Customized targeted massively parallel sequencing enables the identification of novel pathogenic variants in Tunisian patients with Developmental and Epileptic Encephalopathy

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Abstract

Background Developmental and Epileptic Encephalopathies stand for a heterogenous group of epileptic syndromes, where the epileptic activity itself and/or the etiology contribute to cognitive and behavioral impairment. In recent decades, genetic etiology has increasingly been recognized as the cause of Developmental and Epileptic Encephalopathies and numerous genes have been identified, thanks to advances in genetic technologies. These discoveries have enabled precision treatments for several syndromes. Therefore, the identification of the causal variant in a gene is an intrinsic starting point to specify a precision therapy for the patient and an adequate management.

Results We developed a custom panel for Next Generation Sequencing of the coding sequences of 116 genes in individuals with Developmental and Epileptic Encephalopathy from the Tunisian population. Segregation analyses as well as in silico studies have been conducted to assess the identified variants' pathogenicity. We report 12 pathogenic variants in *SCN1A*, *CHD2*, *CDKL5*, *SZT2*, *KCNT1*, *GNAO1*, *PCDH19*, *MECP2*, *GRIN2A,* and *SYNGAP1* in patients with Developmental and Epileptic Encephalopathy. Five of these variants are novel: "c.149delA, p.(Asn50MetfsTer26)" in *CDKL5*; "c.3616C>T, p.(Arg1206Ter)" in *SZT2*; "c.111_113del, p.(Leu39del)" in *GNAO1*; "c.1435G>C , p.(Asp479His)" in *PCDH19;* as well as "c.2143delC, p. (Arg716GlyfsTer10)"in *SYNGAP1*. Additionally, for five of our patients, the genetic result facilitated the choice of the appropriate treatment.

Conclusion This is the first report of a custom gene panel to identify genetic variants implicated in Developmental and Epileptic Encephalopathy in the Tunisian population as well as the North African region (Tunisia, Egypt, Libya, Algeria, Morocco) with a diagnostic rate of 30%. This high-throughput sequencing panel has considerably improved the rate of positive diagnosis of Developmental and Epileptic Encephalopathy in the Tunisian population, which was less than 15% using Sanger sequencing. The benefit of genetic testing in these patients was approved by both physicians and parents.

Background

Epilepsy is a common neurological disease with 50 million affected individuals worldwide. As a result, it is among the most frequent neurological diseases globally. Relying on the World Health Organization, approximately three-quarters of them live in low- and middle-income countries. Additionally, the premature death risk in people with epilepsy amounts to three times higher than for the general population (https://www.who.int/news-room/fact-sheets/detail/epilepsy). Developmental and epileptic encephalopathy (DEE) corresponds to a heterogenous group of epileptic syndromes marked by early-onset, refractory seizures that also take place within the framework of developmental regression [1]. According to the ILAE classification of epileptic syndromes, DEE encompasses several clinically definable epilepsy syndromes including Dravet syndrome (DS), Infantile epileptic spasms syndrome (IESS), Lennox-Gastaut syndrome (LGS) and epilepsy of infancy with migrating focal seizures (EIMFS) [1–3]. Etiologies of DEE are variable. With the advances in sequencing methods, genetic etiologies become more and more frequent and reach more than 50% of patients with early-onset DEE [4]. In Tunisia, DEE constitutes a

