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The Impact Of Genetic Mutations In 5α-Reductase Enzyme And INSL3 Hormone On The Development Of Hypospadias And Cryptorchidism

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Abstract
Congenital malformations of the genitourinary system, such as hypospadias and cryptorchidism, are among the most researched conditions to understand their molecular causes. This study aims to shed light on the role of genetic mutations in the SRD5A2 and INSL3 genes and their impact on the occurrence of these malformations. Cryptorchidism is a condition in which one or both testicles fail to descend into the scrotum. is associated with an increased risk of infertility and testicular germ cell tumors. Testicular descent is a complex process, often described in two separate stages: the transabdominal stage and the inguinal-scrotal stage. In the first stage, which occurs between the 10th and 15th weeks of pregnancy, the testicle migrates from the abdominal area to the area near the deep inguinal ring. The main hormone that drives this stage is insulin-like peptide 3 (INSL3), produced by Leydig cells. Insulin-like peptide 3 (INSL3) is a small peptide hormone of the insulin-relaxin family. It is produced and secreted by embryonic Leydig cells in the testes only and is undetectable in female fetuses. INSL3 synthesis begins in the human fetus immediately after gonadal sex determination, at 7 to 8 weeks after fertilization. This peptide can be detected in the amniotic fluid 1 to 2 weeks later. A mutation in the INSL3 gene causes a defect in the efficiency of producing the INSL3 hormone that this gene encodes, which leads to a defect in the descent of one or both testicles into the scrotum. This is what was identified by the genetic variation found in all samples of patients with cryptorchidism.After the ninth week, under the influence of testosterone, the genital tubercle and urinary folds (penis) are formed, while the labial folds (scrotum) are formed. This process is stimulated by androgen hormones, The conversion of testosterone to dihydrotestosterone is catalyzed by the steroid 5α-reductase type 2 enzyme, which plays a crucial role in the masculinization of the external genitalia. It is encoded by the SRD5A2 gene. Allelic variants in this gene cause XY 46 chromosomal abnormalities. The occurrence of the V89L polymorphism and the G34R mutation in the gene responsible for encoding this enzyme reduces the efficiency of production of this enzyme, causing a decrease in testosterone and dihydrotestosterone levels and causing a defect in the formation of the urethra.The study included a group of children diagnosed with hypospadias or cryptorchidism. DNA samples were analyzed to detect the V89L and G34R mutations in the SRD5A2 gene, in addition to examining changes in the INSL3 gene using RFLP (PCR),Allele-specific (PCR)and DNA sequencing techniques. The results showed that the studied mutations in the SRD5A2 gene lead to impaired function of the enzyme 5α-reductase type 2, resulting in a deficiency in the production of dihydrotestosterone, which is essential for the normal development of the male reproductive organs. Mutations in the INSL3 gene also contributed to impaired testicular descent into the scrotum, leading to an increase in the incidence of cryptorchidism. These findings highlight the importance of early genetic testing for children with congenital reproductive abnormalities, which could contribute to improved diagnosis and early therapeutic intervention, as well as providing accurate genetic counseling to families. The study showed that 14 of the 15 children with hypospadias carried the V89L mutation in the SRD5A2 gene, while one child carried the G34R mutation. A new, previously undocumented recurrent mutation was also discovered in 15 other children with cryptorchidism.
Keywords: *V89L polymorphism, G34R mutation, SRD5A2, congenital malformations, genetic mutations, genitourinary system.*

INTRODUCTION

5-α-Reductase deficiency is a rare genetic disorder that affects the development of the male reproductive organs[1]. The conversion of testosterone to dihydrotestosterone is catalyzed by the steroid 5α-reductase type 2 enzyme[2]. which plays a crucial role in the masculinization of the external genitalia. It is encoded by the SRD5A2 gene [3]. Due to the conversion of the amino acid valine to the amino acid leucine at codon 89 (V89L), a major functional polymorphism of the SRD5A2 gene occurs, with the leucine version being 30%

