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Original Article

TGIFLY mRNA Expression in Human Testis with Spermatogenesis Defects

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Abstract

Background: Hox genes are well-known transcription factors that play essential roles in directing embryonic development. *TGIFLY* is a Y-linked homeobox gene that was originally identified by virtue of its expression in adult testis. The functions of *TGIFLY* in normal and abnormal development are unknown.

Methods: To investigate the potential roles of *TGIFLY* gene in the infertile males, a semi-nested Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed on the testicular samples of 110 patients with non-obstructive azoospermia.

Results: The expression of *TGIFLY* in 59(53.6%) out of 110 patients were undetectable. Patients with different spermatogenesis defects failed to show the expected in *TGIFLY* gene expression as demonstrated in the normal testes.

Conclusion: Our results indicate that *TGIFLY* is expressed in testis and could be associated with infertility in patients with azoospermia. *TGIFLY* may be required for the regulation of spermatogonial stem cell specification and proliferation that is essential to the establishment and maintenance of spermatogenesis in human.

Keyword: *Male Infertility, TGIFLX /Y, Homeobox gene, Semi- RT-PCR*

Introduction

In human, the major causes of male infertility are asthenspermia, oligospermia, teratozoospermia and azoospermia, that account for 20–25% of cases (1). Spermatogenesis contains a complicated process and many genes have been suggested to be involved in it (1-3). So far more than 2,000 genes control spermatogenesis, approximately 30 genes being present on the Y-chromosome (1). Some of these genes are known to be testes-specific and cell-type-specific, while some are ubiquitously expressed.

Homeobox-containing genes consist of a family of regulatory genes encoding transcription factors that are implicated in the control of developmental processes. The homeobox genes encode a 60 amino acid helix-turn-helix type of DNA-binding motif, termed the homeodomain (HD) (4). Homeobox genes were initially identified as genes controlling *Drosophila* development (5) and subsequently were isolated from other organisms such as nematodes, plants and vertebrates (6, 7). In *Drosophila* and other invertebrates, these homeotic genes are organised as a complex (*HOM-C*), which shares significant (8). In humans, thirty-nine homeobox genes belonging to these clusters have thus far been identified (9). Many studies have revealed which changes in the expression patterns, target genes and functions of homeodomain proteins are also thought to be a driving force in animal evolution (10, 11). It has been estimated that homeobox genes account for more than 0.1% of the vertebrate genome (12). Homeodomain proteins are classified into distinct groups. One group of homeodomain proteins, called the TALE group, has a 3-amino-acid insertion between helices 1 and 2 of the homeodomain (13, 14), resulting in a 63, instead of the more typical 60, amino acid homeodomain (14). TALE proteins have been identified in many species and involved in activation and repression of gene expression (15-18).

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Transforming Growth -Interacting Factors (TGIFs) belong to TALE superclass of homeobox-containing genes known to play critical role in developmental process such as cell proliferation, differentiation and cell fate. So far many studies have shown that some homeodomain proteins are involved in fertility (19, 20). For example Wang and Mann (2003) indicated that TGIF-related homeobox genes, *vis* and *achi* are required for *Drosophila* spermatogenesis (21). To obtain this finding of involvement of the TGIF like genes in *Drosophila* development, they generated flies deficient for genes, *vis* (*vismay*) and *ach* (*achintya*). In spite of the fact that the flies homozygous for this deletion were viable, homozygous males were sterile. They suggested that these genes play no essential role in embryonic or larval development. Also, they showed that in the absence of *vis* and *achi*, spermatogenesis was blocked (21).

TGIFLX/Y is contains two genes; *TGIFLX* (X-linked) and *TGIFLY* (Y-linked) which are specifically expressed in human adult testes. *TGIFLX* originated from retrotransposition of *TGIF2*, located on long arm of chromosome 20 (q11.2-12), onto the X chromosome (22, 23). *TGIFLY* is a Y -linked homeobox gene, located on Y chromosome (Yq11-2). Analysis of DNA and amino acids sequences particularly through the first 148 amino acids has revealed that TGIFLY not only has remarkable similarity to *TGIFLX*, but also diverged from *TGIFLX*. It has been suggested that *TGIFLY* originated by retroposition of the autosomal *TGIF2* gene (23).

Here we present the *TGIFLY* mRNA expression in the Iranian men with non-obstructive azoospermia. We also evaluated the level of spermatogenesis by histopathological scoring, to find the effect of gene expression on progression of spermatogenesis.

