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Arab American University-Ramallah

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Identification of mutations that cause Congenital Insensitivity to pain in affected Palestinian families.

M.Sc. Thesis

By

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Identification of mutations that cause Congenital Insensitivity to pain in affected Palestinian families.

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Declaration

I declare that my MSc thesis entitled “Identification of mutations that cause Congenital Insensitivity to pain in affected Palestinian families”. Is the result of my research and has been written independently without sources than those quoted.

Boushra S. Khaled

Signature: 

Date: 13.10.2022

Dedication

I dedicate the product of my work in this research to everyone who believed in me and in my ability to end this challenge, especially my children, as I did not take this step except in order to be the role model in their lives.

Boushra S. Khaled

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Abstract

Congenital insensitivity to pain (CIP) is a rare autosomal recessive disorder(Drissi et al.), characterized primarily by an inability to perceive physical pain from birth, resulting in an accumulation of bruising, inflammation and fractures that affect the patient's life expectancy(Drissi et al.). This disease is caused by mutations in many genes, the most important of these genes and the most common is the *SCN9A* gene, as mutations in this gene cause, in addition to CIP, the patient's sense of smell loss(Xue et al.). And the *NTRK1* gene comes to be the second gene that causes CIP, as mutations in this gene lead to CIP with anhidrosis, which in turn causes additional complications for affected patients(Shatzky et al.). In this study, we tried to detect the mutations that cause CIP in five participating Palestinian families, due to the lack of studies that dealt with this disorder in our community. After performing the required clinical examination, followed by DNA extraction of a whole blood samples from the participated patients and family members, we applied sanger sequencing and Whole Exome sequencing for the PCR products in order to revel of the mutations that cause CIP.

The results of this research showed the presence of two mutations that cause CIPA in the *NTRK1* gene. The mutations were as follows: the first mutation is (c.1931-ins- T) in exon 15, which led to early termination in the formed amino acid sequence, and the second mutation is a missense mutation (c.2170 G>A (G724 S) in exon 16. A new mutation was

also detected in the *SCN9A* gene, in exon 7(c.901A>T, K301*) which led to the replacement of the amino acid that occupies position 301 with a stop codon, leading to the loss of a large part of the protein. Additionally, we performed in silico analysis using different soft wares such as polyphen, Clinvar, and SIFT, to detect the degree of severity of the missense mutation causing CIPA, and the analysis revealed that this mutation is a pathogenic mutation. In conclusion our study revealed of three mutations that caused CIP, and CIPA in Palestinian community which would help in improving the diagnostic and genetic counseling process. And help in building a diagnostic and follow up protocol for the affected individuals, since early diagnosis and medical care interference could prevent a lot of unpleasant complication for CIP, and CIPA patients(Shorer et al.).

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Chapter 1: Introduction & Background

INTRODUCTION

Pain is considered an unpleasant physical or emotional feeling, but it has a very important survival value. Pain sensation differs from one person to another. It, also, depends on many variables including: age, sex, and ethnicity, which makes pain measuring impossible(Personal).It is also called nociceptive pain because it has specialized neurons that are called nociceptors which are responsible for detecting the actual and potential tissue damage (Drissi et al.), which give organisms an early warning for their physiological protective systems. This warning is, for example, essential to detect and prevent contact with dangerous stimuli. So, pain has a protective role in preventing tissue damage by generating attention actions by the organism. It has also a role in the healing process by preventing physical motion and contact(Woolf). In the end, pain has an essential role in our development and survival, so, the question is: What if we lose the ability to feel pain?

1.1 Congenital Insensitivity to Pain(CIP)

CIP is a very rare autosomal recessive disorder that is characterized by losing the ability to perceive physical pain from birth (Sun et al.), which leads to death due to the inability to detect injuries and illness (Nagasako et al.). It was first reported, in the 1930s (Nagasako et al.). CIP is clinically divided into two types. The first one is known as Hereditary Sensory and Autonomic Neuropathy(HSAN), characterized by the inability of nociceptors to develop or die due to the absence of trophic signals. The most common of this type is the HSAN4, which is caused by mutations in the Nerve Growth Factor (*NGF*)gene, or the

Nerve Tropomyosin Receptor Kinase A(*NTRK1*) gene, which lead to the lack of (*NGF/TRKA*) signaling pathway that is involved in responding to Staphylococcus aureus infection for example. The second type of CIP is characterized by inability to activate the tissue damage signals although the nociceptors are found in their correct position. It is also characterized by inability to smell (anosmia) due to mutations in another gene known as Sodium voltage-gated Channel Alpha subunit 9(*SCN9A)* gene, that encodes for the alpha subunit of Nav1.7 Sodium channels, which are found in nociceptors and olfactory sensory neurons that are responsible for transmitting pain signals and smell signals respectively (Drissi et al). More than one gene are involved in CIP disorder, and the CIP patient phenotype is dependent on the affected gene.

Due to inability of perceiving physical pain, CIP patients suffer from accumulation of infections, fractures, bruises, and wounds, which affect their life expectancy, making this disorder a very important concern for physicians around the world, since proper diagnosis and medical interference, from early age, help in overcoming a lot of unpleasant signs and symptoms.

1.2 Congenital Insensitivity to Pain with Anhidrosis (CIPA)

Is known also as Hereditary Sensory and Autonomic Neuropathy Type 4 (HSAN4)(Hanatleh et al.), which is a rare autosomal recessive disorder (Masri et al.), that was first reported in 1963 (Li et al.). It has an incidence rate of 1 in 125 million (Y. Indo). In addition to the common features of CIP, which include Inability to perceive physical pain, self-mutilating behaviors, and musculoskeletal manifestation, there are several special characteristics found in CIPA patients, such as: Unexplained fever,

reduced sweating, and mental retardation(Yasuhiro Indo). Children with CIPA had ,also, complicated manifestation such as: Gastric regurgitation and aspiration, osteomyelitis and avascular necrosis(Hanatleh et al.). CIPA is due to almost 100 mutations in the Neurotrophic Receptor Tyrosine Kinase 1(NTRK1) gene(Hanatleh et al.). NTRK1 is responsible for coding for tyrosine-related kinase A receptor(Hanatleh et al.), which is phosphorylated by the presence of Nerve Growth Factor(NGF)(Mardy et al.), that is essential for the survival of peripheral neurons in brain, whilst the process of embryonic development is occurring (Y. Indo). It is, also, involved with the immune, inflammatory, and stress responses, which occur, after tissue damage. Defect in NGF leads to death of neuronal cells in a large manner during development, which leads to the absence of unmyelinated, myelinated, and peripheral autonomic nerve fibers, that are essential for perceiving pain(Masri et al.), innervation of eccrine sweat glands(Mardy et al.), alteration of fracture consolidation, and affecting bone metabolism.

1.3 Diagnosis of CIP and CIPA

Diagnosis of CIP, was previously done, by first collecting clinical data, performing pharmacological tests, and peripheral-nerve biopsy (Mardy et al.). However, the knowledge about the involved genes and the correlation between genotype and phenotype is very helpful in specifying the affected gene and thus more accurate diagnosis of the disease.

1.4 Involved Genes:

Mutations in several genes were reported in CIP patients. These genes include *SCN9A*, *NTRK1*, *NGF*, *SCN11A*, *PRDM12*, and *CLTCL1* genes(Drissi et al.)..

