Full Length Research Article

Discovery and analysis of a novel mutation (G774E) in the Lysine Demethylase 6A (KDM6A) gene causing congenital heart disease with various neurodevelopmental disorders

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ABSTRACT

Background: Congenital heart defects (CHD) are the most common birth defects, affecting approximately 0.8% of live births worldwide. CHD impairs the function and structure of the heart and blood vessels. Due to this damage, blood flow is impaired, which affects many major organs, including the brain, and causes various neurodevelopmental disorders.

Methods: In this study, we recruited a five-generation pedigree for analysis. The proband was born with congenital heart disease and subsequently developed various neurodevelopmental disorders. To understand the causes of the disease, we performed clinical whole-exome sequencing and applied various bioinformatics tools to determine the pathogenicity of the mutation.

Results: Molecular investigation revealed a novel lethal mutation (c.2321G>A) in KDM6A, causing the substitution of Glycine to Glutamic acid (Gly774Glu). The mutation was further confirmed using Sanger sequencing. Various bioinformatics tools were used to predict the lethality of the mutations. KDM6A disruption causes various diseases, among which Kabuki syndrome is the most prevalent.

Conclusion: Our findings may aid in the further development of genome-based medicines, leading to a reduction in mortality rates and improved healthcare in newborns.

INTRODUCTION

Congenital heart disease (CHD) is defined as the birth of a child with structural abnormalities of the heart or great vessels and is prevalent in almost 0.8% of newborns [1]. CHD is responsible for most infant deaths worldwide [2]. In Saudi Arabia, the incidence remains largely unexplored, but a recent study estimated that the incidence is much higher, that is 1.48% [3]. The presence of genetic underpinnings in CHD is strongly considered, as the disease shows high recurrence risks and familial forms, as well as the presence of chromosomal anomalies in many cases of CHD [4]. To date, studies have confirmed the presence of more than 400 genes associated with CHD pathogenesis. Most genetic mutations are present in transcription factors, cell signaling transducers, and chromatin modifiers that interact with specific cells during differentiation, which are important for structural and functional heart development [5].

Most genes encode proteins that work in coherence and are connected by interactive functional networks, leading to a broad network of proteins that may be associated with CHDs [6, 7]. However, to date, the majority of CHD cases remain to be elucidated (~60%), owing to the complexity of the genetic etiology of CHD in human cases and the presence of genetic heterogeneity associated with CHD [8]. This leads to variable expressivity, and similar variants show different phenotypic variability or variable penetrance in some individuals. Therefore, not all CHD cases follow Mendelian inheritance patterns and are better represented by complex genetics.

The emergence of new techniques, such as whole-exome or whole-genome sequencing, has made it far easier to study complex genetics, such as CHD. Currently, the most common technique used is trio studies (using patient and both parents' samples), where patients are sequenced with their unaffected parents to identify the pathogenic variants that are missing in the parents (de novo mutations). In familial forms of CHDs, multiple members of the same family are sequenced to identify the common variants transmitted to family members. Moreover, in large cohort studies, a large number of patient samples were collected from different regions along with healthy control samples, and sequencing was performed to identify the presence of a single gene or group of genes that were enriched in variants in patients compared to healthy individuals. These studies revealed a high number of genes expressed in the heart or at some stage of heart development [8].

After the successful detection of genes associated with CHD, prenatal diagnosis is the best way to prevent the disease in the next generation. Prenatal diagnosis supplements the family with proper guidance on prognosis, planning adequate obstetric follow-up, better preparation for intrauterine management for complex and uncommon cases, and well before time refers to planning for pregnant women to transfer to tertiary care facilities (capable of providing therapeutic catheterization and, if needed, heart surgery), which will significantly reduce the morbidity and mortality in the newborn [9].

In this study, we examined the genetic causes of congenital heart diseases and neurodevelopmental disorders in a large, inbred family using molecular techniques such as whole exome sequencing and further studied their effects using various bioinformatics tools.

METHODS

Pedigree ascertainment

In this study, we recruited a five-generation pedigree from King Abdulaziz University Hospital (Fig. 1). All clinical information and patient features were obtained by interviewing family members and reviewing the medical records of the hospital. Before the start of the study, ethical approval was obtained from the Ethics Committee of the Center of Excellence in Genomic Medicine Research (07-CEGMR-Bioeth-2020). Moreover, we obtained written informed consent from all the study participants, according to the Declaration of Helsinki. The proband (V-3) was a male, one year old, born full-term via normal vaginal delivery. The patient had various cardiac anomalies, including pulmonic valve stenosis, atrial septal defect, small patent ductus arteriosus, and a patent foramen ovale. The patient had difficulty breathing, showed signs of apnea and cyanosis, was admitted to the neonatal intensive care unit at the time of birth because of bronchopneumonia and poor respiration, and was intubated and linked to mechanical ventilation. Later in life, various issues were observed, such as hypoxic-ischemic encephalopathy, weak muscles, global developmental delay, poor feeding, mild to moderate

intellectual disability, and speech problems. Unfortunately, the child died later at approximately 18 months of age because of multiple cardiac and other complications. The proband's older brother (V-2) also exhibited overlapping symptoms and died due to similar complications.

