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ORIGINAL ARTICLE

Male Health

# Genes associated with testicular germ cell tumors and testicular dysgenesis in patients with testicular microlithiasis

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Testicular microlithiasis (TM) is one of the symptoms of testicular dysgenesis syndrome (TDS). TM is particularly interesting as an informative marker of testicular germ cell tumors (TGCTs). KIT ligand gene (*KITLG*), BCL2 antagonist/killer 1 (*BAK1*), and sprouty RTK signaling antagonist 4 (*SPRY4*) genes are associated with a high risk of TGCTs, whereas bone morphogenetic protein 7 gene (*BMP7*), transforming growth factor beta receptor 3 gene (*TGFBR3*), and homeobox D cluster genes (*HOXD*) are related to TDS. Using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, we investigated allele and genotype frequencies for *KITLG* (rs995030, rs1508595), *SPRY4* (rs4624820, rs6897876), *BAK1* (rs210138), *BMP7* (rs388286), *TGFBR3* (rs12082710), and *HOXD* (rs17198432) in 142 TGCT patients, 137 TM patients, and 153 fertile men (control group). We found significant differences in the *KITLG* GG\_rs995030 genotype in TM ( $P = 0.01$ ) and TGCT patients ( $P = 0.0005$ ) compared with the control. We also revealed strong associations between *KITLG*\_rs1508595 and TM (G allele,  $P = 0.003$ ; GG genotype,  $P = 0.01$ ) and between *KITLG*\_rs1508595 and TGCTs (G allele,  $P = 0.0001$ ; GG genotype,  $P = 0.0007$ ). Moreover, there was a significant difference in *BMP7*\_rs388286 between the TGCT group and the control (T allele,  $P = 0.00004$ ; TT genotype,  $P = 0.00006$ ) and between the TM group and the control (T allele,  $P = 0.04$ ). *HOXD* also demonstrated a strong association with TGCTs (rs17198432 A allele,  $P = 0.0001$ ; AA genotype,  $P = 0.001$ ). Furthermore, significant differences were found between the TGCT group and the control in the *BAK1*\_rs210138 G allele ( $P = 0.03$ ) and the GG genotype ( $P = 0.01$ ). *KITLG* and *BMP7* genes, associated with the development of TGCTs, may also be related to TM. In summary, the *KITLG* GG\_rs995030, GG\_rs1508595, *BMP7* TT\_rs388286, *HOXD* AA\_rs17198432, and *BAK1* GG\_rs210138 genotypes were associated with a high risk of TGCT development.

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**Keywords:** associations; genotype; high risk; testicular dysgenesis syndrome; testicular microlithiasis

## INTRODUCTION

Problems with male reproductive health are extremely common. They can appear in newborns (cryptorchidism, hypospadias), the young (hypogonadism, delayed puberty), and adults (subfertility/infertility, poor sperm quality, testicular germ cell tumors, and testicular atrophy). It has been estimated that one in six young men from European countries suffers from at least one of these disorders.<sup>1</sup>

Testicular microlithiasis (TM) is detected during scrotal ultrasound examination in 5% of men.<sup>2</sup> However, its true prevalence remains unknown because it is often asymptomatic. It is considered that deregulated phagocytic activity of sertoli cells is one of the key events in TM pathogenesis. In this condition, apoptotic testicular germinal epithelium is not destroyed by phagocytosis in the seminiferous tubules but undergoes calcification and then forms microcalculates. It has been hypothesized that this is the mechanism

behind the development of testicular dysgenesis syndrome (TDS).<sup>3</sup> Testicular germ cell tumors (TGCTs), hypospadias, cryptorchidism, hypogonadism, TM, and impaired spermatogenesis could be clinical features of TDS presenting in a combination of congenital disorders and appearing during fetal testis development. These clinical signs are thought to be connected to androgen deficiency in the male embryo.<sup>4</sup> It has also now been clarified that clinical risk factors of testicular germ cell tumors, such as cryptorchidism, TM, and low sperm count and quality, are results of the simultaneous action of adverse genetic and environmental factors. To reveal a correlation between these clinical conditions, long-term observation with detailed anamnesis from birth to adulthood for each patient is required, but this is difficult to perform.

