THE UNIVERSITY of NORTH CAROLINA at CHAPEL HILL

Next- Generation Sequencing to Identify Disease Causing Variants in Individuals with Epilepsy

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INTRODUCTION

The transition from Sanger sequencing to next-generation sequencing (NGS) marks a pivotal shift in genomics. While Sanger sequencing was the standard for decades, its limitations prompted the development of NGS. NGS revolutionized sequencing by allowing millions of DNA fragments to be sequenced simultaneously, enabling high-throughput and cost-effective analysis. This transition highlights the impact of technological innovation in scientific progress, opening new avenues for research in fields like metagenomics and personalized medicine.

What is NGS

Next-generation sequencing (NGS), also known as high-throughput sequencing, revolutionizes genomics by swiftly determining nucleotide sequences in DNA or RNA. Unlike traditional Sanger sequencing, NGS processes thousands to millions of DNA fragments simultaneously, enhancing speed and efficiency. The process involves DNA or RNA sample preparation, attachment of sequencing adapters, and sequencing on platforms like Illumina. NGS generates short reads, which are assembled using bioinformatics tools for various applications, including whole-genome sequencing, transcriptomics, and disease research. NGS is pivotal in genetic variant identification and personalized medicine, propelling genomics research forward.

De Novo and Rare Inherited Variants in Epilepsy

Epilepsy, one of the most common neurological diseases, impacts nearly 1% of people in the U.S., showing high genetic influence with up to 70% heritability. Advances in sequencing technologies have led to the discovery of numerous genes responsible for monogenic forms of epilepsy and have highlighted the significant role of de novo variants, especially in severe forms like developmental and epileptic encephalopathy (DEE), where affected individuals rarely reproduce, limiting hereditary transmission. While common and ultra-rare de novo variants have been extensively studied, the impact of rare variants on epilepsy's genetic architecture is less understood. These variants could provide deeper insights into epilepsy's heritability and pathogenesis, considering. Integrating genetic analyses holds promise for unraveling the De Novo mutation landscape further, paving the way for significant breakthroughs in comprehending epilepsy's genetic complexities.

Research and Methods

In our investigation, DNA samples were obtained from a 7-year-old male diagnosed with Idiopathic West syndrome. The patient displayed a range of symptoms, including infantile spasms beginning at 4 months old, mild developmental delays, fine motor challenges, expressive speech difficulties, hypospadias, and benign skin pigmentary mosaicism. Despite undergoing testing for the GeneDX epilepsy panel with reflex to comprehensive epilepsy panel at 6 months old, the results were negative. Notably, the patient's father experienced a solitary seizure at 12 years old, prompting speculation about a potential correlation with the ASH1L mutation identified in the patient. Our main goal was to identify the specific ASH1L mutation in the patient and determine its origin, whether inherited or de novo. Initially we found the ASHL variant using Next Generation Sequencing but needed to confirm that it was not an artifact and see if it was an inherited variant using Sanger sequencing. We started by meticulously designing primers for PCR amplification of the targeted ASH1L gene region. Leveraging the UCSC Genome Browser and Primer 3 software, we crafted primers adhering to stringent guidelines. The resulting primers, 230 nucleotides long with balanced GC content, were strategically centered for optimal amplification, laying the foundation for exploring the genetic mutation.

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OLIGO            start  len    tm     gc%    any_th  3'_th   hairpin seq
LEFT PRIMER      600    20     57.96  55.00  5.44    0.00    0.00
CCACTCTTAAGATGCCACCC

                 start  len    tm     gc%    any_th  3'_th   hairpin seq
RIGHT PRIMER     829    20     59.18  55.00  0.00    0.00    0.00
TCATCGGAGGCAAGAGTCAG
PRODUCT SIZE: 230

CTCTGAAAAAGAGATGGATCAGATTAGAGATTTAAAAAAATATCAGTCAGAAATAATCTCCA
AGAATATTAAAACCACTCTTAAGATGCCACCCTCTGGTAATTCCCTTATTCCAGCAATTGCC
TCAAAGCATCTCATGAACAGCTTTTGGGGAAAGTCATTAGATAATTATTCACCCTTTTCTTC
AGCTTGTGCTTAGACTTTCTCTTCTCTTTTGACCGTCCAGATTTTTTGTGTGTGGCCATGG
GCTGGCTGTTTTTGCTGCTGGTGAGTCCATTCACACGCTGACTCTTGCCTCCGATGATTC
CTCGACATTTCTCAAAGCCACACTTACAAAGTTGCTTTGAAGGGAGAGAATAATTTTTTTAT
TTTTATTTTTTTAAGGCAGGG
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Figure 1: Figure shows both the primers used (green and blue) as well as the mutation of interest (red) in the ASH1L gene.