Blood sampling and DNA extraction

To investigate the molecular causes of the disease, we collected blood samples from the patient and his parents in EDTA tubes, immediately transferred them to the Center of Excellence in Genomic Medicine Research, and stored them at 4 °C for further investigation. We extracted DNA using a DNeasy® Blood and Tissue Kit (QIAGEN) according to the manufacturer's protocol. The quality and quantity of DNA were measured using a spectrophotometer (Nanodrop 2000; https:// www.thermofisher.com/ order/ catalog/ product/ ND2000CLAPTOP) ensuring an A260/ A280 ratio of 1.7– 2.0. To further confirm DNA integrity, we performed horizontal gel electrophoresis on a 1% agarose gel using SYBR Safe dye (Thermo Fisher, USA).

Whole exome sequencing

After ensuring the quality and quantity of DNA, we proceeded to the next step of the whole exome sequencing (WES) experiment to identify pathogenic mutations. WES was performed using a NovaSeq6000 machine. Sequencing samples were processed according to the manufacturer's protocol (SureSelect_v6 Agilent, USA). For the template we used 50 ng of genomic DNA for each sample. For purity and quality, we used 260/280 absorbance ratio values of 1.7– 2.0 as the standard. To prepare the libraries, we used Illumina DNA Prep with the following enrichment protocol:

1) Tagment Genomic DNA. This step uses enrichment bead- linked transposomes (eBLT) to fragment and tag DNA with adapter sequences to target the DNA.

2) Post Tagmentation Clean Up. In this step, we washed the adapter-tagged DNA on the eBLT. 3) Amplified tagged DNA. We used PCR to amplify tagmented DNA.

4) Clean Up Libraries. In this study, we purified the amplified libraries using a double-sided bead purification system.

5) Pool Libraries. Subsequently, we pooled 12 DNA libraries into a single pool.

6) Hybridize Probes. In this step, DNA-targeted regions were bonded to capture probes.

7) Capture Hybridized Probes. Streptavidin Magnetic Beads (SMB) were used to capture probes for the hybridization of the targeted DNA regions.

8) The enriched library was amplified. PCR was performed to amplify the enriched libraries. 9) Clean Up Amplified Enriched Library. AMPure XP Beads were used to purify and remove unwanted products from the enriched libraries. To assess the quantity of the enriched libraries we ran 1 µl of the sample using the Qubit dsDNA BR Assay Kit, and library yields of ≥ 3 ng/µl were considered good. To assess the quality of the enriched libraries, we used an Agilent Technology 2100 Bioanalyzer with a High Sensitivity DNA kit, and if the fragment size was approximately 350 bp and the overall range from ~200 bp to ~1000 bp was found to be suitable for further processing. Finally, the libraries were diluted to 2 nM starting concentration and 175-185 pM for final loading concentrations and run on NovaSeq6000 to obtain sequencing data.

Raw sequencing data were analyzed using Aziz Supercomputer. Initially, the base calls from BCL files were converted to FASTQ format by "bcl2fastq" and then pre-processed FASTQ files, first removed adaptors, second, low-quality bases were trimmed "<20", third, further filtering short reads "<20 bp". The filtered generated FASTQ files were then mapped and aligned to the reference human genome sequence UCSC hg19 version using BWA mapping software (version 0.7.12) to generate the SAM file format, which was further converted to the BAM file format (compressed binary version of SAM). The resultant allele frequency variants were annotated using ANNOVAR tools and base quality score recalibration (BQSR) was performed. GATK-

Haplotype Caller was used to generate variants in the vcf file format with approximately 40 thousand variants which were further reduced to approximately 3 thousand variants after further application of filtration criteria to eliminate common variants in the general population based on minor allele frequencies (MAF <0.001). The Variant Interpreter (Illumina) was used for annotation, interpretation, and detection of disease variants. The pathogenicity of the resultant variants and their disease associations were extracted from multiple databases, including ClinVar, OMIM, PubMed, and VarSome.

