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Research Article

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Identification of New Variants Related to Epilepsy in the Reanalysis of NGS Data of Patients with Epilepsy and Intellectual Disorders

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Abstract

Objective: Epilepsy is a chronic, noncommunicable brain disorder affecting individuals of all ages, with over 50 million cases worldwide. Advances in next-generation sequencing have enabled the identification of disease-related gene mutations; however, the yield of whole-exome sequencing (WES) for patients with epilepsy remains variable. Given our limited knowledge and the continual updates in genetic databases, reanalysis of older sequencing results may reveal relevant mutations and uncover false negatives.

Materials and Methods: In this study, we reanalyzed the initial WES data and clinical information for 12 patients with previously negative results.

Results: New mutations were identified in 5 patients and associated with 5 genes.

Conclusion: Thus, for patients with a strong suspicion of genetic disease, re-evaluating WES data is recommended even if the initial findings are negative.

Keywords Epilepsy · Mental Retardation · Intellectual Disability · Whole Exome Sequencing · Reanalysis

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INTRODUCTION

Even after a comprehensive diagnostic assessment, between 0.6% and 0.8% of the general population suffers from epilepsy, with a significant number of these cases having an unknown origin.[1] In between 20% and 30% of instances, acquired disorders, including stroke and head trauma, are the primary cause; in the remaining cases, hereditary factors are significant.[2] The most frequent co-occurring conditions with epilepsy are intellectual impairment (ID) and developmental delay (DD).[3] ID/DD and epilepsy are genetically diverse illnesses. It remains difficult to diagnose epilepsy and ID/DD disorders genetically. It is believed that the genesis of epilepsy, DD, and ID involves more than hundreds of genes.[4] Next-generation sequencing (NGS) has made it possible to find disease-associated gene variations at a pace never before possible. One NGS method, whole-exome sequencing (WES), has a diagnostic rate of 25%–50%. The reported yield of WES for patients with epilepsy varies (28–70%), partly because of phenotypic variations among the cohorts under analysis.[5,6] New genes and broader phenotypes of existing genes have been found thanks to NGS. However, NGS is not without its difficulties, especially regarding variant interpretation and clinical correlation, which may both need Multiple sources. Most people contain more than 2500 non-synonymous variations, according to studies from control populations. These variants include approximately 150 loss-of-function variants and 20–40 variants that are believed to be pathogenic. Numerous variations discovered by NGS are variations of Uncertain Significance (VUS) or occur in genes with unclear clinical significance, although certain variants are readily classified as benign or pathogenic.[7]

Following reanalysis, several studies found that novel gene-disease connections, resequencing singletons as trios, searching for copy-number variations, and case matching using Matchmaker Exchange resulted in increased diagnosis rates of 10%–36%. We performed a reanalysis of WES data from 12 raw WES data and clinical information for patient's trios whose initial reports returned negative results. Due to the updating of gene databases and targeted analysis, we expect to be able to identify phenotype-related variants in patients. Reanalysis of previously negative data in patients with epilepsy and intellectual disability as genetic databases are updated could reveal important findings and hidden diagnoses—a topic that remains under-researched in the scientific literature.

MATERIALS AND METHODS

Sample Collection

We took into account patients who met the conventional inclusion criteria for epileptic ID/DD while collecting the sample, which are as follows: 1. limitations in at least two of the following skill areas: functional academic skills, job, leisure, self-care, self-direction, communication, home living, health, safety, and social/interpersonal skills. 2. frequent seizures or epilepsy before the age of eighteen; 3. diagnosed with drug-resistant epilepsy and progressive developmental delay or with a known epileptic encephalopathy syndrome; 4. multiple epileptiform discharges with severely disorganized background activity on electroencephalography (EEG); 5. speech disorders and hypotonia; 6. intelligence quotient (IQ) below 70 by measured standardized method; 7. Drug-induced epilepsy and fibril epilepsy were the exclusion criteria. Twelve individuals whose WES were originally negative were contacted from the Farabi Clinic in Erbil, Iraq. Reanalysis was performed at 6–12 months after the first negative diagnosis; for reanalysis, the raw data of the probands were gathered from the genetic testing facilities. Pediatric epileptologist thoroughly examined the EEGs and detailed clinical characteristics. The Ethics Committee of the Kurdistan Higher Council of Medical Specialties Vice President's Office approved the study (Approval Number: 4454). The parents of all patients provided written, informed permission. The institutional review boards gave their approval for this research.

