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ORIGINAL ARTICLE

The G178A polymorphic variant of INSL3 may be linked to cryptorchidism among Egyptian pediatric cohort

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Abstract

Cryptorchidism (CO) is a genital disorder of multifactorial etiology, with serious remote complications. Mutations in insulin-like 3 hormones (INSL3) G/A variant remain a matter of inquiry. We aimed to investigate the association between G178A-INSL3 polymorphism and undescended testis in a cohort of Egyptian children. In this study, a total of 160 children, including 80 cases with primary non-syndromic undescended testes and 80 healthy children with normal external genitalia as controls, both, were analyzed after detailed history, physical examination and imaging for mutations of G178A polymorphism of INSL3 gene by restriction fragment length polymorphism (RFLP) technique. We found most of the undescended testes were inside the inguinal canal mainly on the left side. Genetic analysis revealed that the mutant A allele of G178A INSL3 variant was significantly detected in the patient group with a frequency of 26.2% against 12.5% for control subjects, especially among cases with an evident family history of similar cases as shown by p value = 0.001 and odd's ratio (CI95%) of 0.13 (0.04–0.723). In conclusion, G178A—INSL3 gene polymorphism could be a susceptibility factor for testicular maldescent in Egyptian children. Also, family history of similar cases was considered as significant predictive risk for cryptorchidism, added to the shared genetic links to consanguinity in our locality.

Keywords Cryptorchidism · INSL3_G178A · PCR/RFLP · Undescended testis

Introduction

The undescended testis is considered the most common disorder of sex differentiation within boys; at birth, about 4.5% of them have an undescended testis. Because testicular descent occurs during the period of 7–8 months' gestation, 30% of premature male infants have an undescended testis, with an incidence of 3.4% at near term and an incidence decline to about 0.8% as a result of spontaneous testicles descent [1].

Testicular normal descent is thought to take place during two phases in fetal development between 8 and 15 weeks (the first phase of decent) and another second phase at 25–35 weeks gestation.[2].

The role of INSL3 on gubernaculum enlargement, anchoring the testes passively downward became more evident at early weeks' gestation. Between 25 and 35 weeks of gestational period, hormonal, neuronal and mechanical factors are incorporated to complete the anatomical descent of both testes [3].

Apart from the congenital type, acquired forms could also exist at which scarring is likely an important etiological factor [4].

A specific association of *G178A-INSL3* gene polymorphism with cryptorchidism had been identified through animal and human studies in the recent years. The later polymorphism is shown to be localized to the C peptide region of the involved gene, resulting in amino acid change of an alanine to threonine. This amino acid alteration was thought to be responsible for interrupting the normal physiological sequence of testicular descent [5].

The undescended testicle may be prone to a variety of harmful consequences as trauma, torsion and inguinal

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hernias, and later on male infertility, atrophy, malignant transformation and psychological stress. Infertility ratios could be increased up to 40% in unilateral cryptorchidism, compared to a possibility of 70% in case of bilateral cryptorchidism.

Orchiopexy had been the mainstay line of treatment for placing the cryptorchid testicle into the scrotum in affected cases [6, 7].

In respect to the above considerations of possible remote serious complications, our study aimed to evaluate the association between *G178A-INSL3* gene polymorphism and the development of undescended testis, and whether or/not it contributeed to its clinical appearance.

Patients and methods

Participants

Following the approval of the Institutional Ethical Committee of Menoufia University Hospital, and after obtaining written consents from the caregivers of all children to be included in this research, the study was carried out between April 2018 and March 2020, with all of its steps being done in Genetic Laboratory of the Genetics and Endocrinology Unit, Pediatric Department, Faculty of Medicine, Menoufia University Hospitals.

In this case–control study, a total of 160 children who were equally divided into two groups were included. Group (I) comprised 80 unrelated children with primary non-syndromic cryptorchidism; their phenotypic pattern was: 28 children with bilateral cryptorchidism, 28 children with unilateral left cryptorchidism and 24 children with unilateral right cryptorchidism, their ages ranging from 1 to 12 years with mean age of (3.89 ± 3.03) years.