Sanger's Sequencing

To confirm the pathogenic mutation in the patient and family, we performed Sanger sequencing on an ABI XL3500 sequencer using BIGDye Terminator® technology. Primers (forward and reverse) were designed using the primer3 software (https:// primer3.ut.ee/). We used forward primer sequence 5'CTGGCTCTGGTATTCAGAATC3' and reverse primer sequence 5'GAGCTGAGGATTGTCTGAACTTA3'. KDM6A gene sequence was retrieved using accession number NM_001291415. Sequencing was performed according to the manufacturer's instructions, and the PCR products were purified using a master mix of 100% ethanol (2.5 volume), NaOAc (1:10 volume), and EDTA (1:10 volume), which were distributed and mixed in all sample tubes. The mixture was vortexed and centrifuged at 2146 x g for 45 min. Then 100 µl of 70% ethanol was added, and the mixture was vortexed and centrifuged at 2146 x g for 10 min. The supernatant was discarded and dried at 50°C for 20 min, nuclease-free water was added for elution, and the cycle sequencing step was initiated. We prepared a master mix of 10 µl as 5x sequencing buffer 2 µl, Primers (forward and reverse) 1 µl each, Big Dye Terminator 1 µl, template DNA 1 µl, and H2O 5 µl. The samples were then run on the thermocycler for initial denaturation at 95°C for 5 min, followed by 25 cycles of 95°C for 10 s, 50°C for 5 s, 60°C for 4 min, and held at 4°C, followed by a second purification step. The second purification was the same as the first, and after drying the samples, we added 10-12 µl of Hi-Di formamide to each sample. The Hi- Di formamide samples were denatured at 95 °C for 5 min and immediately placed on ice.

To read the resultant sequencing peaks and for analysis, we used BioEdit Sequence Alignment Editor Version 7.2.5.

In Silico Predictions

To evaluate the specific genetic variant G774E in KDM6A, we used the Combined Annotation-Dependent Depletion (CADD) method. The genomic coordinates and alleles for the G774E variant in *KDM6A* were entered in CADD available online at https:// cadd.gs.washington , and selected human genome assembly UCSC hg19 and then submitted the input for further processing [10,11].

Because the complete three- dimensional structure of the KDM6A protein is not available, we used the SwissModel server to build homology models of both wild- type (WT) and mutant KDM6A proteins, as described previously [12] using the AlphaFold DB model of KDM6A_HUMAN (gene: KDM6A, organism: Homo sapiens (Human)) (https:// swissmodel.expasy.org/ templates/ afdb_v4/O15550).

To evaluate the impact of G774E on the WT protein, we used the missense 3D tool (http:// missense3d.bc.ic.ac.uk/ missense3d/). As an additional confirmation, we used the mutant homology structure to check whether the replacement of E with G reversed the deleterious impact using the missense 3D tool [13, 14].

Furthermore, STRING software (version 12.0) was used to predict protein- protein interaction networks and perform functional enrichment analysis (https:// string-db.org/). STRING provides both physical and functional associations generated through different computational predictions and interactions gathered from other databases [15].

RESULTS

The proband was a child of one year old (12 months) and his two siblings were the result of a consanguineous marriage. The entire family showed multiple cousin marriages, suggesting the concentration of alleles and genes in the same family (Fig. 1). The proband's elder brother also

died in early childhood due to cardiac complications and multiple neurodevelopmental issues. From birth, the child had congenital heart disease, including pulmonic valve stenosis, atrial septal defect, patent ductus arteriosus, and patent foramen ovale. The child had bronchopneumonia and poor respiration, which may have resulted in hypoxic- ischemic encephalopathy due to lack of oxygen supply to the brain. With increasing age, multiple disease features have been noted, such as weak muscles, global developmental delay, poor feeding habits and intellectual disability. Unfortunately, the proband died within a few months after donating blood for genetic analysis due to cardiac complications.

Whole exome sequencing, based on filtration criteria and disease manifestations, revealed a pathogenic mutation in the *KDM6A* gene, where guanine at position c.2321 was substituted with adenine (c.2321G>A). Thus, in the protein, the amino acid glycine is replaced by glutamic acid at position 774 (Gly774Glu or G774E). Furthermore, the results were confirmed by Sanger sequencing (Fig. 2). The *KDM6A* gene is located on the X chromosome; therefore, one affected allele on the X chromosome is sufficient to cause disease in male patients. The mother was heterozygous for this allele, whereas the father was hemizygous for the normal allele. This confirmed the transfer of the disease allele from the mother to the patient.

CADD software was used to study the lethality of the substitution, and it was predicted to be “deleterious” based on the Sorting Intolerant From Tolerant (SIFT) category (SIFTcat) with a SIFT value (SIFTval) of 0.02 (Table 1). The results of this analysis provide insights into how the G774E mutation affects the KDM6A protein's demethylase activity, its interactions with other proteins or DNA, and its overall role in epigenetic regulation. Such information is essential for elucidating the potential pathogenic effects of this mutation and its implications in related disorders. PHRED-scaled C-scores > 20 for G774E SNV in KDM6A predicted that it was in the top 1% of deleterious variants. Furthermore, we utilized other tools, such as ExAC and 1000 genome variants, but it was not found in either ExAC or 1000G.

