and loss of sweating. They were evaluated for inherited neuropathy through different approaches including nerve conduction studies, radiological examinations including ultrasound of the abdomen and the pelvis. X-ray of skull, ankle, leg and foot were performed to evaluate additional skeletal features including abnormal dentition. Head circumference was measured to document microcephaly in family A.

Genomic DNA extraction and molecular analysis

Blood samples were collected from the both families after written informed consent. Genomic DNA was extracted by standard phenol-chloroform method. The Trusight One Sequencing Panel by Illumina covering of >4800 clinically relevant genes was performed. This panel covers 12 MB of genomic content including 4813 genes associated to a clinical phenotype. This enabled us to focus on genes with proven relevance, rather than wading through excess data that may not be of immediate value. The panel sequencing identified homozygous mutations of NTRK1 gene (NM_002529.3-ENSG00000198400) in two families associated with CIPA. Specific sequence primers of exon 15 (family B) and exon 16 (family A) for PCR amplification were obtained using on-line application of the Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm). PCR amplification was conducted by using *Taq* DNA polymerase (Applied Biosystems). Purification of the PCR-amplified product was performed with commercially available kits (Marligen Biosciences, Ijamsville, MD, USA). Bi-directional sequencing was executed for both strands of the amplified DNA fragments by using the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems on an ABI Prism 3130 Genetic Analyzer according to the manufacturer's instructions. Analysis of sequence variants was carried out via BIOEDIT sequence alignment editor version 6.0.7.

In-silico analysis

Pathogenicity prediction and evolutionary conservation of p.P695L mutation

KinMut Random Forest (http://kinmut2.bioinfo.cnio.es/) is kinase-specific software to predict the pathogenicity of mutations. In this prediction tool, two important independent methods, namely SIFT (Sorting Intolerant from Tolerant) and KinMut estimate the pathogenicity of mutations.[14] A threshold value for each predictor tool is applied to define that mutations are prospective to be damaging. The methods have been validated on a large set (60k) of disease associated (OMIM) and polymorphic variants. The conservation of the respective NTRK1 residue was analyzed for P695L mutation by constructing a multiple sequence alignment (MSA) and the phylogenetic tree of NTRK1 proteins from different species using the MEGA 5 software.[15]

Molecular modeling of NTRK1 mutations

Protein modeling was conducted with the help of the PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC (http://www.pymol.

org) and 4PMS pdb file (resolution 2.80 Å) for the crystal structure of the cytoplasmic domain of NTRK1. The p.P695L and p.Y681X mutations were introduced using the Discovery Studio Version 3.1. The amino acid change is visualized in the rotamer form with side chain orientations incorporated from Dunbrack backbone dependent rotamer library with maximum probabilities.