Research and Methods

In the next phase, we optimized primer conditions by creating six distinct mixtures, varying in temperature or the inclusion of di-methyl sulfoxide (DMSO). Each mixture, totaling 20 microliters, included "My TAQ 2x Mix," forward and reverse primers, blood from a control sample, and distilled water. Three mixtures contained DMSO. PCR cycling conditions were uniform across all mixtures except for annealing temperature. After denaturation at 95°C for one-minute, subsequent steps involved denaturation at 95°C for 15 seconds, annealing at 54°C, 56°C, or 58°C for 15 seconds, and extension at 72°C for 10 seconds. Gel electrophoresis followed PCR cycling, where a gel was prepared with agarose and SYBR Safe gel stain. Once solidified, the gel was submerged in 1 x TAE buffer, and PCR samples were loaded into slots along with a ladder. Electrophoresis was conducted at 120 volts for 40 minutes. Analysis of the gel revealed successful PCR reactions, with the optimal conditions observed at 56°C without DMSO, guiding subsequent steps in the experimental process.

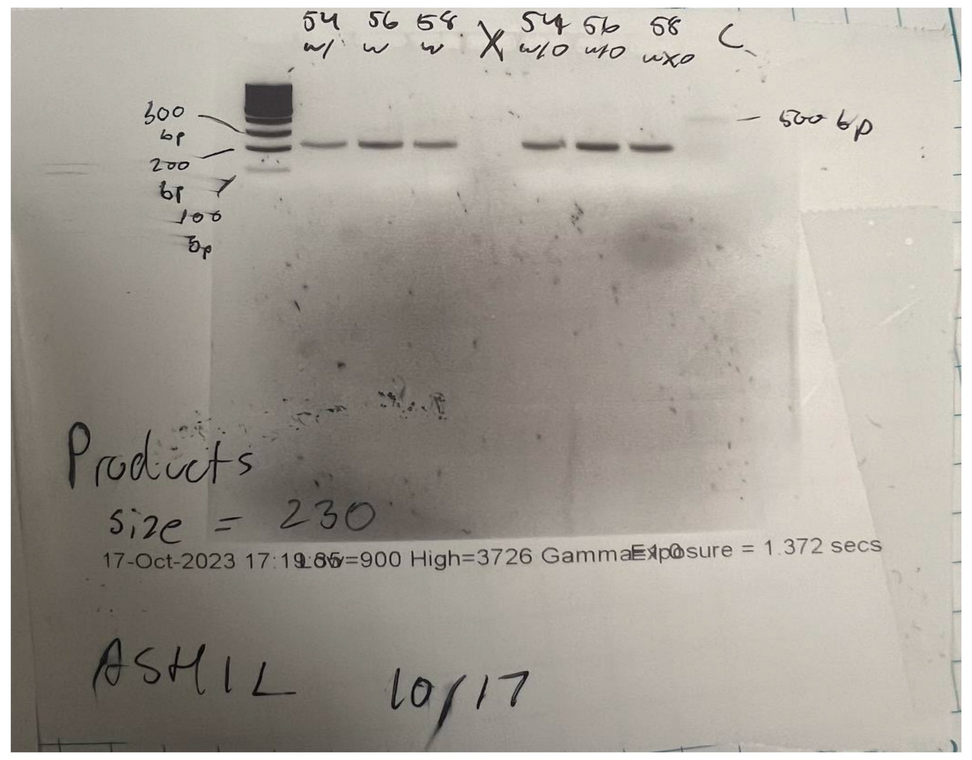

Figure 2- Figure shows the Gel Electrophoresis results for the Primer validation. The most prominent band can be seen at 56 C w/o DMSO and shows that all bands lie between the 200 and 300 length mark indicating successful validation

Following the successful validation of primer conditions, we proceeded with replicating the PCR procedure using blood samples from the patient and parents. This iteration focused on real blood samples rather than control blood used in primer verification. Three samples were generated, each designated for the patient, father, and mother, and subjected to PCR under consistent conditions: annealing at 56°C without DMSO. Gel electrophoresis results confirmed distinct bands in expected positions for all samples, indicating successful reactions and reinforcing the reliability of our experimental protocol. The purified samples were then forwarded to the Sanger Sequencing facility for comprehensive DNA analysis. Renowned for its accuracy and reliability, Sanger Sequencing determined nucleotide sequences, unraveling the genetic information within the samples with precision.