The 3D analysis of the missense mutation (G774E) using the WT homology model of the KDM6A protein obtained using the SwissModel server (Fig. 3) indicated a clash, as the introduction of the mutant Glutamic Acid (E) at position 774 resulted in a significantly higher clash score (76.02), compared to the Wild Type Glycine (55.21). This is based on the criteria that the mutant KDM6A homology model has a MolProbity clash score ≥ 30, which is > 18 compared to the wild type (Table 1).

Furthermore, we used the STRING software to generate a protein-protein interaction network and performed functional enrichment analysis of the KDM6A gene. STRING resulted in an interaction map that clearly showed the close association between KDM6A and KMT2D (both appear to cause Kabuki syndrome, showing similar and overlapping features as our patients). Moreover, the KDM6A gene shows bonding with NKX2-5, GATA4, and TBX5, which are exclusively involved in cardiogenesis, and if this interaction is inversely affected by one of the genes, it may result in overall impaired functioning and result in reduced or non-functional heart and body organ development (Fig. 4).

Figures

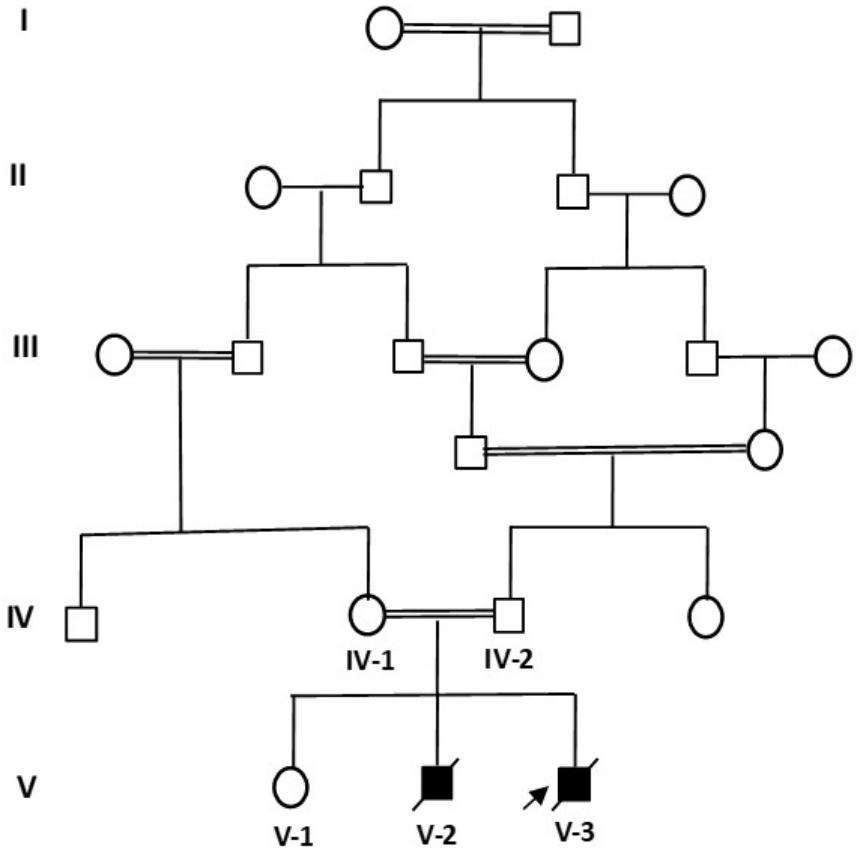

Figure 1: Pedigree of a consanguineous family showing features of CHD and neurodevelopmental disorders.

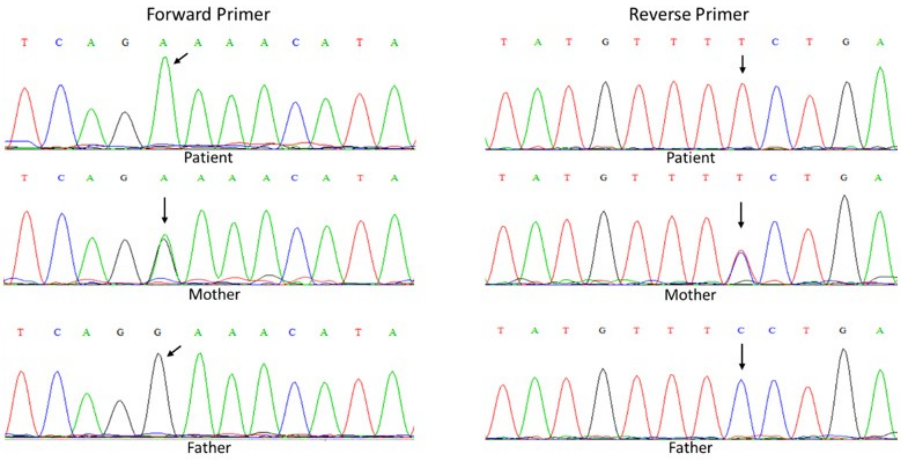

Figure 2: The chromatograms from Sanger sequencing of the patient and both parents showed the presence of the disease allele in the patient, while the mother was heterozygous and the father had a normal allele for this X-linked KDM6A gene c.2321 G>A (GGA>GAA) mutation.