RESULTS

Phenotype characteristics of Family A

Patient IV:3 is a 17 year old girl seen for recurrent threatening osteomyelitis of the limbs. She was diagnosed to suffer from hereditary sensory and autonomic neuropathy type 4. Her parents were first cousins, and her brother and sister were normal (Figure 1A). Birth history was normal but gross motor functions and speech development was delayed. She was mentally handicapped and did not attend the school. Her parents reported self-mutilations, tongue biting and insensitivity to pain at age of 1 year. On examination, she had a low frontal hair line, a beaked nose, and dystrophic nails and teeth (Pictures of the patient were not provided by family members). Her occipital-frontal circumference was 46 cm (-5 SD). Due to multiple infections and cyanosis of
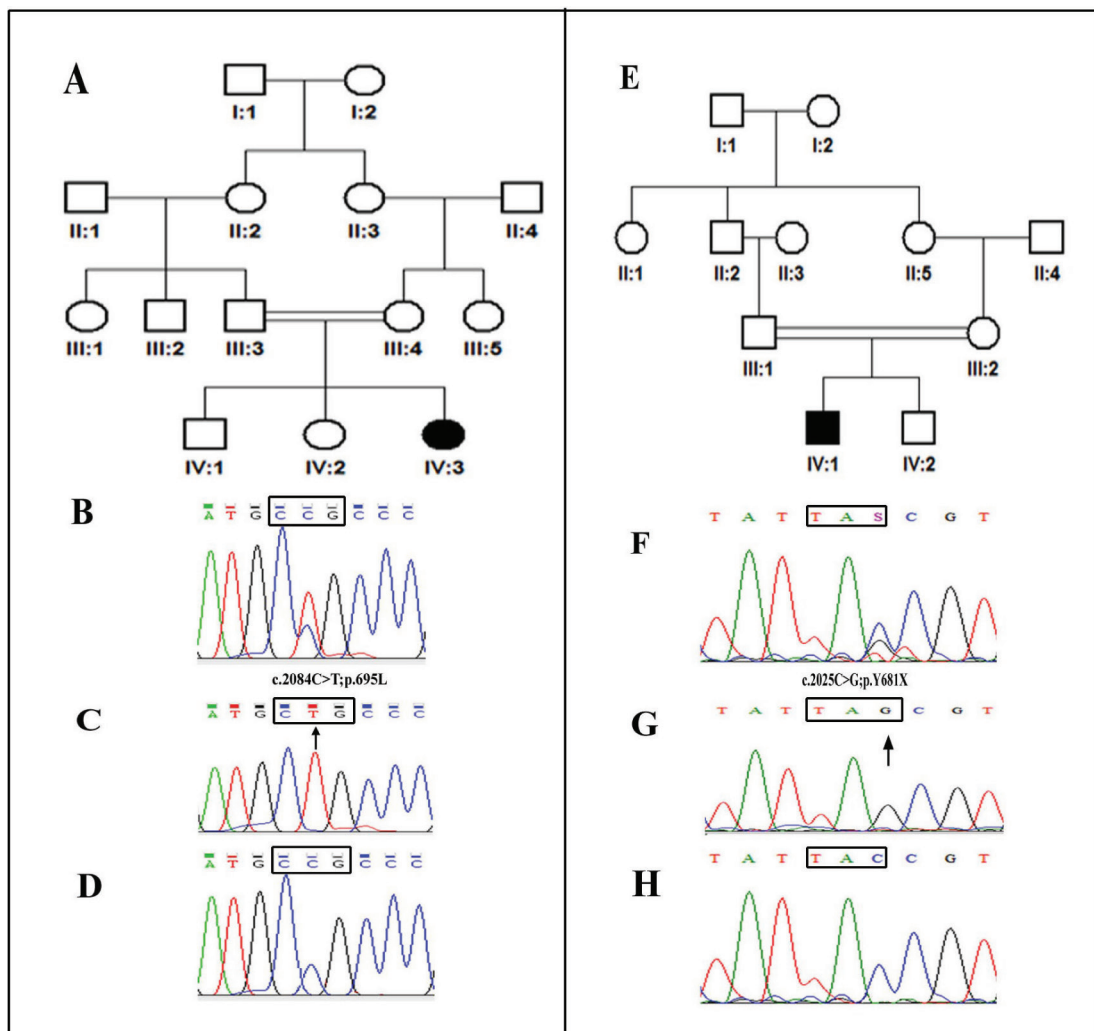

Figure 1. Pedigree of a family A with member suffering from congenital insensitivity to pain with anhidrosis (**A**), Sequence analysis of exon 16 of the NTRK1 gene in the carrier parent (**B**) affected daughter IV:3 with c.2084C>T variant in NTRK1 (**C**) and unaffected individual IV:2 with wild sequence (**D**). The pedigree of family B is outlined showing affected and unaffected individuals (**E**). DNA sequence of exon 15 of NTRK1 gene represents the carrier father III:1 with heterozygous peaks (**F**), and affected individual IV:1 with novel c.2025C>G mutation (**G**) and wild sequence in unaffected individual (**H**) of family B.

the tissue, her right leg was amputated at Shifa International Hospital, Islamabad, Pakistan. The joints of the left leg were also deformed (Figure 2A, B). She had deep plantar skin ulcers and premature loss of teeth (Figure 2C). X-ray of the skull showed multiple convolutions (Figure 2D). Neurological examination showed brisk arm jerks bilaterally, diminished lower limb reflexes with intact vibration and proprioception in all limbs. However, sympathetic skin response or sweat provoking tests were not carried out due to unavailability of the tests at the hospital. Nerve Conduction Study (NCS) was within normal limits apart from a mild decrease in sural sensory and peroneal motor nerve amplitude. Taken together her symptoms were consistent with Congenital Insensitivity to Pain with Anhidrosis (CIPA) with microcephaly and severe intellectual disability.

Phenotype characteristics of Family B

A 4 year old boy (IV:1) was examined in the neurology clinic, presenting with a history of inability to feel pain since birth and loss of sweating characteristics. According to his father, patient had been unable to feel pain since the birth. He had multiple injuries and a fracture but he was unable to feel pain and did not cry at all. He also complained that patient did not sweat in the hot summer season and had a history of recurrent episodes of fever. His parents are first cousins and his brother is normal (Figure1E).