1.41 *SCN9A* (Sodium voltage-gated channel alpha subunit 9)

SCN9A is the most common gene involved in congenital insensitivity to pain (Drissi et al.). It is a member of gene family that encodes for sodium channel proteins. *SCN9A* is gene located on chromosome 2(q24.3), has 27 exons and encodes protein for the alpha subunit of the Nav 1.7 sodium channel(Sun et al.) that is found on the cell surface of nociceptors and olfactory neurons, which are responsible for transmitting pain, and smell signals respectively. Nav 1.7 channel, regulates Sodium ion flow from the exterior of the cell to the interior, which plays a key role in generating, and transmitting electrical signals. Loss of function mutations in the *SCN9A* gene, lead to producing nonfunctional sodium channels, which prevents transmitting of pain in addition to touch and smell signals to the central nervous system, causing congenital insensitivity to pain with anosmia. Most of these mutations, are frameshift, nonsense, and splicing mutations (Drissi et al.).

1.42 *NTRK1 (*Neurotrophic Receptor Tyrosine Kinase 1)

NTRK1 is located on chromosome 1(q21-22)(Y. Indo) and has 17 exons (Li et al.), that encodes for Tropomyosin Receptor Kinase (*TRKA*) protein which is essential for the development and survival of neurons (Drissi et al.). TRKA protein is found on the surface of cells. Which binds Nerve Growth Factor (NGF) protein leading to autophosphorylation and activation of TRKA. The activated TRKA protein, phosphorylates other proteins, that are essential for transmitting signals for the growth and survival of neurons which are

responsible for transmitting touch and pain signals to nervous system (Drissi et al.) . Loss of function mutations, in *NTRK1* gene affect the survival of neurons and cause inability to the perceive physical pain, in addition to inability to sweat (anhidrosis), which are characteristics of CIPA(Drissi et al.).

1.43 *NGF* (Nerve Growth Factor)

The *NGF* gene is located on chromosome 1(p 13.2). It encodes for the *NGF* protein which was the first identified nerve growth factor (Drissi et al.). It plays a major role in different levels of cell survival and apoptosis processes. It is involved in the maintenance and survival of peripheral neurons by stimulating homodimer formation and activation of TRKA receptor(Y. Indo) (Usai et al.). . Mutations in the *NGF* gene affect the disulfide cysteine bond in the protein structural motif leading to *NGF* protein structure instability that affects *NGF* intracellular processing (Drissi et al.).

1.44 *PRDM12* (PR domain zinc finger protein 12)

PRDM12 gene is located on chromosome 9 (q33-q34) and contains 5 exons. This gene encodes for a single protein that has a PR domain (Drissi et al.,), involved in methyltransferase activity, a zinc finger domain, and a C-terminal polyalanine tract. PRDM12 protein, directly or indirectly, influences histone modification, by recruiting histone-modifying enzymes, which play a major role in the proliferation and differentiation of neuronal cells (Rienzo et al.) and thus, loss of function mutations in *PRDM12* gene leads to CIP disorder(Drissi et al.).

1.45 *SCN11A* (Sodium voltage-gated channel alpha subunit type 11)

SCN11A gene is located on chromosome 3(p 22.2), and encodes for protein that is essential for the voltage-gated sodium channel Nav 1.9, which plays a major role, in initiation of action potential. It does so by altering the resting membrane potential, in excitable cells, either in , positive manner that leads to easier action potential generation or in a negative manner that leads to a more difficult action potential generation. Although it is known to have a minor effect on pain perception, loss of function mutations in *SCN11A,* is reported to cause CIP with a specific characteristic which is gastrointestinal hypomobility(Drissi et al. .

1.46 *CLTCL1* (Clathrin Heavy Chain Like 1)

CLTCL1 gene is located on chromosome 22 (q 11.21). It has 34 exons that encode for Clathrin Heavy Chain protein (CHC22), which plays a major role in intracellular trafficking(Drissi et al.). It is considered as a negative regulator of sensory neuron differentiation. It is, also, involved in the formation of GLUT4 storage compartment. The CHC22 protein is highly expressed in muscle cells(Nahorski, Borner, et al.). Mutation in *CLTCL1* gene, causes a defect in central nervous system, and peripheral nervous system (Nahorski, Al-gazali, et al.), leading to inability to perceive pain signals in combination with learning difficulties (Drissi et al.,).

1.5 The relationship between the phenotype and the genotype in CIP

The above mentioned genes, are considered to be the most common genes involved in CIP disorder. Each gene encodes for a protein or proteins that function at different levels, in transmitting pain signals (Fig.1). Mutations in these genes, is transmitted

recessive mode of inheritance except for *SCN11A*, which is transmitted in dominant mode of inheritance(Drissi et al.).Each mutation causes CIP, with special features , in addition to the common clinical features, that defines the CIP condition. Table 1 summarizes the function, location, and the special features for these genes. The phenotype is, directly related to the genotype, which will help in defining the gene of concern in each case.

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Table.1: CIP involved genes

Gene	*SCN9A*	*NTRK1*	*NGF*	*PRDM12*	*SCN11A*	*CLTCL1*
Location	Chr2(q24.3)	Chr1(q21-22)	Chr1(p13.2)	Chr9(q33-34)	Chr3(p22.2)	Chr22(q11.21)
Inheritance Pattern	AR	AR	AR	AR	AD	AR
Function	Transmitting pain and smell signals	Development and survival of neurons	Survival of neurons	Proliferation and differentiatio n of neuronal cells	Initiation of action potential	Intracellular trafficking
Phenotype	CIP with anosmia	CIP with anhidrosis	CIP with anhidrosis	CIP	CIP with gastrointestina l hypomobility	CIP with learning difficulties

1.6 CIP in the literature

Several studies have been performed , to define mutations that cause CIP by using different experimental approaches. Results of these studies, showed that the most common gene causing CIP is *SCN9A*. Different mutations in *SCN9A* have been reported. One study a 1.8 years old girl reveled a missense mutation in exon 10, and caused

replacement of Lysine at position of 376 with Glutamine, leading to a loss of function in the Nav1.7 protein (Shorer et al.). In the same study, a frameshift mutation at amino acid 375 that caused for stop codon after 5 amino acids, leading to tra protein synthesis (Shorer et al.). Intron8/exon 9 junction mutation, was shown to alter splicing of *SCN9A* gene and leads CIP(Klein et al.) . Further studies have identified missense mutations. One was p.Cys1339Arg in exon 21, in the gene (Xie et al.). Other missense mutations were reported in another study.(c.296G>C), , (c.2749T>G) mutation in exon 3and 16. Both of these missense mutations caused amino acid substitutions that lead to a loss of function in Nav 1.7 channel(Sun et al.). .

Although most of CIP cases are related to *SCN9A* mutations worldwide, in Palestine nearby countries, most CIP cases are diagnosed with anhidrosis (CIPA). In different publications, the diagnosis of CIPA cases were diagnosed based on the clinical picture only. For example, a CIPA case was reported in a study from Egypt that included a 15 months old female born to a first degree relative parents. In this study the diagnosis of the CIPA case was based on the clinical picture only (Abdel-hafez and Awad). Another study involved a four years old female that suffered from painless lesions and fractures. Where CIPA was diagnosis was based on clinical examination and family history. The same diagnosis protocol was followed, in another study, that included a 10 months old girl form a Saudi family, who has insensitivity to painful stimuli noticed, when she needed insertion for an intravenous line at the hospital. Also, by performing clinical testing, and taking the family history, CIPA was diagnosed in a 22 years old man, who has, insensitivity to pain. In addition, three relatives from the same grandfather, a suffered from the same conditions and were diagnosed with CIPA(Karkashan et al.).