significant burden for the family and the healthcare system. It represents 43% of the epileptic syndrome in the Sfax department of child neurology (not published data). In Tunisia, as well as in North Africa, data are scarce regarding the genetic basis of epilepsy. In fact, in the whole African continent, only one study has recently been published. It was concerned with the development of a DEE panel in the South African region [5]. Therefore, exploration of the etiology of DEE as well as the early diagnosis of causal variants by Next Generation Sequencing (NGS) assists to a great extent in terms of alleviating social and economic problems. Scrutinizing through literature, multiple research works tackled the use of gene panels in epilepsy with variable diagnostic yields [6]. So far, this variability made it difficult to identify an appropriate gene pool whose screening might enable accurate diagnosis and generate higher diagnostic yields in people displaying heterogenous epilepsy phenotypes. From this perspective, wide-ranging approaches, such as whole exome sequencing (WES) or whole genome sequencing (WGS), are basically adopted as alternative genetic testing methods. Yet, the interpretation of gene variants with WES and WGS is not only time-consuming but also a more sophisticated task. Currently, panel-based sequencing is still preferred in certain clinical centers, owing to its depth of coverage, speed of data analysis as well as cost saving [7].

In our study, we built up a comprehensive NGS panel interrogating 116 genes implicated in DEE. The panel covered the coding exons as well as the exon-intron junctions, providing a high throughput assay. Our basic objective is to clarify the frequency of disease-causing genes among the Tunisian population.

Methods

Subjects and sample preparation

We collected 40 Tunisian children from the region of Sfax and southern Tunisia. Recruitment of patients was undertaken between 2020 and 2022 in the Department of Child Neurology of Hedi Chaker Hospital in Sfax. All patients were examined and diagnosed by a pediatric neurologist. Clinical data, including age at the onset, frequency, and type of seizures, as well as the presence of developmental delay or regression, acquired or congenital microcephaly, abnormal movements or stereotypies, and dysmorphic syndrome were analyzed. Seizure types and epileptic syndromes have been diagnosed and classified relying on International League Against Epilepsy classification [1–3].

Whole blood was collected from all the patients. Samples from additional family members were invested whenever possible to conduct segregation analysis of the sequence variants identified in the index patient. We extracted DNA from all samples through the use of the phenol-chloroform standard method.

Capture design and Targeted Next generation sequencing

We designed a hybridization-based multi-disease gene panel using Design Studio which is a web application from Illumina to devise a custom target enrichment library design (San Diego, CA 92122 USA). The design rested on GRCh37/hg19 reference sequences, with target sources obtained from the RefSeq database. All coding exons in this custom design were targeted including 25 bp of the flanking

intronic sequence of 116 genes. The criteria for including a gene on the panel were that it should have been reported more than once in patients with epilepsy. All selected genes are included in Supplemental Table 1. Most of these genes have an autosomal dominant or an autosomal recessive inheritance mode (Supplemental Fig. 1). Libraries were prepared relying on the “Illumina DNA Prep with Enrichment sample preparation reference guide” (Diego, CA 92122 USA). The libraries were sequenced on the Miseq system (Illumina).

Bioinformatic pipeline

We adjusted MiSeq Reporter software settings (Illumina) to generate VCF files for index reads. VarAFT was used for variant annotation and filtering [8]. Variants with minor allelic frequency (MAF) < 0.1% were retained. Nonsense variants and small deletions or insertions inducing a frameshift of the coding sequence were regarded as the most harmful, as they necessarily altered the amino-acid sequence of the protein. We also examined the pathogenicity of the different variants in accordance with ACMG (American College of Medical Genetics and Genomics) standards and guidelines [9]. Variants were classified into five types, namely “Benign”, ”Likely Benign”, “Pathogenic”, ”Likely Pathogenic” and “Uncertain significance”. Moreover, AF in the genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/) was invested to assess the variant’s frequency.

We estimated the pathogenicity of missense variants through the use of SIFT and PROVEAN algorithms [10]. Additionally, we specified the pathogenicity of the substitution variants by Mutation Taster [11]. To check if the sites of the variants are conserved, we used PhyloP100way whose scores rely on many alignments of 99 vertebrate genome sequences to the human genome. The higher the score, the more conserved the site.

Validation by Sanger sequencing

We analyzed variants suspected to be pathogenic using Sanger sequencing. We amplified DNA fragments involving the variants by PCR with specific primers and we sequenced them on both strands using the Big Dye 3.1 Terminator Sequencing Kit. We analyzed purified sequence products on a 3100 ABI instrument (Applied Biosystems, Foster City, CA). We conducted a segregation analysis in cases where DNA samples of relatives were obtainable.