Inclusion criteria included apparently normal male infants and children, as signed in the karyotype XY in the wholly analyzed lymphocytes, presented with unilateral or bilateral undescended testis.

Those with the following non-satisfactory criteria: infants less than 6 month's age, patients with abnormal syndromic features or having multiple congenital anomalies, those whose testes were retractile, or had a positive history of other surgical operation in the inguinal region were excluded from the work.

Group (II) included 80 apparently healthy non-cryptorchid male children collected from the general pediatric population who were chosen as control subjects. their ages ranged from 2 to 12 years with mean age of 4.13 ± 3.21 years.

All of the patients and normal control children were subjected to a detailed history taking, general examination, anthropometric measurements, and examination of the genitalia under the patient's authorization for complete ensured safety procedures. Size determination of the palpable testis was done in cases of unilateral UDT using a Prader Orchidometer, and data were referred to external masculinization score (EMS) for assessing the child external genetalia to provide a score out of 12 [8, 9]. Control subjects were assessed for their testicular size as well.

In addition to basic laboratory investigations and hormonal studies, pelvi-abdominal and inguino-scrotal ultrasound was done to show the site of each testis and the parenchymal structure and dimensions of the testes to calculate the testicular volume based on the following formula:

$$\text{Testicular volume (TV)}\ \left[\text{cm}^3\right] = 0.52 \times \text{width [cm]} \times \text{length [cm]} \times \text{height [cm]}$$

In the present study, the testicular size obtained for each case by ultrasonography was compared to the normal values reported by Lawal et al. (2016) [10]. Further radiological assessment was done accordingly whenever needed.

Molecular analysis of *INSL3* gene polymorphism was applied through sequential steps as follows.

DNA was extracted from 2 ml (ml) of anti-coagulated peripheral blood collected into an EDTA tube by using a DNA extraction kit (Gene JET Whole Blood Genomic DNA Purification Mini Kit). Genomic DNA was then amplified using PCR with different forward and reverse primers (forward 5-AAA GAC TCG TTG CCC AGT GCT CCC T-3; reverse 5_-GCA TCT GCG CCT ACG TGC AC-3). The polymerase chain reaction (PCR) conditions for the amplification of exon 1 with primers F2/R1 were 95 °C for 5 min, followed by 33 cycles of 95 °C 40 s, 61 °C 35 s, 72 °C 45 s.

Restriction endoclease assay was applied for detecting non-silent gene alterations through the specifically used EagI restriction enzyme according to the manufacturer's conditions. The digested products were visualized on 2%agarose gel electrophoresis, after being stained with ethedium bromide, where the targeted enzyme recognized the wild G/G genotype certainly at the position 178(exon1) of *INS3L gene*.

Statistical analysis

IBM SPSS software version 20.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The data were given as the mean ± the standard deviation and also as a range. The categorical data were analyzed by the Chi-square test. The latter was also performed for analyzing data about genotype differences and allelic distribution between patients and control groups. Continuous variables were compared using the *t* test. A P value < 0.05 was considered to be significant.

Results

One hundred and sixty full-term infants and children were categorized in this study with mean ages of 3.89 ± 3.03 years and 4.13 ± 3.21 years representing patients and control groups in order.

A statistically significant difference regarding history of similar cases with other group data is shown in Table 1. Most of the undescended testes were found inside the inguinal canal [47.5% on the right side and 57.5% on the left side] (Table 2). The genotype results and allele frequencies of *G178A _ INSL3* gene polymorphism revealed a statistical significant difference between the patients and the

Table 1 Demographic data of the studied groups

Variables	Groups				Test	*P* value
	Patient no. (80)		Control no. (80)			
	N	%	*n*	%		
Age Mean ± SD	3.89 ± 3.03		4.13 ± 3.21		$t = 0.481$	0.631
Consanguinity						
Positive	21	26.3	18	22.5	$\chi^2 = 2.48$	0.06
Negative	59	73.7	62	77.5		
Family history of similar cases	12	15	2	2.5	$\chi^2 = 8.28$	0.04*
Paternal age						
Mother's age: mean ± SD (years)	29.71 ± 5.68		29.50 ± 5.61		$t = 0.238$	0.812
Father's age: mean ± SD (years)	35.60 ± 6.41		35.95 ± 6.59		$t = -0.340$	0.734