less efficient than the valine version, leading to lower levels of the hormone DHT, which affects the stimulation of external genital growth [4].The conversion of glycine to arginine at codon 34 (G34R) alters the spatial structure of the enzyme, potentially reducing its efficiency or halting its function altogether[3]. This results in a decrease in the level of dihydrotestosterone in the body[5]. This deficiency leads to delayed development of the male reproductive organs during the fetal period, leading to the birth of male children with underdeveloped reproductive organs[3]. Both affect the activity of the enzyme 5α-Reductase, but in different ways. The V89L mutation is a polymorphic point mutation located in the SRD5A2 gene, where valine is replaced by leucine at codon 89. It is considered more common and less pathogenic and has less severe effects. This mutation contributes to individual differences in DHT levels without causing a clear pathological condition. This mutation has been detected in many populations and ethnicities, such as Mexico, Sicily, Japan, and Vietnam. The G34R mutation is highly pathogenic and causes almost complete loss of enzyme activity and a marked deficiency of DHT, leading to marked male sexual dysfunction[6]. This mutation is located in the first exon of the SRD5A2 gene, where glycine is replaced by larknine at codon 34[7]. in a sensitive site of the enzyme. This mutation was isolated from five unrelated Egyptian families distributed across different geographical areas[8]. Insulin-like peptide 3 (INSL3) is a small peptide hormone of the insulin-relaxin family[9]. It is produced and secreted by embryonic Leydig cells in the testes only and is undetectable in female fetuses[10]. INSL3 synthesis begins in the human fetus immediately after gonadal sex determination, at 7 to 8 weeks after fertilization. The role of the INSL3 gene in the male fetus is to cause an increase in the thickness of the pouch that holds the testes in the inguinal region, while the rest of the abdominal organs grow in an anterior-dorsal direction. This represents the first stage of the descent of the testes into the scrotum through the inguinal canal[11].

MATERIALS AND METHODS

Study area and data collection

This research was carried out in the Department of Biology/ Faculty of Science/ University of Mosul, Iraq. The study included 45 male children aged from 6 months to 12 years with a body mass index ranging from 6 kg to 50 kg.The subjects were stratified into three cohorts of 15 individuals each, as follows:

1. Group 1 served as the control group Those who do not have a family history of the disease.
2. Group 2 included patients who have cryptorchidism.
3. Group 3 included patients who have hypospadias.

1 ml of venous blood withdrawn from the kids and divided into two parts. The first was put in an EDTA tube for DNA extraction.

The “Materials and Methods”

V89L Polymorphism

The ratios of V/V, V/L and L/L genotypes among all samples in this study, Include The primer that have been used in DNA sequence and PCR reaction

(Wi-34f)	5 AAGCCCTCCGGCTACG3
(Wi-34-r)	5 GGAAAAACGCTACCTGTGGA3
(Mut-34f)	5 AAGCCCTCCGGCTACA3
(Mut-34-2r)	5 CAAGGGAAAAACGCTACCTG3
V89L-FO	ATGAGGTCCTGGGGGAGTGAA
V89L-RO	GAAGCACACGGAGAGCCTGAAG

G34R Mutation Detection

Two forward primers i.e. wi-34 and mut-34 for wild type and mutated alleles, respectively, as well as two reverse primers, 34-r and 34-2r, were designed using the freeware primer 3.

DNA sequence

The sequences of the nitrogenous bases of the INSL3 gene were determined for the study samples to ensure the validity of the designed primer used in the PCR technique and to detect the presence of additional variations in the target genes.

The products of the PCR reaction for the INSL3 gene were sent with the primers. The sequence was then read at the Psomagen Center in the United States of America. After that, the gene sequences were matched with the gene sequences documented at the National Center for Biotechnology Information (NCBI), and the results