RESULTS

Clinical findings

We collected 40 Tunisian children with DEE. Pathogenic variants were identified per patient and their respective clinical presentations are illustrated in Table 1.

Genetic findings

A total of 12 variants were identified and predicted to be pathogenic or Likely pathogenic (Two frameshift, five missense, four nonsense, and one in-frame variants). The commonest gene in which

positive findings were identified was *SCN1A* (3 patients). Additionally, variants were identified in *CDKL5*, *GNAO1*, *KCNT1*, *CHD2*, *PCDH19*, *SZT2, MECP2, GRIN2A,* and *SYNGAP1.* Among these variants, five are novel and seven were previously reported in the literature. Table 2 shows the number of pathogenic variants detected in the study as well as the results of the software's predictions.

Treatment adaptations offered to patients after the genetic diagnosis

According to the World Health Organization, it is recorded that up to 70% of people affected with epilepsy can live seizure-free if they are properly diagnosed and treated (https://www.who.int/news-room/fact-sheets/detail/epilepsy). In the current study, 12 pathogenic or likely pathogenic variants were identified. Identification of a specific underlying genetic variant can guide precision medicine in terms of preventing paradoxical aggravation of certain epilepsies. In fact, it is crucial to manage seizures carefully so as to shun disability and injuries and minimize the risk of life-threatening complications, like sudden unexpected death in epilepsy and status epilepticus. In this study, five patients benefited from treatments after genetic diagnosis. The ages of these patients in the study range from 2 to 17 years and the ages of the first symptoms range from 1.3 to 11 months (Table 1). As far as DS is concerned, its early suspicion by means of *SCN1A* loss of function variants' identification may be extremely beneficial. Sodium channel-blocking drugs need to be avoided, as they may even aggravate the seizures or are likely to be ineffective [12, 13]. In such cases, alternative treatments involve benzodiazepines, valproate, stiripentol, cannabinoids, fenfluramine, and the ketogenic diet [13-17]. For our patients (SEED.0009 and SEED.0198) carrying "c.3094G>T, p.(Glu1032Ter)" and "c.1837C>T,p.(Arg613Ter)" variants in *SCN1A*, these alternative treatments were prescribed. Additionally, given the efficient role of Quinidine in patients carrying variants in *KCNT1*[18], SEED.0060 patient with the "c.2714G>A,p.(Arg905Gln)" variant in *KCNT1*, benefited from this treatment. In this respect, it was reported that Levetiracetam corresponds to a powerful and reliable therapy for females with *PCDH1*9-Girls Clustering Epilepsy and has to be considered early in the management of the highly refractory clusters of seizures that characterize this genetic disease [19]. Moreover, Ganaxolone was reported to significantly reduce the frequency of CDKL5 Deficiency Disorder-associated seizures [20]. For these reasons, SEED.0074 and SEED.0021 benefited from Levetiracetam and Ganaxolone, respectively.

DISCUSSION

In Tunisia, the analysis of DEE remains confined to a few cases based on candidate gene approach or clinical exome sequencing instead of targeted sequencing [21-30]. Yet, in terms of DEE for which more than 100 candidate genes were reported with a phenotypic heterogeneity, investing in targeted sequencing may be an optimal method for choice as this approach facilitates the analysis of generated sequencing data, reduces sequencing cost, increases sequencing depth, and does not include any ethical concerns in terms of the return results. This report corresponds to the first one tackling a custom gene panel to identify genetic variants implicated in DEE in the Tunisian population with a positive diagnostic rate of 30% (12/40). This diagnosis rate was less than 15% before the development of the panel (Unpublished data).

Expanding the spectrum of DEE variants in Tunisian patients

In this research work, we successfully identified five novel pathogenic mutations and seven preceding reported pathogenic variants in the Tunisian population for the first time.