Materials and Methods

Patients Testicular biopsies were acquired from 110 unrelated infertile men undergone Testicular Sperm Extraction (TESE) in Avesina Infertility Clinic, Tehran, Iran during 2004-2005. The estimated size of testicular samples was 2_x 2_x2 mm and all samples were stored in -70˚ C. Single testicular biopsy was performed to obtain the samples, unless the patient agreed to have multiple biopsies.

The inclusion criterion was diagnosis of azoospermia based on a semen analysis confirmed by an andrologist. Patients' information including clinical and laboratory findings were obtained from database of Avesina Infertility Clinic laboratories. Approval from the Avesina Research Institute's ethics & human rights committee was obtained for the use of specimen and study protocol. Before biopsy, all participants were signed the informed consent form.

Histopathological Assessment The testicular biopsies from patients with infertility problems were sent to the pathology laboratory of Avesina Research Institute in Bouin's fixative and after processing, prepared slides were stained by hematoxilin and eosin. To define the level of spermatogenesis in which *TGIFLY* was expressed, the slides were evaluated by a pathologist and scored using the modified Holstein's scoring system for testicular biopsy (as shown in Table1).

RNA Extraction & RT-PCR Total RNA was extracted by the RNA-Bee™ (Tel-Test, Protech Technology Enterprises Co., Ltd, Friendswood, TX, USA) following the manufacturer's instructions with minor modifications and stored in -70° C for further tests. Quantification of RNA was performed by absorbance at 260 nm using spectrophotometer (Biophotometer, Eppedorf, Berlin, Germany). The mean RNA concentration was 410.2 µg /µL and OD 260/280 ratio was about 1.8.

For the synthesis of complementary DNA, 10µL of total RNA was heated in 80° C for 10 min followed by cooling on ice. Master mixture included 4 µL of 5x reverse transcriptase (RT) buffer containing Tris-HCl (pH= 8.3) (Fermentase, Burlington, Canada), 10 mM of each dNTP, 20 pmol/µL random primer, 20 U RNase inhibitor (Roche, Mannheim, Germany), 200 U of Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (RevertAid™, Fermentas, Burlington,

Canada), and 1.5 µL of DEPC-treated water. RT temperature profile was 42° C for 60 min, 70° C for 10 min, and final cooling to 4° C. cDNA was checked for the integrity using the house keeping gene phosphoglucomutase 1 (PGM1) primers (as shown in Table 2) which amplify region 1718-2104 (from exon 10 to exon 11). Samples with similar cDNA quality through PGM1 PCR were stored in -20° C for following investigations.

TGIFLY PCR Semi -nested PCR was performed for samples using 1 µl of cDNA and DNA polymerase (CinnaGen Inc.) by Thermocycler PCR machine (eppendorf). Amplification of cDNA was performed during 30 cycles (30 seconds at 94 °C, 30 seconds at 54 °C and 30 seconds at 72 °C). Table 2 shows the sequence of primers used in PCR. The PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light. The results were considered as positive or negative.

Statistical Analysis The Statistical Package for Social Sciences (SPSS) version 11.2 and Microsoft Excell spreadsheat were used for data entry and analysis. Differences between m-RNA expression groups in the mean of continuous variables were assessed using student t-test and analysis of covariance (ANCOVA) for adjustments for possible confounders. An alpha level of 0.05 was used to indicate statistical significance.

Results

Histopathological findings of testicular biopsies from 54(49.1%) patients showed germ cell aplasia (Sertoli cell-only syndrome) [SCOS], (3> Johnsen score) (24). Thirty nine (32.7% testicular biopsies showed maturation arrest (MA) (Fig 1) corresponding to a Johnsen score of 3-7. Twenty patients (18.2%) had spermatozoa in their histopathological examination (Johnsen score 8, 9) (Table 1).

Table 3 shows demographic characteristics of 110 infertile males, were studied for *TGIFLY* gene expression using semi-nested RT-PCR.

TGIFLY mRNA expression in Testicular Biopsy:

To determine the expression of TGIFLY gene in various stages of spermatogenesis (Fig. 1), a semi-nested PCR was performed for 110 samples. TGIFLY was expressed in 51 patients (46.4%), whereas 59 patients had no expression of TGIFLY. 21(19.1%) had the expression of TGIFLY, representing positive results in both rounds of PCRs (Fig. 2). Table 4 presents a summary of clinical indications of patients with infertility, the status of TGIFLY gene expression and the situations of cDNA template used in semin-nested RT-PCR (Fig. 2).

FSH and LH serum levels were significantly higher within the patients who showed no TGIFLY expression than normal controls. Also reduced testis size was observed in cases with no TGIFLY expression (*t*-test: $P< 0.0001$).

