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Research article

Biallelic *HMGXB4* loss-of-function variant causes intellectual disability, developmental delay, and dysmorphic features

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ABSTRACT

Background: HMGXB4 (additionally known as HMG2L1) is a non-histone DNA-binding protein that contains a single HMG-box domain. HMGXB4 was originally described in Xenopus where it was seen to negatively regulate the Wnt/β-catenin signaling pathway.

Materials and methods: In this study, we conducted a genetic and clinical evaluation of a single family with three affected individuals suffering from intellectual disability (ID), global developmental delay (GDD) and dysmorphic facial features.

Whole genome sequencing (WGS) and Sanger sequencing were performed on the affected individuals' DNA to identify genetic variations. Additionally, a reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to assess gene expression in both the affected and unaffected individuals in the family.

Result: WGS identified a homozygous frameshift variant c.1193_1196del p. (Lys398Argfs × 25) in exon 5 of the *HMGXB4* gene (OMIM 604702), which completely segregated the disease phenotype in the family. Furthermore, RT-qPCR revealed a substantial decrease in the *HMGXB4* gene expression in the affected individuals as compared to the unaffected individuals of the family.

Conclusions: The current study is the first evidence linking a genetic variant in the *HMGXB4* gene to ID, GDD, and dysmorphic facial features. Therefore, it is possible that *HMGXB4* contributes significantly to developmental milestones and may be responsible for neurodevelopmental disorders in humans.

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1. Introduction

HMG-box is an acronym for "high mobility group-box" and refers to a family of HMGB proteins known for their DNA-binding properties and their ability to act together with proteins that promote the formation of complex nucleoprotein structures [1]. They are further divided into four subtypes, HMGB1, HMGB2, HMGB3, and HMGB4, which are grouped according to their DNA-binding preferences [2]. They are ubiquitously expressed and highly abundant non-histone chromatin proteins found in eukaryotic chromosomal proteins and transcription factors [3]. Three distinct physiological groups are associated with the HMGB family, including class I, class II, and class III [4]. Class I proteins act by binding the DNA in a sequence-specific manner and contain a single HMC box, which can bind to four-way DNA junction and duplex DNA targets [5]. Class II includes non-histone chromosomal proteins and class III includes nuclear and mitochondrial transcription factors, where both bind DNA in a non-sequence-specified manner [6].

In mammals, the three α-helix stretches contain two domains that are classified based on their function, abundance, and a sequence-specific to DNA binding, which depends on charged surface residues between the two HMG [high mobility group] boxes [6]. These domains contain two tandem DNA-binding domains, A and B, linked via a short flexible linker which allows both domains to act independently [1]. The differences between the two domains are likely to contribute to the high affinity and strong preference for HMG-box A, which is more positively charged in helices I and II in comparison to HMG-box B [3].

HMGB contains a shorter loop and bends helix 90–100° within a single double-helical turn, a conformation stabilized by the basic region, and it acts on untwisting the DNA by binding to minor grooves [7]. HMGB interacts with various proteins, including DNA repair proteins, silencing complexes, transcription factors, and site-specific recombinant proteins, facilitating the efficient assembly of regulatory nucleoprotein complexes. This interaction enables specific chromatin site interactions [2].

HMGXB4 (also known as HMG2L1) is an abundant non-histone DNA-binding protein that has been shown to negatively regulate the Wnt/β-catenin signaling pathway that regulates chromatin and the expression of neuronal differentiation markers [8,9]. This protein