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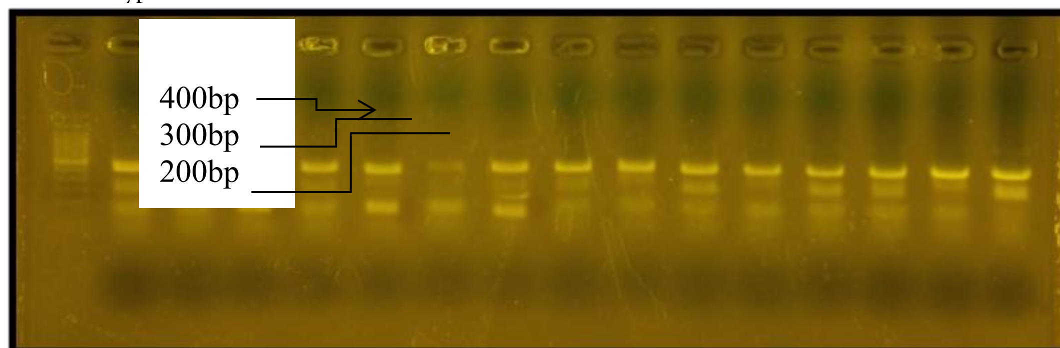
were analyzed using the BLAST program.
Digested with HaeIII enzyme
INSL3 F ACTAGCTACTCTCAGGCTGGTGA
INSL3 R TGTCTGGGGACAGTTTTAGATGT

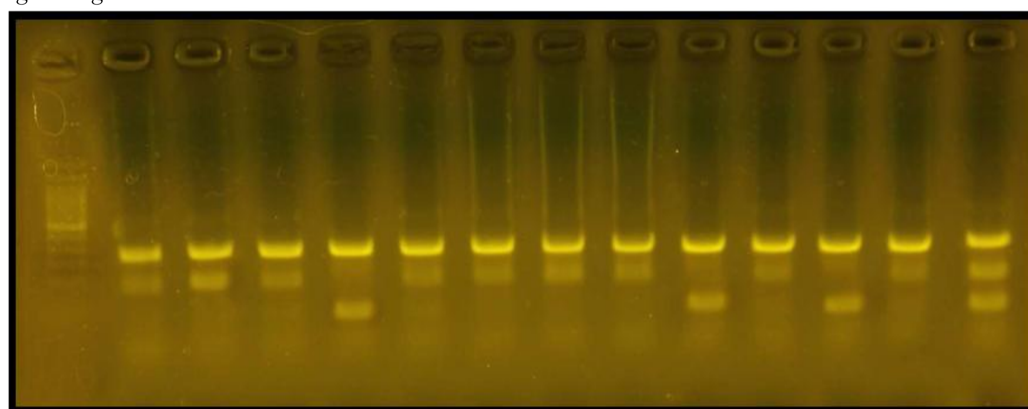
Statistical Analysis was conducted using a T-test at the probability level ($P \leq 0.05$), and the allelic frequency was calculated based on the following equation:
Allelic frequency of the normal allele = 2 (number of homozygous individuals) + (number of heterozygous individuals) / 2 (total number)
Allelic frequency of the mutant allele = 2 (number of homozygous individuals) + (number of heterozygous individuals) / 2 (total number)

RESULTS

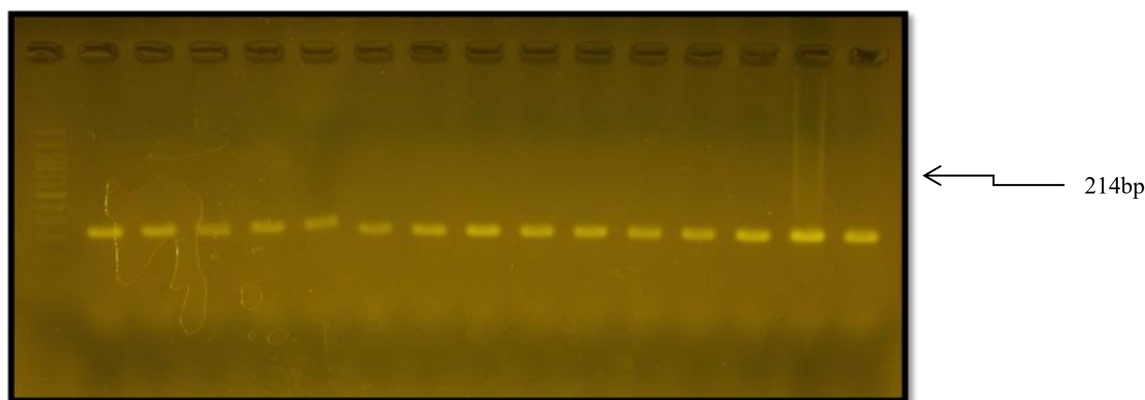
Determination of the polymorphism of the steroid 5 alpha-reductase type 2 gene at the V89L mutation site using RFLP-PCR. The results of the RFLP-PCR, as shown in Figure(1), revealed a relationship between children with hypospadias and the genetic variation of the SRD5A2 gene at the V89L site. The PCR results revealed the presence of genetic variation of the SRD5A2 gene in three different genotypes (LL, VL, and VV) at varying rates. The results of the current study, which included analysis of the V89L mutation in the steroid 5 alpha-reductase type 2 (SRD5A2) gene, 46.7% of children with hypospadias whose parents were related were heterozygous carriers of the mutation, 46.7% of those with hypospadias were homozygous mutant, and 6.6% were wild type.