No number, *SD* standard deviation, χ^2 Chi-square

*Significant: < 0.05

Table 2 Testicular site and size by pelvi-abdominal and inguino-scrotal ultrasound of the studied patients

Variables	Patients (No. = 80)	
	N	%
Testicular site		
Unilateral UDT	52	65.0
Right inguinal UDT	24	30.0
Left inguinal UDT	26	32.5
Left pelvic UDT	2	2.5
Bilateral UDT	28	35.0
Bilateral inguinal UDT	24	30.0
Bilateral pelvic UDT	4	5.0

Testicular size

Right testes ($n = 80$)	Normal scrotal right testes ($n = 28$)		Right inguinal UDT($n = 48$)		Right Pelvic UDT ($n = 4$)	
	No	%	No	%	No	%
Normal	28	100.0	45	93.7	0	0.0
Smaller than normal	0	0.0	3	6.3	4	100.0
Testicular size						
Left testes ($n = 80$)	Normal scrotal left testes ($n = 24$)		Left inguinal left UDT($n = 50$)		Left Pelvic UDT ($n = 6$)	
	No	%	No	%	No	%
Normal	24	100.0	46	92	0	0.0
Smaller than normal	0	0	4	8	6	100.0

UDT undescended testes

apparently healthy normal control children ($p=0.003$). GG genotype was more frequently observed among the control children compared with cases (80.0% versus 55.0%), respectively, while heterozygous GA and homozygous AA genotypes were more frequent in cases compared with the control group (37.5% versus 15.0% and 7.5% versus 5.0%), respectively (Table 3). The presence of mutant A allele and positive history of similar cases were significant predictive risk factors for occurrence of cryptorchidism using regression analysis as shown by odd's ratio (95% CI) 0.13 (0.04–0.723) and p value of 0.001 for A allele frequency.

The distribution of *G178A_INSL3* polymorphisms (GG, GA, AA) in participant children with CO with respect to parents' ages, consanguinity and testicular site is shown in Table 4, where ten children at a percentage of 22.7% of those with GG genotype were born to a consanguineous marriage compared to eight children of GA genotype (26.6%) and three out of six affected children accounted for 50% of those children with AA genotype. The latter subgroup had a consanguinity of fourth degree.

Gel electrophoretic pattern of PCR-digest products on 2% agarose gel for *G/A_INSL3* is shown in Fig. 1.

Discussion

Undescended testis is the most common urogenital problem in pediatrics. Normally intrauterine testicular descent occurs in two stages: transabdominal, then transinguinal. plenty number of intervening factors are responsible for undescended testis: genetic, hormonal and mechanical factors [7].

INSL3 gene comprised two exons in addition to one intron, contained in a single copy within the human genome. A specific association of detected mutations in the INSL3 gene in cases with bilateral cryptorchidism had been identified in human and animal studies, including the analyzed G/A SNP that leads to alanine (GCC) to the other amino acid threonine (ACC), at codon 60 of the C-peptide region [11, 12].

The study focused on the essential role of insulin-like 3hormone, derived from Leydig cells, in detecting a precise molecular pathogenic mechanism involved in integral gubernaculum differentiation and testicular descent [13]. Detailed background reviews from formal studies were directed to the identification of multiple defects within that gene [14–20]. Furthermore, it has recently gained great attention owing to the suggested paracrine-related role of INSL3 in male germ cell survival [21, 22].

While analyzing the results of molecular study of *G178A_INSL3* gene polymorphism, in the present study it was found that a group difference of statistical significance was evident regarding the frequency distribution of A allele among patients with CO as shown by an odd's ratio (95% confidence interval) of 0.13 (0.04–0.723), p value = 0.001, in comparison to the reference G allele that was frequently observed in the group of control children at a percentage of 80%.

Also, the GG genotype was significantly linked to non-cryptorchid normal children ($p<0.05$).