Genome-wide association studies (GWASs) have enabled the identification of a number of genes (KIT ligand gene [*KITLG*], sprouty RTK signaling antagonist 4 gene [*SPRY4*], BCL2 antagonist/killer 1

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gene [*BAK1*], bone morphogenetic protein 7 gene [*BMP7*], transforming growth factor beta receptor 3 gene [*TGFBR3*], and homeobox D cluster genes [*HOXD*]) involved in embryonic development of the testis and human spermatogenesis. These genes could be responsible for the clinical appearance of TDS and reproductive disorders associated with TDS and TGCT development.<sup>5</sup>

To evaluate common genetic factors related to the development of both TGCTs and TM, we investigated *KITLG*, *SPRY4*, *BAK1*, *BMP7*, *TGFBR3*, and *HOXD* and performed a comparative analysis of allelic variants and genotypes of patients with TGCTs/TM and those of fertile men.

## PATIENTS AND METHODS

The examined cohort featured 432 men, consisting of 142 patients with TGCTs, 137 patients with TM, and 153 fertile men (control group). The TM patient group consisted of young men, aged 16 to 26 years, who had undergone a medical examination before military service. Individuals with cryptorchidism, hydrocele, and varicocele were excluded from the study. The TGCT group included 142 patients (seminomas,  $n = 53$ ; nonseminomas,  $n = 74$ ; and mixed tumors,  $n = 15$ ) recruited from male patients, aged 25–45 years, undergoing postsurgical treatment at the chemotherapy department of N. N. Blokhin Russian Cancer Research Centre (Moscow, Russia). Control group consisted of fertile men with no testicular tumors or other andrology diseases.

The study was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The Institutional Ethics Committee of Russian Medical Academy of Postgraduate Education approved all studies. Study participants gave informed consent to the presented study.

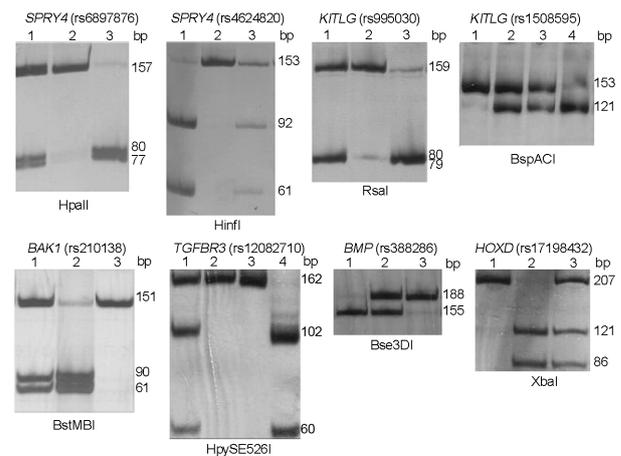
Testicular microlithiasis was diagnosed by scrotal ultrasound examination using Voluson 730 PRO V (General Electric Company, Vienna, Austria) and Mindray DC-7 (Shenzhen Mindray Bio-Medical Electronics, Shenzhen, China) equipment with a high-frequency linear array transducer (7–12 MHz). The diagnostic criterion for TM was the detection of more than three inclusions (with a diameter of 1–3 mm) without acoustic shadowing in testicular parenchyma.<sup>6</sup>

Peripheral blood samples from patients and fertile men (semen donors) were used for DNA analysis. Genomic DNA was extracted from peripheral blood leukocytes using a standard method, as described previously.<sup>7</sup>

In polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, PCR amplification was performed in 25- $\mu$ l reaction mixtures (Syntol, Moscow, Russia), containing 1–2  $\mu$ l of DNA, 2.0 mmol l<sup>-1</sup> MgCl<sub>2</sub>, 1.5 mmol l<sup>-1</sup> of each dNTP, 2 pmol l<sup>-1</sup> of each primer, 2.5  $\mu$ l of PCR buffer, and 1 unit of HotStarTaq DNA polymerase. PCR conditions, primer sequences, temperatures, and the lengths of PCR products are listed in **Table 1**. Subsequently, PCR products were treated with a restriction enzyme (Syntol) at 37°C for 2–5 h (**Supplementary Table 1 and 2**).