On the other hand, several studies tried to identify CIP related gene mutations. A study from Saudi Arabia that involved two brothers, one of them suffered from unexplained fever, anhidrosis, and inability to perceive physical pain and the other showed a delayed mental development Genetic testing revealed a novel deletion of three nucleotides in exon 7(c.783-785 del GAA) of *NTRK1* gene. This mutation caused deletion of lysine number 261 in the protein, leading to nonfunctional protein (Algahtani et al.). In another study that included seven Jordanian CIPA cases from five families, genetic testing revealed three different mutations in the *NTRK1* gene. The first mutation was a frameshift mutation (c.1860-1861 ins T) in exon 15. The second mutation was a novel missense mutation (c.2170G>A; p. Gly724Ser) . The third case involved , was a missense mutation (c.2125G>T; p. Val709Leu) in exon 16(Masri et al.).

To the best of our knowledge, there are no CIP-related studies that were conducted in the Palestinian community except a study that was conducted on Arab- Bedouins . The study , included 13 males, and 12 females from the southern area. Patients were diagnosed as CIP cases with anhidrosis. They were diagnosed in the first month after birth based on several clinical features, such as: Inability to perceive physical pain, anhidrosis, unexplained fever, Charcot joint, self –mutilation, corneal injures, and mental retardation. The study also involved three affected individuals from the northern area(a male, and two females) who have recurrent episodes of fever, and soft tissue infections. Genetic analysis of samples from these cases revealed frameshift mutation due to insertion of T at position 1931, leading to shortening of the amino acid chain due to premature stop codon (c.2150 ins T; p. Pro689Leu) (Shatzky et al.).

Based on the number of samples that we were able to collect, , . In this study , we aimed to define the causative mutations of CIP among Palestinian families, which would help in developing testing protocols and provide better genetic counselling for the affected families. The study focused on investigating CIP causative mutations in five affected families with multiple affected individuals. The results confirmed the presence of a previously identified founder mutation in *NTRK1* gene, in addition two mutations in *NTRK1* gene and one novel mutation in *SCN9A* gene were identified.

Chapter two: Materials and Methods

This research was designed to detect mutations that cause CIP disorder in five Palestinian families. Detailed clinical examination was first conducted for the indicated patients before starting genetic testing, since Phenotype is directly related to Genotype.

2.1 Patients and samples

All , the necessary measures were taken to ensure the confidentiality and privacy of the participants in this research, who signed a consent form that includes an explanation about the research and contact information in case any explanation or clarification were needed from them. The necessary personal information including , (Date of birth, gender, age, and clinical signs.. etc) were also recorded.

Eight patients from five different families participated in this research. The participated families were as follows:

Family number one (CIPA):

It is the product of consanguineous marriage and belongs to the Palestinian Bedouin Arabs. The family contains two males with CIP, and a female who died at age 8 years, the father and the mother. The uncle on the father's side and the aunt on the mother's side were included in this study to examine the segregation of the mutation causing CIP in this family(fig2.A). Everyone has their own code to ensure privacy and confidentiality.

Family number two (CIPA):

This family is also the product of consanguineous marriage and belongs to the Palestinian Bedouin Arabs. A girl with CIP belongs to this family and only the father and his affected daughter agreed to participate in this study (fig2.B).

Family number three (CIPA):

This family is the result of a consanguineous marriage and consists of the father, the mother, three females, one of whom has CIP, and two males, one of whom has CIP as well. All the family members were agreed to participate (fig2.C).

Family number four (CIPA):

It is a family of Palestinian Bedouin Arabs, resulting from a consanguineous marriage. A female with CIP belongs to this family. Only the father, in addition to her, participated in this study (fig2.D).

Family number five (CIP):

It is a family resulting from a consanguineous marriage and it consists of a mother and a father and two males, one of whom has CIP, and a female who suffers from mixed symptoms (fig2.E).

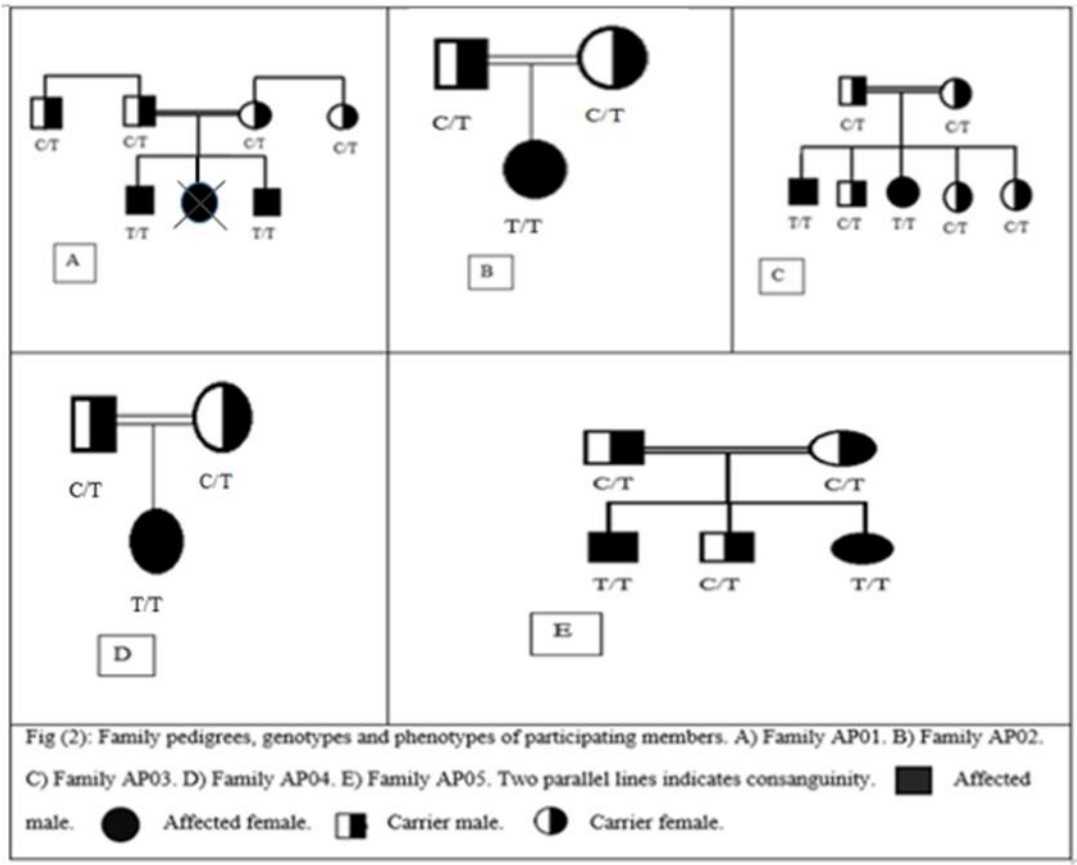

Fig (2): Family pedigrees, genotypes and phenotypes of participating members. A) Family AP01. B) Family AP02. C) Family AP03. D) Family AP04. E) Family AP05. Two parallel lines indicates consanguinity. Affected male. Affected female. Carrier male. Carrier female.

The affected individuals were presented to an orthopedic specialist, who in turn examined the patients and looked at the symptoms they were suffering from and did x-rays when necessary. After conducting a clinical examination, it was found that six patients suffer from symptoms consistant with CIPA, which suggested that the genetic cause is a mutation in *NTRK1* gene. As for the patient from family no 5, it was suggested based on the clinical examination, that he suffers from CIP, which is caused by a mutation in the *SCN9A* gene.