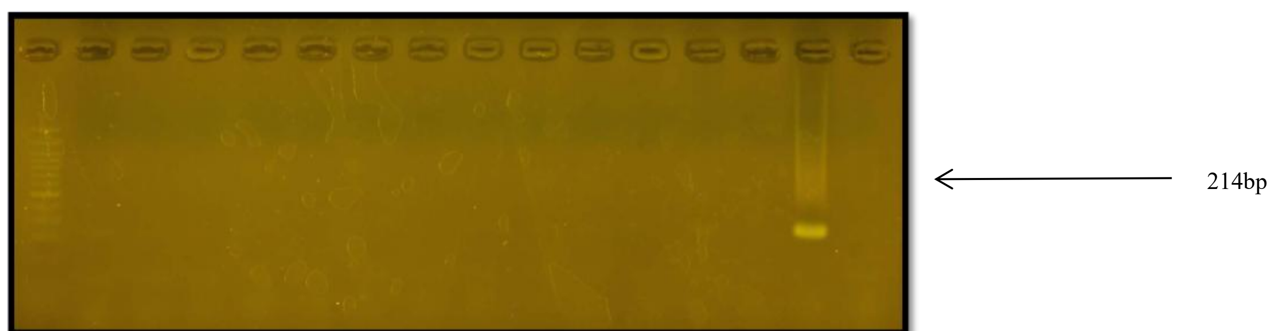
Figure(1) RFLP-PCR product of the V89L mutant gene (steroid 5 alpha-reductase type 2) carried over a 2% agarose gel.



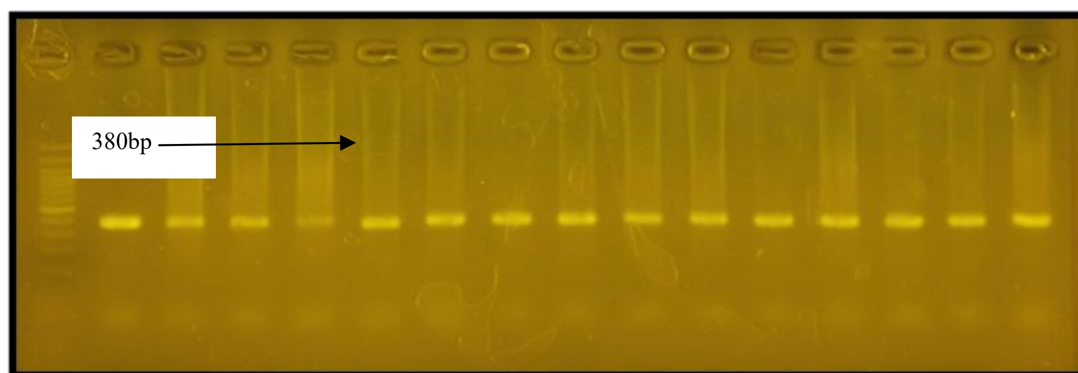
Figure(2) RFLP-PCR product of the V89L mutant gene (steroid 5 alpha-reductase type 2) carried over a 2% agarose gel. G34R Mutation Detection of the Steroid 5 alpha-reductase type 2 gene using Allele-specific PCR The results of the G34R mutation analysis in the Steroid 5 alpha-reductase type 2 (SRD5A2) gene, as shown in Figure (3), which used the Allele specific-PCR technique to interact with the normal allele and the mutant allele, showed that all samples had the normal allele, while Figure (4) showed that only one sample carried the mutant allele, and there was no infection with the mutant type (Homozygous mutant).