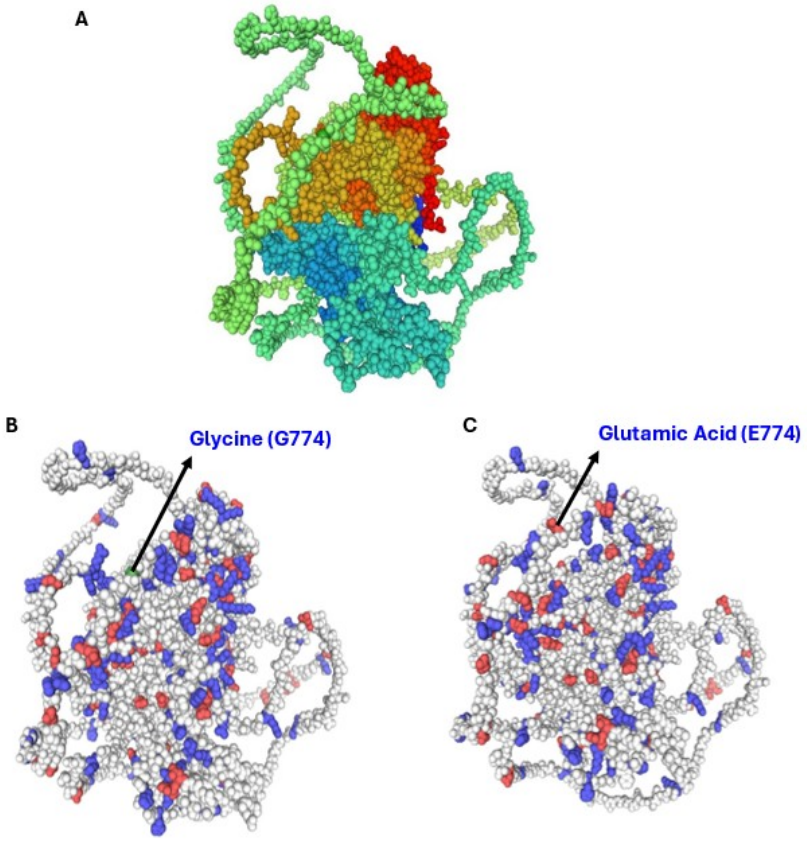

Figure 3: Homology models of wild-type (WT) and mutant (MT) KDM6A proteins. (A) Homology model of WT KDM6A represented by space-filling with a rainbow color scheme. (B) WT KDM6A homology model represented by spacefill with a color scheme based on charge, indicating the location of glycine at position 774 in the protein. (C) Mutant KDM6A (G774E) homology model represented by spacefill with a color scheme based on charge, indicating the location of glutamic acid at position 774.