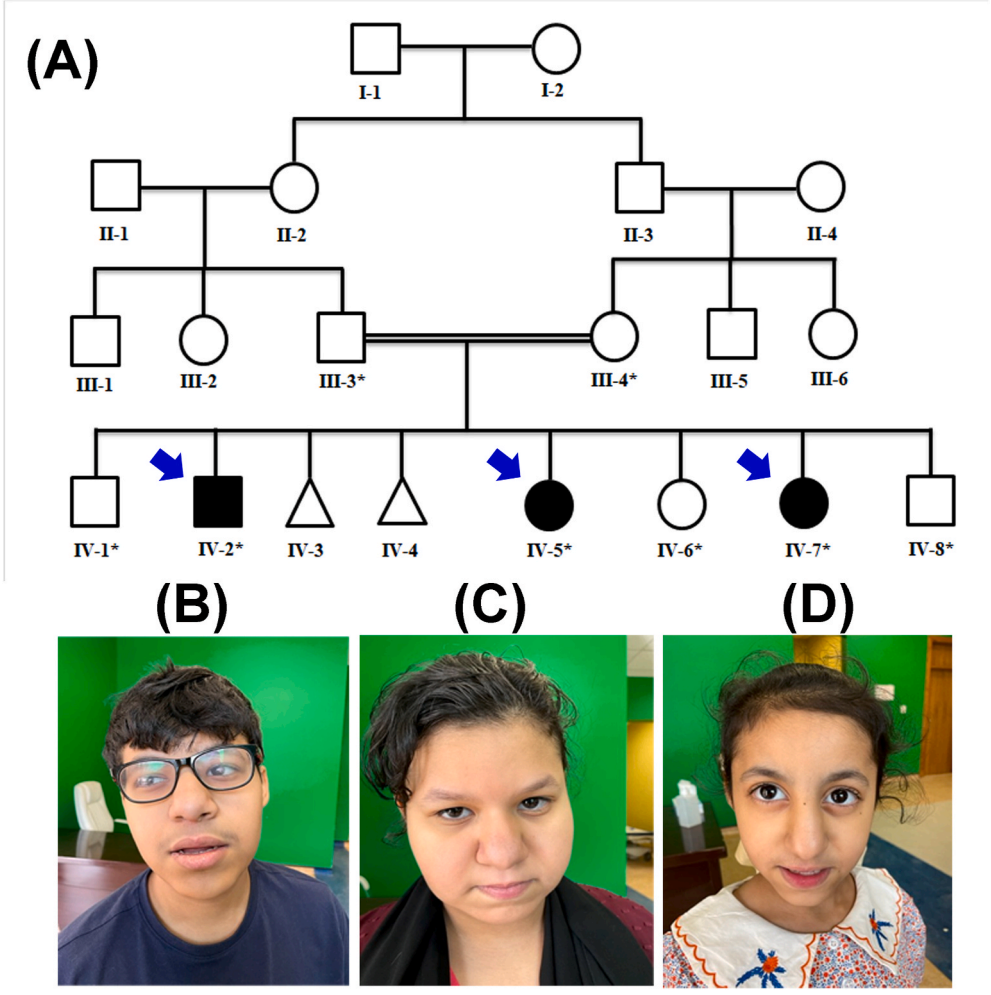

Fig. 1. (A) Pedigree of the investigated family, showing autosomal recessive inheritance. The individuals available for the present study were represented with an asterisk. **(B–D)** Facial photographs of the three affected individuals (IV-2, IV-5, IV-7) showing dysmorphic facial features.

(isoform 1) has restricted expression patterns and is mainly expressed in euchromatin, particularly in late spermatocytes and round spermatids. During early embryology, HMGXB4 is expressed in the brain and kidneys, but during the adult period, its expression in the brain is reduced [9]. Additionally, HMGXB4 is a factor that acts on regulating the growth of transformed cells, and the development of brain cells, regulates chromatin, and expresses neuronal differentiation. Furthermore, it affects the maintenance of neuroplasticity by regulating the expression of neuronal precursors allowing the cell to advance to the neuroblast stage [9].

Even though the expression profile of HMGXB4 plays an important role during early development, it is activated by specific transcription factors to coordinate the pluripotency and self-renewal pathways, but it is also suppressed by epigenetic repression machinery [10]. Furthermore, HMGXB4 is regulated by the expression of PPP1R14A a potent marker that regulates the genes that are involved in the maturation and differentiation of neuronal precursor cells during embryogenesis [11]. Inhibiting the endogenous expression of HMGXB4 in neural progenitor cells can result in the downregulation of the Neuri D1 gene affecting the regulation of neuronal differentiation via modulating gene expression in neuronal cells [9]. A *de novo* missense variant in the *HMGXB4* has already been described in the literature in association with autism spectrum disorder (ASD) [12]. In this article, we report three affected family members with unique characteristic dysmorphic features associated with intellectual disability and developmental delay.