Variants in *SCN1A* were reported previously in Tunisian patients with DS or generalized epilepsy with febrile seizures plus (GEFS+) [24, 26, 27]. However, this is the first report of "p.(Glu1032Ter)", "p.(Arg613Ter)" and "p.(Gly1586Arg)" variants in Tunisia. According to the literature, the "p.(Glu1032Ter)" variant in *SCN1A* is reported twice in DS patients [31, 32], and "p.(Arg613Ter)" variant is reported several times in patients with DS [33-38] (Supplemental Table 2). Our patients (SEED.0009 and SEED.0198) present also a DS (Supplemental Table 2), which goes in good consistency with previous studies showing that this syndrome is associated with loss of function variants [39]. The "p.(Gly1586Arg)" variant was previously reported twice in an Italian and a Turkish patient with DS and unclassified epilepsy, respectively [40, 41] (Supplemental Table 2). However, our patient presents DEE with fever-sensitive epilepsy. Our findings are consistent with those reported in previous research suggesting that variants cluster in regions of channel inactivation (S4-5 and D3-4 linker) are often associated with Gain of function [42].

Additionally, the "p.(Arg905Gln)" variant is detected in *KCNT1*. This variant was recorded previously in affected persons with DEE[43, 44], Childhood-Onset Epilepsy [32, 45], EIMFS with or without IESS[46-51], Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE)[52], Autosomal dominant forms of Sleep-related hypermotor epilepsy (ADSHE) [53, 54] and Early Onset Epileptic Encephalopathy (EOEE) [47] in many populations. Referring to our patient (SEED.0061), the "p.(Arg905Gln)" variant is responsible for Sleep-related Hypermotor Epilepsy (Supplemental Table 2). The coexistence of different phenotypes for the same genetic variation is indicative that possibly genetic or environmental modifiers exist, which suggests the need for extended research into gene variants.

We equally identified a truncating variant "p.(Arg900Ter)" in *CHD2* causing LGS in the patient. This variant has never been described in the literature but it has been reported in the ClinVar database as being involved in DEE 94. So far, according to the Human Gene Mutation Database, 86 disease-causing variants have been reported in *CHD2* in patients with epileptic encephalopathy, intellectual disability ranging in severity, autism spectrum disorder, seizures myoclonus, status epilepticus, and ataxia (clinicalgenome.org). The majority of pathogenic variants are truncating (>72%)[55]. However, in spite of the high number of previously reported *CHD2* pathogenic or likely pathogenic variants, no clear genotype-phenotype correlation was found. Our study characterizes the tenth patient worldwide carrying a variant in *CHD2* with LGS as a phenotype [55, 56]. The identification of variants implicated in the same phenotype can help find a correlation between genotype and phenotype in the future.

The frame-shift variant "p.(Asn50MetfsTer26)" was not previously reported in *CDKL5*. So far, more than 265 variants in this gene have been reported [57]. As far as we know, we judge that our novel frame-shift variation corresponds to the second frame-shift *CDKL5* variant recorded in Tunisian patients after the "p.(Glu930GlyfsTer9)" [21]. In this population, six other variants are identified at the level of this gene which

all correspond to missense variants [22]. The identified variant in this study, as well as all previously reported variants in *CDKL5* in Tunisia, were associated with IESS. Our data offer soundproof and more comprehensive information to confirm that *CDKL5* is a potential gene for IESS in our population.