2.2 Primers Design

For genotyping, primers were designed to amplify the 17 exons of *NTRK1* gene and exon 7 in *SCN9A (*Table.2) including flanking intronic regions . This was done first by using the reference mRNA sequence from NCBI https://www.ncbi.nlm.nih.gov/website , accession number NM_002977.3 for *SCN9A* gene and NM_002529.3 for *NTRK1* gene, using primer3 software http://primer3.ut.ee/, and UCSC in silico PCR tool https://genome.ucsc.edu/cgi-bin/hgPcr.

Table.2: *NTRK1* and *SCN9A* primers

Fragment	Exon	Forward Primer	Reverse Primer	Product size
F1	1	5'-GCGGCTGGGTCTTTAACA-3'	5'-CATCCAGGCACTGACCATAA-3'	520 bp
F2	2+3	5'-TGGCATGTGCATGTGTATTG-3'	5'-CATTAGCAGCCCAAGTCTGG-3'	605 bp
F3	4	5'-CACCCCACCATCTACACACA-3'	5'-CACTGGCATGCACATAGTCC-3'	377 bp
F4	5+6	5'-TCTGTGTCCTCCCTTTCACC-3'	5'-CTGAGCCCTGTGGGTCTAAA-3'	820 bp
F5	7	5'-ACATTCTCTCCCACCCCTCT-3'	5'-GCAGCTGTGGAGACACACAT-3'	396 bp
F6	8	5'-CCTGTGGGGCTGTGACTTAT-3'	5'-ACCAGGCACTCCAATTTCTG-3'	701 bp
F7	9+10+11	5'-CGTCCCATGAAGGAATGAGT-3'	5'-ACACTCACAAGCCTCACAGC-3'	904 bp
F8	12	5'-TCAGTCTCTCCCCTGCAAGT-3'	5'-ACATGGTTTGGGCTAGCTGT-3'	482 bp
F9	13+14	5'-GGGGTGCAGGTTGAATTTTA-3'	5'-GTGTCTCCCCTTGGTTTGAA-3'	740 bp
F10	15	5'-CCAGTCTCCTCTCCCATCAC-3'	5'-TGAATCTCAGACCCATGCAG-3'	458 bp
F11	16	5'-TGAACCACCGAGCTTGTGTA-3'	5'-CACAGTGGAGGGGACACAGT-3'	426 bp
F12	17	5'-CAGCTAAGAAGCCCAGACGA-3'	5'-CCACATGCTGAGGGTGAAT-3'	432 bp
SCN9A				
F1	7	**5'**-ACAGCTGTTCATGGGAAACC-**3'**	5'TGACCCAAATTCACACTGTAGC3'	253 bp

2.3 DNA extraction

DNA was extracted from peripheral blood samples of affected individuals and their family members who agreed to participate. DNA was prepared by following the protocol of Wizard® genomic DNA purification kit (Promega, USA). In brief, 300 µL of peripheral blood were mixed with 900 µL of lysis solution to lyse red blood cells. After centrifugation at 15.000 xg for 20 seconds, 300 µL Nuclei lysis solution was used to lyse white blood cells. Afterwards, DNA was precipitated using 500ul absolute isopropanol (manufacturer) and centrifuged at 15.000 xg for 1 minute. The, DNA pellet was washed using 70% ethanol, dried and hydrated with 100 µL of dehydration solution. The DNA concentration and purity was then measured by using NanoDrop 2000 (Thermo scientific).

2.4 PCR

Amplification of the indicated DNA fragments was carried out in 20 µL reaction mix that contained 10 µL PCR Master mix (Go Tag®, Promega, USA), 3 µL of DNA (100 ng), 2 µL of 0.4µmol each forward and reverse primers and 5 µL nuclease free water. The PCR reaction was then ran on Thermal cycler (Flex Cycler, analytikjena) using the following program; Initial denaturation at 95°c for 10 minutes followed by 34 cycles of denaturation at 95°c for 20 seconds, annealing at 57.6°c (for NTRKA) and at 56°c (for SCN9A) for 30 seconds, and extension step 72°c for 1 minute followed by a final extension step for 10 minutes at 72°c.

2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to confirm the amplification of PCR products. 5 µL of PCR mix were run in 1.5% agarose gel that was prepared by dissolving 1.5g agarose powder (Sea Kim®LE Agarose) in100 ml 1X TAE buffer (thermo scientific). The suspension was heated in microwave until the agar was molten and the suspension became clear. Then 50 µL ethidium bromide (thermo scientific) was add for DNA visualization. 1000bp DNA ladder was used in all gels as molecular size marker. Electrophoresis was carried on for 30 minutes at constant voltage (100 V). UV transilluminator (Chemi Doc, BIO RAD) was used to visualize and photograph PCR products.

2.6 Sanger sequencing

PCR products were cleaned from leftover primers and dNTPs using EEPIC Fast kit (A&A biotechnology), then 1 µL of EEPIC Fast enzymatic solution was added to 5 µL of cleaned PCR product and placed in thermal cycler at 37°c for 10 minutes, followed by 1 minute at 80°c. Sanger sequencing was done by using BigDye Terminator v3 kit (Applied biosystem) according to the manufacturer's instructions.

2.7 Whole Exome Sequencing

2.71 Library preparation

Based on the results of the clinical examination that was done for the affected individuals, it was found that one of the patients who belongs to family no 5 suffers from CIP with anosmia which is due to mutation in the *SCN9A* gene, and due to the lack of

studies about this gene in the region, it was decided to apply WES on DNA from this proband. DNA library was first prepared from 500ng of genomic DNA by using Illumina® DNA Prep with Enrichment- (s) Tagmentation, 16 samples kit, and following the manufacturers instruction in catalog # 20025523. First, a tagmentation master mix containing of Enrichment Bead-Linked Transposomes (eBLT) and Tagementation Buffer 1 TB1, was added to DNA sample on 96-well PCR plate, in order to fragment and tag DNA to specific adaptor sequence. After tagmentation, ST2 was added for each reaction to perform post tagmentation clean up, followed by using TWB buffer for two round of wash of the adaptor tagged DNA. The amplification for the tagged DNA was done in a limited cycle PCR reaction which included the addition of 10 µl Index 2 (i5) adaptors, Index 1 (i7) adapters, and sequences required for cluster generation. In the next step the plate was placed in thermal cycler and (eBLT) program was applied as the follows: 72°C for 3 min, 98°C for 3 min, and 9 cycles of 98°C for 20 secs, 60°C for 30 secs, and 72°C for 60 secs with a final extension of 72°C for 3 min. Double-sided bead purification method was applied to clean up the amplified libraries using AMPure XP magnetic beads. First a nuclease free water was adding to a new MIDI plate containing supernatants, then 88 µL of AMPure XP Beads was added to each well before mixing and incubating. During incubation, 20 µL of the AMPure XP Beads was added to a new MIDI plate. 80% ethanol was then used to perform washing before placing the plate on magnetic stand to air dry. The beads were resuspend in the next step by adding RSB, then a single library concentration was measured pooled by volume using 12-plex enrichment for preparing single library. After pooling, the single library was denatured and biotinylated oligonucleotide probes were hybridized to the targeted regions of the library

fragments that was done by adding NHB2 Enrichment probe panel and EHB2 sequentially to the pooled libraries. In the next step, streptavidin Magnetic Beads (SMB3) were used in order to capture the targeted library fragments at the regions of interest. Preheated EEW was used to wash captured DNA libraries before adding an elution mix containing EE1 and HP3 to elute the captured DNA libraries from the beads. After that, EPM and PPC were added to each well in order to perform post capture PCR amplification. Thermal cycling conditions were as follows: 98°C for 60 secs, and 12 cycles of 98°C for 20 secs, 60°C for 30 secs, and 72°C for 30 secs with a final extension of 72°C for 5 min. At the end, cleanup the amplified enriched library was performed using AMPure XP beads before applying two round of wash using 80% ethanol.