On examination, he had mutilated fingers. Extra ocular movements and vision were normal. There was no facial asymmetry and tongue movements were normal. He had no weakness of his limbs bilaterally and tone was normal. The deep tendon reflexes were absent bilaterally and plantar responses were bilateral flexor. He had bilateral deformities of feet but was able to walk normally. He was unable to feel pain to his knee joints in the lower limbs and his wrist in the upper limbs (X-rays are not available). Nerve conduction studies showed moderately reduced compound muscle action potential amplitude of the left peroneal extensor digitorum brevis nerve and an absent right peroneal extensor digitorum brevis response. Bilateral peroneal motor responses at tibialis anterior were within normal limits. Bilateral median, ulnar, radial and sural sensory responses were also within normal limits. Bilateral median and ulnar motor responses were within normal limits. Needle EMG of the left tibialis anterior, gastrocnemius, vastus lateralis, biceps, and deltoid was within normal limits. He

appeared to be suffering from sensory/autonomic neuropathy or congenital insensitivity to pain with anhidrosis.

Mutation detection

DNA sequence analysis of the *NTRK1* gene in family A revealed the carrier parent (III:3) showed heterozygous peak (Figure1B), the homozygous peak with mutation c.2084C>T (p.P695L) in patient IV:3 (Figure1C) and unaffected family member (IV:2) was wild type (Figure 1D) . In family B, the carrier father (III:3) was heterozygous (Figure1F), the affected boy IV:1 was homozygous for a novel c.2025C>G (p.Y681X) mutation (Figure 1G) and unaffected family member IV:2 was of wild sequence (Figure 1H) in *NTRK1*. The variant was absent in 100 control chromosomes of the Pakistani ancestral population.

Damaging and conservation prediction of p.P695L mutation

The online prediction analysis wKinMut was used to study the biological effect of the respective mutation of NTRK1 protein (NP_002520). KinMut score was 0.558 (cutoff <0) and SIFT mutation score was 0.0 (cutoff score <0.05). Moreover, conservation of amino acid P695 was studied using the ClustalW tool. Proline 695 is conserved in most of the species analyzed (Figure 3).

Protein Modeling Studies of NTRK1 mutations in CIPA families

The p.P695L and p.Y681X are located at the end of a regulatory segment (amino acids 671-696) in the upper edge of the tyrosine kinase domain of NTRK1 protein, where it defines transition spelling of a loop into α-helix structure (Figure 4A-D). Substitution of proline by leucine causes steric hindrance with loss of rigidity within this important tyrosine kinase domain. In the case of the p.Y681X mutation, structural analysis revealed that the mutant adopts an altered conformation as predicted by a model generated through PyMOL software.

DISCUSSION

In the present study, we described patients of two consanguineous Pakistani families with CIPA caused by the homozygous mutations p.P695L and p.Y681X in *NTRK1*. Clinically, the affected individuals of these families (A and

Figure 2. In family A, X-ray of leg showed joint deformity and amputated foot (**A**, **B**). Mandible shows the loss of premature teeth (**C**), and skull shows various convolutions (**D**).

B) suffered from loss of pain perception and sweating, dental anomalies (progressive loss of teeth), nail dysplasia, beak shaped nose, low hair line, self-mutilating behavior (tongue biting). In family A, an additional phenotype of congenital microcephaly was also co-associated with CIPA in this family.

Presently, about 73 mutations have been reported in *NTRK1* in different populations. Out of these mutations, 31 are missense and 12 mutations are non-sense while others are frameshift, small/large deletion, insertion or duplications. We also reviewed the previously reported non-sense mutations given in Table 1. Although mutations in *NTRK1* are distributed in many populations but these mutations are more frequent in Asian populations including Chinese and Japanese groups.[7,9,16-17] Previously, non-sense mutations were identified in consanguineous as well as sporadic cases. In the current study, the
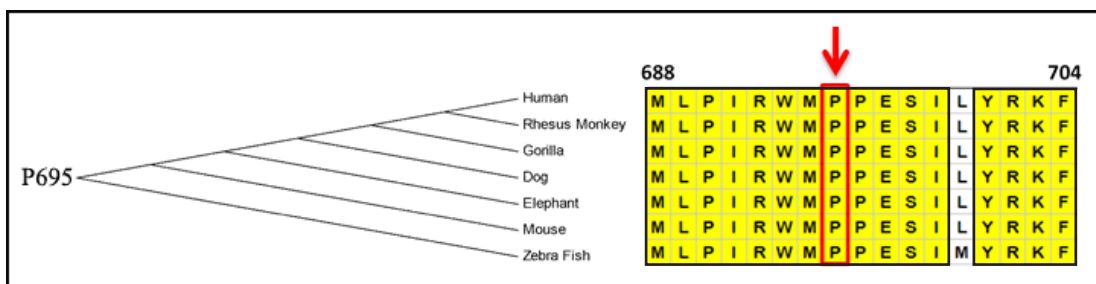