Restriction products were separated by vertical electrophoresis in 8% polyacrylamide gel (Acros Organic, Morris Plains, NJ, USA; **Figure 1**). To visualize the PCR products, the gel was stained with 1.1 mmol l<sup>-1</sup> AgNO<sub>3</sub> (Merck, Darmstadt, Germany) for 10–15 min, and then with 0.75 mol l<sup>-1</sup> NaOH, 0.5 mol l<sup>-1</sup> HCHO, and 2.3 mmol l<sup>-1</sup> NaBH<sub>4</sub> (Merck) for 10–15 min.

Statistical analysis of the data was performed using GraphPad Prism version 6.0 software (GraphPad Software, San Diego,



**Figure 1:** PCR-RFLP analysis of allelic variants of *SPRY4* (rs4624820, rs6897876), *KITLG* (rs995030, rs1508595), *BAK1* (rs210138), *TGFBR3* (rs12082710), *BMP7* (rs388286), and *HOXD* (rs17198432). Genotypes: *SPRY4* (rs6897876): 1 – CT, 2 – TT, 3 – CC; *SPRY4* (rs4624820): 1 – GG, 2 – AA, 3 – AG; *KITLG* (rs995030): 1 – AG, 2 – GG, 3 – AA; *KITLG* (rs1508595): 1 – AA, 2 – AG, 3 – AG, 4 – GG; *BAK1* (rs210138): 1 – AG, 2 – GG, 3 – AA; *TGFBR3* (rs12082710): 1 – CT, 2, 3 – CC, 4 – TT; *BMP7* (rs388286): 1 – TT, 2 – CT, 3 – CC; and *HOXD* (rs17198432): 1 – AA, 2 – CC, 3 – AC. *SPRY4*: sprouty RTK signaling antagonist 4 gene; *KITLG*: KIT ligand gene; *BAK1*: BCL2 antagonist/killer 1 gene; *TGFBR3*: transforming growth factor beta receptor 3 gene; *BMP7*: bone morphogenetic protein 7 gene; *HOXD*: homeobox D cluster genes; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism.

CA, USA). Associations were evaluated by comparison of allele and genotype frequencies among the TGCT, TM, and control groups. A test for Hardy–Weinberg equilibrium was performed using Chi-square criteria  $\alpha = 0.05$  and degree of freedom (df) = 1. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated. Associations of disorders with allelic variants (genotypes) were determined using the two-sided Fisher's test, the Chi-square test with Yates's correction for continuity (df = 2, mode of inheritance), or the Mann–Whitney U test, when comparing the allele frequency and genotype distribution for each polymorphism in the TGCT and TM groups versus the control. The false discovery rate (FDR) was controlled using the Benjamini–Hochberg method (FDR test). A comparative analysis of genotype frequencies was performed using a two-sided Fisher's test. Statistical significance was assumed for  $P < 0.05$  in all tests.

## RESULTS

Using PCR-RFLP analysis, we developed a detection method (**Figure 1**) and investigated the *KITLG* (rs995030, rs1508595), *SPRY4* (rs4624820, rs6897876), *BAK1* (rs210138), *BMP7* (rs388286), *TGFBR3* (rs12082710), and *HOXD* (rs17198432) loci in 142 patients with TGCTs, 137 patients with TM, and 153 fertile men. The alleles and genotypes revealed in the test and the control groups are presented in **Table 1** and **2**.

We found significant differences in the prevalence of the *KITLG* genotype GG rs995030 in patients with TM ( $P = 0.01$ ) and TGCTs ( $P = 0.0005$ ) compared with the level in the control group. Strong associations of TM with *KITLG* (rs1508595) were detected for both the G allele ( $P = 0.003$ ) and the GG genotype ( $P = 0.01$ ) and also for TGCTs with the G allele ( $P = 0.0001$ ) and the GG genotype ( $P = 0.0007$ ). For *BMP7* (rs388286), significant differences

**Table 1: Allele and genotype frequencies of KIT ligand gene (rs995030, rs1508595), sprouty RTK signaling antagonist 4 gene (rs4624820, rs6897876), BCL2 antagonist/killer 1 gene (rs210138), bone morphogenetic protein 7 gene (rs388286), transforming growth factor beta receptor 3 gene (rs12082710), and homeobox D cluster genes (rs17198432) in testicular germ cell tumor patients**