NGS Data Analysis

For the elimination and filtration of low-quality reads, the fastp tool was applied. Each sample's quality measurements were computed using the TEQC package and the FastQC program. For data analysis, we created a pipeline tailored to each WES result. In a nutshell, BaseStudio software (Illumina) was used to cross-check the small nucleotide variations identified by Genome Analysis Toolkit (GATK). The Burrows-Wheeler Aligner approach was used to map the raw sequence data to GRCh37 (hg19). Then, GATK software (version 4.1.7) was used to remove duplicate reads, realign insertions and deletions, recalibrate the base quality, and identify variants. By visually inspecting the Bam file using Integrated Genomics Viewer version 2.3 (IGV; Broad Institute, Cambridge, MA, USA), every variation presumed to be VUS, possibly pathogenic, or pathogenic was verified.

ExomeDepth software was used for the read-depth detection of structural rearrangements. For split-read detection of large insertions and deletions, the Pindel and Manta algorithms

were used. The CopywriteR software was used for off-target analysis of chromosomal copy-number alterations. The ExomeDepth algorithm was used to detect chromosomal copy-number variations.

For variant annotation, common databases such as ClinVar, Human Gene Mutation Database (HGMD), Exome Aggregation Consortium (ExAC), 1000 Genome, Exome Sequencing Project, Korean Reference Genome Database (KRGDB), dbSNP, and OMIM are used.

Polymorphism Phenotyping v2 (PolyPhen-2), Mutation Taster, Mutation Assessor, and Sorting Tolerant from Intolerant (SIFT) were used to identify the pathogenicity of missense variants. Functional Analysis through Hidden Markov Models (FATHMM) implemented in dbNSFP version 3.0a. The effects on splicing were predicted using SPIDEX version 1.0 and dbscSNV version 1.1. 2-3. Annotation and interpretation of variants.

In order to optimize the diagnostic process, variants that did not occur in the general population were deemed to have moderate evidence of being pathogenic, whereas those that did occur in any of the population subgroups and had a minor allele frequency (MAF) greater than 5% were classified as absolutely benign. Second, using the HGMD professional database and the Alamut Visual 2.6 program (Interactive Biosoftware, France), literature and database searches were conducted for prior reports and functional investigations.

We used a methodical approach to analyze variations in accordance with the 5-tier categorization scheme suggested by the Association for Molecular Pathology and the American College of Medical Genetics and Genomics (ACMG/AMP). The variants identified were characterized using the Human Genome Variation Society's recommended nomenclature (http://www.hgvs.org/mutnomen). *In silico* studies are considered to indicate benign or harmful variations when all predictions are consistent.

Phenotype Review and Consensus Discussion

For each patient, clinical findings were carefully assessed by pediatric epileptologist, neurologists, and medical geneticists in a multidisciplinary environment. All phenotypic details were cross-checked with reported gene-disease associations in the OMIM and ClinVar databases. If an identified variant was similar to a patient's phenotype, the case was submitted for variant reclassification by consensus discussion. VUS were filtered for analysis according to phenotypic similarity, inheritance mode, *in silico* prediction, and parental segregation analysis.

Confirmation Using Other Methods

All pathogenic, likely pathogenic, and high-priority VUS variants identified by WES were confirmed by Sanger sequencing. Each specific primer was designed using Primer3 software. PCR amplifications were performed under standard conditions: 95°C for 5 min, 35 cycles of 95°C for 30 s, 55–60°C annealing for 30 s, and 72°C extension for 1 min, with a final 72°C for 10 min. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on a 3730 DNA Analyzer, and sequences were edited using Sequencher 5.3 software.

RESULTS

Patient Demographic Information and Clinical Features

Based on the inclusion and exclusion criteria, 12 previously WES-negative patients with the main clinical manifestations of DD/ID and epilepsy were identified. The mean age was 12.25 years (±3.2 CI:95%), with eight males and four females. For the definition of the congenital route, we investigated the history of seizures and DD/ID in parents or their family, and more than a third/fourth of the patients (9 cases) demonstrated the congenital pattern. All patients exhibited frequent permeant seizures. To better classify the multiple clinical conditions of our patients, we introduced six general clinical manifestations. All clinical and demographic data of the patients are summarized in Table 1.

Molecular Diagnosis Yields

The WES results were reconstructed to discriminate epileptic MR/DD gene variants. Five patients (5/12=41.6%) were distinguished into the pathogenic and like-pathogenic groups associated with five gene variants. For VUS genomic alterations, seven patients had nine different gene mutations related to other congenital disorders (Table 2).

