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Observational Study

Screening the *UBE3A* gene referred for Angelman Syndrome followed by a genetic approach to identify UBE3A putative targets

Screening of *UBE3A* gene in Tunisian patients

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Abstract

BACKGROUND

Angelman syndrome (AS) is caused by maternal chromosome 15q11q13 deletions, imprinting defects, paternal uniparental disomy (UPD) 15, and UBE3A gene mutations. UBE3A is a brain-specific imprinting gene that encodes a ubiquitin-protein ligase. Here, we describe the variants in the UBE3A coding region detected by sequencing analysis in 50 AS Tunisian individuals with a normal bi-parental inheritance and methylation pattern of 15q11q13. Seven polymorphisms were found in our patients, including three novel variants.

Screening for UBE3A mutations in AS patients has proven useful both for confirming or refuting the diagnosis, for genetic counselling, and for studying Angelman-like syndromes in negative patients. Sequencing analysis has been proven to be a sensitive and effective sample screening method for these polymorphisms. To identify bi-allelic recessive mutations that give rise to AS-like phenotypes, we considered consanguineous families, as they are more likely to develop such a recessive disease. These consanguineous families are common in North Africa and the Middle East but rare in Western Europe.

AIM

In the present study, screening for the *UBE3A* gene was performed in 50 patients referred with a strong suspicion of AS and for whom classical molecular diagnostic tests had failed to provide the diagnosis. We described the difficulties encountered in determining the genetic etiologies of AS and suggested some solutions.

METHODS

2.1. Clinical Description of patients and sample collection

Fifty patients (33 males and 17 females, aged 1–7 years) with a probable diagnosis of AS were referred to the Laboratory of Human Cytogenetics, Molecular Genetics, and Reproductive Biology of Farhat Hached University Hospital between 2006 and 2021. These patients showed severe mental retardation, severe speech impairment, epileptic seizures, abnormal electroencephalogram (EEG) findings, and dysmorphic facial features. The clinical characteristics of these patients are presented in **Table 1**.

Clinical geneticists evaluated all patients. The blood samples were collected after obtaining written consent from the parents and after the approval of the ethics committee of Farhat Hached University Hospital.

Combined cytogenetic, Fluoresence In Situ Hybridization (FISH), Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA), and microsatellite analyses excluded deletions, uniparental disomy (UPDs), and imprinting defects (IDs) in all patients (data not shown).

2.2. DNA extraction, polymerase chain reaction and sequencing analyses

Genomic DNA was extracted and purified from peripheral blood leukocytes using the Qia-amp DNA blood mini kit (Qiagen, Valencia, CA, and USA). Protocol was performed according to the manufacturer's instructions. DNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3. PCR amplification

Exon 7 to exon 16 of the *UBE3A* gene **(Table 2)** were amplified by polymerase chain reaction (PCR) of a 25µl reaction mixture containing: 1µl of genomic DNA (150ng/ul), 1µl of forward primer (20pg/µl), 1µl of reverse primer (20pg/µl), 0.2µl of Taq DNA polymerase recombinant (Invitrogen), 5µl MgCl2 in the Taq buffer (MgCl2 = 7.5 mM), and 16.8µl of deionized water.

PCR was performed in thermocycler Gene Amp PCR System 9700 from Applied Biosystems, Foster City, CA.

2.4. *UBE3A* gene sequencing

The *UBE3A* gene was analyzed by direct sequencing of exons 7 to 16 and flanking exon/intron boundaries in patients and their parents (Transcript: ENST00000232165). The primers and conditions are summarized in **Table 2.**

The amplicons were purified and directly sequenced. Sequencing reactions were prepared using an ABI Big Dye Terminator v3.1 cycle sequencing kit and separated by a 3500 Genetic Analyzer 16-Capillary Array. Sequencing data were analyzed with the Seqscape V2.0 software (Applied Biosystems) and compared to the reference sequence of the exon *UBE3A* gene downloaded from the Genome Browser Gateway (http://www.genome.ucsc.edu/).

In-silico softwares such as PolyPhen-2 and Mutation Taster were used to calculate variable effects.