Figure(3)Allele-specific PCR product of the 214 bp natural allele was electrophoresed on a 2% agarose gel.



Figure(4)Allele-specific PCR product of the 214 bp mutant allele as detected by 2% agarose gel electrophoresis.
Determination of genetic variation in the INSL3 gene using DNA sequencing technology
DNA sequencing and polymerase chain reaction (PCR) results showed an association between patients with cryptorchidism and genetic variation in the INSL3 gene using DNA sequencing technology.



Figure(5)PCR product of the 380 base pair INSL gene separated by 2% acarose gel electrophoresis.

Determining the matching of nucleotides of the INSL3 gene in the study samples with the gene sequence on the NCBI website The aim of conducting the nucleotide sequencing test is to definitively confirm that the primers used in this study belong to the INSL3 gene, as well as to identify new variations in the gene that may directly or indirectly affect the gene's activity, which is one of the main reasons for the development of the disease. The results of the sequencing test for the amplification of the INSL3 gene showed differences including (deletion, addition, mutation, and transfer) compared to the gene sequence in NCBI, shown in the following figures(6)

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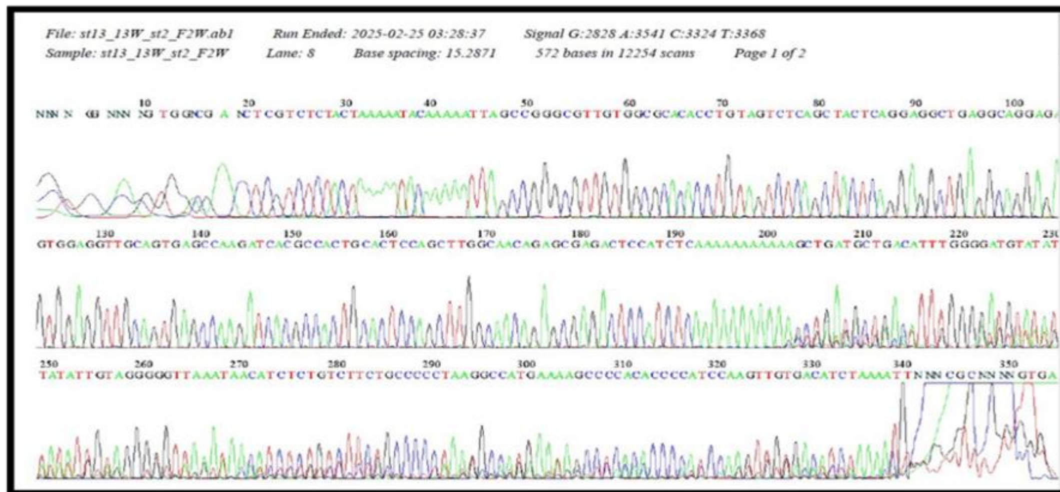

figures(6)

The results of matching the nucleotide sequences of the INSL3 gene for the samples included in the study showed that they were 98% identical to the nucleotide sequences on the NCBI website. This indicates the accuracy of the designed primer used in this study, as shown in the following figures(7)

figures(7)

Matching result of the INSL3 gene sequences in the control samples with the gene sequences on the NCBI website

Study samples compared with the original gene sequence on the NCBI website.

