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ORIGINAL ARTICLE

Identification of a new mutation in the *ACTL9* gene in men with unexplained infertility

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Abstract

Background: Infertility is defined as the failure to achieve pregnancy after one year of unprotected intercourse within a marital relationship. Approximately 10%–15% of couples worldwide experience infertility issues, with nearly half of these cases attributed to male factors. Among men with unexplained infertility, genetic mutations have been identified as a potential cause. Studies have indicated that mutations affecting the function of the protein encoded by the *ACTL9* gene may play a role in male infertility.

Methods: The purpose of this research was to identify mutations in the *ACTL9* gene associated with male infertility in a sample of 40 infertile men with unknown causes. Genomic DNA extraction and PCR amplification were carried out on samples from each individual. The genetic material was then analyzed using Sanger sequencing, followed by bioinformatics and segregation analysis to determine the potential effects of the observed variations.

Result: A novel genetic variant, c.376G>A (p.Glu126Lys), was identified in an infertile male individual, representing a previously unreported finding that was validated through segregation analyses. This specific variant induces a change from glutamate to lysine at the amino acid level by replacing the nucleotide G with A in the genomic DNA sequence, consequently impacting the secondary structure and function of the protein.

Conclusions: The conclusive analysis of the procedure indicated that this alteration has the potential to interfere with the process of fertilization, ultimately resulting in the complete failure of fertilization (TFF) and causing male infertility.

KEYWORDS

ACTL9 gene, male infertility, mutation, total fertilization failure

1 | INTRODUCTION

Among the common human reproductive system disorder, infertility is characterized by the inability to pregnant after 1 year or more of frequent, unprotected sexual activity. Since one in every six people of reproductive age experiences infertility at some point in their lives, this issue is becoming more and more acknowledged as a severe

global public health issue (World Health Organization (WHO), 2018).

For decades, women were thought to be the main cause of infertility, but recent studies show that about 33% of infertility is only due to men (Division of Reproductive Health, n.d.). Male infertility may be attributed to several factors, such as deficiencies in sperm count, hormonal imbalances, obstructions in sperm motility, anti-sperm antibodies, reproductive pathologies, varicocele, and endocrine and genetic abnormalities (American Urological Association, 2020; Bracke et al., 2018). However, research has consistently shown that particular genetic causes such as chromosomal abnormalities and single-gene mutations are involved in some cases of severe male infertility (Ferlin et al., 2006).

Over the previous years, research has been undertaken on phospholipase C zeta (PLCz) and has demonstrated that it constitutes a specialized sperm protein that contributes to the triggering of egg activation in the aftermath of fertilization (Amdani et al., 2013). As of late, scholars have directed their attention toward a particular gene known as *ACTL9*, characterized by its actin-like nature (ACTL9 Gene, n.d.). The database and reverse transcription-polymerase chain reaction (RT-PCR) analysis indicate that the expression of *ACTL9* is prominently manifested solely in the testicular tissue of humans. Moreover, the immunohistochemical staining of human sperm has revealed that the ACTL9 protein is predominantly situated in the equatorial region of the acrosomal portion of the sperm (Dai et al., 2021a). The *ACTL9* gene serves a pivotal function in facilitating the amalgamation of proacrosomal vesicles, the creation of the Perinuclear Theca, and the appropriate positioning of PLCz. The disruption in the ACTL9 protein gives rise to anomalies in the Perinuclear Theca (PT), resulting in the repositioning of PLCz from its typical location within the head of the sperm to the neck of the mutant sperm. Consequently, this alteration impedes the occurrence of regular calcium oscillations in eggs, and total fertility failure (TFF) will ensue, leading to the inability to achieve fertility via assisted reproductive techniques like intracytoplasmic sperm injection (ICSI) (Dai et al., 2021b).

Given the significance of infertility as a prevalent issue among couples experiencing reproductive complications and the important role of the *ACTL9* gene in contributing to this problem, the need for comprehensive investigations into this genetic factor becomes crucial. However, the currently available body of research on this gene remains limited and fails to provide a comprehensive understanding of its impact on infertility. Thus, this inquiry aims to explore the mutations associated with the *ACTL9* gene, to contribute to a more comprehensive understanding of the genetic causes of infertility. It is anticipated that the findings derived from this investigation will contribute to improvement in *ACTL9*-related databases, as well as facilitate the effective resolution of infertility concerns and the provision of genetic counseling to families.