2.5. Genetic analysis and Exome sequencing

We used Illumina Human OmniExpress 700K single nucleotide polymorphism (SNP) arrays for both linkage and copy number analysis with DNA isolated from venous blood, assuming an autosomal recessive model. Linkage analysis was aimed to identify chromosomal regions shared by all affected family members. Allegro embedded in EASY Linkage was used to perform linkage analysis to identify chromosomal regions shared by all affected family members.

Copy number variations analysis was performed using NEXUS Discovery Edition, version 7 (Biodiscovery, El Segundo, CA). Whole-exome sequencing was performed on affected patients and their unaffected parents **(Figure 2).**

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Seq 2000 sequencer. Reads were aligned to the human reference genome version 19 using the Burrows-Wheels Aligner. Genome Analysis Toolkit (GATK) was used for SNPs and indels. We used Cartagenia software (Cartagenia Bench Lab, Agilent Technologies) to filter the variants.

We filtered the heterozygous variants based on the following criteria: a) present within the shared genomic regions; b) predicted to affect protein-coding (nonsense, missense, splice site, frameshift); and c) have a minor allele frequency (MAF) of <0.1% in the more recent databases (10000G, ExAC).

RESULTS

All our patients exhibited significant traits, including severe mental retardation, inability to speak, abnormal EEG findings, epileptic seizures and dysmorphic facial features **(Table 1)**. The consanguinity was observed in four families. Additionally, we examined patients from six unrelated families, originating from different regions in Tunisia. To identify rare coding variants associated with the disease within these families, we performed linkage analysis in conjunction with exome sequencing in carefully selected consanguineous patients.

Identification of *UBE3A* variants:

To investigate mutations in the coding regions of UBE3A, we examined 50 cases diagnosed with probable AS. Based on clinical observations, the patients displayed characteristics consistent with the AS phenotype. We ruled out deletion, UPD, and imprinting defects in these individuals (data not provided). After sequencing all ten coding exons of UBE3A, we identified seven polymorphisms. There were four previously known mutations and three novel mutations, but none of them was identified as causal **(Table 3).**

The six suspected AS patients were from sporadic families, with sometimes one affected member, such as an uncle or cousin, suffering from a learning disability with an unknown syndrome **(Figure 3).**

Copy number variant analysis and parametric linkage analysis:

The analysis conducted using NEXUS software did not identify any copy number variants associated with the disease. Shared genomic regions on chromosomes 4, 6, 10, and 11 (around 50 Mb) were detected using the parametric linkage analysis under an autosomal recessive model. Exome sequencing was employed to investigate variant discovery in all shared regions.

Exome sequencing:

Exome sequencing was done for two separate families; every family had a consanguineous relationship with their relatives. The two families had more than one affected member **(Figure 3).**

The analysis of the exome sequencing data did not reveal any homozygous variants, but it did reveal the presence of compound heterozygous variants in six genes for the patient within the selected family (**Figure 3,** Family 1) **(Table 4)**: *SHPRH*(c.4331C>T p.A1444V), *SLC30A9* (c.528-7T>C), *HBS1L* (c.2043+5T>G), *TAAR6* (c.865C>T p.P289S), *TAAR2* (c.467C>T p.T156I), *SASH1* (c.1126C>T p.P376S), *LOC100287896* (c.4C>T p.R2C), *PCF11* (c.3355C>T p.H1119Y), *ANKRD42* (c.676A>G p.N226D), *PDGFD* (c.7C>G p.R3G), and *DIXDC1* (c.226G>A p.G76S). Due to the high polymorphism observed in the SLC30A9 and HBS1L genes, we did not consider these variants as plausible candidates in our analysis.

The analysis of the exome sequencing for the patient in the second consanguineous family (**Figure 3**, Family 2) revealed the presence of the following variants: *KMT5A* (c.904T>C (p.(C302R))), *KMT5A* (c.995T>C (p.(L332))), *PSTK36* (c.2516G>A (p.(R839Q))), *PIK3CB*

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CONCLUSION

Angelman syndrome still lacks a cure. It is possible to generate pluripotent stem cells (iPSCs) line derived from skin fibroblasts of AS patients. These iPSC models for genomic imprinting disorders will enable the exploration of AS processes. Moreover, they provide an opportunity to explore the developmental mechanism and timing of *UBE3A* repression in human neurons [17,18].

The rapid advancement of iPSC technology has turned these cells into versatile tools for both basic and clinical research. Several studies have already developed this method, which may be very interesting to investigate for our patients.