D sequence	Nucleotide	Location	Mutation type	Identity	Gaps
(NG-012092.1)	T→ G	(7689)	(Transversion)	(98%)	(0)
(NG-012092.1)	G → A	(7694)	(Transversion)	(98%)	(0)
(NG-012092.1)	→ T G	(7722)	(Transversion)	(98%)	(0)
(NG-012092.1)	T→ A	(7731)	(Transversion)	(98%)	(0)
(NG-012092.1)	T→ C	(7736)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7689)	(Transversion)	(98%)	(0)

(NG-012092.1)	G → A	(7694)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7722)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7755)	(Transversion)	(98%)	(0)
(NG-012092.1)	A → T	(7657)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → A	(7661)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7689)	(Transversion)	(98%)	(0)
(NG-012092.1)	G → A	(7694)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7722)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → A	(7731)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → A	(7661)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7689)	(Transversion)	(98%)	(0)
(NG-012092.1)	G → A	(7694)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7689)	(Transversion)	(98%)	(0)
(NG-012092.1)	G → A	(7694)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → C	(7734)	(Transversion.)	(98%)	(0)
(NG-012092.1)	A → C	(7736)	(Transversion)	(98%)	(0)
(NG-012092.1)	T → G	(7755)	(Transversion)	(98%)	(0)

It is worth noting that the T→G variation at position 7689 appeared in all studied samples, indicating that it is the pathogenic mutation causing the infection. After conducting the sequencing test and matching the INSL3 gene in the study samples with the control sample and matching it with the gene sequence on the NCBI website, it became clear to us that there were many different genetic variations, most of which were transversion variations and their locations depended on the type of heterozygous bases, and that these variations may reduce the gene's effectiveness in producing the INSL3 hormone and thus increase the risk factors for cryptorchidism in children.

Registration of a new genotype of the INSL3 gene in the NCBI database for study samples:

In this study, a new mutant genotype of the INSL3 gene in Mosul was registered on the global genome database NCBI and given the identification number GeneBank:PV395589.1

National Library of Medicine
National Center for Biotechnology Information

UNVERIFIED: Homo sapiens sequence
GenBank: PV395589.1

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ACCESSION   PV395589
VERSION     PV395589.1
KEYWORDS    UNVERIFIED.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
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            Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 531)
  AUTHORS   Salih,Z.Y.
  TITLE     Genetic Causes of Genital Abnormalities in Some Children in the
            City of Mosul
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 531)
  AUTHORS   Salih,Z.Y.
  TITLE     Direct Submission
  JOURNAL   Submitted (21-MAR-2025) Department of Biology, University of Mosul/
            College of Science, DNA LAB street, Mosul, Ninava 09334, Iraq
COMMENT     GenBank staff is unable to verify sequence and/or annotation
            provided by the submitter.

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      301 ccaagttgtg acatctaaaa ttnnncgcnn nngtgangcc ctgcgccctc nccttttggag
      361 gatttgagaa tcccccccgc ttatgcaaaa actttccaag gccnccccg gctcaccaag
      421 ttgaaaaaga aaaaattnaa cagtaatgtc gccctctctc cgcgatgtac tatataaccc
      481 caaatgggag tatcccctca aaattactat agaacaggtt ttaagcntgn t
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FOLLOW NCBI

DISCUSSION

Molecular analysis revealed that 93.4% of samples from children with hypospadias carried the V89L mutation, either as carriers or as affected individuals, while only one case was normal. This high percentage indicates the widespread prevalence of this mutation within the geographic area where the samples were collected. These results may indicate the presence of a common genetic ancestry or recurrent genetic factors in this population. When these results were compared to a 2019 study conducted in China on 14 patients with steroid 5 alpha-reductase deficiency, most of whom had hypospadias, undescended testicles, and varying degrees of penile size reduction, nine different mutations were found in these 14 patients, two of whom carried the V89L polymorphism, and 11 of whom had the V89L polymorphism [12]. Laboratory studies conducted on children in China and India have shown that the most common and widespread polymorphic mutation in the SRD5A2 gene is V89L, which results from a change from a guanine base to a cytosine base in exon 1, leading to the substitution of a valine for a leucine at position 89. This mutation reduces enzyme activity by approximately 30%, increasing the risk of hypospadias[13]. Glycine is one of the smallest amino acids and is characterized by its structural flexibility, allowing the protein to adopt the precise folds necessary for its function. In contrast, arginine is larger, carries a positive charge, and has a complex side chain tail that can cause significant structural changes. The effect of the G34R mutation is summarized in the structural folding disturbance of the protein. Replacing the small, flexible glycine with the bulky, charged arginine may disrupt proper protein folding, affecting enzyme stability. Multiple mutations distributed throughout the codon have been identified in patients with steroid 5 alpha-reductase deficiency. To date, the majority of these mutations are point mutations. One such point mutation, at codon 34, converts glycine to arginine and reduces enzyme activity to less than 5% of its original level. This mutation has been reported sporadically in isolated cases in Mexico, Vietnam, Sicily, and Japan during 1992, 1993, 1997, and 2003. On the other hand, It was reported in five unrelated Egyptian families from different geographical areas, which means that the G34R mutation is the most common mutation among Egyptians[8].