2.72 Purification and quality control

Qubit dsDNA HS Assay Kit was used to determine library yield, (catalog # Q32850). An Agilent Technology 2100 Bioanalyzer with a High Sensitivity DNA kit, (catalog #5067-4626) was also used to determine the mean fragment size. Resuspension Buffer (RSP) was used to dilute the libraries to the required molarity for a starting concentration. Denaturation and final loading concentration of the libraries were performed according to the Denature and Dilute Libraries Guide for NextSeq 500 and NextSeq 550 Sequencing Systems. The library pool was denatured using 0.2 N NaOH before neutralization, that was done by adding equal volume(5 µl) of 200 mM Tris-HCl pH 7.0. Then, HT1used to dilute the denatured libraries to 20 pM before dilution to a final loading concentration of 1.5 pM with a total volume of 1.3 ml. PhiX was diluted to 4 nM with RSB followed by denaturation with 0.2 N NaOH and Tris-HCl to be used as a sequencing control. Finally

denatured PhiX was diluted to a final loading concentration of 1.5 pM with a total volume of 1.3 ml.

2.73 Sequencing

Mixture containing the diluted library and PhiX was loaded onto reagent cartilage for sequencing on a NextSeq550 platform (Illumina, USA).

2.74 WES analysis

The SnpEff tool was used to prepare a VCF file containing annotations of variant effects. The VCF file included information such as chromosome number, position, reference and variant nucleotide, gene name, genomic context, synonymy, amino acid change, the clinical significance of the variant, phenotypes related to the variant according to HPO and OMIM, predicted pathogenicity scores according to some tools like Polyphen2 and SIFT, and the Phenotype correlation score from exomiser. Finally, the variant that was considered to be related to pain disorder was filtered.

2.8 In silico Analysis

To examine the degree of severity of the missense mutation, we used different soft wares, such as, SIFT, COBALT, Polyphen, PhD-SNP, Clinvar, and Mutation Taster software's in order to obtain a comprehensive picture about the impact of this mutation on the affected individuals

Chapter Three: Results

3.1 Family description and Clinical Examination

This research involved five unrelated Palestinian families, all involved consanguineous marriages as shown in fig (2). First, clinical examination was performed for the eight patients and their family members who participated in this study. The clinical examination findings for all participants are summarized in table 3. Six of the patients (families A-D) suffered from inability to perceive physical pain, unexplained fever, mild mental retardation, and they were all unable to sweat, therefore, they were diagnosed as CIPA patients. The last two patients (family E) had mixed clinical signs, they were unable to perceive physical pain, able to sweat, and they are mentally normal. One of them was unable to smell which suggest the involvement of SCN9A gene, but based on literature, there is no reported cases with mutation in SCN9A gene in Palestine other countries in the region, which suggested to perform NGS to detect the putative mutation in one of the two since they are offspring's of the same family.

Table.3: The participated family's clinical examination results.

Family number	Family code	Participant code	Age (years)	Sex	CIP	CIPA	Phenotypic features	Mental Retardation	Self-mutilation	infections	Avascular Necrosis
1	AP01	AP01-01	42	Male	-	-	-	-	-	-	-
		AP01-02	38	Female	-	-	-	-	-	-	-

		AP01-03	18	Male	-	+	Anhidrosis	+	+	+	+
		AP01-04	17	Male	-	+	Anhidrosis	+	+	+	+
		AP01-05	23	Female	-	-	-	-	-	-	-
		AP01-06	24	Male	-	-	-	-	-	-	-
2	AP02	AP02-01	-	Male	-	-	-	-	-	-	-
		AP02-02	-	Female	-	+	Anhidrosis	+	+	+	+
3	AP0	AP03-01	47	Male	-	-	-	-	-	-	-
		AP03-02	46	Female	-	-	-	-	-	-	-
		AP03-03	23	Male	-	-	-	-	-	-	-
		AP03-04	20	Female	-	-	-	-	-	-	-
		AP03-05	18	Female	-	+	Anhidrosis	+	+	+	-
		AP03-06	16	Male	-	+	Anhidrosis	+	+	-	-
		AP03-07	11	Female	-	-	-	-	-	-	-
4	AP04	AP04-01	-	Male	-	-	-	-	-	-	-
		AP04-02	12	Female	-	+	Anhidrosis	+	+	+	+
5	AP05	AP05-01	34	Male	-	-	-	-	-	-	-
		AP05-02	28	Female	-	-	-	-	-	-	-

		AP05-03	8	Female	+	-	Anosmia	-	-	+	-
		AP05-04	7	Male	+	-	Anosmia	-	-	+	-
		AP05-05	4	Male	-	-	-	-	-	-	-

3.2 Congenital Insensitivity to Pain with Anhidrosis(CIPA)

In 6 patients, (families A-D), the clinical data suggested that they were suffering from CIPA which is usually related to mutations in NTRK1 gene. Thus, to detect the causative mutation in these patients, we first mixed the DNA of the CIPA patients and did Sanger sequencing for all NTRK1 gene exons. This primary analysis revealed that, in these families, there were two mutations in the NRTK1 gene; a frameshift mutation in exon 15 (c.1931-ins- T), and a missense mutation in exon16(c.2170 G>A) that changes glycine amino acid at position 724 to serine (p. G724 S). Of note, this mutation was not identified in CIPA patients before. Based on these results, each patient was tested for these two identified mutations. The results revealed that the two patients from family AP01 the patient from family AP02, and the patient from family AP04, were homozygous (T/T) for (c.1931-ins- T) mutation (Fig. 3 A). The last two patients from family AP03 with CIPA were homozygous (T/T) for (c.2170 G>A) mutation. The insertion of the T nucleotide, leads to formation of an early termination codon after ten amino acids which affect the protein structure and thus the protein function. Fig .4 show the location of the inserted T and the formed termination codon.

In order to prove that that the identified mutations are the ones responsible for CIPA in our families, we did segregation analysis for these mutations. We genotyped all

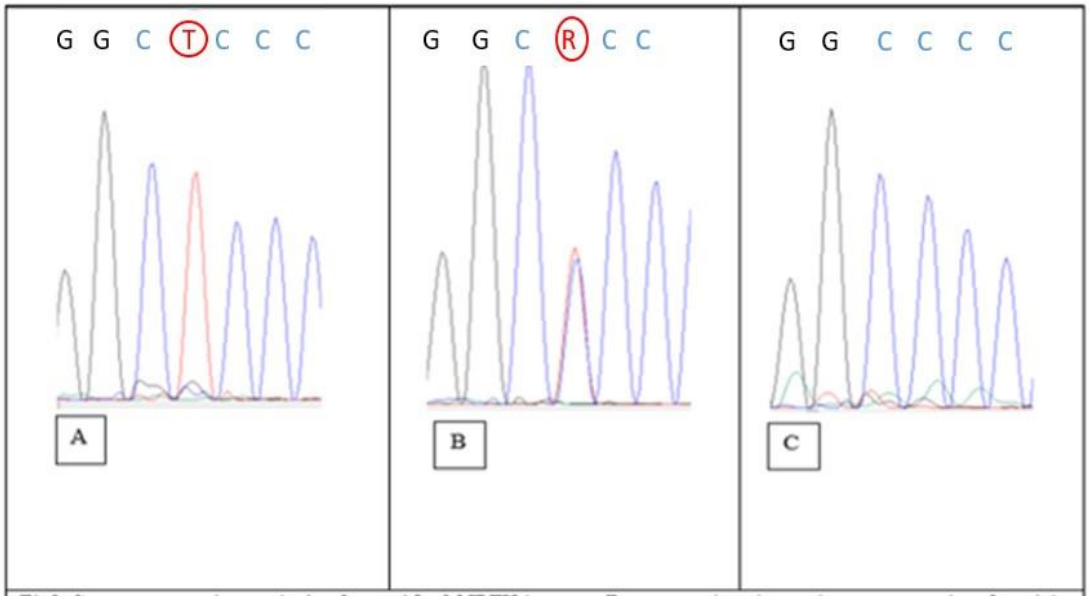

Fig3. Sanger sequencing analysis of exon 15 of NRTKA gene . Representative electropherogram results of partial sequences of exon 15 showing A. Homozygous c.1931-ins-T mutation in affected siblings. B. Heterozygous c.1931-ins-T mutation in parents of affected siblings. C. Wild type normal control from unrelated family.