Gene Variants

Autosomal recessive (AR) and autosomal dominant (AD) or inherited from a parent (XL) were classified as homozygote, heterozygote, and hemizygote pattern. The eight genes *NDUFS3, NEU1, CLN3, CLN5, TRAPPC9, GJC2, BTD*, and *CNTNAP2* were identified in the AR group; three genes, such as *CLN3*, *BTD*, and *CNTNAP2,* were associated with pathogenic and pathogenic-like groups. Moreover, three gene mutations, including *KCNQ2*, *CPLANE1*, and *MUTYH*, are transmitted by autosomal dominance, where *CPLANE1* and *MUTYH* are associated with pathogenic and pathogenic-like groups,

Table 1. Case Information.

Case	Age	Consanguinity	Gender	Clinical Information
1	13	Yes	M	Abnormal facial shape, Dysarthria, Hyperactive deep tendon reflexes, Infantile onset, Intellectual disability, Left hemiplegia, Seizures, Strabismus, Upper limb spasticity
2	13	Yes	F	Generalized myoclonic seizures, Lower limp muscle weakness, Muscle weakness, nystagmus, and proximal muscle weakness in upper limbs
3	17	Yes	M	Dysarthria, Seizures, visual impairment, Visual loss
4	14	Yes	M	blindness, delayed speech and language development, dementia, seizures
5	15	No	M	Childhood-onset, Generalized tonic-clonic Seizures
6	9	Yes	M	Abnormality of movement, Ataxia, Delayed speech and language development, Developmental regression, Difficulty walking, Dysarthria, EEG abnormality, EMG, myopathic abnormalities, Intellectual disability, Myopathy, Seizures
7	10	Yes	F	Abnormal aggressive, impulsive or violent behavior, Cataract, Global developmental delay, Low-set ears, Nystagmus, Seizure
8	9	Yes	F	Mental deterioration, Nystagmus, Polydactyly, Seizure, Slurred speech
9	9	No	F	Cerebral atrophy, Delayed speech and language development, Failure to thrive, Hyporeflexia, Intellectual disability, Microcephaly, Micrognathia, Seizure
10	18	Yes	M	Abnormality of the cerebral white matter, Difficulty walking, Hydrocephalus, Memory impairment, Seizure
11	11	Yes	M	Aphasia, Behavioral abnormality, Global developmental delay, Mental deterioration, Seizure
12	9	No	M	Aphasia, Bowel incontinence, Brain atrophy, Difficulty climbing stairs, EEG abnormality, Intellectual disability, Seizure, Urinary incontinence

Table 2. Gene mutations related to epilepsy.

	Variant	Gene	Zygosity	Inheriting Pattern	Classification	Disease
1	c.79C>T	*NDUFS3*	Hom	AR	VUS	Autosomal recessive mitochondrial complex one deficiency nuclear type 8
2	c.1766T>C c.451G>A	*ALG13 NEU1*	Het Hom	X-linked AR	VUS	X-linked early infantile epileptic Encephalopathy type 36 and autosomal recessive sialidosis
3	**c.1127del**	***CLN3***	**Hom**	**AR**	**P**	**Autosomal recessive neuronal period lipofuscinosis type 3**
4	**c.1127del**	***CLN3***	**Hom**	**AR**	**LP**	**Autosomal recessive neuronal period lipofuscinosis type 3**
5	c.68G>A	*KCNQ2*	Het	AD	VUS	Autosomal dominant KCNQ2-related seizures
6	c.623G>A	*CLN5*	Hom	AR	VUS	Autosomal recessive neuronal period lipofuscinosis type 5
7	c.3162A>T c.1598C>T	*HUWE1 TRAPPC9*	Hem Hom	X-linked AR	VUS	X-linked syndromic Mental retardation Turner type. Autosomal recessive mental retardation type 13
8	c.706G>C	*GJC2*	Hom	AR	VUS	Autosomal recessive hypomyelination Leukodystrophy type 2
9	**c.1336G>C c.3577C>T**	***BTD CPLANE1***	**Hom Het**	**AR AD**	**P P**	**Biotinidase deficiency Joubert Syndrome**
10	**c.1336G>C c.734G>A**	***BTD MUTYH***	**Het Het**	**AR AD**	**P P**	**Biotinidase deficiency AUTYII-associated polyposis**
11	**c.785G>A**	***CNTNAP2***	**Hom**	**AR**	**LP**	**Autosomal recessive Pitt-Hopkins-like syndrome type 1**
12	c.3997c>g	*SHROOM4*	Hem	X-linked	VUS	Stocco dos Santos type X-linked syndromic intellectual developmental disorder

respectively. *NEU1*, *HUWE1*, and *SHROOM4* are X-linked genes classified into the VUS group.