2 | MATERIALS AND METHODS

2.1 | Samples

This research involved the selection of 40 infertile men who were not related to each other and had been referred to infertility centers located in the southwestern region of Iran. The data collection period spanned from July 2022 to March 2023. All participants in this study presented with primary infertility lasting for a minimum of 1 year. The average age of the participants was 35.02 ± 5.71 (range: 26–55 years old). The selection criteria for the participants included a normal karyotype (46, XY) and the absence of microdeletions in the Y chromosome. Furthermore, the participants' semen parameters were in accordance with the latest update of the WHO protocol in 2021, and they did not have any underlying medical conditions that could cause infertility.

Prior to commencing the study, ethical clearance was sought from the Research Ethics Committee of Tehran Islamic Azad University of Medical Sciences (Approval number: IR.IAU.PSREC.1401.345). Meanwhile, after obtaining written informed consent from the patients, a questionnaire regarding their personal and clinical information was completed by them.

2.2 | Exclusion criteria

The medical records and diagnostic findings of all subjects were comprehensively examined. Those male participants who had a medical history of chromosomal anomalies, varicocele, Y chromosome microdeletion, genital tract infection, anti-sperm antibodies, and abnormal seminal fluid analysis were excluded from the research.

2.3 | Genomic DNA extraction

Blood samples of 5 mL were collected from 40 male patients and stored in tubes containing ethylenediaminetetraacetic acid (EDTA). Subsequently, genomic DNAs were extracted utilizing the Favorgen kit (Catalog Number: FABGK 001, 50 Preps) in accordance with the prescribed protocol and conserved at a temperature of −20°C until their utilization.

2.4 | Polymerase chain reaction (PCR)

To achieve amplification of the whole *ACTL9* gene, including its exon and promoter region, specialized primers were employed to specifically target the regions of interest. The sets of two pairs of PCR primers designed for the detection of mutations in a single reaction comprise forward primer A_F (ACCCATCCTCTTGGCTGCATTG), reverse primer A_R (ACAGACAGCACCGACTGCGATG) for the first part of gene, forward primer B_F (ACCTACACAGTGCCCGTCTTC), and reverse primer B_R (AGGGACCCTGTTCCTCGTACTG) for the second part of gene.

In this experiment, a total volume of 25 microliters was prepared for the PCR reaction. The reaction mixture consisted of 1 microliter of template DNA, 0.3 microliters (10 pmol) of forward primer, and 0.3 microliters (10 pmol) of reverse primer. These were added to 12.5 microliters of PCR master mix (Red PCR master mix 2x AMPLIQON). To achieve the final volume of 25 microliters, 10.9 microliters of deionized water were added to the reaction mixture.

The thermal cycling program involved an initial preheating step at 95°C for 4 min, followed by a denaturation step at 95°C for a duration of 25 s. Subsequently, annealing was carried out for part A at a temperature of 65°C and for part B at a temperature of 67°C, both for 30 s. Extension was then performed at 72°C for a duration of 30 s for a total of 30 cycles. The protocol was concluded with a final extension step at 72°C for a duration of 5 min.

Following PCR amplification, gel electrophoresis was conducted using a 1% agarose gel at 100 V for a duration of 45 min to identify the amplified regions of DNA. The agarose gels were subsequently exposed to UV light using a Vilber Gel DOC E-Box CX5 imager (Vilber, Germany). Interpretation of the results was carried out by comparing the observed bands to the anticipated product size, which were as follows: Part A *ACTL9* (734 bp) and Part B *ACTL9* (619 bp).

2.5 | Sequencing

The Sanger sequencing of all PCR products was carried out using the ABI Prism Big Dye termination cycle sequencing Ready Reaction Kit and by ABI–Prism 3500 Genetic analyzer (Applied Biosystems).