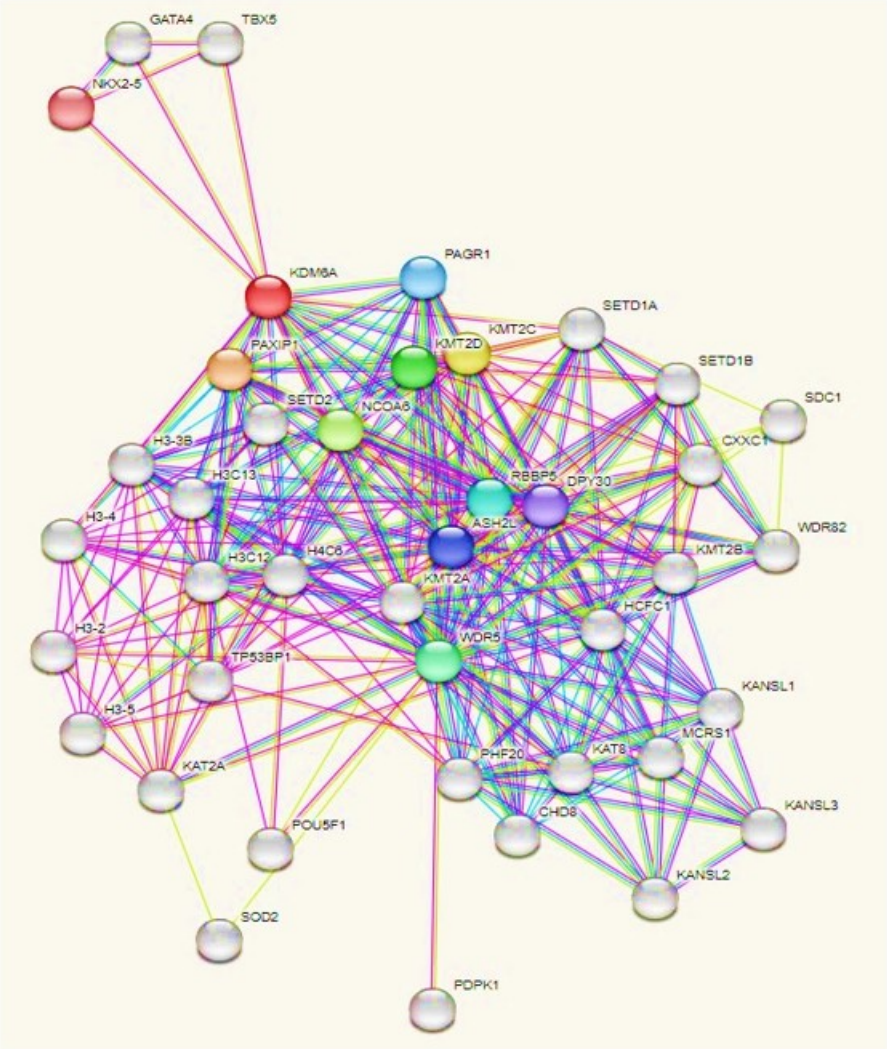

Figure 4: STRING predicted the protein-protein interaction map of KDM6A with other proteins (https://string-db.org/).

Tables

S.No	Parameters	Description
1	Chrom	X
2	Pos	45069820
3	Ref	G
4	Alt	A
5	Type	SNV
6	Length	0
7	AnnoType	CodingTranscript
8	Consequence	NON_SYNONYMOUS
9	ConsScore	7
10	ConsDetail	missense
11	GeneID	ENSG00000147050
12	FeatureID	ENST00000611820
13	GeneName	KDM6A
14	CCDS	CCDS94599.1
15	SIFTcat	deleterious
16	SIFTval	0.02
17	PolyPhenCat	benign
18	PolyPhenVal	0.014
19	RawScore	3.860662
20	PHRED	23.7
21	Missense 3D	This substitution triggers clash alert. The local clash score for wild type is 55.21 and the local clash score for the mutant is 76.02. Criterion: The mutant structure has a MolProbity clash score ≥ 30 and the increase in clash score is > 18 compared to the wild type.

Table 1: Analysis results of the G774E mutation in KDM6A using the different bioinformatics software and Missense 3D tools[A4.1][A4.2]. The table also provides detailed information about the position of mutation in the gene, reference sequence and the predicted consequences of the mutation.

DISCUSSION

Kabuki syndrome is a very rare disorder that affects many organs and systems in newborns, and it is estimated that 1 in 32000 newborns have this disease [16,17]. To date, approximately four genes have been reported to cause Kabuki syndrome, and one gene has features similar to those of Kabuki syndrome [18]. However, pathogenic variants in the two genes, *KDM6A* and *KMT2D*, have been reported in the literature to be the most common cause of Kabuki syndrome, where 28-80% of patients suffer from CHDs [17-19]. The first gene discovered to cause Kabuki syndrome was *KMT2D* (MLLe), located on chromosome 12q13.12 [20]. The second most frequently involved gene is KDM6A, which is present on the X chromosome at location Xp11.3 [21]. KDM6A and KMT2D encode proteins involved in chromatin transcription; KDM6A demethylates H3K27 (lysine-27 histone-3), and KMT2D methylates H3K4 (lysine-4 histone-3) [2, 22]. Pathogenic mutations in these two genes lead to impaired histone methylation and result in

the malfunctioning of multiple organ systems [23, 24]. *KMT2D* gene mutations are mostly transmitted in an autosomal dominant pattern and are responsible for most cases of Kabuki syndrome (KS). The *KDM6A* gene, located on the X- chromosome, is transmitted from the unaffected carrier female to the patient, where the father mostly shows a normal allele, and many patients have de novo mutations rather than those inherited from the parents [15, 16]. In our case, the c. 2321G > A mutation was transmitted through the carrier mother, who had an affected allele, while the father had a normal allele (Fig. 2).

Faundes et al. (2021), in one of the major studies that involves 80 patients, analyzed the molecular basis of heterozygous and hemizygous gene alterations in the *KDM6A* gene and found that patients with protein- altering variations (PAVs) had shorter birth spans than those with protein termination variants (PTVs). Patients with protein- altering variants (PAVs) have shorter birth lengths than those with protein- truncating variants (PTVs). They found that intellectual disability and impaired central nervous system were more common in PTVs than in PAVs (97.6% vs. 80% and 71.4% vs. 28.6%, respectively), although the differences were not statistically significant. Hence, they concluded that PTVs had more lethal effects on the patient's phenotype, and patients with PAVs showed a more variable phenotype [25].

The third gene involved in Kabuki syndrome is *RAP1A*, which is responsible for a minority of patients [26] and is located in the region of the 12-MB duplication of chromosome 1p13.1-p22.1, that is known to be involved in Kabuki syndrome [27]. The fourth gene, *RAP1B*, is closely related to *RAP1A* [26]. The last gene that is considered to cause overlapping features, such as Kabuki syndrome with nodular heterotopia, is HNRNPK [28, 29].