2. Materials and methods

Ethical approval

In the present investigation, a single family was clinically and genetically evaluated. In compliance with the Helsinki Declaration, written informed consent was obtained from the parents for the publication of data and any accompanying images. The institutional review board (IRB) of King Abdullah International Medical Research Center (KAIMRC) approved the research study recorded under project RC19/120/R.

2.1. Genomic DNA extraction and quantification

A detailed pedigree was constructed showing an autosomal recessive inheritance pattern (Fig. 1A). The parents had a consanguineous marriage. DNA was extracted and quantified from all the available family members using standard protocols, as previously described [13].

2.2. Whole genome sequencing

Enzymatic fragmentation of genomic DNA was used, and libraries were created by the PCR-mediated addition of Illumina-compatible adapters. On an Illumina platform, the libraries were paired and sequenced, yielding an average coverage depth of 30×. An in-house bioinformatics pipeline was used to read the sequenced alignment to the GRCh37/hg19 genome assembly, variant calling and annotations were performed using standard methods. The DRAGEN pipeline from Illumina was used to call the structural variants. (SVs). All the variants with a minor allele frequency (MAF) of less than 1 % in the gnomAD database, as well as disease-causing variants reported in HGMD®, ClinVar, or CentoMD®, were considered significant. While coding exons and flanking ± 30 intronic bases were the primary focus, the entire gene region was searched for candidate variants with a plausible association with the phenotype. As the pedigree depicted autosomal inheritance patterns, the homozygous and compound heterozygous variants were filtered. Furthermore, the provided family history and clinical information were used to assess the pathogenicity and causality of the identified variants. They were classified into five groups: pathogenic, likely pathogenic, variant of uncertain significance (VUS), likely benign, and benign. All the variants related to the patient's phenotype were investigated. Orthogonal methods confirmed the variants of low-quality and/or unclear zygosity. The identified variant was screened in gnomAD, 1000genomes and in-house (2000) genomes/exoms from the Saudi population.

2.3. Sanger sequencing

After WGS filtration, the screened variant was Sanger sequenced in all the available family members using standard methods. Primers were designed using Primer3 version 0.4.0. The primers sequence used for the Sanger sequencing was *HMGXB4*-1193-F1: AGCGACACTCCAAGTCCAAG, *HMGXB4*-1193-R1: AATCTCTTACCCCCACCACA (Product size: 243bp).

2.4. RT-qPCR

To functionally validate the variant, total RNA was extracted from all available family members to investigate the relative mRNA HMGXB4 expression using GAPDH as the internal control. After isolating total RNA from PBMCs with a TRIzol® reagent (Invitrogen), 200 μl of chloroform was used to separate the organic and aqueous phases. Following a 15-min centrifuge at 4 °C, the RNA was washed out with isopropanol, precipitated with 75 % ethanol, and quantified using standard protocols. cDNA libraries were prepared using standard methods from total RNA using a standard cDNA reverse transcription kit. The Primer Bank database (https://pga.mgh.harvard.edu/Parabiosys/) was used to design the primer pair (HMGXB4-cDNA-F1: CCTCCCAGCATCCCATAC; HMGXB4-cDNA-R1: CACAATGGTCACGCGATACT) having a product size of 194 bp. The qPCR experiment, which comprised a PCR SYBRGreen Master Mix (Thermo Fisher, MA USA), was carried out using the QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems). No template

control was used as a negative control in all experiments. The ExpressionSuite software version 1.1 (Applied Biosystems) was used to analyze the results after each reaction was carried out independently and in duplicate [14]. An analysis was conducted on the quantitative real-time PCR findings using GraphPad Prism (version 8.1). A one-way ANOVA (Analysis of variance) statistical test was used to analyze the variance, and a value of $p < 0.05$ was deemed significant.