These findings were in agreement with those of Yamazawa et al. [23], who studied the *G178A_INSL3* gene polymorphisms in 62 cryporchid patients and 60 control males and reached the result that the frequency of the *G178A_INSL3* gene polymorphism genotypes (GG, GA and AA) was distributed between the cryptorchid patients and the controls as follows; 40.3%, 32.2%, 27.4% in patients versus 55%, 38.3%, 6.6% in controls, respectively, with a statistically significant difference ($p=0.0094$).

Table 3 Distribution of G178A_INSL3 genotypes, G and A alleles among patients and control children

Variables	Groups				Test	OR (95%CI)	*P* value
	Patients no. (80)		Controls no. (80)				
	No	%	No	%			
Genotype					$\chi^2=11.818$	–	0.003
AA	6	7.5	4	5.0			
GA	30	37.5	12	15.0			
GG	44	55.0	64	80.0			
Alleles (frequency number)					$\chi^2=0.426$	–	0.001
A allele	42	26.2	20	12.5		0.13 (0.04–0.723)	
*G allele	118	73.5	140	87.5		_	

**G* reference allele

OR odd's ratio, *CI* confidence interval

χ^2 Chi square

$P<0.05$: significant

Table 4 Distribution of G178A_INSL3 genotypes in relation to the characteristic data of the studied patients

Variables	Groups						*P*value value	Post hoc value
	GG no. (44)		GA no. (30)		AA no. (6)			
	n	%	*n*	%	*n*	%		
Consanguinity	10	22.7	8	26.6	3	50	0.126	I vs II = 0.98 I vs III = 0.11 II vs IIII = 0.15
Family history of similar cases (%) Paternal age:	4	9	6	20	2	33.3	0.004	I vs II = 0.21 I vs III = 0.04 II vs IIII = 0.06
Mother: mean ± SD (years)	29.73 ± 6.56		29.13 ± 4.31		29.67 ± 4.23		0.904	I vs II = 0.91 I vs III = 0.87 II vs IIII = 0.85
Father: mean ± SD (years)	36.32 ± 7.01		35.40 ± 6.45		36.00 ± 4.47		0.844	I vs II = 0.82 I vs III = 0.91 II vs IIII = 0.79
Testicular site	*n*	%	*n*	%	*n*	%		
Bilateral inguinal UDT	13	29.5	7	23.3	4	66.6	0.057	I vs II = 0.473 I vs III = 0.029 II vsIIII = 0.233
Bilateral pelvic UDT	0	0	4	13.3	0	0	0.003	I vs II = 0.004 I vs III NA II vsIIII = 0.722
Unilateral left inguinal UDT	17	38.6	9	30	0	0	0.248	I vs II = 0.377 I vs III = 0.232 II vsIIII = 0.252
Unilateral right inguinal UDT	14	31.8	8	26.6	2	33.3	0.376	I vs II = 0.27 I vs III = 0.467 II vs IIII = 1.00
Unilateral left pelvic UDT	0	0	2	6.6	0	0	0.058	I vs II = 0.064 I vs III NA II vs IIII = 1.00

No number, *SD* standard deviation, *UDT* undescended testes

*Significant: < 0.05

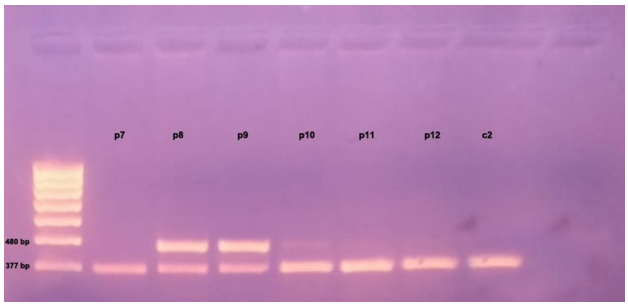

Fig. 1 Gel electrophoresis of G178A-INSL3 polymorphism. Interpretation, from left against 100 bp ladder. *P* patient, *C* control. GG genotype gave a band at 377 bp (lane 2). GA genotype gave two bands at 480 bp and 377 bp (lanes 3, 4). AA genotype gave a band at 480 pb (lanes 5, 6, 7, 8)

On the other hand, Koskimies et al. [24] in their studies conducted on 30 cryptorchid boys and 89 controls for the G178A mutation found that the A allele frequency in both the homo- and heterozygous states was slightly higher (38.3%) than in the control subjects (29.7%) with otherwise non-significant difference.