The major cardiac features of Kabuki syndrome include atrial and ventricular septal defects, double-outlet right ventricle, aortic coarctation, pulmonary stenosis, dysplastic mitral valve, and tetralogy of Fallot [18, 30-32]. In our study, we found overlapping features, as described in the literature, in our studied patient, who also showed various cardiac anomalies, including pulmonic valve stenosis, atrial septal defect, small patent ductus arteriosus, and patent foramen ovale.

In addition to heart features, patients with Kabuki syndrome show developmental delay, poor and difficult feeding, various growth defects, renal impairment, skeletal deformities, and distinct facial features such as sparse eyebrows, eversion of the lateral third of the lower eyelids, long palpebral fissures, and large everted ears [18, 32-34]. Our patient also showed global developmental delay, poor and difficult feeding, mild to moderate intellectual disability, speech problems, and difficulty in breathing.

In our study using STRING analysis, we found that KDM6A interacts closely with KMT2D, while it is also associated with NKX2-5, GATA4, and TBX5, which are important CHD- associated transcription factors that have been identified in the majority of CHD patients and show multiple cardiac structural and functional impairments [5]. These transcription factors are responsible for the transcriptional activation and regulation of cardiac- specific genes encoding cardiac-specific regulatory or structural proteins [35, 36].

NKX2-5 is one of the earliest transcription factors expressed in cardiac cell differentiation, atrial compartment formation, atrioventricular outflow, and conduction system formation [37, 38]. NKX2-5 is located on chromosome 5q35.1, with two coding exons, and encodes a protein of 324 amino acids. Many studies using linkage analysis and single- candidate gene research have investigated the roles of NKX2-5 and GATA4 in heart disease in large cohorts [39]. GATA4 transcription factor is expressed in almost all types of cardiac cells and is required for cardiac differentiation, proliferation, morphogenesis, heart tube formation, and proepicardium generation [40-43]. GATA4 is located on chromosome 8p23.1- p22, has seven exons, and encodes a protein of 442 amino acids. GATA4 is essential for normal cardiogenesis because of its significance during embryonic development and its continued expression in the adult myocardium [44]. TBX5 (T- box transcription factor 5) is a member of the T- box transcription factor family and is involved in forelimb and cardiac development. Pathogenic mutations in this gene impair the cardiac septum and cardiac conduction system, resulting in Holt– Oram syndrome [45]. TBX5 gene is located on chromosome 12q24.21, contains nine exons, and encodes a protein of 518 amino acids with a T-box domain of 180 amino acids located between amino acid residues 56 and 236 [46].

KDM6A, also known as UTX, is a histone demethylase that plays a crucial role in epigenetic regulation and embryonic development [47]. This gene, located on the X chromosome, encodes a protein that specifically demethylates di- and trimethylated lysine 27 on histone H3 (H3K27me2/3), thereby promoting gene activation. The significance of KDM6A in human development and disease has been underscored by its association with Kabuki syndrome, a rare genetic disorder characterized by distinctive facial features, intellectual disability, and various congenital anomalies [48]. KDM6A belongs to the Jumonji C (JmjC) domain-containing histone demethylases, which are essential for maintaining the proper epigenetic landscape in cells. By removing methyl groups from H3K27, KDM6A counteracts the repressive effects of Polycomb Repressive Complex 2 (PRC2), which is responsible for depositing methyl marks [47]. This dynamic interplay between histone methylation and demethylation is critical for the regulation of gene expression during development and in adult tissue. Kabuki syndrome is a heterogeneous condition caused by mutations in several genes, with KDM6A being one of the primary causative genes, along with KMT2D [49]. Pathogenic variants in KDM6A account for approximately 5-8% of Kabuki syndrome cases, particularly in females, owing to its X-linked inheritance pattern [50]. The identification of KDM6A mutations in patients with KS has provided valuable insights into the molecular mechanisms underlying this complex developmental disorder. The clinical presentation of Kabuki syndrome is highly variable, with affected individuals displaying a wide range of symptoms and severity. Common features include distinctive facial characteristics (such as elongated palpebral fissures, arched eyebrows, and large ears), skeletal abnormalities, cardiac defects, immunological problems, and developmental delays [48, 51]. This variability in clinical presentation may be attributed to the diverse roles of KDM6A in the regulation of gene expression across different tissues and developmental stages. The involvement of KDM6A in Kabuki syndrome emphasizes the critical role of epigenetic regulation in human development. As a histone demethylase, KDM6A influences gene expression patterns by modifying the chromatin structure, which is essential for proper embryonic development and tissue-specific gene regulation [52]. Disruption of KDM6A function can lead to aberrant gene expression, resulting in diverse clinical manifestations observed in patients with Kabuki syndrome. Recent studies have demonstrated that KDM6A is involved in various developmental processes, in addition to those affected by Kabuki syndrome. For example, it has been implicated in cardiac development, neurogenesis and hematopoiesis [48]. The wide-ranging effects of KDM6A on multiple organ systems highlight its importance as a master regulator of cellular development and differentiation. Furthermore, KDM6A plays a role in the biology of cancer. Mutations or dysregulation of KDM6A have been associated with various cancers, including bladder cancer, renal cell carcinoma, and acute myeloid leukemia [52]. These findings suggest that KDM6A may function as a tumor suppressor in certain contexts, further emphasizing its significance in the maintenance of cellular homeostasis. Understanding the role of KDM6A in Kabuki syndrome has not only advanced our knowledge of the etiology of the disorder but has also opened new avenues for potential therapeutic interventions in Kabuki syndrome. Research on KDM6A function and its downstream targets may lead to the development of targeted treatments for Kabuki syndrome and other related developmental disorders [48]. Current therapeutic approaches to Kabuki syndrome primarily focus on managing individual symptoms and providing supportive care to patients. However, the identification of KDM6A as a causative gene has sparked interest in developing targeted therapies. One potential avenue of research involves exploring the use of epigenetic modifiers to compensate for the loss of KDM6A function. For example, inhibitors of histone deacetylases (HDACs) or DNA methyltransferases (DNMTs) could potentially be used to alter the epigenetic landscape and partially restore normal gene expression patterns in affected individuals [50]. Additionally, advances in gene therapy and genome editing techniques, such as CRISPR-Cas9, offer promising possibilities for correcting KDM6A mutations and modulating their expression in affected tissues. Although these approaches are still in the early stages of development, they represent exciting potential avenues for future therapeutic interventions in Kabuki syndrome and other disorders caused by epigenetic dysregulation [53].