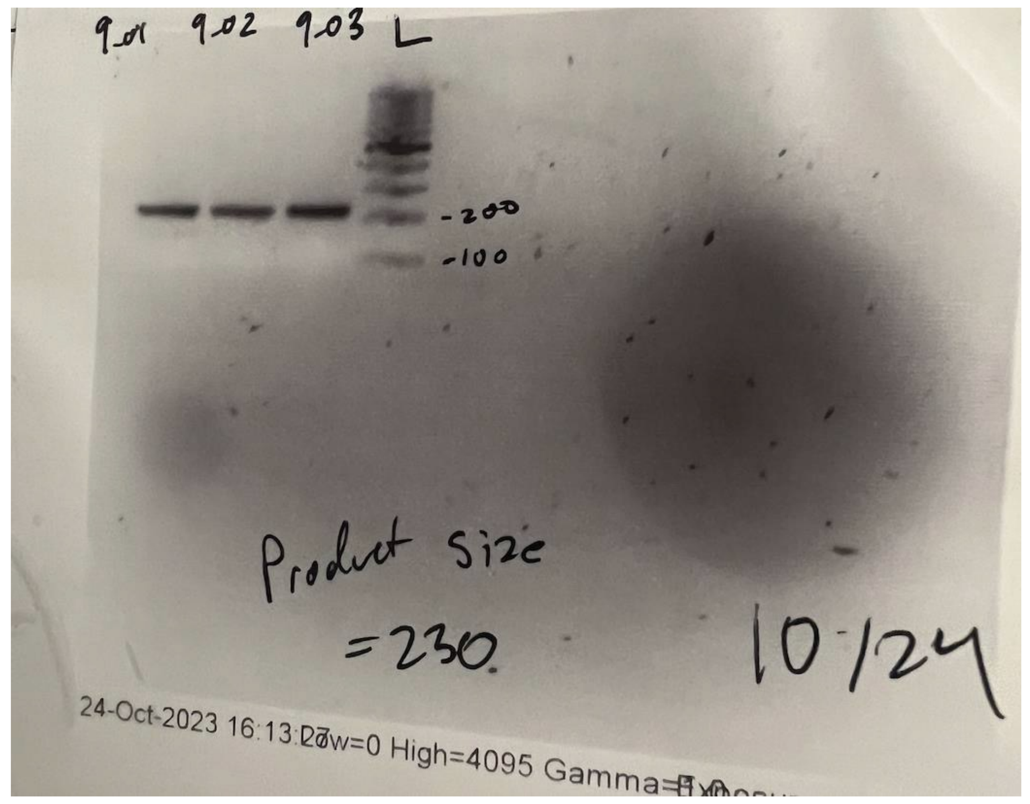

Figure 3- Figure shows the successful PCR reaction of the 3 samples and the successful validation of the primers for each sample.

RESULTS

Following the successful completion of Sanger Sequencing, the results undergo meticulous analysis to identify specific mutations. Each sample is scrutinized for genetic nucleotide variations within a particular region of the genome, revealing crucial information on genetic inheritance. In this case, the mutation absent in the maternal genetic profile but present in the paternal makeup strongly suggests paternal inheritance, potentially contributing to epilepsy development, especially considering the father's seizure history. This finding underscores the importance of genetic factors in epilepsy and paves the way for further research into neurological disorders.

Figure 4- Figure shows the genetic analysis of the patient (child with the disease) and shows the presence of a mutation from a typical G genotype to an A genotype at the region of interest (highlighted)