Regarding the c.1127del mutation in the *CLN3* gene, according to the ClinVar database, this mutation causes a frame-shift, which ultimately leads to RNA degradation via nonsense-mediated decay. This change reduces the expression of this gene at the protein level, and this receptor loses its function. According to the MEDLINE database, mutations in this gene cause CLD3 disease. CLN3 disease is a hereditary condition that mostly affects the neurological system. After 4–6 years of normal growth, children with this disorder experience eye impairment, intellectual disabilities, mobility impairments, speech difficulty, and seizures, which worsen over time. Single nucleotide mutations were the main type of genomic alterations observed in 14 cases (14/16 = 87.5%). In addition, only one kind of nucleotide deletion was identified in two cases. The whole genomic analysis is summarized in Table 2.

DISCUSSION

As NGS methods advance quickly and become more economical, more patients with suspected genetic illnesses will be able to afford WES rather than gene panel testing. In recent investigations, the overall diagnosis rate with proban-only WES was 22%~28.8%.[8-10] Positive yields of 16%–62% have been reported in many publications using a trio-based WES method.[11] According to these findings, proband-only WES testing was inferior to trio-WES testing.[12] Mostly due to its ease of usage in identifying proband de novo variations as well as compound heterozygous or homozygous variants. Some examples of Mendelian disorders did not yield favorable results. This may have occurred because the mutations were not identified properly because of insufficient information on the link between the phenotype and genotype or because the available tools and tactics for mutation hunting were insufficient.

The ClinVar database indicates that the c.1127del mutation in the *CLN3* gene results in a frame-shift, which in turn causes RNA degradation via nonsense-mediated decay. This alterations result in decreased gene expression at the protein level and the loss of function of this receptor. The MEDLINE database indicates that this gene mutation results in CLD3 disease, a hereditary condition that mostly affects the neurological system. Children with this syndrome experience mobility issues, speech difficulties, intellectual impairments, visual impairments, and seizures after 4–6 years of normal development. These symptoms intensify with time.[13] Individuals with CLN3 illness often have seizures and strange movements throughout their adolescent years. These anomalies include clumsiness, hypokinesia (slow or decreased movement), stooped posture, and muscular rigidity or stiffness.[14] Affected people are eventually unable to walk or sit on their own and need help from a wheelchair. A twisted impression of reality (psychosis) or erroneous perceptions (hallucinations) is uncommon in patients with CLN3 illness. Later in adulthood, some people experience an irregular heartbeat (arrhythmia). Most CLN3 illness sufferers survive into their early adult years.[15] Another mutation that was found in two patients to be both homozygous and heterozygous was the missense variant c.1336G>C in the *BTD* gene. This single nucleotide variation is located in the gene's coding region, resulting in the conversion of amino acid histidine to aspartate. Data from the ClinVar and OMIM databases indicate that this mutation is associated with biotinidase deficiency.[16] This impairment is a hereditary condition in which the body is unable to recycle the vitamin biotin, according to the MEDLINE database. The signs and symptoms of this ailment usually appear within the first few months of birth, but they may also appear later in childhood if not identified and treated. The most severe version of the illness, known as profound biotinidase deficiency, may lead to a variety of symptoms, including skin rashes, hair loss (alopecia), breathing difficulties, hearing and vision loss, seizures, hypotonia, breathing issues, skin rashes, and fungal infections like candidiasis. Children affected also have developmental delays. Chronic care may either prevent these problems from developing or help them if they have already started.[17,18] An additional pathogenic mutation in a patient was c.3577C>T in the *CPLANE1* gene. At position 1193, this single nucleotide alteration results in the amino acid conversion of arginine to cysteine in the coding region of this gene. Joubert syndrome is the sole illness associated with this variation in the ClinVar database[19] based on the MEDLINE database. A condition known as Joubert syndrome affects several bodily components. Even among members of the same family, afflicted people exhibit different indications and symptoms of this disorder.[20] Joubert syndrome is characterized by several brain abnormalities. The majority of newborns with Joubert syndrome have hypotonia or low muscle tone, which may lead to ataxia or trouble coordinating movements in early-life Ocular motor apraxia, irregular eye movements, and periods of abnormally rapid (hyperpnea) or slow (apnea) breathing throughout infancy are further characteristics of this illness. The majority of those afflicted have moderate to severe intellectual disabilities and delayed development. More than 30 genes may have mutations resulting in Joubert syndrome.[21] In addition to the previously described variation c.1336G>C in the *BTD* gene, case number 10 also included the identification of a heterozygous variant in the *MUTYH* gene, c.734G>A, which was classified as pathogenic based