2.5. Protein structure determination

In this study, *in silico* methodologies such as homology modeling for wild-types and mutants were carried out. The crystal structure of the human HMGXB4 protein was retrieved from the AlphaFold Protein Structure Database (AlphaFold DB; AF-Q9UGU5-F1)[https://alphafold.ebi.ac.uk/entry/Q9UGU5], having uniport ID (https://alphafold.ebi.ac.uk/entry/Q9UGU5) and the SWISS-MODEL was used to create the structure of the mutated protein. The protein structure from the AlphaFold DB was used as a template to yield the mutated protein structure and the obtained model was then run through the Ramachandran plot server and ERRAT. The PDBsum tool (http://www.ebi.ac.uk/pdbsum/) was used to analyze the secondary structure and the topology. Errat and Pr0Check were used as evaluation tools for the assessment of protein structure (Fig. 3A–D).

3. Results

3.1. Clinical examination

A 16-year-old male (IV-2), a known case of obesity, presented with complaints of speech delay and hyperactivity since early childhood. His developmental history showed intellectual disability. His neonatal history was unremarkable. Symptoms started at age 3 with hyperactivity and delayed speech, without seizures or neurological deficits. The family sought medical advice at age 5. He developed normally in gross and fine motor skills, but delays were noted in social and language skills. He initially attended integration class till grade 12 with poor performance, then attended a rehabilitation center for special needs education with poor improvement. He failed the Leiter International Performance Scale and Stanford Binet intelligence scale for IQ assessment due to limited ability. The Vineland Adaptive Behavior Scales (Vineland-3) showed severe deficits in communication, activities of daily living, social relationships, and adaptive behavior. He was evaluated for hyperactivity using ADHD rating scales, which showed severe ADHD and started on Aripiprazole 5 mg once daily. His parents were first-degree cousins. He had two sisters (12 years and 8 years) with the same phenotype and two healthy siblingsTable 1.

On physical examination, the patient was obese (weight: 64 kg and BMI: 27.4), with normal height and head circumference for age and gender. He had dysmorphic features, including a bulbous nose, flat nasal root with flushed full cheeks, deep-set eyes, arched eyebrows, epicanthal folds, thick lips, protruding teeth, and a high-arched palate. Other systemic examinations were normal, including the central nervous system examination. Workup for his manifestation showed normal results, including lipid profile, TSH/T4, HbA1c, PAA, CDT, acylcarnitine profile, and urine organic acid. His brain MRI was within normal limits. Prader-Willi methylation testing and array comparative genomic hybridization returned negative.

Table 1
Detailed clinical description of the affected individuals identified in the present study.

Clinical Phenotype		Affected individual (IV-2)	Affected individual (IV-5)	Affected individual (IV-7)
Neurological manifestations	Global developmental delay	+	+	+
	Delayed ability to walk	+	+	+
	Delayed gross motor development	Normal	Normal	Normal
	Delayed speech and language development	+	+	+
	Failure to thrive			
	Feeding difficulties			
	Intellectual disability	+	+	+
	Hyperactivity	+	+	+
Facial dysmorphism	Abnormal facial shape	+	+	+
	Abnormal nasal morphology	+	+	+
	Bulbous nose	+	+	+
	Epicanthus	+	+	+
	Depressed nasal bridge	+	+	+
	Highly arched eyebrow	+	+	+
	Dental crowding	+	+	+
	Protruding tongue	+	+	+
Obesity		+	+	+
Skeletal abnormalities		–	–	–
Age		16 years	12 years	9 years
Mutation type		Frameshift	Frameshift	Frameshift
cDNA position		c.1193_1196del	c.1193_1196del	c.1193_1196del
Protein position		p. (Lys398Argfs × 25)	p. (Lys398Argfs × 25)	p. (Lys398Argfs × 25)
Gene		*HMGXB4*	*HMGXB4*	*HMGXB4*

His 12-year-old sister (IV-5), also a known case of obesity, presented with complaints of hyperactivity and intellectual disability. Her developmental history showed global developmental delay and cognition with speech delay without seizures or neurological deficit. The family noted her delay after two years of age, along with her poor intellectual skills when she joined 1st grade. She is currently in 6th grade at a special needs school with poor performance. On physical examination, she had the same dysmorphic features. Her growth parameters showed a weight of 62 kg and a BMI of 30.8 with normal height and head circumference for age and gender. Her other systems were unremarkable. No formal assessment was done for her IQ. Her investigations were unremarkable, including TSH, ACTH, growth hormone, and IGF-1. Additionally, genetic testing for Prader-Willi syndrome and array CGH were normal.