The nonsense variant “p.(Arg1206Ter)” in *SZT2* was detected in a homozygous state in our patient from a consanguineous family. This variant has not been previously reported in the homozygous state in control databases. Our study was conducted on the first set of siblings with homozygosity for the “p.(Arg1206Ter)” variant in *SZT2.* This result further indicates biallelic variants in this gene as a cause of DEE. According to the literature, only 24 patients carrying variants in *SZT2* have been reported with a wide phenotypic spectrum from mild Intellectual Disability without epilepsy to DEE [58]. Other relevant characteristics refer to such macrocephaly and radiological findings as neuronal migration disorders and corpus callosum abnormalities, which might refer to the hyperactivation of mTORC1 signaling [58]. Though the genotype-phenotype correlation in *SZT2* variants remains ambiguous, frameshift variants proved to result in hyperactivation of mTORC1 signaling [59-61]. The identification of a homozygous variation in a consanguineous family reveals the role of inbreeding in the onset of autosomal recessive DEE. A study conducted on a non-consanguineous Caucasian population revealed a high contribution of recessive inheritance in DEE but with compound heterozygous variants [62]. In our study, despite the consanguinity, we have only one patient with autosomal recessive inheritance. This can be explained by the fact that we did not screen the whole genome and the structural variations in our patients. Thus, it is possible that variations which are not covered with our panel or copy number variations are responsible for an autosomal recessive DEE in negative patients.

The detected in-frame variant in *GNAO1* “p.(Leu39del)” was classified as “Likely Pathogenic” referring to ACMG and was not found in control population databases. Furthermore, leucine at position 39 was highly conserved between species with a PhyloP100 conservation score equal to 7.53. So far, 50 *GNAO1* variants have been found in patients with movement disorders and epileptiform encephalopathy, where three variants are in-frame: “p.(Ala301del)”, “p.(Ala338del)”, and “p.(Ile344del)”[63]. These three reported patients didn’t present epilepsy or presented a single seizure, and they all share problems in motor and language development, with normal EEG findings and brain MRI, with or without intellectual disability [64, 65]. SEED.0020 presents similar clinical findings. In fact, epileptic seizures appear notably at the age of 1 month, but these seizures disappear completely at the age of 2 years and 9 months with problems in motor and language development as well as normal EEG findings at this age. A second variant “p.(Leu23Pro)” was reported previously in the same domain containing the p.Leu39del variant (N-terminus domain, prior to the first G-motif), in a patient with the same phenotype [66] (Supplemental Table 3).

Furthermore, the “p.(Asp479His)” in *PCDH19* is a newly discovered variant that proves to be severe. This novel variant is located in the fifth cadherin domain of the PCDH19 protein. This domain contains six reported missense variants involved in epilepsy [67-71] (Supplemental Table 3). An alternative variant at the same position “p.(Asp479Asn)” was classified as ‘likely pathogenic‘ by ClinVar. The affected mother possessed also the variant in the heterozygous state. The presence of variants with maternal inheritance in *PCDH19* was described four times in state of art works [72-74]. Moreover, it was emphasized that

variants in *PCDH19* are associated with seizures occurring in clusters, provoked by fever with cognitive impairment [75]. Our patient SEED.0074 presents also this clinical phenotype (Table 1).

A previously reported variant p.(Arg504Trp) was detected in *GRIN2A*[76, 77] in a patient presenting DEE with Spike-Wave-Activation in Sleep (SWAS). The same variant was reported in patients with similar phenotypes presenting Landau-Kleffner syndrome (LKS) or Epileptic Encephalopathy with continuous spike-and-wave during sleep (CSWS) and Attention deficit and hyperactivity disorder (ADHD). However, this is the first report of *GRIN2A* variants in the Tunisian population.

According to the varsome database (https://varsome.com), the p.(Arg145Cys) missense variant in *MECP2*, is reported more than 140 times in the literature. This variant is present in a hot spot region [78] and classified as Pathogenic by ClinVar and UniProt. Additionally, there are 4 alternative variants in the same position((p.(Arg145Lys);p.(Arg145Leu);p.(Arg145His);p.(Arg145Gly)), classified as “Pathogenic” by ClinVar and implicated in Rett syndrome or Intellectual developmental disorders. In Tunisia, other variants were reported in *MECP2* in patients with Rett syndrome, but this is the first report of p.(Arg145Cys) variant [79-87].