Subsequent research on iPSC holds the promise of advancing drug discovery, enhancing cell therapy, and introducing novel diagnostic approaches for neurogenetic disorders.

Key Words: Angelman syndrome, Ubiquitin-protein ligase E3A, exome, consanguinity, polymorphism

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Core Tip:

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To identify bi-allelic recessive mutations that give rise to AS-like phenotypes, we considered consanguineous families, as they are more likely to develop such a recessive disease.

INTRODUCTION

The introduction of Next-Generation Sequencing (NGS) technology, which enables the comprehensive reading of all protein-coding nucleotides in the genome (the exome), or the entire genome, has brought about a revolution in the field of human molecular genetics. When combined with family-based linkage analysis, this technological advancement has played a pivotal role in identifying numerous novel variants responsible for rare Mendelian forms of human diseases. The present study describes genetic analyses in three families suspected to have AS with a high incidence of intellectual disability and genetic disorder. In our study, we conducted linkage analysis alongside exome sequencing in specific patients to pinpoint rare coding variants that segregate with the disease within the family.

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Clinical results:

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DISCUSSION

AS is a severe neurodevelopmental disorder that affects 1 in 20,000 children. There is no effective treatment for cognitive defects, and the treatment of seizures is often ineffective. Lack of insight into the fundamental mechanisms underlying AS hinders drug development for the condition. This syndrome is caused by the lack of a functional maternal *UBE3A* gene. This gene encodes a protein known as UBE3A or E6AP (E6-associated protein), which can modify other proteins through a process called ubiquitination. However, it is unclear which proteins are modified by UBE3A. We sought

to identify these proteins, referred to as 'targets', which is a crucial stage in the development of treatments.

In this study, we examined 50 patients from unrelated Tunisian families who presented with a profile consistent with AS phenotype.

The patients were referred to different pediatric departments, due to unidentified etiology of severe mental retardation, abnormal EEG findings or epileptic seizures, severe speech impairment and dysmorphic facial characteristics and the genetic anomaly was confirmed for all of them.

Cytogenetic analysis demonstrated a 46,XY and 46,XX karyotype in all analyzed cells from the patients. The parent's karyotypes were found to be normal as well. After that, microsatellite marker PCR analysis was conducted using the conventional methodology, employing polymorphic markers situated within the 15q11q13 region. The purpose was to authenticate the duplication and ascertain the parental source of the duplicated chromosome 15. All our patients were normal. We performed MS-MLPA for diagnosis of AS associated with deletions, UPD15, or rare duplications. After all these tests, the deletion, UPD, and imprinted defects were excluded in all 50 patients.

Molecular analysis by direct sequencing (exons 7 to 16 and flanking exon/intron boundaries) of the *UBE3A* gene performed on all patients revealed negative results.

We found seven polymorphisms, three were novel, and four had been described in the literature. In Family 1 (**Figure 2**), we found a variation in intron 13 (c.2064+9T>C), which is known in the NCBI database as rs79328837 (**Figure 4**). This variant was described by Bekim et al. 2014 [12]. It manifests at a poorly conserved position in the protein. The variant is anticipated to be benign according to multiple in silico algorithms, and its population frequency is incongruent with the disease.

The second patient (**Figure 2**, Family 2) showed two known polymorphisms (**Figure 5**). The third patient (**Figure 2**, Family 3) showed a single nucleotide variation in exon 9 with an uncertain significant allele (**Figure 6**). The patient presented with severe microcephaly (-5 standard deviation (SD)), aggressive behavior, and an abnormal EEG.

To our knowledge, there is limited information in the literature about the c.2220+14 T>C (intron 14), c.30-47_30-46 insT (Exon 7), and c.2507+43 T>A (Exon 15) variants. It is unclear whether these mutations directly caused AS or they were just non-synonymous polymorphisms with low effect.

The allele frequency of the c.2064+9T>C variant in UBE3A is 0.4% in gnomAD, a level deemed high enough to classify it as benign, according to thresholds set by the ClinGen Rett/Angelman-like Expert Panel for Rett/AS-like conditions. Splice prediction analysis, employing multiple computational biology tools does not indicate an impact on splicing. In summary, the c.2064+9T>C variant in UBE3A is classified as benign based on the ACMG/AMP criteria.