The youngest sister is nine years old (IV-7), with a similar clinical phenotype of dysmorphic features, hyperactivity, and intellectual disability. Her growth parameters showed a weight of 41 and a BMI of 23, with normal height and head circumference for age and gender. Her investigations, including HbA1c, lipid profile, TSH, T4, and chromosomal analysis, were all unremarkable. Her IQ assessment by Stanford Binet intelligence scale (4th edition) was 55 (mild deficit).

3.2. Molecular testing

Whole genome sequencing was performed for the affected individuals for molecular diagnosis. The variant filtration criteria were based on the inheritance, pedigree analysis, homozygous and compound heterozygous variants, MAF using gnomAD, splice site (± 30bp), and the variants that affect the function.

WGS revealed a homozygous frameshift variant [c.1193_1196del; p. (Lys398Argfs × 25)] (rs767967598) in exon 5 of the *HMGXB4* (NM_001003681.2; OMIM 604702) (Fig. 2A). The identified variant revealed an MAF of 0.0000294 in the gnomAD/Exomes; however, the variant was not reported in the homozygous form in gnomAD or in a homozygous state in in-house (2000) genomes/exoms from the Saudi population. In addition, the identified variant was suggested as being a disease using different Insilco tools. The gene is not under significant constraint for either missense or LOF variation (pLI = 0) although there are no homozygous variants in gnomAD.

Sanger sequencing revealed that the variant is perfectly segregated within the family. Sanger testing showed heterozygous variants in the parents and the eldest brother, while the two healthy sisters were carrying a wild-type genotype (Fig. 2A). The identified variant is present in exon 5 of the *HMGXB4* gene (Fig. 2B) and before the HMG domain; thus, the loss-of-function variant at such a position might be associated with disease pathophysiology (Fig. 2C). Depending on the isoforms, *HMGXB4* has 5, 11 and 12 exon transcripts. The variant identified in the present study affects only the transcripts with 11 exons (the variant affecting exon 5) and 12 exons (the

