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Design and optimization of 18-gene Ion AmpliSeq panel of Next-generation sequencing for gene mutation analysis causing pain insensitivity

Mohammad Athar [a, b, *], ***Faisal A. Al-Allaf*** [a, b], ***Zainularifeen Abduljaleel*** [a, b], ***Mohiuddin M. Taher*** [a, b], ***Abdellatif Bouazzaoui*** [a, b]

[a] *Science and Technology Unit, Umm Al-Qura University, Makkah, Saudi Arabia.*
[b] *Department of Medical Genetics, Faculty of Medicine, Umm Al-Qura University, Makkah, Saudi Arabia.*

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ABSTRACT

Pain insensitivity is a unique phenotypic condition inherited in an autosomal dominant or recessive Mendelian form. Affected individuals suffer the loss of feeling to pain after exposure to high or low temperature. Recurrent mouth ulcers, face flushing, recurrent periods of elevated body temperature, and unexplained sweating are signs of a sensory neuropathy of mild autonomic activity in kids. Pain sensitivity deficiency is characterized by a loss of deep pain and temperature sensation, but natural reasoning skills and most other neural responses remain unchanged. Unfortunately, the spectrum of mutations causing pain insensitivity in Arabs, which is the prerequisite for prenatal and pre-implantation prevention regimes, is not yet determined. Therefore, this study's goal was to develop a high-throughput mutation screening method for gene mutation analysis causing pain insensitivity using the Ion Torrent Next-generation personal genome analyzer based on semiconductor nano-chips technology. We've created a panel that includes 18 genes linked to pain insensitivity. The panel was created using Ion Torrent's Ion AmpliSeq next-generation sequencing technology (NGS). Furthermore, two healthy human control samples were used to optimize all NGS procedures successfully. The molecular method should offer a fast, easy, and cost-effective, reliable mutation scanning/screening method that may also be applied in the future for prenatal and pre-implantation genetic diagnosis to offer families at risk of having a child with pain insensitivity opportunity to give birth to an unaffected child.

1. Introduction

Pain is a highly conserved sensory modality that protects all vertebrate species from harmful and dangerous stimuli. Loss of pain sensation is linked to a variety of injuries that placed one's life in jeopardy. Pain insensitivity can range from pain syndromes linked to neurological dysfunction, such as severe peripheral neuropathy, consciousness, and mental disabilities, to pure analgesia [1]. In most pain insensitivity cases, an underlying neuropathy causes an inability to perceive pain, which is referred to as pain insensitivity. Congenital indifference to pain is the term applied to this condition [2]. Peripheral neuropathy is now used to differentiate between congenital indifference to pain and congenital insensitivity to pain [3]. There are currently no genes known to cause pure forms of pain indifference. For peripheral neuropathies, several genes are known today, including pain insensitivity especially in combination with other neurological dysfunctions such as hereditary sensory and autonomic neuropathies (HSAN).

HSAN is a group of peripheral neuropathies characterized by sensory and/or autonomic abnormalities and the loss of pain sensation [3, 4]. There are five different types of HSAN. HSAN I has been linked to distal pain loss and heat sensitivity, ulcers, and deafness in some families [5]. HSAN I is caused by a mutation in the Serine Palmitoyl Transferase, Long Chain Base Subunit 1, *SPTLC1* gene [6]. There is still no causative gene for HSAN II, which causes autonomic disruptions and seriously impaired sensory functions, resulting in trophic lesions in infant stages [7]. HSAN III, also known as familial dysautonomia or Riley–Day syndrome, is characterized by a variety of symptoms, which include reduced pain and heat sensitivity, cardio-vascular destabilization, reoccurring pneumonitis, nausea catastrophes, and gastro-intestinal impairment [8]. HSAN III is caused by a mutation in the IkB kinase complex associated protein, *IKBKAP*, gene [9, 10]. HSAN IV, also widely recognized as CIPA, seems to be a congenital anhidrosis with pain perception. HSAN IV is marked by significant failure of pain perception, which can result in serious injury, self-mutilation, osteomyelitis, anhidrosis, as well as mental disabilities [11]. Variants in the nerve growth factor receptor gene *TRKA* have been discovered in HSAN IV patients, affecting the *NGF/TRKA* pathway in the disease's pathophysiology [12, 13]. With only a few cases documented, HSAN V is a relatively uncommon disorder. Symptoms of the disease include loss of pain sensitivity, impeded temperature stability, ulcers, and, in some cases, self-mutilation. The degree of autonomic involvement varies [7]. HSAN V's genetic background is still unknown. Except for the first type, which is autosomal dominant, all HSAN types are autosomal recessive. The genes targeted in this genetic study have all been directly or indirectly linked to HSAN [7, 14-17].