Interestingly, sixteen patients had at least one infertile male in family history of which eight patients had no expression of the gene. In order to demonstrate the potential roles of TGIFLY in above patients, we are currently analyzing their pedigrees.

Patients with Hypospermatogenesis (Johnsen's Score: 8, 9):

Seventeen patients had spermatozoa in testicular biopsies. The expression of TGIFLY was detected in eight patients with hypospermatogenesis, but not detected in nine.

Patients with Spermatogenesis Arrest (Johnsen's Score: 3-7)

Forty six patients showed partial tubular atrophy with maturation arrest at the level of spermatogonia, spermatocytes or spermatids in histopathology. Results from semi-nested PCR revealed that the TGIFLY expression was detectable in 18 cases. 25 of the samples from spermatogenesis arrest patients showed undetectable *TGIFLY mRNA* expression in the RT-PCR.

Patients with Sertoli Cell-only Syndrome (Johnsen's Score: 1, 2)

22 out of 41 patients with SCOS samples had no TGIFLY gene expression. The expression of gene was not significantly related to Johnsen's score.

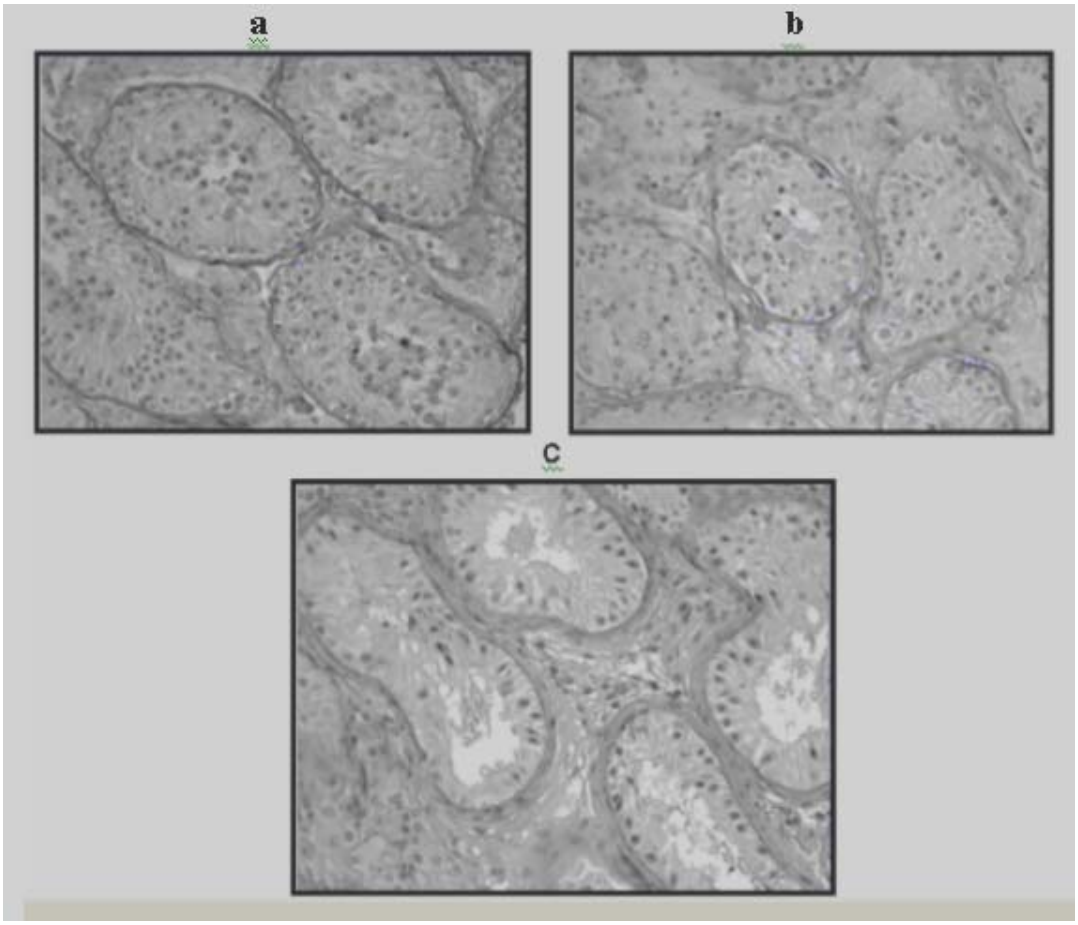

Fig. 1: Histopathology of testicular samples stained with hematoxylin and eosin (x 200). (a) Maturation arrest; (b, Hypo-spermatogenesis; (c), Sertoli-Cell- only Syndrome