Additionally, we identified the novel frameshift variant p.(Arg716GlyfsTer10) in *SYNGAP1*. Approximately 200 cases were reported worldwide, and most of these patients are from Europe [88]. The phenotype detected in our patient is in good agreement with previous studies showing that mutations in *SYNGAP1* are responsible for a clinical syndrome characterized by intellectual disability, developmental delay, and epilepsy[89]. This is the first report of a variant in this gene on the African continent. Therefore, the *SYNGAP1* gene should be analyzed in the future in patients with this syndrome.

Significance of genetic studies in different populations for the assessment of population-based disease-causing epilepsy genes

Since 2014, several studies in various populations in America and Europe have used targeted NGS-based epilepsy gene panels to identify the responsible genes for DEE. These panels may include between 5-580 genes, with a diagnostic yield ranging between 18% and 46% (Table 3). In Africa, only one study has recently been published which was concerned with the South African population [5]. However, in North Africa (Tunisia, Egypt, Libya, Algeria, Morocco), no study on this issue has been undertaken before. In the Arabic countries, only one study was published in the Saudi Arabia region using WES/WGS sequencing rather than panels [90]. From this perspective, this is the first study that uses a personalized panel of high throughput sequencing in the Tunisian as well as the North African region with a diagnosis rate of 30% (Table 3). In our study, we specified Pathogenic or Likely Pathogenic variants in ten genes (*SCN1A, CHD2, CDKL5, SZT2, KCNT1, GNAO1, PCDH19, MECP2, GRIN2A, SYNGAP1*) involved in DEE, and *SCN1A* seems to be the most frequently affected gene in Tunisia (Table 2).

Conclusions

This study allows a deeper and better insight into the underlying causative genes and variants of DEE in Tunisian children. The 30% diagnostic yield goes in good conformity with previously reported international pediatric cohorts. Gene-directed therapies will be further enhanced in the future in a way that the management of all patients with DEE would be highly facilitated.

Abbreviations

DEE
Developmental and epileptic encephalopathy
DS
Dravet syndrome
IESS
Infantile epileptic spams syndrome
LGS
Lennox-Gastaut syndrome
EIMFS
Epilepsy of infancy with migrating focal seizures
NGS
Next Generation Sequencing
WES
Whole exome sequencing
WGS
Whole genome sequencing
MAF
Minor allelic frequency
ACMG
American College of Medical Genetics and Genomics
gnomAD
Genome Aggregation Database
ADNFLE
Autosomal dominant nocturnal frontal lobe epilepsy
ADSHE
Autosomal dominant forms of Sleep-related hypermotor epilepsy
EOEE
Early Onset Epileptic Encephalopathy

Declarations

Ethics approval and consent to participate

This study was approved by the Ministry of higher education and scientific research of Tunisia. All procedures performed in studies involving human participants were in accordance with the guidelines of the Regional Committee of the Protection of Persons, Sfax, Tunisia (CPP SUD reference number 28/2019).

Consent for publication

Not Applicable.

Availability of data and materials

The data that support the findings of this study are available in the article. If additional data were required, they might be requested to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Author's contribution

Mariem Ben Said, Chahnez Triki and Olfa Jallouli wrote the manuscript. Chahnez Triki, Fatma Kamoun, Olfa Jallouli and Abir ben Issa performed the collection of family members and the acquisition of clinical data. Mariem ben said and Amal Souissi performed library preparation. Mariem ben said and Ikhlas ben Ayed interpreted the NGS data. Chahnez Triki, Fatma Kamoun, Saber Masmoudi, Faiza Fakhfakh and Ikhlas ben Ayed have contributed to the acquisition of the financial support for the project leading to this publication. Chahnez Triki, Mariem Ben Said, Saber Masmoudi and Ikhlas ben Ayed commented and substantively revised the article. All authors read and approved the final manuscript.

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