on pathogenicity. This single nucleotide alteration, which changes arginine to histidine, was observed in the coding region and exon 9 of the *MUTYH* gene. The association between this variation and familial adenomatous polyposis 2 and hereditary cancer predisposition has been reported in several studies.[22] MUTYH is a base excision repair enzyme that may be considered a cell protection factor. It is essential for repairing DNA mistakes caused by guanine oxidation. In both salivary gland secretory carcinoma and sporadic gastric cancer, the existence of *MUTYH* pathogenic mutations is an independent predictor of poor prognosis, and its suppression has been shown to decrease the survival of pancreatic ductal adenocarcinoma cells.[23] Additionally, a few MUTYH SNPs have been linked to increased risks of cervical, hepatocellular, and lung cancer. A further function of MUTYH can be its involvement in the prevention of many other conditions that have an inflammatory or degenerative base, such as illnesses of the nervous system and eyes. In case 11, the c.785G>A variation in the *CNTNAP2* gene was ultimately identified as a novel mutation. This mutation, which likewise occurred in the gene's coding region and resulted in the change of arginine for glycine in the protein's 228 amino acid sequence, was probably pathogenic. The transmembrane protein contacting-associated protein-like 2, encoded by NTNAP2, is expressed throughout the brain, with the cortex, hippocampus, substantia nigra, interpeduncular nucleus, pontine nucleus, and medial mammillary nucleus exhibiting the highest levels of expression.[24,25] It facilitates axo-glial contact in mature myelinated axons. The first known mutation causing cortical dysplasia-focal epilepsy syndrome was found in a consanguineous Old-Order Amish family with the homozygous *CNTNAP2* frameshift variation, c.3709delG.[25] The homozygous splice acceptor mutation c.1671-1G>T was discovered by Zweier and colleagues[26] in a consanguineous family of Pakistani descent with Pitt-Hopkins-like syndrome. Furthermore, individuals with epilepsy, intellectual impairment, and/or autistic spectrum disease have also been shown to have homozygous deletions of different sizes within CNTNAP2. Our findings are in agreement with previous studies that have highlighted the clinical significance of reinterpretation of negative WES findings in patients with epilepsy and intellectual disability. For example, Li et al. (2019) reported that reanalysis increased the diagnostic yield by uncovering newly classified pathogenic variants and making use of the latest genetic databases, resulting in further diagnoses among patients who had not been previously diagnosed.[8] While such studies had reanalysis diagnostic yields of 10% to 36%, in our study the reanalysis identified pathogenic or likely pathogenic variants in 41.6% (5 out of 12) of patients, which is a relatively high yield compared to other published reports. This discrepancy may be attributed to the selection of highly specific patient population with elevated clinical suspicion of genetic epilepsy and intellectual disability and extensive clinical re-phenotyping before reanalysis. Furthermore, we observed that the majority of variants were in genes such as *CLN3*, *BTD*, and *CNTNAP2*, which have been described as having overlapping phenotypes with neurodevelopmental disorders and epilepsy, highlighting the importance of diligent genotype-phenotype re-matching upon reanalysis. This is consistent with other reports like Demos et al. (2019), who observed that the diagnostic yield in epilepsy was optimal with a detailed clinical review along with trio-WES.[10] These observations collectively emphasize the pivotal role of continuous database enrichment, along with multidisciplinary team discussions, in maximizing the diagnostic yield of WES, especially in genetically diverse disorders such as epilepsy. A reanalysis of previously negative data may still provide positive findings because of the progress made in bioinformatics and the identification of additional genes that cause illness. Our data revealed that reanalysis at 6–12 months after the first negative diagnosis is critical for enhancing the diagnostic yield and utility of trio-based WES. For patients with suspected Mendelian illness, successful reanalysis requires improved communication between genetic testing facilities and doctors. All genetic testing institutions should promptly update their databases.

CONCLUSION

There is a chance of false-negative findings in this condition because epilepsy is a multigene disorder, and gene databases connected to the disease are updated at a remarkable rate. This research demonstrated how, over time and with the upgrading of gene databases (genes linked to illness), negative findings from NGS analysis might become erroneous results. Regular database updates, along with a more thorough analysis and comparison of the findings with recent research, are some possible solutions. This research recommends that diagnostic centers reanalyze test findings between 6 and 12 months after the first test in situations where the results were reported as negative to confirm the results obtained.

Ethics Committee Approval	The study was approved by the Ethics Committee of Kurdistan Higher Council Of Medical Specialties Vice President Office, (Approval Number: 4454).
Informed Consent	Informed consent was obtained from the parents of all patients participating in the study.
Peer Review	Externally peer-reviewed.

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