Table 1: Histopathological scoring of testicular biopsies based on modified Holstein's system

Score	Modified Holstein's scoring system
10	Intact spermatogenesis: many spermatozoa and zones of spermiation
9	Modest reduced spermatogenesis; reduced number of spermatozoa, a few zones of spermiation
8	Distinct reduced spermatogenesis; few spermatozoa, no spermiation
7	Considerably reduced spermatogenesis; no spermatozoa, only spermatids, no spermiation
6	Severely reduced spermatogenesis; only few spermatids, reduced height of germinal epithelium
5	Arrest of spermatogenesis at the stage of primary spermatocytes; many spermatocytes border the lumen of the seminiferous tubule
4	Arrest of spermatogenesis at the stage of primary spermatocytes; a few primary spermatocytes are present
3	Arrest at the stage of spermatogonia; a type spermatogonia multiplicate but do not develop to maturing cells of spermatogenesis
2	No germ cells, only Sertoli cells are present
1	No germ cells, no Sertoli cells. The seminiferous tubule is replaced by connective tissue ground substance

Table 2: Sequences of primers used in TGIFLY gene expression in male infertility

Name of Primer	Sequences
PGM F	5'-GCCCGCAGGTCCTCTTTCCCTCACA-3'
PGM R	5'-TCCGACTGAGCGGCACTGGGAGTGC-3'
TYINF	5'-CTCTGTTAGCAAAGCTGTTTGTC-3'
TYR1	5'-CATTGATAAACCAGTTAGAAATCC-3'
TYR2	5'-CATGATTGAGGTGTCTTGGGCTG-3'

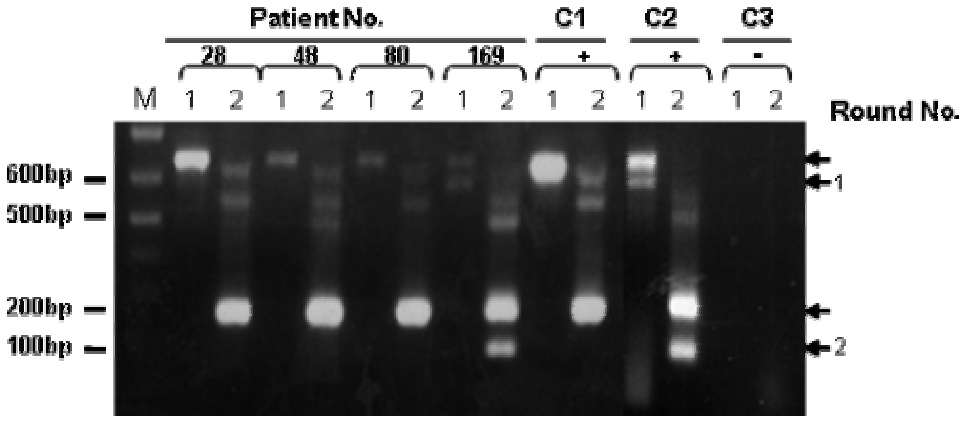

Fig. 2: TGIFLY expression in male infertility. Nested- Reverse Transcriptase-Polymerase Chain Reaction products of TGIFLY genes in infertile male samples on 1.5% agarose gel stained with ethidium bromide. Patient number: 28 , 48 and 80, positive in rounds 1 and no TGIFLY expression; 169, expressed TGIFLY; C1; positive control using gDNA; C2, positive control using cDNA template which was contaminated with gDNA, C3, negative control (ddH_2O) for rounds 1 and 2, M; (100bp) molecular weight marker; PCR conditions and the primer sequences are detailed in materials and methods. Numerated arrows indicate the real signals (1and 2); by contrast, arrows without numbers show contaminated cDNA with genomic DNA.

Table 3: Demographic characteristics of IBD patients

Age (y), mean (95% CI)	**35.4 (34.1–36.7)**
Duration of infertility (y), mean (95% CI)	7.9 (6.8–8.9)
Family history of male infertility	16 (14.7%)
History of testis developmental problems	
Cryptorchidism	10 (9.1%)
Gynecomastia	1 (0.9%)
Testis examination	
Size (cm), mean (95% CI)	10.8 (9.6–12.1)
Consistency	
Normal	70 (63.3%)
Firm	21 (19.3%)
Soft	19 (17.4%)
Serum FSH(IU/L), mean (95% CI)	21.2 (17.3–25.2)
Serum LH Level(IU/L), mean (95% CI)	10.2 (8.1–12.4)
Serum T level(ng/mL), mean (95% CI)	5.8 (4.5–7.1)
Semen analysis	
Volume, mean (95% CI)	2.4 (2.0–2.7)
PH, mean (95% CI)	7.36 (7.28–7.45)
Fructose (mg/dL), mean (95% CI)	247 (247.1–301.0)