Figure 3. Phylogenetic tree and alignment of NTRK1 orthologs showing conservation of P695. The P695 of human NTRK1 is evolutionarily conserved in the seven representative species as indicated by the red frame and the arrow. All conserved residues within this region are highlighted in yellow and the numbers indicate amino acid positions in the NTRK1 protein.
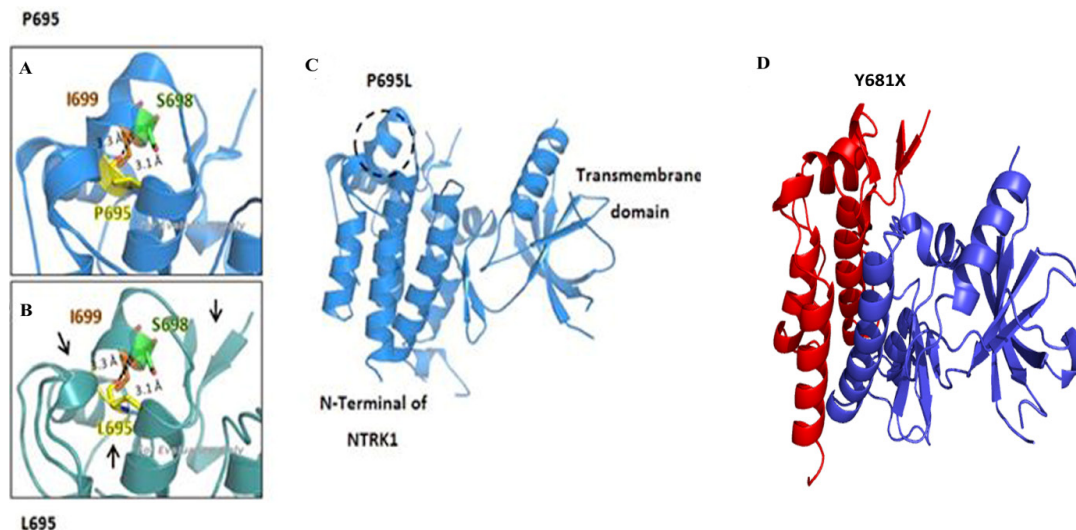
Figure 4. Molecular representation of the p.P695L and p.Y681X mutation within the NTRK1 cytoplasmic (Protein Kinase) domain. The black open circle localizes the p.P695L and p.Y681X substitution, while inserts show the changes induced by p.P695L and p.Y681X mutation. The P695 (**A**) results in a sharp change of loop structure into an alpha helix on the upper edge of the NTRK1 cytoplasmic domain. Its replacement by L695 (**B**) introduces changes in the domain structure of that region, evident as shortening of alpha helix involved and other conformational changes indicated by black arrows, thus bringing instability in the overall structure. Amino acids involved in hydrogen bonding are shown as sticks (distances also indicated) with C atoms for P695, S698 and I699 in yellow, green and orange respectively, N atoms in blue, O atoms in red and S atoms in orange. On the right side (**C**) is the ribbon diagram showing the normal and mutated cytoplasmic domain of NTRK1 protein with the positions of trans-membrane domain and N-terminal of NTRK1 indicated and the Y681 also resides in loop structure of alpha helix of NTRK1 domain. Its replacement by X695 (**D**) consequences conformational change in the domain structure of that region and causes the disruption/damaging of protein domain.