participating family members (who are mainly the parents of affected siblings) for the mutations by using Sanger sequencing. As shown by the representative electropherogram in Fig. 3B, all parents in families AP01,02 and 04 are heterozygous (C/T) for the c.1931-ins- T mutation. In family AP03 our mutation segregation analysis showed that the parents and unaffected siblings are positive for c. 2170G>A mutation, confirming that this is the CIPA causative mutation in this family. which was present in two patients from the same family (Family number 3). the mutation lead to a change in the Glycine amino acid at position 724 to Serine, due to a substitution of the G to A in the

5`-GAGGATGTGGCTCCAGGCCCCCTGGGTCTGGGGCAGCTGCTGGCCGTGGCTAGCCAGGTCGC-3`
5`-GAGGATGTGGCTCCAGGCTCCCCTGGGTCTGGGGCAGCTGCTGGCCGTGGCTAGCCAGGTCGC-3`

Fig 4: Schematic present an alignment between partial sequences of Exon 15 in NTRK1 gene in wild type and mutated gene to show the location of the inserted T base pair and the formed stop codon(red).

position of 2170 (fig.5A). The segregation of the mutation was tested and the analysis revealed that all the unaffected family members are carrier (C/T) for this mutation (fig.5B) .Altogether, these results confirm that in families AP01,02 and 04 c.1931-ins- T is the CIPA causative mutation. The (c.1931-ins- T) mutation was found in patients who are Arab Bedouin, which is consistent with the results of previous studies that dealt with Arab Bedouin people in the occupied territories.

3.3 In silico analysis of c. 2170G>A mutation

Since this mutation was identified for the first time in CIPA patients, we wanted to predict its pathogenicity by using different in silico analyses online software. The analysis revealed that the c.2170G>A mutation is a disease causing mutation according to PhD-SNP software, and probably damaging according to polyphen software. Comparing

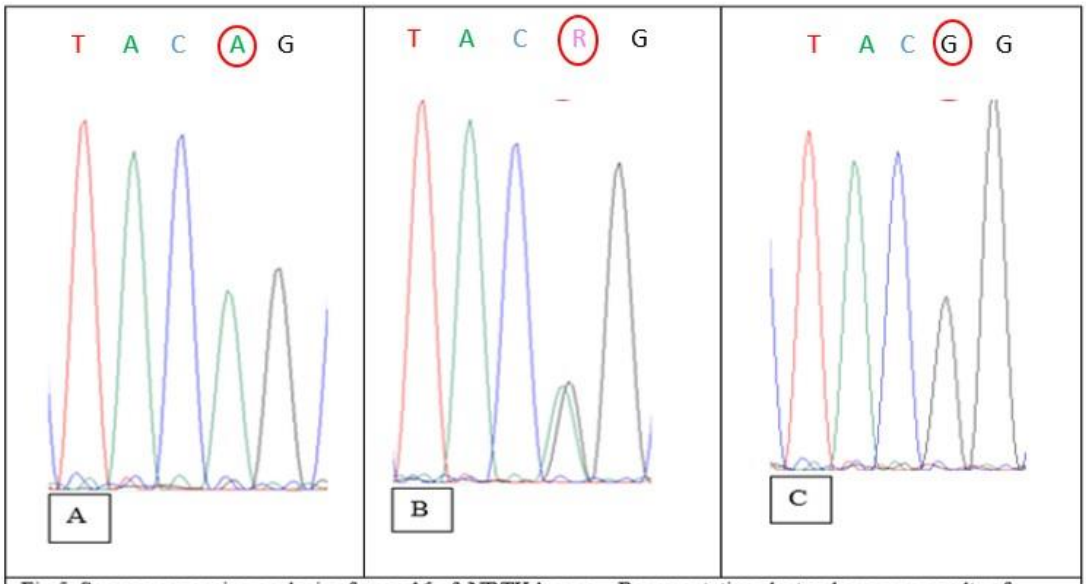

Fig 5. Sanger sequencing analysis of exon 16 of NRTKA gene . Representative electropherogram results of partial sequences of exon 16 showing A. Homozygous c.2170 G>A mutation in affected siblings. B. Heterozygous c.2170 G>A mutation in parents of affected siblings. C. Wild type normal control from unrelated family.

the gene in different species using COBALT software, the alignment showed that the c.2170G>A mutation occurs in the conserved region in *NTRK1* gene (fig6.B). So more than one software reported this mutation as pathogenic mutation which call for more research about this mutation and the importance of NTRK1 protein in transmitting neuronal signals. Fig(6.A) present the c.2170G>A mutation report by using different in silico analyses online software.

Software	(c.2170G>A) mutation report
COBALT	Conserved
Polyphen	Probably damaging
Mutation Taster	Disease causing
PhD-SNP	Disease- related polymorphism
SIFT	Predicted to be damaging
Clinvar	Pathogenic

A

B

Fig 6.A: Table present c.2170 G>A mutation report in online software. Fig 6.B Amino Acid Glycine 724 conservation analysis. Multiple protein sequence alignment showing the conservation of Gly 724 between different species including Homo sapiens, Danio rerio, Mus musculus, Panthera uncia, Epinephelus fuscoguttatus, Canis lupus dingo, Macaca fascicularis, Macaca mulatta, Rattus rattus and Rattus norvegicus. The alignment was performed using COBALT

3.4 Congenital Insensitivity to Pain without Anhidrosis:

For family AP05, our clinical examination diagnosed the proband to have CIP without anhidrosis. In order to elucidate which is the causative mutation in this family, we did Whole exome sequencing (WES) for the proband. Our results, confirmed the presence of a novel c.901A>T mutation that creates a stop codon at lysine 301 (pK301*) in *SCN9A* gene which is known to be related to CIP(Drissi et al., “Understanding the Genetic Basis

of Congenital Insensitivity to Pain") (Fig. 7A). To further confirm the causation relationship between the identified mutation and family phenotypes, we did segregation analysis by Sanger sequencing the gene area flanking the mutation position. Our analysis revealed that the parents and an unaffected brother were heterozygous (C/T) for the mutation (Fig. 7B), while our proband and his affected sister were homozygous (T/T) for c.901A>T mutation. Altogether, our results prove that the novel c.901A>T mutation is the variant that causes CIP in family AP05.