High-throughput next-generation sequencing (NGS) is increasingly being used to allow for the simultaneous and rapid evaluation of various genes at a low price [18]. This cutting-edge technique, which involves preferential sequencing of pre-specified genes, whole-exome sequencing, or whole-genome sequencing, is revolutionizing the molecular assessment of a variety of genetic disorders. NGS has a relatively high sensitivity for detecting low-frequency variants, as well as a faster processing time for large sample quantities with extensive genomic coverage [18]. Its application, however, is limited to diagnostics, and while it is becoming more common in clinical trials, its significance persists in the field of study. As a result, for NGS, we developed a panel of specific genes renowned to possess genetic variants associated with pain sensitivity. The goal of this study was to

* **Corresponding Author**
Science and Technology Unit, Umm Al-Qura University, Makkah, Saudi Arabia.
E-mail address: athar80@gmail.com (*Mohammad Athar*)

adapt an NGS-based approach for genetic characterization of pain insensitivity patients in Saudi Arabia.

2. Materials and Methods

2.1. DNA isolation and custom panel design

The Invitrogen PureLink genomic DNA mini kit was used to isolate genomic DNA from whole blood samples, as instructed by the manufacturer (Thermo Fisher Scientific). We used Ion AmpliSeq Designer, an online primer modeling platform that lets you create custom panels for Ion Torrent next-generation sequencing, to create an Ion AmpliSeq panel for 18 genes. To generate BED files, these chosen targets (containing coding exons and flanking intron regions) were entered into the online tool. To maximize target specificity, the online designer separated the generated amplicons into two primer pools. There are 729 amplicons in total, with 367 (Pool 1) and 362 (Pool 2) amplicons in each pool.

2.2. Optimization of genes mutations analysis by Next-generation sequencing (NGS), Ion Torrent (PGM) platform

We used two healthy human control samples to optimize the Ion AmpliSeq-based technique. The PGM Ion Torrent NGS protocol consists of three steps: (1) library preparation, (2) template preparation on the Ion One touch, and (3) PGM sequencing. We've optimized methods for sequencing unique genes. AmpliSeq library preparation kit and customized primers pool were used to build the libraries. With an Agilent bioanalyzer and the high sensitivity DNA kit, DNA concentrations and size distributions were determined and analyzed. Ion one-touch template kit v2.0 was used for emulsion PCR and Ion Sphere Particles (ISP) enrichment (Life Technologies). Finally, using the Ion PGM 200 sequencing kit, sequencing was performed on PGM (Life Technologies). All steps were carried out according to the instructions given by the manufacturers of the kits used in the experiment. Using the Torrent Suite Program (TSS), version 2.0, the sequences or readouts created on the Ion Torrent PGM were mapped to the human reference genome sequence. CLC-work bench software was used to evaluate the mapped readout sequences. The schematic representation of the NGS workflow on Ion Torrent PGM is shown below (Figure 1).