Table 1: Non-sense mutation of *NTRK1* associated in families/cases with congenital insensitivity to pain with anhidrosis (CIPA)

NTRK1 Exon	DNA variation	Protein variation	Ethnic Origin	Consanguinity	Reference/s
Exon 1	c.25C>T	p.Q9X	Italian	Yes	[21]
Exon 1	c.44G>A	p.W15X	Chinese	No	[17]
Exon 5	c.490G>T	p.E164X	Japanese	No	[7,22]
Exon 5	c.496G>T	p.G166X	Turkish	Yes	[22,23]
Exon 5	c.526C>T	p.Q176X	Japanese	No	[16,22]
Exon 8	c.1077C>A	p.Y359X	Japanese	No	[7,22]
Exon 14	c.1654C>T	p.Q552X	French	No	[22,24]
Exon 14	c.1786C>T	p.R596X	Japanese	No	[7,22]
Exon 15	c.1825G>T	p.E609X	Polish	No	[22,25]
Exon 15	c.2025C>G;	p.Y681X	Pakistani	Yes	Present study
Exon 17	c.2253C>G	p.Y751X	Japanese	No	[16]
Exon 17	c.2290C>T	p.Q764X	Turkish	Yes	[23]
Exon 17	c.2326C>T	p.Q776X	Swedish	No	[26]

mutation p.P695L is a recurrent variant which was already reported in a consanguineous family from Northern Israel suffering from CIPA.[13] The other mutation p.Y681X is a novel mutation identified in family B of the present study.

NTRK1 protein contains a cytoplasmic (tyrosine) kinase domain which is highly relevant for signal transduction.[18] Previous studies have shown that the tyrosine kinase domain of NTRK1 contains a regulatory segment (amino acids 671-696) which is important in regulating the TRKA activity.[19] The functional role of TRKA is to maintain nociceptive reception and thermo-regulation through interaction with NGF. The p.P695L and p.Y681X mutations identified in our study are also located in this particular regulatory segment. Two other mutations (D674Y and R686H) of this gene implicated in CIPA also lie within this domain.[3,20] A mutation in this regulatory domain suggests that the tyrosine kinase activity is lost due to abnormalities in ATP accessibility and inefficient substrate binding as a result of conformational changes induced by the mutation. The *in silico* studies including the molecular modeling analysis for p.P695L mutation also confirmed the deleterious effects of the P695L substitution on NTRK1 structure and function.

In a previous study, this mutation (p.P695L) was identified in an Israeli family with similar clinical pattern except for microcephaly; which is reported in our study only.[13] In Northern Israeli Bedouin family, the mutation found; c.2150C>T, resulted in substitution of a proline at position 689 into a leucine (new nomenclature is c.2084C>T; p.P695L).[13] Repeat finder software excluded presence of any mutation hotspot in the region of mutation. We do not have access to the DNA of Israeli family for haplotype analysis; therefore, we cannot propose a single mutation event to be responsible for this disease in these two families (Israeli and Pakistani families).

As shown by the molecular modeling analysis of NTRK1 tyrosine kinase domain, the presence of a pair of proline residues at positions 695 and 696 drives the transition from a loop to a helix conformation in the upper edge of the NTRK1 cytoplasmic domain. This domain is very important and more than thirty mutations (about 60% of total) are located within the tyrosine kinase domain of the NTRK1 protein.[17] The replacement of proline at 695 by leucine affects this sharp loop to helix transition, inducing flexibility in the mutated segment. Although the hydrogen bonds with S698 and I699 are not disturbed in the process, this induced flexibility brings conformational changes in the overall secondary structure of the protein kinase domain which is predicted to disturb the normal structure and function of NTRK1 receptor kinase. In addition, this proline residue lies close to catalytic core of the kinase domain, right next to an "activation loop" that regulates kinase activity through prevention of binding ATP and inhibition of basal kinase activity by acting as a pseudo-substrate, supporting a crucial role of P695 in NTRK1 kinase function (http://www.uniprot.org/uniprot/P04629). Some other bioinformatics tools are used for pathogenicity prediction, including KinMut combined with SIFT. All these features demonstrated that c.2084C>T (p.P695L) change significantly affects protein structure and function. On the other hand, due to truncated mutation p.Y681X, there are conformational changes and deleterious effects on terminal loop in the domains and in consequence resulted in a prematurely disrupted protein.

In conclusion, we described two families of CIPA for *NTRK1* homozygous mutation p.P695L (recurrent) in family A and p.Y681X truncated mutation in family B which are co-segregated precisely in respective family. Different bioinformatics servers predicted that this missense variant has a pathogenic and damaging influence on NTRK1 protein function. The proline at position 695 is conserved throughout mammalians. Protein modeling analysis of both mutations (p.P695L and p.Y681X) suggested in steric hindrance with a loss of the rigidity within this important tyrosine kinase domain of NTRK1 that lead to conformational changes and has deleterious effect on protein function. It is interested that most of *NTRK1* mutations are associated within this domain. Furthermore, CIPA is a rare heterogeneous disorder which is dispersed worldwide and the diverse phenotypes are associated which may determine the involvement of complex molecular pathway.

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