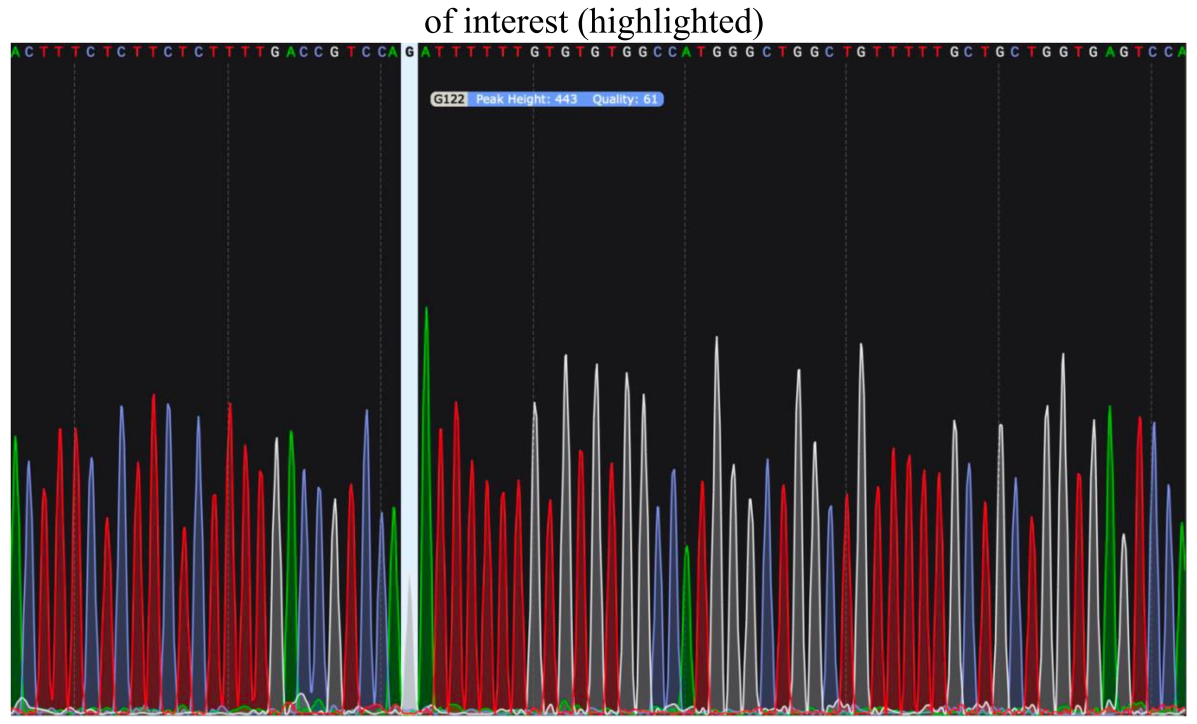

Figure 5- Figure shows the genetic analysis of the mother of the patient DNA and shows that there is the typical G genotype present at the region of interest (highlighted) indicating no mutation.

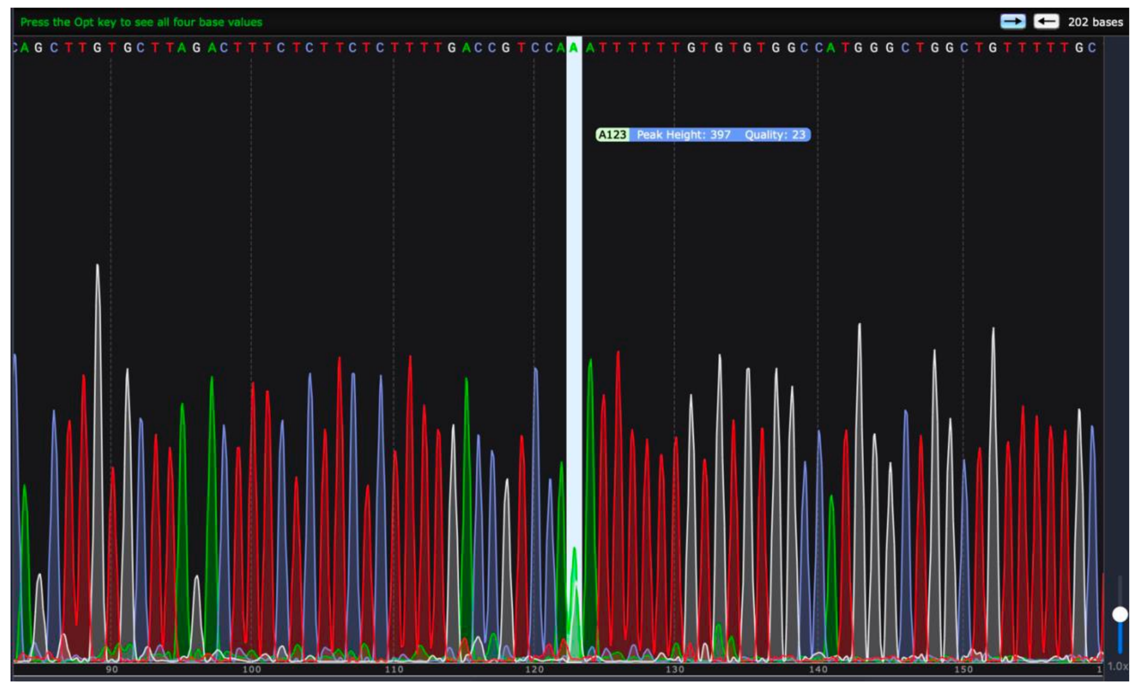

Figure 6- Figure shows the genetic analysis of the father of the patient and shows the presence of a mutation from a typical G genotype to an A genotype at the region of interest (highlighted)