CI= confidence interval

Table 4: A summary of clinical indications of patients with infertility, the status of TGIFLY gene expression and the situations of cDNA template used in semin-nested RT-PCR

Patient No	Infertility Type	TGIFLY	gDNA Contamination
35	MA	-	+
144	HYPO	+	+
17	HYPO	+	-
21	MA	+	+
-	SCO	-	-

MA, Maturation arrest; Hypo, Hypospermatogenesis; SCO, Sertoli- Cell only Syndrome.

Discussion

Human male infertility is a main health problem that affects about 13–18% of couples in the human population (25, 26) regardless of race and ethnic group. It has been suggested that male factor infertility plays a critical role in about 50% of

infertile couples. Male infertility is caused by various exogenous and endogenous factors. The main causes are asthenospermia, oligospermia, teratozoospermia and azoospermia, which account for 20–25% of cases (27, 1). Despite of the fact that different genetic factors such as chromosomal abnormalities and other genetic factors (follicle stimulation hormone (FSH) receptor mutation, etc.) are important (1, 2, 25, 27) in male infertility, 30% of male factor infertility is still unknown.

The homeobox gene family encodes helix-turn-helix transcriptional regulators that are spatially and temporally expressed during embryonic development and regulate pattern formation and development in vertebrates (9). Many homeodomain-containing factors, such as members of the Hox family, play critical roles in development al processes (28-30). In the mouse, *Hoxa10* expression is essential for fertility. Transgenic mice carrying a targeted disruption of the *Hoxa10* gene exhibit uterine factor infertility (16, 31). Additionally, it has been proposed that *HOXA10* may have a similar function in the human (20).

Due to their pivotal role in animal development, numerous human diseases and genetic disorders are linked to genetic alterations in homeodomain proteins (32-34).

Zhaohui and Mann (21) demonstrated that two nearly identical homeobox genes of the TGIF, *vismay* and *achintya* are essential for spermatogenesis in *Drosophila*. They found that these genes play no essential role in embryonic or larval development. On the other hand, homozygous males were sterile as results of blocking of spermatogenesis.

This automatically raises the possibility that TGIFLY as a homeodomain protein may associate with male infertility via interactions with both DNA and its partner proteins.

This study is, to our knowledge, the first study investigating *TGIFLY* mRNA expression in 110 testicular samples of patients with non-obstructive azoospermia. Semi- nested RT-PCR was used to detect the strength of gene expression. We also evaluated the expression level of *TGIFLY* during spermatogenesis with the histopathological scoring.

We observed that *TGIFLY* mRNA is expressed in testis. This confirms the findings of Blanco et al. which described *TGIFLY* as a testis- specific gene (23). However, we could not find any significant relationship between the gene expression and spermatogenesis progression. We had some patients with Sertoli cell-only syndrome who had the expression. On the other hand, some patients with hypospermatogenesis were found to have no expression. We could not assume any significant role for *TGIFLY* during human spermatogenesis. Present and absent of TGIFLY mRNA expression in different stage of spermatogenesis could be due to the genetic heterogeneity of male infertility. It has been suggested that more than 2000 genes may acts in the spermatogenesis and man infertility (1).

The elevated serum levels of FSH and LH as well as reduced testis size were observed in patients without *TGIFLY* expression. The analysis of covariance showed that it can be due to other parameters like lack of germ cells and azoospermia rather than the gene expression, as observed in previous studies (3).

TGIFLY expression was negative in half of 16 patients with family history of male infertility. Although the relationship between *TGIFLY* and family history was not significant in this study, the gene is still suspected to be responsible for infertility in above patients. However, further study regarding the gene expression in the patients' family and pedigree analysis are necessary.

Several studies have shown that molecular assays may be useful for assessing disorders of spermatogenesis and predicting the presence of testicular spermatozoa in men with non-obstructive azoospermia (35-37). We have also reported *Synaptonemal Complex Protein 3 (SYCP3)* as a possible molecular marker which is expressed at the level of primary spermatocytes (38). This study shows that *TGIFLY* is not an appropriate molecular marker for spermatogenesis. However, the potential role of this gene and other homeobox genes in human spermatogenesis should be investigated in further studies. In conclusion, although the results presented in this paper indicate

significant correlation between infertility in human and aberrant expressions of TGIFLY genes, the precise mechanisms by which this gene involved remain to be elucidated.

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