Along with the spectrum of studies that favored the significant association of *INSL3 G178A* variant to primary non-syndromic CO was that of Huang et al. [25]. In this study, the sequencing of PCR-amplified coding regions of INSL3 in 97 azoospermic patients with a positive history of bilateral cryptorchidism represented the patient group, which they further divided into two subgroups according to the results of testes spermatic extraction, in contrast to a comparable group of 49 obstructive azoospermic males (control individuals). They reached conclusive findings of significant association of the studied variant to CO, whereas the damaged spermatogenic process could not be directly associated with G178A of INSL3 polymorphism among males with bilateral CO.

The distribution of cases with primary CO showed significant association with positive family history as shown

in our results where the patients with (GG, GA, AA) genotypes showed that four (9%), six (20%) and two (33.3%) had positive family history of similar cases, respectively, with statistically significant difference between patients with GG and AA genotypes ($p = 0.$ 0.004). This was not agreed by Mamoulakis et al. [26], who stated that the non-wild type (178A) was found at percentages of 52.9% and 42.3% of patient alleles with documented family history and sporadic CO patients ($P = 0.240$), showing no significant association between that polymorphism and positivity of family history. Burgu et al. similarly agreed with clustering of CO within certain families [27], taking into account the possibility that 178 A variant polymorphism may be a marker of consanguinity, as shown by an increased frequency of mutations made obviously because of genetic links in parents of children harboring the mutant allele.

Many authors reported that the association of inguinal hernia with cryptorchidism varied in the literature. Several studies suggested that cryptorchidism was almost always associated with an indirect inguinal hernia. Among those, Al-Abbadi and Smadi [28] conducted a study on 37 children, where undescended testis associated with indirect inguinal hernia was reported in 75.68% of patients. These findings were also concordant with that of Ravikumar et al. [29]. The processus vaginalis (PV) is a conduit, extending from the peritoneum down to the scrotum, usually obliterated after the end of the testicular migration. In cases where the vaginal processus did not close, the child could develop inguinal hernias or communicating hydrocele as a result of vaginal process persistence [30]. On the other hand, Tanyel et al. [31] clarified that despite the patency of that conduit in boys with undescended testis, clinical inguinal hernia is only encountered in 10–15% of cases.

Well seen in our study participants was the distribution of 178A variant among non-cryptorchid control infants and children at percentage of 5% versus 7.5% in CO patients, the matter that was extensively viewed in related research studies that interpreted the relevance of G178A nucleotide substitution to be claimed as a common polymorphic variant of human gene; INSL3 not linked to CO [32]. Mamoulakis et al. also studied the *G178A_INSL3* gene polymorphisms in 170 cryptorchid patients and 50 control males and found that the wild-type nucleotide (178G) was found in 55.9% of patient and 55.0% of control alleles. Thus, the substitution represents common polymorphism unrelated to testicular maldescent ($P = 0.876$) [26].

It is possible that the *G178A_INSL3* gene polymorphism represented one of the genetic factors associated with the existence of cryptorchidism, so that it can be detected in some patient populations, but not in others, depending on the attribution of other genetic, environmental and ethnic factors.

It is of great importance to discuss the clinical aspects and the diagnostic approaches with the family through counseling sessions, for implementing adequate intervention strategies, to avoid the possible complications or adverse proven testicular atrophy: an issue that was in accordance with Zvizdic et al., who reported that reduction of testicular size correlated with increasing the distance of maldescended testes away from the scrotum [33, 34].

Conclusion

The findings of this study indicated significant relevance of INSL3 178A allele to constitute a risk for susceptibility to cryptorchidism development with consideration to cases with positive family history along with worth continuation of molecular advances, thus, implying a new evidence for relation to testicular maldescent among Egyptian children.

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Compliance with ethical standards

Conflict of interest None.

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