The Chromas 2.6.6 software was employed for sequence analysis, then the sequence reads were aligned against the *ACTL9* gene using the reference sequence ENSG00000181786 (GRCh38.p14, NM_178525.5). The alignment of the sequences was performed using the NCBI Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi).To check the alignment, the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) database was used. To facilitate the genomic alignment for a novel variation, MEGA 7.0.26 software was utilized to verify the region across different species.

2.6 | In silico analysis of novel variant

The assessment of the novelty of variants was conducted by utilizing MutationTaster (https://www.mutationtaster.org/), Clinvar (https://www.ncbi.nlm.nih.gov/clinvar/), and Related findings from previously published research.

In addition, checking the pathogenicity of the variant was conducted through the use of Mutation Tester (https://www.mutationtaster.org/), SIFT (https://sift.bii.a-star.edu.sg/), and Polyphen2 (http://genetics.bwh.harvard.edu/pph2/) databases. Additionally, the Swiss Model database (https://swissmodel.expasy.org/) was employed to examine the alterations in the secondary structure of the protein. The MutPread2 (http://mutpred.mutdb.org/) database was employed to prognosticate the impact of the aforementioned alteration in the amino acid sequence on the protein.

2.7 | Segregation analysis

The Segregation study aimed to ascertain the existence of the identified variant among the members of the available family. After receiving the blood sample of the patient's parents and performing PCR for them, sequencing was done by the Sanger method.

3 | RESULTS

This study involved the examination of a cohort consisting of 40 infertile males, aged between 26 and 55 years, who had a documented history of infertility for more than 1 year. Out of this cohort, 12 individuals had a body mass index (BMI) exceeding 25. It is worth mentioning that the results of the sperm analysis conducted on these participants were found to be within the established normal range (Table 1). Among the subjects studied, a pathogenic heterozygous variant identified as c.376G>A was observed in one individual. The variant was detected in a 35-year-old man with normal sperm parameters (proband III-8, Figure 1a, Table 2) who had been experiencing unexplained infertility for a period of 7 years. Also based on the patient's medical records, it is indicated that the patient's female partner did not have any fertility-related health issues. Based on the patient's account, the individual underwent in vitro fertilization (IVF) on a single

TABLE 1 The summary of clinical information and sperm parameters of 40 infertile men in this study.

Issues	Infertile men group
Clinical information	
Age (years-old)	26–55 ($\overline{X} = 35.02 \pm 5.71$)
Infertility history (year)	1–12
BMI (kg/m^2)	19–35.5 (12 infertile men > 25)
Sperm parameters	
Sperm count (million/mL)	20–56
Volume (mL)	2–6
Sperm DNA Fragmentation Assay (SDFA)	5–13%
Normal sperm morphology	46–98%
Total progressive (Class A + B) (%)	44–81%

TABLE 2 The summary of clinical information of patients with *ACTL9* gene mutation.

Issues	Patient[a]
Clinical information	
Age	35 years-old
Infertility history	7 years
BMI	>25
Sperm parameters	
Sperm count	26×10^6/mL
Volume	2 mL
Sperm DNA Fragmentation Assay (SDFA)	10%
Normal sperm morphology	58
Total progressive (Class A + B) (%)	72

[a]Proband III-8 (Figure 1-a).