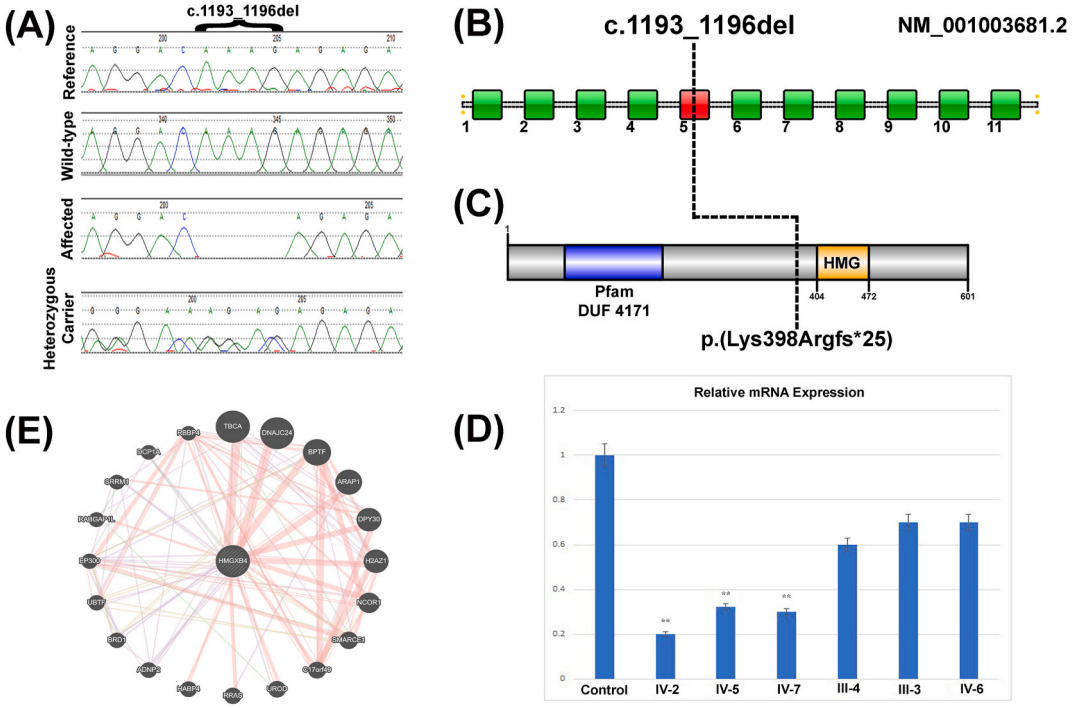

Fig. 2. **(A)** Sanger electrograms of the frameshift variant identified c.1193_1196del p. (Lys398Argfs × 25) in the exon 5 of the *HMGXB4* gene. Sanger sequencing showed perfect segregation of the identified variant. **(B)** Schematic representation of the 11, 12 and 5 exons of the *HMGXB4* gene and location of the variant identified in different transcripts of the *HMGXB4* gene. Blue arrows represent the location of the primers used in the RT-PCR. **(C)** Schematic representation of the HMGXB4 protein, showing the position of the identified variant. **(D)** Relative mRNA expression of the *HMGXB4* gene in the patients, parents, and siblings, suggests a significant decrease in the mRNA expression in the affected individuals. **(E)** Interaction pathway of the HMGXB4 with other proteins using GeneMANIA.

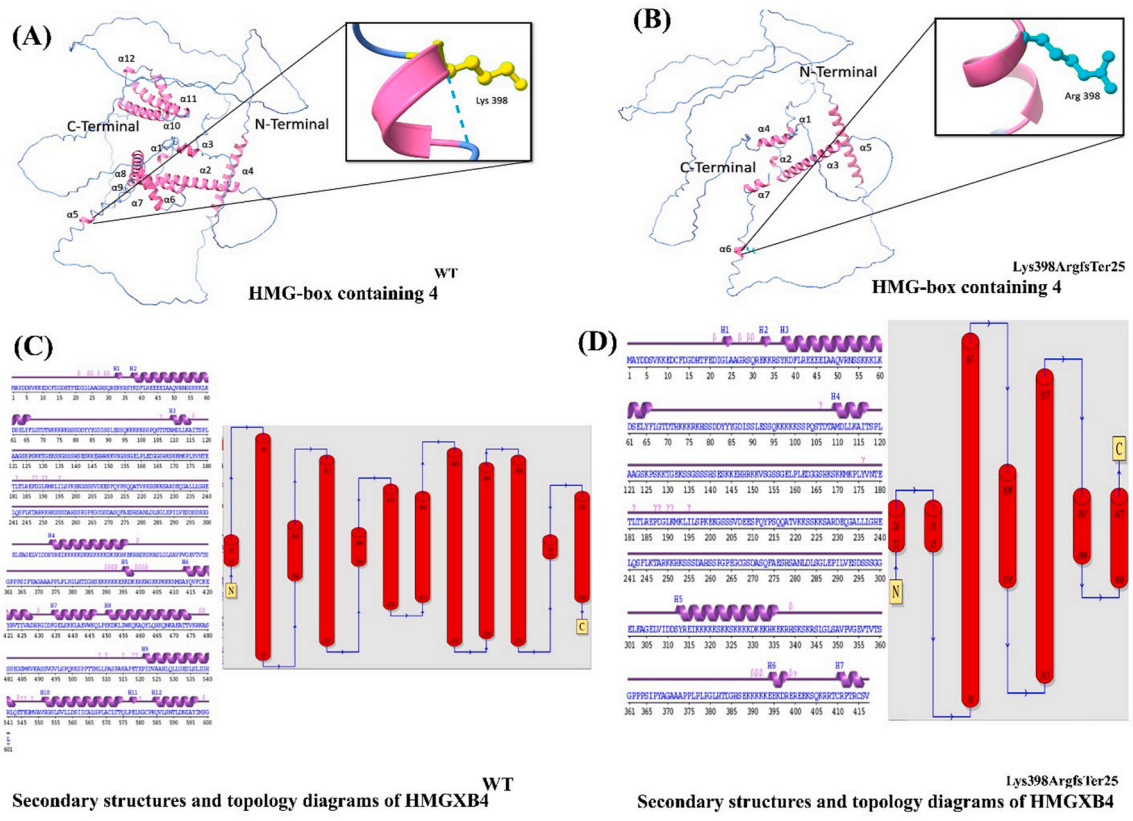

Fig. 3. Protein Homology and Secondary structures and topology of HMGXB4. **(A, B)** Protein homology structure of wild-type and mutant (p. Lys398Argfs × 25) of HMGXB4. **(C, D)** Secondary structures and topology of wild-type and mutant (p. Lys398Argfs × 25) of HMGXB4.

variant affecting exon 6).