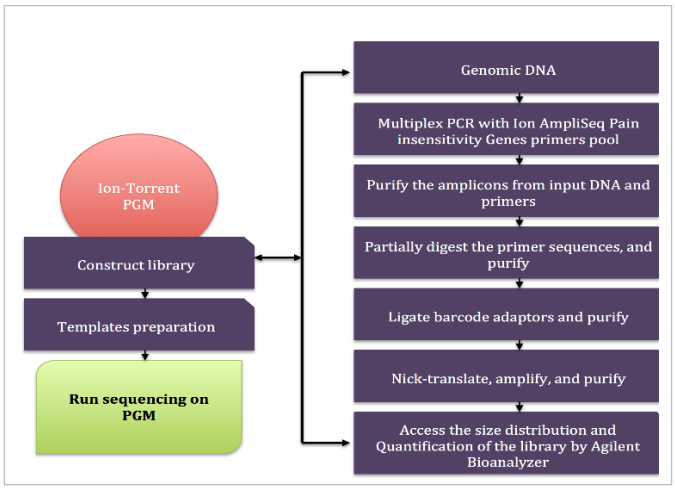

Figure 1. Flow chart describing the process of NGS sequencing on Ion Torrent PGM

2.3. NGS data analysis optimization

Adapter trimming, eliminating reads shorter than 20 bp, removing exact duplicates, and accuracy trimming was performed on binary alignment mapping (BAM) raw reads. CLC Genomics Workbench v7 (http://www.clcbio.com) was used to match the pre-processed reads against reference sequences corresponding to the personalized genes (ref. Genome Hg19). The identification of SNV and indel was then achieved. The Ion Torrent PGM raw reads were compared against the human reference genome (Hg19) for each version using the CLC genomics workbench. The aligned data were used with additional base quality score recalibration to detect variants. For subsequent analysis, all observed variants within the coding exons of personalized genes were taken into account using a probabilistic variant detection process. Similarly, we've refined and developed an NGS methodology for identifying variations in many inherited genetic disorders [18-21].

3. Results

We have designed a customized panel for 18-targeted genes associated with pain insensitivity (Table 1). The tailored panel primers were created to cover the coding exons as well as flanking intron regions of the genes. Two genomic DNA samples were used to optimize the NGS assay of the proposed collection of 18 human genes linked to pain insensitivity. Both DNA samples were loaded onto a single chip, with a chip loading percentage of 71 %. The chip loading value should be greater than 60 % to produce a sequencing output with a high density of ISPs on a sequencing chip (Life Technologies, United States). There were 2.9 x 10^6 high-quality reads provided on average. After mapping with the hg19 reference human genome, approximately 92 per cent of clean reads distinctively corresponded to the target genes, with about 96 % of the desired area. Exons from two samples had an overall depth coverage of 98 %, enough to identify DNA variants mainly within the regions targeted accurately. The analysis of the Ion Torrent Suite should include determining the Ion Sphere Particle (ISP) loading, the quantity of usable reads, and thus the length of sequencing reads. Figure 2 illustrates the overview of NGS sequencing data annotation.

Table: 1: List of 18-targeted genes associated with pain insensitivity.

Request ID	Type	Name	Chr	No. Amplicons	Total Bases	Covered Bases	Missed Bases	Overall Coverage	No. Exons
IAD72389	GENE	*NTRK1*	Chr1	39	2703	2445	258	0.905	19
IAD72389	GENE	*NGFB*	Chr1	9	736	736	0	1	1
IAD72389	GENE	*PKLR*	Chr1	25	1852	1829	23	0.988	12
IAD72389	GENE	*OPRD1*	Chr1	8	1149	789	360	0.687	3
IAD72389	GENE	*HSN2*	Chr12	102	8943	8559	384	0.957	32
IAD72389	GENE	*GCH1*	Chr14	10	925	812	113	0.878	8
IAD72389	GENE	*SCN9A*	Chr2	83	6194	5999	195	0.969	26
IAD72389	GENE	*COMT*	Chr22	9	856	762	94	0.89	5
IAD72389	GENE	*CACNA2D2*	Chr3	64	3849	3321	528	0.863	40
IAD72389	GENE	*CCT5*	Chr5	24	1736	1690	46	0.974	11
IAD72389	GENE	*OPRM1*	Chr6	31	2275	2216	59	0.974	18
IAD72389	GENE	*ABCB1*	Chr7	58	4113	4088	25	0.994	27
IAD72389	GENE	*TRPA1*	Chr8	53	3630	3336	294	0.919	27
IAD72389	GENE	*OPRK1*	Chr8	13	1173	939	234	0.801	4
IAD72389	GENE	*SPTLC1*	Chr9	29	1739	1739	0	1	17
IAD72389	GENE	*WNK2*	Chr9	70	7230	5042	2188	0.697	31
IAD72389	GENE	*IKBKAP*	Chr9	60	4359	4328	31	0.993	36
IAD72389	GENE	*NTRK2*	Chr9	42	2784	2764	20	0.993	20