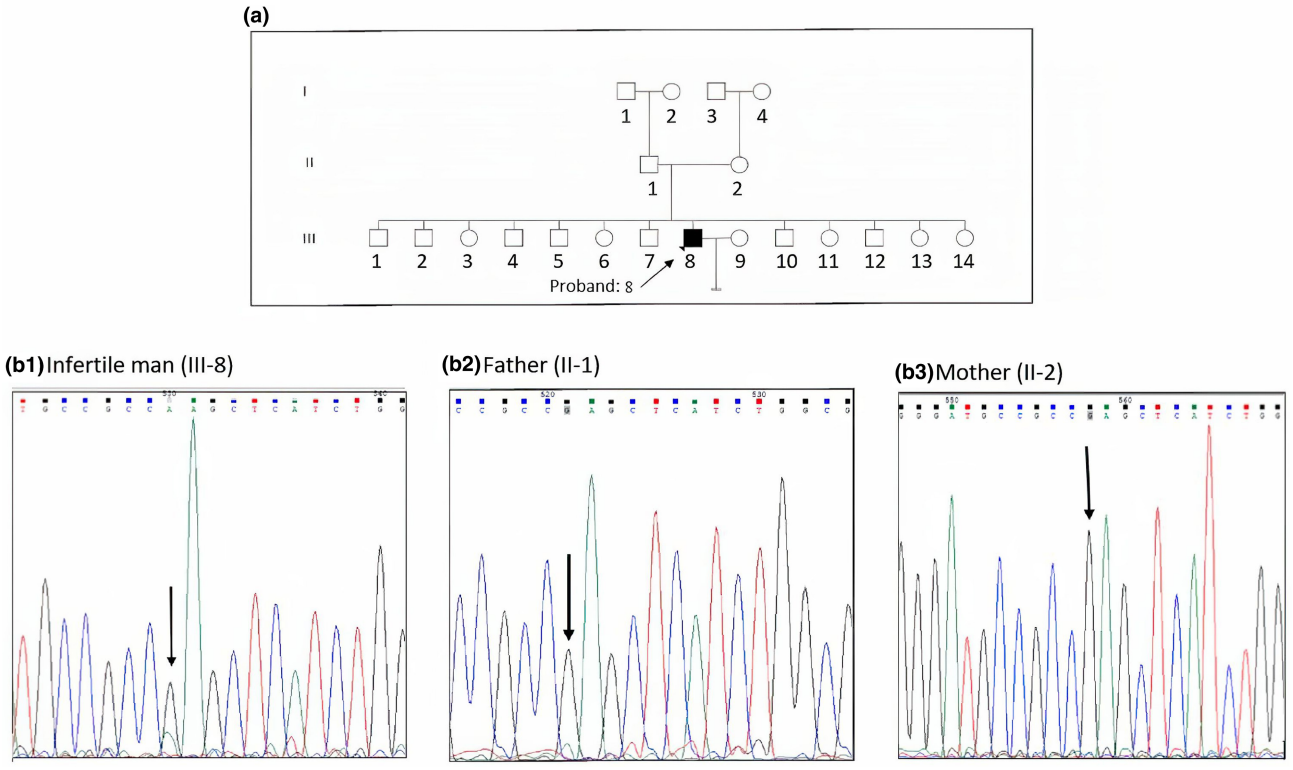

FIGURE 1 Pedigrees and Sequence chromatography of the *ACTL9* gene. (a) the pedigree of Proband's family. (b1) sequence of the c.376G>A variation in proband and (b2) sequence of the *ACTL9* gene in father's proband and (b3) sequence of *ACTL9* gene in mother's proband.

occasion; however, the outcome proved to be unsuccessful. It is important to highlight that the patient's medical records were not accessible in this particular instance. This particular variation was found to be novel and had not been previously reported in the Clinvar, ExAC, 1000 Genome, HGMD database, or any previously published literature. Therefore, this study represents the first report of c.376G>A as a pathogenic variation associated with male infertility.

Through segregation analysis, it was determined that the desired mutation in the *ACTL9* gene was absent in the genomic sequence of both parents of the proband. Furthermore, while interviewing with the patient and his family members, it came to light that all of the proband's

siblings were married and had one or more children. This observation provides evidence that the mutation arose de novo. (Figure 1-b2,b3).

The bioinformatics analysis revealed that the genomic DNA sequence of the proband exhibited a heterozygous state, with a nucleotide substitution from G to A at position 376 in the reference gene. This substitution resulted in a single base exchange and a missense mutation in the individual. The Mutation Tester database classified this mutation as pathogenic, as presented in Table 3. Furthermore, this mutation caused a change in the amino acid sequence, with glutamate (E) being replaced by lysine (K) at position 126. The Swiss Model database analysis indicated that this amino acid substitution altered the folding of the second structure of the ACTL9 protein (Figure 2-a1,a2). Furthermore, the analysis conducted on this protein utilizing the MEGA 7.0.26 software, as depicted in Figure 2b, revealed its conservation across various species throughout the course of evolution.