The alteration c.1713A>G (RS34670662) is deemed benign through a comprehensive assessment, considering several factors. These include population frequency, absence of segregation with the disease, intact protein function, co-occurrence, analysis of RNA, in silico models, amino acid conservation, absence of disease association in case-control studies, incongruence with a known cause of pathogenicity in terms of the mechanism of disease or impacted region.
(https://www.ncbi.nlm.nih.gov/clinvar/variation/96258).

According to the Global Variome shared Leiden Open Variation Database (LOVD) for *UBE3A* (ubiquitin protein ligase E3A), the variant c.2221-40_2221-38delGTA (RS149854051) in intron 14 marks the initiation of clinical classification. For the polymorphism, c.486A>T; p.Ala162 (RS28528079 (exon9A)), the clinical significance was considered benign, as described by Nykamp et al. [13].

Approximately 10% of patients clinically diagnosed with AS or AS-like conditions do not exhibit an identifiable molecular defect [14]. Some of these patients have genetic variations that exhibit overlapping features with AS. Hence, we need next-generation sequencing as one of the fastest techniques to screen other genes responsible for the development of these disorders.[15]. The integration of whole-exome and whole-genome

sequencing, along with high-throughput genotyping and linkage analysis, may contribute to the identification of new genes linked to AS-like syndromes.

Here, we aimed to use a genetic approach to identify putative *UBE3A* targets. We hypothesize that a mutation in a *UBE3A*-target protein may result in a syndrome resembling AS. However, it is likely that such a syndrome only arises when a mutated gene is inherited from both the father and mother.

The results of exome sequencing revealed that patients in the first family (**Figure 3**, **Family 1**) present several gene mutations, such as *TAAR6* (*TRAR4*), that are expressed in low abundance in various human brain tissues, especially frontal cortex, substantia nigra, amygdala, and hippocampus.

The gene expression for the first family were detailed in **Table 6**.

The exome sequencing of the second patient in family 2 (**Figure 2**) gave different genes.

The gene expression for the second family were detailed in **Table 6**.

We identified several consanguineous families where multiple children present symptoms of AS, but no mutation was identified. In this project, we propose to use state-of-the-art genetic technologies to identify mutated genes. The identified genes in these families will be studied for their pathological effect on neural development in laboratory cell-culture experiments and in mouse models. Moreover, we will test if these genes are targets of *UBE3A*. By using this approach, we hope to generate fundamental insight into the neurobiology underlying AS and AS-like syndromes, which may lead to the development of therapeutic interventions.

The 50 patients studied had been previously found negative for 15q11q13 deletions, paternal uniparental disomy, and in imprinting defects.

Their clinical characteristics with suspected AS are presented in **Table I**. All patients demonstrated severe speech impairments or a total absence of speech, behavioral abnormalities, movement difficulties and severe developmental delays. Thirty-eight of them (76%) had the capacity to sit unsupported, 21 (42%) could walk with support, and 29 were unable to walk. Forty of them (80%) couldn't speak, while 10 (20%) could speak a few meaningful words. Dysmorphic facial characteristics like protruding tongue,

occipital groove, prognathism and wide-spaced teeth were most common in our patients (**Table 1**). All patients had an ataxic gait. Moreover, all patients were on antiepileptic therapy because of the presence of seizures. Epileptic attacks were completely controlled in five patients, and partially in one. The clinicians performed to all patients the cranial magnetic resonance imaging. Only two patients showed minimal cerebral atrophy, and eight were normal. Metabolic screening tests showed normal results for every patient.

The genetic etiologies of AS are unknown in many populations [16]. However, implementing a next-generation sequencing (NGS) based approach will reduce cost reduction, increased efficiency, save time, and facilitate the exploration of novel pathways that contribute to the pathophysiology of AS.

The introduction of Next-Generation Sequencing (NGS) technology, which enables the comprehensive reading of all protein-coding nucleotides in the genome (the exome), or the entire genome, has brought about a revolution in the field of human molecular genetics. When combined with family-based linkage analysis, this technological advancement has played a pivotal role in identifying numerous novel variants responsible for rare Mendelian forms of human diseases. The present study describes genetic analyses in three families suspected to have AS with a high incidence of intellectual disability and genetic disorder. In our study, we conducted linkage analysis alongside exome sequencing in specific patients to pinpoint rare coding variants that segregate with the disease within the family.