We also identified a heterozygous variant [c.1026del p. (Glu342Aspfs × 2)] in the *CENPF* gene (NM_016343.3) located on chromosome 1q41. Homozygous variants in *CENPF* have been associated with Stromme syndrome. The *CENPF* variant [c.1026del p. (Glu342Aspfs × 2)] did not segregate with the disease phenotype within the family. There was no other variant found that might be disease-causing or associated with the disease phenotype.

No copy number variations (CNVs) were observed in the present study that might be relevant to the disease phenotype of the patients.

3.3. HMGXB4 mRNA expression

The relative expression data of the *HMGXB4* gene in the affected individuals, parents, and unaffected control individuals were examined. The results showed that the affected individuals (IV-2, IV-5, IV-7) bearing the disease-causing variant [c.1193_1196del; p. (Lys398Argfs × 25)] had a substantial reduction in the *HMGXB4* gene expression as compared to the wild-type (control) and carrier (parents; III-3, III-4) (Fig. 2D). The relative mRNA expression levels were calculated for a control sample and individuals were labeled IV-2, IV-5, IV-6, IV-7, III-4, and III-3. The corresponding fold changes concerning the control were determined to be 5, 2.87, 1.43, 3.33, 1.67, and 1.43, respectively.

3.4. Protein homology

Using 3D-homology modeling, wild-type and mutated HMGXB4 proteins (p.Lys398Argfs × 25) were predicted and evaluated (Fig. 3A and B). The Ramachandran plot indicated that 89–90 % of residues in the wild-type and mutant structure lay in the permitted regions of torsion angles. Errat provided 85.182 and 89.33 quality factors of wild-type and mutant structures (Fig. 3C and D). The wild-type HMGXB4 protein structure contains 12 alpha helices while Lys398Argfs × 25 resulted in a truncated protein with only 07 alpha helices. The truncated protein structure indicated that the variant would greatly destabilize the protein structure and, hence, result in disrupted function. The identified variant (p.Lys398Argfs × 25) resulted in a stop codon 25 amino acids (a.a) downstream of the Lys398. This means a 423 a.a position that will result in the loss of the HMG domain and the entire C terminal. Thus, the truncated HMGXB4 protein may be unstable and degraded. Thus, the location of the frameshift within the protein is expected to result in both a

shortened protein and nonsense-mediated decay.

4. Discussion

Many neurodevelopmental disorders have been linked to early life inflammation where the presence of autoantibodies, neuro-inflammation, and T-cell response which contribute to neurodevelopmental and neurodegenerative disorders [13,14] exist. Furthermore, the pathogenesis of neuro-inflammation may occur via the upregulation of several selective proinflammatory cytokines (such as IL-1b, IL-6, TGF-B1, interferon-y, and NF-kB) [13]. Specifically, the expression of IL-1 strongly contributes to inhibiting long-term potentiation by crossing the blood-brain barrier, thereby reducing the strength of the synapse [15]. NF-kB plays an important role in regulating the development and maturation of the nervous system because of its essential role in several molecular cascades resulting in the inflammation of residual immune cells in the brain. Thus, the disruption of neuroimmunology homeostasis can contribute to the loss of neuronal cells and reduced connectivity which can impair the abilities in regression and skills [16]. The neuroinflammatory process, initiated by microglial cells, can cross the blood-brain barrier by modulating gene expression and promoting cytoskeletal rearrangement and tight junction reorganization. Additionally, the influx of peripheral immune cells is facilitated by the upregulation of CXCL12 on endothelial cells, enabling binding and transmigration into the brain. This process leads to vascular endothelial dysfunction and structural tissue damage [13,17].