In our study, the links between KDM6A and other cardiac-related genes can potentially address important questions regarding cardiac function and structural development, along with neurodevelopmental and other disorders [54-56]. The missense mutation at position 774 in the KDM6A protein, resulting in the substitution of glycine with glutamic acid, has significant implications for the development of Kabuki Syndrome. This mutation affects the catalytic domain of KDM6A, a histone demethylase that is crucial for epigenetic regulation and gene expression. The replacement of a small, non-polar amino acid (glycine) with a larger, negatively charged amino acid (glutamic acid) likely alters the protein structure and function in multiple ways. Primarily, the introduction of a negatively charged residue in place of a neutral one may disrupt the local electrostatic environment within the protein. This alteration could potentially

affect the protein's ability to interact with its substrates or cofactors, possibly modifying its catalytic efficiency. Furthermore, the larger size of glutamic acid compared to that of glycine may introduce steric hindrance, further compromising protein function. Structural modifications induced by this mutation may impair the enzyme's capacity to remove methyl groups from histone proteins, a process critical for regulating gene expression. Histone demethylation is a key mechanism of epigenetic control that facilitates dynamic changes in chromatin structure and accessibility. Disruption of this process can lead to aberrant gene expression patterns throughout the genome. The implications of this mutation extend beyond the immediate biochemical effects of KDM6A protein. The altered epigenetic landscape resulting from impaired histone demethylation can have far- reaching effects on cellular function and development. Genes that are normally tightly regulated during embryonic and postnatal development may be inappropriately expressed or silenced, leading to a cascade of developmental abnormalities. These disruptions in normal developmental processes contribute to the characteristic features of Kabuki Syndrome. Hence, the missense mutation (G774E) in KDM6A serves as a critical example of how subtle molecular changes can have profound effects on human health and development. Continued research on this and similar mutations is essential for advancing our understanding of epigenetic regulation and developing targeted therapies for Kabuki Syndrome and related disorders.

In conclusion, we identified a novel mutation in the *KDM6A* gene (G774E) responsible for Kabuki syndrome. The patient exhibited various cardiac abnormalities, breathing issues, hypoxic-ischemic encephalopathy, weak muscles, global developmental delay, poor feeding, mild to moderate intellectual disability, and speech problems. Furthermore, in the future, large cohorts of patient recruitment are needed, in combination with next-generation sequencing technologies combined with animal and cell models, to shed light on molecular insights into this disease and enhance our awareness and knowledge for the development of molecular/ genomic therapies and to improve clinical practice.

CONFLICT OF INTEREST

The authors declare that they have no competing interests

AUTHOR CONTRIBUTIONS

Mahmood Rasool, Sherin Bakhashab and Isam M. Abu Zeid designed and supervised the study. Majed Alsulami, Ayat Mohammed Shorbaji, Loubna Siraj Mira and Mohammad Basabrain performed experiments, Mohamed Nabil Alama provided samples. Absarul Haque wrote and edited manuscript. Sajjad Karim, Hisam N. Altayb and Peter Natesan Pushparaj performed in silico analysis.

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