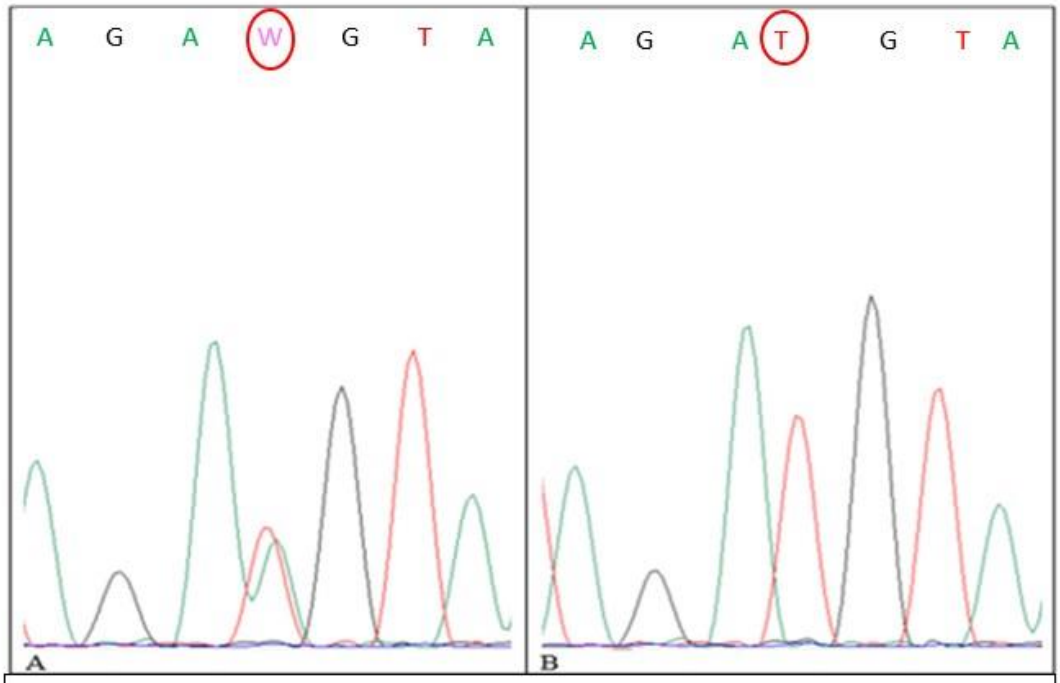

Fig7: Sanger sequencing analysis of exon 7 in SCN9A gene. Representative Electrophergram results of partial sequence of exon 7 show A) Heterozygous (c.901A>T) mutation in parents of affected siblings. B) Homozygous (c.901A>T) mutation in the affected siblings.

The replaced base pair is the last one in exon 7, and form with the first two base pairs from exon 8 a codon (AAA) that code for Lysine amino acid. The new base pair which is T in this mutation form a stop codon with the two base pairs from exon 8, which causes early termination in SCN9A protein synthesis, at amino acid 301. Figure 8 present an

alignment between partial CDC sequence of SCN9A wild type and mutated gene, which show the replaced base pair, and the formed stop codon.

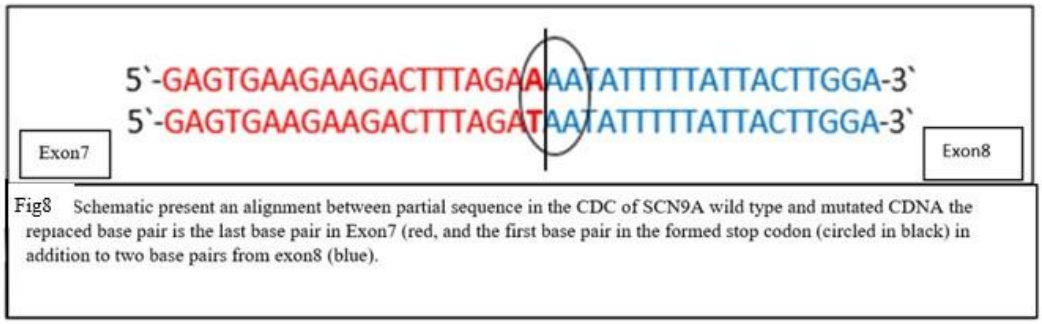

Fig8 Schematic present an alignment between partial sequence in the CDC of SCN9A wild type and mutated CDNA the replaced base pair is the last base pair in Exon7 (red, and the first base pair in the formed stop codon (circled in black) in addition to two base pairs from exon8 (blue).

Chapter four: Discussion

Congenital Insensitivity to Pain (CIP), is defined as an autosomal recessive disorder, that is characterized with inability to perceive physical pain from birth due to mutations in several genes. The most common gene involved in CIP worldwide is *SCN9A* gene,(Drissi et al.). Loss of function mutations in this gene lead to the formation of nonfunctional Nav 1.7 sodium channel, that is responsible for regulating sodium ions flow, which affects pain signal initiation(Bar-On et al.). The second common gene is *NTRK1* gene. where, mutations in this gene affect pain signal transmission, causing CIP with anhidrosis, which is known as CIPA(Masri et al.). Individuals with CIP and CIPA suffer from accumulation of injuries, fractures, infections, and bruises, which affect their life expectancy (Shatzky et al.). In this study we aimed to detect mutations that cause CIP, and CIPA in five affected unrelated Palestinian families.

since in this disorder the phenotype is directly related to the genotype(Drissi et al., , the clinical examination for the eight affected individuals were evaluated. We did so to define which type of CIP our cases have because different CIP type correlates with different genes(Drissi et al.,). The examination revealed six cases of CIPA and thus we hypothesized that mutations in *NTRK1* gene is the cause of the complication in these cases. In fact, most of CIP reported cases in Palestine and nearby countries are CIPA cases (Shatzky et al.). In one of the studied cases, the loss of pain was characterized as CIP without anhidrosis which suggested an *SCN9A* gene is the causative mutation(Drissi et al.,). Since, *SCN9A* gene mutation- related CIP cases were not reported before in our region, we performed WES for the CIP without anhidrosis case.

In all studied CIPA cases, as expected, our genetic analysis of all proband showed mutations in *NTRK1* gene. Two mutations in *NTRK1* gene were found, the first mutation was insertion (c.1931ins-T) in exon 15, which lead to the formation of stop codon after ten amino acids which caused premature termination of the NTRK1 protein. The termination affects the protein function because it is located in the intracellular domain of NTRK1 protein. NTRK1 intracellular domain contains Juxta membrane region, a tyrosine kinase domain, and a short carboxy terminal tail (Y. Indo). The phosphorylation of the intracellular domain in response to NGF binding is important for intracellular signaling since the phosphorylated tyrosine residue in NTRK1 cytoplasmic domain form a binding site for downstream signaling molecules (Y.Indo) . Premature termination due to (c.1931insT) mutation leads to the loss of tyrosine residues Tyr-670, Tyr-674, and Tyr-675 that form autophosphorylation sites (Mardy et al.). In addition to these phosphorylation sites, other tyrosine residues such as Tyr-751 and Tyr-785 are important for NTRK1 function (Mardy et al.). For example, Tyr-751 is part of the consensus sequence motif YXXM which interacts with phosphatidylinositol-38 kinase, and Tyr-785 which is located 15 amino acids from the carboxy-terminal tail, is involved in the biological activities of EGF, and CSF-1 receptors (Mardy et al.).

In present study, all cases were inherited in Bedouin families, the c.1931ins-T mutation was also reported in a Palestinian Bedouin patient. Similarly, the same mutation was also reported in a study that was conducted on Bedouin families . which confirms that this is a founder mutation that segregates in our Bedouin community.