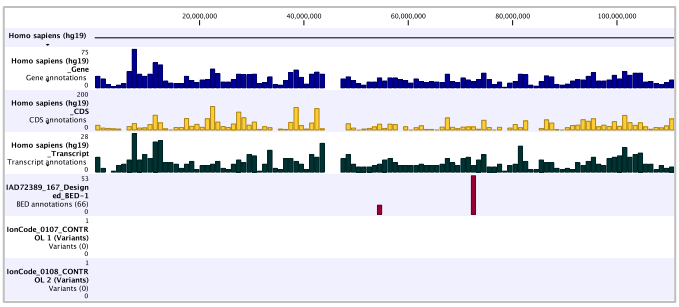

Figure 2. Overview of NGS sequencing data annotation. This different colour bar diagram image shows the annotation of targeted genes amplicons (red) bed file with the hg19 genes (blue), coding sequences (yellow), and transcript mRNA (black). No variants were detected in the respective genes when annotating the above with the variant calling file of two control samples. This diagram represents some segments of the targeted panel annotation's area.

4. Discussion

This studies showed the use of a custom designed pain insensitivity targeted NGS panel for patient molecular diagnosis. The targeted genomic regions designed with the Ion AmpliSeq Designer tool were successfully enriched during the library preparation method.

Sequencing runs presented sufficient sequencing quality and data output, as well as validated the appropriate library preparation and panel design. The panel provided sufficient coverage to reliably identify variants associated with pain sensitivity. The creation and validation of a novel Ampliseq NGS assay for the coding regions and boundary sections of 18 genes that qualify as candidate modulators of pain insensitivity were outlined in this study. NGS assay significantly decreased the laboratory effort needed to collect genetic data and provides the prerequisites for use in high-throughput environments. The presented NGS approach, in particular, is ideal for small to large-scale setups. The opportunity to validate the diagnosis of pain insensitivity at the molecular level will help differentiate peripheral neuropathies and ensure that patients receive adequate treatment. Other significant advantages of molecular diagnosis, in this case, include the ability to determine carrier status and the ability to diagnose prenatally.

HSAN is a disorder with a wide range of phenotypes. Some individuals only have sensory symptoms, while others have sensory, autonomic, and motor abnormalities and still others have purely autonomic findings [22]. Based on the age of onset, mode of inheritance, and leading clinical findings, five distinct types of HSAN have been described [22, 23]. *SPTLC1* gene mutations have been linked to HSAN I, with disease onset occurring in childhood or adulthood [14]. HSAN II-V and HSAN with spastic paraplegia have congenital or early childhood onset of symptoms and are caused primarily by mutations in *WNK1, IKBKAP, NTRK1,* and *NGF* [22, 23].

The method of transforming raw instrument signal data into nucleotide base call sequence data. The primary research typically occurs onboard the NGS Ion torrent, such as translating raw Binary Base Call (BCL) files to biological sequence data in the form of millions of fast reads on an Ion torrent sequencer [24]. The signal processing varies between platforms; Ion Torrent instruments process signals in the form of a pH transition translated to voltage, while Illumina instruments translate fluorescent signals into nucleotide base calls. After NGS reads have been translated from raw signals, primary research requires pre-processing them. This was achieved to ensure that only the highest-quality, longest-length reads were used for downstream research. Following the generation of high-quality sequence reads, the amplicons are matched against a reference genome or assembled from scratch, and any variants discovered are named. During secondary research, several different file types are used and produced. The tertiary examination in the NGS analysis workflow discusses the important consequence of making sense of the observable data. In human genetics, this means determining the fundamental link between variant data and the phenotype observed in a patient. Variant annotation is the first step in the tertiary analysis, and it adds more information to the variants found in the previous actions.