The Polyphen2 databases confirmed the possibility of pathogenicity of this mutation, also SIFT reported its impact on the function of the resulting protein. According to the MutPread2 database, the observed amino acid substitution results in an altered metal binding, as evidenced by a *p*-value of 1.9 and a probability of 64%. Additionally, the substitution is associated with a gain of an allosteric site at position E126, with a *p*-value of 2.2 and a probability of 30% (Table 4).

During the course of the study, a cohort of 40 patients was examined, and a total of five polymorphisms were identified, specifically rs62117992, rs2340550, rs10410943, rs4804079, and rs73507819. It is noteworthy that no prior research has been conducted to investigate the potential correlation between these polymorphisms and infertility.

TABLE 3 Prediction of the effect of the detected variations using MutationTaster.

Analyzed issue	Mutation detected
DNA changes[a]	c.376G>A g.497G>A
Amino acid changes	E126K
Prediction	disease causing
Alteration (Phys/location)	chr19:8808676C>TN/A
Alteration type	single base exchange
phastCons (flanking)[b]	1

[a]Ensembl transcript ID: ENSG00000181786, GenBank transcript ID: NM_178525.5.

[b]The closer the value is to 1; the more probable the nucleotide is conserved.

4 | DISCUSSION

Male infertility accounts for approximately 40 to 50% of infertility cases, with a significant portion remaining undiagnosed (Kumar & Singh, 2015). In this study, we have identified and confirmed a novel heterozygous missense mutation c.376G>A in the *ACTL9* gene through Sanger sequencing. This mutation leads to the substitution of glutamate (E) with lysine (K) at position 126. Notably, this variant of *ACTL9* has not been observed in any population analysis and has been predicted to be pathogenic by various in silico analyses, including Mutationtester, Sift, and Polyphen2. Furthermore, this substitution alters the secondary structure of the ACTL9 protein and is expected to impact its function based on bioinformatic analyses.

Of particular significance is the identification of this mutation in a family where only the proband exhibits this specific *ACTL9* variant. Segregation studies have been conducted, confirming that the gene sequence of the parents (II-1, II-2) aligns completely with the reference sequence. Additionally, the other sons in the family have demonstrated normal reproductive health, further supporting the association of this mutation with male infertility. To strengthen the validity of these findings and obtain additional evidence, we recommend conducting further functional studies.

In this regard, previous studies showed that actin-related proteins are frequently observed in pairs or multiple protein complexes. Several actin-related genes have been identified in the testis, including *ACTRT1*, *ACTRT2*, *ACTRT3*, *ACTL7A*, and *ACTL7B* (Chadwick et al., 1999; Heid et al., 2002; Hisano et al., 2003). Of particular importance is *ACTL7A*, which is a significant paralog of *ACTL9*. Research has indicated that the actin-like protein ACTL9 in the testis plays a crucial role in the PT structure and interacts with *ACTL7A* in sperm, as confirmed by laboratory experiments. Mutations in *ACTL9* have been shown to weaken or completely disrupt the interaction with *ACTL7A*. These findings suggest that *ACTL9* and *ACTL7A* may form complexes that are involved in PT formation and acrosome anchoring. Pathogenic mutations in *ACTL9* may interfere with the function of these complexes, leading to the separation of the acrosome inner membrane from the nuclear envelope and the formation of an abnormal PT structure (Dai et al., 2021b). Hence, the alteration in protein function resulting from the manifestation of mutation in this investigation is in alignment with prior research endeavors.

Furthermore, after scrutinizing the sequencing outcomes of the *ACTL9* gene in the subjects examined with the reference gene, five polymorphisms were detected in infertile men who did not exhibit mutations in the *ACTL9* gene. These polymorphisms include rs62117992,