The second mutation identified in *NTRK1* gene of CIPA patient was c.2170G>A (p. G724S), in exon 16. The mutation is a missense mutation that replaces Glycine 724 with

Serine. The replaced amino acid is located between two tyrosine residues in the intracellular domain of the *NTRK1* gene; Tyr-675, and Tyr-785 residues (Mardy et al.). The mutation occurs in the conserved region of the *NTRK1* gene, close to the catalytic core of the kinase domain, and near the activation loop, that prevents ATP binding and acts as a pseudo substrate to inhibit the basal kinase activity and thus regulates its activity(Cunningham et al.). All in silico prediction tools suggested that this mutation is deleterious. The c.2170G>A mutation was reported before in two studies. In one study, the mutation was revealed by whole exome sequencing study that involved consanguineous Palestinian and Arab families in 48 area with suspected neurogenetic disorder (Hengel et al.). In the other study, the mutation was identified in 7Jordanian CIPA patients. Here, the identification of the same founder mutations in Palestinian and Jordanian families is expected since more than 50% of the Jordanian community has Palestinian roots(Deprived).

Most mutations that cause CIP without anhidrosis were identified in the SCN9A gene (Drissi et al) . Therefore , WES was performed for the patient case with CIP without anhidrosis. the data revealed a novel mutation in exon 7 in *SCN9A* gene (c.901A>T). This mutation replaces the last nucleotide in exon 7. This change replaces Lysine 301 with a stop codon. The mutation is close to another reported mutation (c.1124delG) that causes frame shift at amino acid 375 and formation of a stop codon after five amino acid, leading to early termination in the sixth transmembrane domain in the Nav1.7 channel(Shorer et al.), *SCN9A* gene encodes for the alpha sub unite of Nav1.7 sodium channels that regulate sodium ions flow from the exterior of the cell to its interior through central pores and generating action potential in nociceptor neurons(Xue et al.).

Consequently, bi-allelic mutations in *SCN9A* gene are known to form non-functional Nav1.7 channels and failure to generate pain signals (Drissi et al.).

Although CIP and CIPA are considered to be rare disorders, it was noticed that the frequency of the disease is much higher than in other populations worldwide. since CIP cases are product of consanguineous marriages, which are common in our community (Assaf and Khawaja). In fact, consanguineous marriage cases in Palestine seems to represent one of the highest in the world since almost half of married women that age between 15-54 are related to their husbands (Assaf and Khawaja). Several studies showed that high consanguinity is related to higher frequency of rare diseases in rural communities including Bedouin communities (Assaf and Khawaja). This was very obvious in our study in which three out of the five participating families were Bedouin families. This was also the case in other studies were CIP cases were reported in Bedouin families. Moreover, our study as well as other studies confirmed the presence of the same founder mutation in different Bedouin communities residing in different geographical areas which confirms that Bedouins in the Palestinian society belong to the same ancestors. These findings support the need of genetic counseling about CIP and other rare diseases that circulate in such communities in order to prevent the spread of such diseases. This claim is supported by that fact that during the course of our study three couples from the same tripe were referred to us to test them for the c.1931ins-T mutation before they get married. Two out of the three couples were heterozygous for this mutation. The couples were advised about the potential consequences of such marriages and referred to the relevant medical services.

In addition to genetic counseling, early mutation detection is very important because it can prevent a lot of unpleasant complications (Shorer et al.). This can be achieved by providing the affected family with the knowledge and preventive care instructions that are needed to deal with the affected siblings. Also the orthopedic need to deal with these cases in more care and regular follow up in order to prevent any complications at the level of bone injuries which are common between CIP patients(Hanatleh et al.). The early and regular medical interference, such as radiological imaging after injury, revealed to be the best choice in dealing with CIP cases, and improve the health condition for the affected individuals.

Clearly more studies are needed to detect other possible mutations that cause CIPA and CIP in our population. This will help in building a protocol for genetic testing of affected individuals. Such a protocol will make the diagnosis process and genetic counselling more efficient, shorter and cheaper for affected families. Awareness about CIP and CIPA in the population and the medical care providers and the importance of genetic testing and genetic counselling is very important to increase the life quality and expectancy of affected individuals and to prevent the spread of such a deadly disease.

Conclusion

This study aimed to investigate mutations that cause CIP, and CIPA among the Palestinian community. Our results revealed two mutations in the NTRKA gene causing CIPA in four Palestinian unrelated families and a novel mutation in the SCN9A gene causing CIP in one Palestinian family. Further investigation and collaboration between the community and medical services supplier is needed in order to detect infected patients to help increasing the life quality of these patient and to design proper diagnostic protocol , and to set up a follow up plan to deal with CIP, and CIPA patients in our population .

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الملخص

تعد متلازمة عدم الاحساس بالألم (CIP) اضطراب وراثي جسمي متنحي نادر ، يتميز بشكل أساسي بعدم القدرة على إدراك الألم الجسدي منذ الولادة (Drissi et al.) ، مما يؤدي إلى تراكم الكدمات والالتهابات والكسور التي تؤثر على متوسط العمر المتوقع للمريض. يحدث هذا المرض بسبب طفرات في العديد من الجينات ، أهمها جينSCN9A المسؤول عن انتاج بروتينات ضرورية لتشكيل قنوات الصوديوم و التي تتواجد بالخلايا العصبية الخاصة بنقل الاشارات العصبية الحسية و كما تتواجد أيضاً بالخلايا المسؤولة عن نقل الاشارات الشمية للدماغ , و تؤدي الطفرات بهذا الجين الى فقدان القدرة على الاحساس بالألم وفقدان القدرة على الشم ايضاً(Xue et al.) . ويأتي الجين NTRK1 ليكون الجين الذي يسببCIPA ، حيث تؤدي الطفرات في هذا الجين إلى مع عدم التعرق ، مما يؤدي بدوره إلى مضاعفات إضافية للمرضى المصابين(Drissi et al.). في هذه الدراسة، حاولنا الكشف عن الطفرات التي تسبب في خمس عائلات فلسطينية مشاركة، بسبب نقص الدراسات التي تعاملت مع هذا الاضطراب في مجتمعنا. بعد إجراء الفحص السريري المطلوب، متبوعًا باستخراج الحمض النووي لعينات دم كاملة من المرضى المشاركين وأفراد الأسرة، طبقنا تسلسل

سانجر و WES لناتج تفاعل PCR من أجل الكشف عن الطفرات التي تسبب CIP، CIPA.

أظهرت نتائج هذا البحث وجود طفرتين تسببان في جينNTRK1 . كانت الطفرات على النحو التالي: الطفرة الأولى هي(C.1931-ins- T) ، والطفرة الثانية (C.2170G>A). تم اكتشاف طفرة جديدة أيضًا في جين SCN9A، في مما أدى إلى استبدال الحمض الأميني الذي يشغل الموضع 301بكودون إيقاف، مما أدى إلى فقدان جزء كبير منه. بالإضافة إلى ذلك، أجرينا Insilco analysis باستخدام الأواني اللينة المختلفة مثل ,polyphenوClinvarلاكتشاف درجة شدة الطفرة الخاطئة التي تسبب، وكشف التحليل أن هذه الطفرة هي طفرة ممرضة. في الختام كشفت دراستنا عن ثلاث طفرات تسببت في وفي المجتمع الفلسطيني والتي من شأنها أن تساعد في تحسين عملية الاستشارة التشخيصية والوراثية. وتساعد في بناء بروتوكول تشخيص ومتابعة للأفراد المتضررين لأن التشخيص المبكر وتداخل الرعاية الطبية يمكن أن يمنع الكثير من المضاعفات غير السارة لمرضى(.Xue et al) .