Overall variant annotation refers to the process of predicting the biological effect or function of genetic variants, whether for a human clinical case or pain insensitivity resistance mutations. Annotation tools converted the VCF generated by variant calling pipelines into a report of annotated variants and their biological effects [25]. Among these tools are comprehensive software packages such as the CLC bio platform, which can perform sequence analysis from variant calling to variant annotation and reporting. The generic variant calling pipeline was processed using an arbitrary number of variant calling tools. After that, the variant lists are normalized and combined into a single list (in vcf format). The functional annotation of the resulting list of variants was performed using SIFT, Polyphen2, and SNPeffect [26]. From the bam file, the bam-read count was used to calculate sample-specific quality parameters (like allelic frequency, base quality, and so on).

One of the unique approaches for the molecular diagnosis of pain insensitivity was Sanger sequencing of distinct candidate genes [27]. Sanger sequencing is more expensive and time-consuming when focusing on a large number of variations or genes, such as those addressed in this panel, than NGS approaches, which have a higher throughput for detecting variants. Targeted NGS panels, such as this customized AmpliSeq panel, are a great alternative to Sanger sequencing for studies with many target variations. Although whole-exome sequencing is a popular alternative to custom NGS panels, it is more expensive. Exome sequencing generates a greater volume of sequencing data, necessitating more storage space and bioinformatic analysis, which can be costly. When using entire exome sequencing, incidental results become even more challenging to detect. This NGS panel is a low-cost, efficient molecular method that requires little hands-on time. This study's main limitation was the unavailability of patients' samples to validate the designed gene panel. We will be able to overcome this limitation in our future research.

5. Conclusion

Our designed targeted next-generation sequencing panel could be used to characterize pain insensitivity at the molecular level efficiently. Our findings show that this approach for scanning genetic variants and able to diagnose pain insensitivity patients in Saudi Arabia might become a reliable and consistent, reproducible, precise, cost-effective, and faster molecular and genetic test.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

References

[1] Nagasako EM, Oaklander AL, Dworkin RH. Congenital insensitivity to pain: an update. Pain 2003;101(3):213-219.
[2] BAXTER DW, OLSZEWSKI J. Congenital universal insensitivity to pain. Brain 1960;83:381-393.
[3] Dyck PJ, Karnes J, O'Brien PC, Swanson CJ. Neuropathy Symptom Profile in health, motor neuron disease, diabetic neuropathy, and amyloidosis. Neurology 1986;36(10):1300-1308.
[4] Thomas PK. Autosomal recessive hereditary motor and sensory neuropathy. Curr Opin Neurol 2000;13(5):565-568.
[5] Wright A, Dyck PJ. Hereditary sensory neuropathy with sensorineural deafness and early-onset dementia. Neurology 1995;45(3 Pt 1):560-562.
[6] Dawkins JL, Hulme DJ, Brahmbhatt SB, Auer-Grumbach M, Nicholson GA. Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. Nat Genet 2001;27(3):309-312.
[7] Hilz MJ. Assessment and evaluation of hereditary sensory and autonomic neuropathies with autonomic and neurophysiological examinations. Clin Auton Res 2002;12 Suppl 1:I33-43.
[8] Axelrod FB. Familial dysautonomia. Muscle Nerve 2004;29(3):352-363.
[9] Anderson SL, Coli R, Daly IW, Kichula EA, Rork MJ, Volpi SA, et al. Familial dysautonomia is caused by mutations of the IKAP gene. Am J Hum Genet 2001;68(3):753-758.
[10] Slaugenhaupt SA, Blumenfeld A, Gill SP, Leyne M, Mull J, Cuajungco MP, et al. Tissue-specific expression of a splicing mutation in the IKBKAP gene causes familial dysautonomia. Am J Hum Genet 2001;68(3):598-605.
[11] Rosemberg S, Marie SK, Kliemann S. Congenital insensitivity to pain with anhidrosis (hereditary sensory and autonomic neuropathy type IV). Pediatr Neurol 1994;11(1):50-56.
[12] Indo Y, Tsuruta M, Hayashida Y, Karim MA, Ohta K, Kawano T, et al. Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis. Nat Genet 1996;13(4):485-488.
[13] Indo Y. Genetics of congenital insensitivity to pain with anhidrosis (CIPA) or hereditary sensory and autonomic neuropathy type IV. Clinical, biological and molecular aspects of mutations in TRKA(NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor. Clin Auton Res 2002;12 Suppl 1:I20-32.
[14] Auer-Grumbach M. Hereditary sensory neuropathy type I. Orphanet J Rare Dis 2008;3:7.
[15] Foulkes T, Wood JN. Pain genes. PLoS Genet 2008;4(7):e1000086.