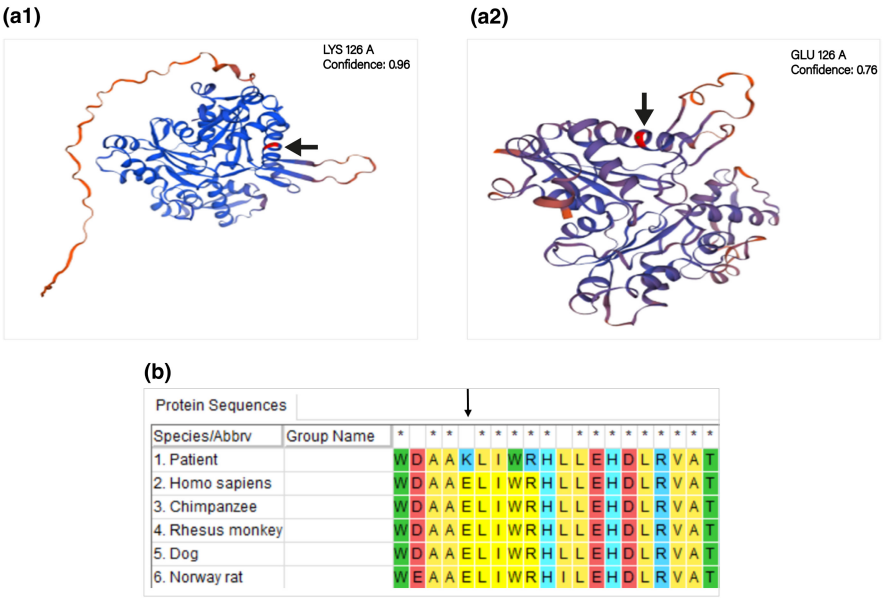

FIGURE 2 Investigating the effect of the c.376G>A variant on the protein's secondary structure, specifically the conversion of the amino acid GLU to LYS at position 126. (a1) mutated form. (a2) wild form. (b) Alignment of *ACTL9* gene sequence in five different species by MEGA7, showing this variation is in conserved sequences.

TABLE 4 Results of other databases to check pathogenicity.

Other databases check pathogenicity	Score	Summary
SIFT[a]	0.00	Affect Protein Function
Polyphen2[b]	0.868	Possibly Damaging
MutPread2		
Altered Metal binding	Probability: 64%	
Gain of Allosteric site at E126	Probability: 30%	

[a]The closer the value is to 0.00; the more probable the pathogenicity is conserved.

[b]The closer the value is to 1; the more probable the pathogenicity is conserved.

rs2340550, rs10410943, rs4804079, and rs73507819. Genetic polymorphisms are crucial determinants of phenotypic alterations and may moderate or even cause the risk of various diseases, including genetic disorders and multifactorial diseases. Additionally, they can be employed to trace genetic variants linked to disease in families. Given the shortage of research on the *ACTL9* gene and the absence of exploration of its polymorphisms in relation to the incidence of infertility, it is imperative to investigate the aforementioned polymorphisms.

5 | CONCLUSION

In summary, the present investigation has successfully identified a novel pathogenic variation within the *ACTL9* gene. The in-silico analysis conducted in this study has revealed that this variant has the potential to disrupt the function of the protein and certain crucial factors, which may ultimately lead to alterations in the expression of the *ACTL9* gene and consequent male infertility.

AUTHOR CONTRIBUTIONS

Ali Hossein Saberi was responsible for the overall planning and guidance of the project. Roya Sinaei and Ali Hossein Saberi collaborated in designing the research study, developing the experimental methods, analyzing and interpreting the data, and preparing the initial draft of the article. Maryam Eslami contributed to the review of the project topic, data interpretation, and article writing. Mohammadreza Dadfar played a role in patient selection, clinical examinations, medical record review, and article writing. Roya Sinaei took charge of designing the figures in the article, conducting experiments, and writing

the manuscript with input from all authors. All authors provided valuable feedback, contributed to the research, analysis, and manuscript, and actively participated in discussions regarding the results, ultimately shaping the final manuscript.

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CONFLICT OF INTEREST STATEMENT

None of the authors has a conflict of interest to report.

DATA AVAILABILITY STATEMENT

Data are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Ethical guidelines as stipulated by the regulations were adhered to during the course of this study. Approval for ethical clearance was obtained from the Research Ethics Committee at Tehran Islamic Azad University of Medical Sciences (approval number: IR.IAU.PSREC.1401.345). Furthermore, all patients involved in the study provided written informed consent.

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