CONFLICT OF INTERESTS

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.

REFERENCES

1. Han, B., Zhu, H., Yao, H., Ren, J., O'Day, P., Wang, H., ... & Qiao, J. (2022). Differences of adrenal-derived androgens in 5α-reductase deficiency versus androgen insensitivity syndrome. Clinical and Translational Science, 15(3), 658-666.
2. Ali, K., Li, W., & Wu, G. (2024). Evolution, classification, structure, and functional diversification of steroid 5α-reductase family in eukaryotes. Heliyon, 10(14).
3. Batista, R. L., & Mendonca, B. B. (2020). Integrative and analytical review of the 5-alpha-reductase type 2 deficiency worldwide. The Application of Clinical Genetics, 83-96.
4. Zhang, K., Li, Y., Mao, Y., & Ma, M. (2017). Steroid 5-alpha-reductase type 2 (SRD5A2) gene V89L polymorphism and hypospadias risk: a meta-analysis. Journal of Pediatric Urology, 13(6), 630-e1.
5. Alukal, J. P., & Lepor, H. (2016). Testosterone Deficiency. Hypogonadism, An Issue of Urologic Clinics of North America, 43(2), 203.
6. Mazen, I., Gad, Y. Z., Hafez, M., Sultan, C., & Lumbroso, S. (2003). Molecular analysis of 5α-reductase type 2 gene in eight unrelated Egyptian children with suspected 5α-reductase deficiency: prevalence of the G34R mutation. Clinical endocrinology, 58(5), 627-631.
7. Gong, L., Bui, M. M., Zhang, W., Sun, X., Zhang, M., & Yi, D. (2021). H3F3A G34 mutation DNA sequencing and G34W immunohistochemistry analysis in 366 cases of giant cell tumors of bone and other bone tumors.
8. Gad, Y. Z., Khairt, R., Mazen, I., & Osman, H. G. (2007). Detection of the G34R mutation in the 5 alpha reductase 2 gene by allele specific PCR and its linkage to the 89L allele among Egyptian cases. Sexual Development, 1(5), 293-296.
9. Mita, M. (2021). Relaxins. In Handbook of Hormones (pp. 297-300). Academic Press.
10. Bay, K., & Andersson, A. M. (2011). Human testicular insulin-like factor 3: in relation to development, reproductive hormones and andrological disorders. International journal of andrology, 34(2), 97-109.
11. Ivell, R., Mamsen, L. S., Andersen, C. Y., & Anand-Ivell, R. (2022). Expression and role of INSL3 in the fetal testis. Frontiers in Endocrinology, 13, 868313.
12. Cheng, T., Wang, H., Han, B., Zhu, H., Yao, H. J., Zhao, S. X., ... & Qiao, J. (2019). Identification of three novel SRD5A2 mutations in Chinese patients with 5α-reductase 2 deficiency. Asian Journal of Andrology, 21(6), 577-581.
13. Avendaño, A., Paradisi, I., Cammarata-Scalisi, F., & Callea, M. (2018). 5-α-Reductase type 2 deficiency: is there a genotype-phenotype correlation? A review. Hormones, 17, 197-204.