The expression of HMGB4 is induced through the NF-kB signaling pathway, leading to the expression of the proinflammatory genes NOS2 and ICAM-1, and the production of NO and monocyte adhesion via the HMGXB4-dependent factor is responsible for tissue damage, vascular hyperpermeability, and organ failure. Moreover, the deficiency of HMGXB4 has been shown to protect against systemic inflammation-induced endotoxemia in knockout mice [18]. Likewise, the serum HMGB1 level is positively correlated with the severity of neurodevelopmental disorders such as autism. This correlation is due to the upregulation of NADPH oxidase 2 (NOX-2)-dependent reactive oxygen species production, leading to leukocyte infiltration into nerve cells, resulting in persistent neuroinflammation [19].

In the present study, we investigated a Saudi family exhibiting hallmark features of ID, GDD and facial dysmorphism. WGS identified a homozygous frameshift variant c.1193_1196del p. (Lys398Argfs × 25) in exon 5 of the *HMGXB4* gene which completely segregated with the disease phenotype in the family. Furthermore, RT-qPCR revealed a substantial decrease in the *HMGXB4* gene expression in the affected members as compared to the unaffected members of the family. To date, no Online Mendelian Inheritance in Man (OMIM) phenotype has been associated with pathogenic variants in the *HMGXB4* gene (OMIM®:604702). In the literature, Kim et al. [12] associated a *de novo* missense variant in the HMGXB4 with autism spectrum disorder (20). Additionally, recent studies have uncovered a proinflammatory role of HMGXB4 in orchestrating a systemic inflammatory response in vivo and suggest that HMGXB4 could be a potential therapeutic target for the treatment of sepsis (20). According to the HGMD database, 05 *de novo HMGXB4* variants have been associated with diseases such as autism spectrum disorder, developmental disorder, autism, and elevated waist-hip ratio (12, 22–24).

Similarly, in the DECIPHER database (https://decipher.sanger.ac.uk/), several patients with *de novo* CNVs in the *HMGXB4* have been reported to exhibit neurodevelopmental phenotypes (https://www.deciphergenomics.org/gene/HMGXB4/patient-overlap/cnvs). Variants listed in DECIPHER for HMGXB4 encompass multiple genes with deletions and duplications. However, in one of the patients, a *de novo* missense variant (VUS) was observed with phenotypes such as nervous system abnormality, musculoskeletal abnormality, and limb abnormality (https://www.deciphergenomics.org/gene/HMGXB4/ddd-research-variant-overlap). HMGXB4 interacts with several proteins including BPTF (Bromodomain PHD Finger Transcription Factor); variants in this gene are associated with neurodevelopmental disorders with dysmorphic facies and distal limb anomalies (OMIM 617755). It also interacts with *SMARCE1*, which, when mutated, results in Coffin-Siris syndrome 5 (OMIM 616938) [https://genemania.org/; Fig. 2E]. This further supports the evidence that homozygous variants in the *HMGXB4* in the patients described in this study might be the underlying cause of ID, GDD and dysmorphic facial features.

In conclusion, this study is the first to define the phenotypic spectrum of *HMGXB4*-associated disorder, exhibiting ID, GDD and dysmorphic facial features. Our work provides the first evidence that biallelic variants in *HMGXB4* may lead to neurodevelopmental disorders in humans.

Consent for publication

Written informed consent was obtained from the patients.

Availability of data and materials

The data supporting this study's findings are available from the corresponding author upon reasonable request.

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CRediT authorship contribution statement

Fuad Al Mutairi: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Faisal Joueidi:** Writing – original draft, Data curation. **Maha Alshalan:** Visualization, Data curation. **Essra Aloyouni:** Validation, Methodology, Investigation. **Mariam Ballow:** Visualization, Methodology, Investigation. **Mohammed Aldrees:** Visualization, Validation, Investigation. **Abdulkareem Al Abdulrahman:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Abeer Al Tuwaijri:** Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Safdar Abbas:** Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Muhammad Umair:** Visualization, Software, Formal analysis, Data curation, Conceptualization. **Majid Alfadhel:** Visualization, Supervision, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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