[16] Williams FM, Scollen S, Cao D, Memari Y, Hyde CL, Zhang B, et al. Genes contributing to pain sensitivity in the normal population: an exome sequencing study. PLoS Genet 2012;8(12):e1003095.

[17] LÛpez-CortÈs A, Zambrano AK, Guevara-RamÌrez P, EcheverrÌa BA, Guerrero S, Cabascango E, PÈrez-Villa A, et al. Clinical, genomics and networking analyses of a high-altitude native American Ecuadorian patient with congenital insensitivity to pain with anhidrosis: a case report. BMC Med Genomics 2020;13(1):113.

[18] Athar M, Ghita IS, Albagenny AA, Abduljaleel Z, Shadab G, Elsendiony A, et al. Targeted next-generation sequencing reveals novel and known variants of thrombophilia associated genes in Saudi patients with venous thromboembolism. Clin Chim Acta 2021;519:247-254.

[19] Al-Allaf FA, Athar M, Abduljaleel Z, Taher MM, Khan W, Ba-Hammam FA, et al. Next generation sequencing to identify novel genetic variants causative of autosomal dominant familial hypercholesterolemia associated with increased risk of coronary heart disease. Gene 2015;565(1):76-84.

[20] Alnouri F, Athar M, Al-Allaf FA, Abduljaleel Z, Taher MM, Bouazzaoui A, et al. Novel combined variants of LDLR and LDLRAP1 genes causing severe familial hypercholesterolemia. Atherosclerosis 2018;277:425-433.

[21] Edrees BM, Athar M, Al-Allaf FA, Taher MM, Khan W, Bouazzaoui A, et al. Next-generation sequencing for molecular diagnosis of autosomal recessive polycystic kidney disease. Gene 2016;591(1):214-226.

[22] Rotthier A, Baets J, Timmerman V, Janssens K. Mechanisms of disease in hereditary sensory and autonomic neuropathies. Nat Rev Neurol 2012;8(2):73-85.

[23] Auer-Grumbach M, Mauko B, Auer-Grumbach P, Pieber TR. Molecular genetics of hereditary sensory neuropathies. Neuromolecular Med 2006;8(1-2):147-158.

[24] Pereira R, Oliveira J, Sousa M. Bioinformatics and Computational Tools for Next-Generation Sequencing Analysis in Clinical Genetics. J Clin Med 2020;9(1).

[25] Roy S, Coldren C, Karunamurthy A, Kip NS, Klee EW, Lincoln SE, et al. Standards and Guidelines for Validating Next-Generation Sequencing Bioinformatics Pipelines: A Joint Recommendation of the Association for Molecular Pathology and the College of American Pathologists. J Mol Diagn 2018;20(1):4-27.

[26] Cingolani P, Platts A, Wang lL, Coon M, Nguyen T, Wang L, et al. A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 2012;6(2):80-92.

[27] Johnson JO, Chia R, Miller DE, Li R, Kumaran R, Abramzon Y, et al. Association of Variants in the SPTLC1 Gene With Juvenile Amyotrophic Lateral Sclerosis. JAMA Neurol 2021;78(10):1236-1248.