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Angelman syndrome still lacks a cure. It is possible to generate pluripotent stem cells (iPSCs) line derived from skin fibroblasts of AS patients. These iPSC models for genomic imprinting disorders will enable the exploration of AS processes. Moreover, they provide an opportunity to explore the developmental mechanism and timing of *UBE3A* repression in human neurons [17,18].

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ARTICLE HIGHLIGHTS

Research background

the most important goal in our study is to investigate all the genes that may be responsible for the Angelman-like syndrome, since all the samples present the clinical features of Angelman syndrome without any genetic abnormalities responsible for the disease.

Research motivation

Exome sequencing of patients suspect to have Angelman syndrome, showed the presence of different genes that may be responsible for the disease
clinicians that may have patients suspect to have Angelman syndrome, cannot look into UBE3A gene but also to other genes already described and that may be responsible for AS.

Research objectives

the student of Angelman syndrome cohort patients is to our knowledge the first research study in the Tunisian patients, this study may help physicians to know how to diagnose the patients in case of the absence of all genetic alterations for Angelman syndrome and to look further for the Angelman-like syndromes. it help also to do functional studies that may be interesting for further treatment in the future

Research methods

2.1. Clinical Description of patients and sample collection

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2.5. Genetic analysis and Exome sequencing

We used Illumina Human OmniExpress 700K single nucleotide polymorphism (SNP) arrays for both linkage and copy number analysis with DNA extracted from venous blood, following an autosomal recessive model. Linkage analysis was aimed to identify chromosomal regions shared by all affected family members. Allegro embedded in EASY Linkage was used to perform linkage analysis to identify chromosomal regions shared by all affected family members.

Copy number variations analysis was conducted using NEXUS Discovery Edition, version 7 (Biodiscovery, El Segundo, CA). For affected patients and their unaffected parents **(Figure 2)**, whole-exome sequencing was carried out. The exome sequencing utilized the Agilent SureSelect V4 Human 50MB kit (Agilent Technologies) for capture, paired-end sequencing on an Illumina Hi-Seq 2000 sequencer, and alignment of reads to the human reference genome version 19 using the Burrows-Wheeler Aligner. SNPs and indels were analyzed with the Genome Analysis Toolkit (GATK), and Cartagenia software (Cartagenia Bench Lab, Agilent Technologies) was employed for variant filtering. Heterozygous variants were filtered based on the following criteria: a) presence within shared genomic regions; b) predicted impact on protein-coding (nonsense, missense, splice site, frameshift); and c) a minor allele frequency (MAF) of <0.1% in more recent databases (10000G, ExAC).

Research results

2.1. Clinical Description of patients and sample collection

Between 2006 and 2021, fifty patients (33 males and 17 females, aged 1–7 years) with a likely diagnosis AS were referred to the Laboratory of Human Cytogenetics, Molecular Genetics, and Reproductive Biology at Farhat Hached University Hospital. The individuals demonstrated profound cognitive impairment, severe speech challenges, abnormal EEG findings, epileptic seizures, and distinctive facial characteristics. The clinical characteristics of these patients are presented in **Table 1**. Clinical geneticists evaluated all patients, and blood samples were collected following written consent from parents and approval from the ethics committee of Farhat Hached University Hospital.

Combined cytogenetic, FISH, MS-MLPA, and microsatellite analyses excluded deletions, UPDs, and IDs in all patients (data not shown).

Research conclusions

the physicians, geneticists and researchers have to investigate very carefully the suspected Angelman syndrome patients. in case of all the molecular and cytogenetics tests were negative for Angelman syndrome, they must go further with exome sequencing and think more about AS-like syndromes that may be responsible for the disease in the patients.

Research perspectives

Angelman syndrome still lacks a cure. It is possible to generate pluripotent stem cells (iPSCs) line derived from skin fibroblasts of AS patients. These iPSC models for genomic imprinting disorders will enable the exploration of AS processes. Moreover, they provide an opportunity to explore the developmental mechanism and timing of *UBE3A* repression in human neurons.

Subsequent research on iPSC holds the promise of advancing drug discovery, enhancing cell therapy, and introducing novel diagnostic approaches for neurogenetic disorders.

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