Alleles and genotypes	Allele and genotype frequencies (absolute value/frequency)		P	OR (95% CI)
	TGCTs (n=142)	Control (n=153)		
<b>SPRY4 (rs4624820)</b>				
A	175/0.616	167/0.546	0.08 <sup>a</sup>	1.34 (0.96–1.86)
G	109/0.384	139/0.454		0.75 (0.54–1.04)
AA	55/0.387	44/0.288	0.18 <sup>b</sup>	1.57 (0.96–2.55)
AG	65/0.458	79/0.516		0.79 (0.50–1.25)
GG	22/0.155	30/0.196		0.75 (0.41–1.38)
<b>SPRY4 (rs6897876)</b>				
C	178/0.627	174/0.569	0.15 <sup>a</sup>	1.27 (0.92–1.77)
T	106/0.373	132/0.431		0.78 (0.56–1.09)
CC	56/0.394	51/0.333	0.35 <sup>b</sup>	1.30 (0.81–2.10)
CT	66/0.465	72/0.471		0.98 (0.62–1.54)
TT	20/0.141	30/0.196		0.67 (0.36–1.25)
<b>KITLG (rs995030)</b>				
A	49/0.173	46/0.239	0.05 <sup>a</sup>	0.67 (0.44–1.00)
G	235/0.827	148/0.761		1.50 (1.00–2.25)
AA	8/0.056	3/0.020	0.0005 <sup>b,*</sup>	2.99 (0.78–11.48)
AG	33/0.232	67/0.438		0.39 (0.23–0.64)
GG	101/0.711	83/0.542		2.08 (1.28–3.37)
<b>KITLG (rs1508595)</b>				
A	55/0.194	102/0.333	0.0001 <sup>a,*</sup>	0.48 (0.33–0.70)
G	229/0.806	204/0.677		2.08 (1.43–3.04)
AA	10/0.070	20/0.131	0.0007 <sup>b,*</sup>	0.50 (0.23–1.12)
AG	35/0.246	62/0.405		0.48 (0.29–0.79)
GG	97/0.683	71/0.464		2.49 (1.55–4.00)
<b>BAK1 (rs210138)</b>				
A	217/0.764	256/0.837	0.03 <sup>a,*</sup>	0.63 (0.42–0.95)
G	67/0.236	50/0.163		1.58 (1.05–2.38)
AA	82/0.577	103/0.673	0.01 <sup>b,*</sup>	0.66 (0.41–1.07)
AG	53/0.373	50/0.327		1.23 (0.76–1.98)
GG	7/0.049	0/0		16.99 (0.96–300.32)
<b>BMP7 (rs388286)</b>				
C	152/0.535	214/0.699	0.00004 <sup>a,*</sup>	0.50 (0.35–0.69)
T	132/0.465	92/0.301		2.02 (1.44–2.83)
CC	36/0.254	69/0.451	0.00006 <sup>b,*</sup>	0.41 (0.25–0.68)
CT	80/0.563	76/0.497		1.31 (0.83–2.07)
TT	26/0.183	8/0.052		4.06 (1.77–9.31)
<b>TGFBR3 (rs12082710)</b>				
C	118/0.415	129/0.422	0.88 <sup>a</sup>	0.98 (0.70–1.35)
T	166/0.585	177/0.578		1.03 (0.74–1.42)
CC	23/0.162	32/0.209	0.33 <sup>b</sup>	0.73 (0.40–1.32)
CT	72/0.507	65/0.425		1.39 (0.88–2.20)
TT	47/0.331	56/0.366		0.86 (0.53–1.38)
<b>HOXD (rs17198432)</b>				
A	54/0.190	25/0.082	0.0001 <sup>a,*</sup>	2.64 (1.59–4.37)
C	230/0.810	281/0.918		0.38 (0.23–0.63)
AA	9/0.063	2/0.013	0.002 <sup>b,*</sup>	5.11 (1.08–24.07)
AC	36/0.254	21/0.137		2.13 (1.18–3.87)
CC	97/0.683	130/0.850		0.38 (0.22–0.67)

\*Statistically significant ( $P < 0.05$ ) differences; <sup>a</sup>the two-sided Fisher's test; <sup>b</sup>Chi-square test. *KITLG*: KIT ligand gene; *SPRY4*: sprouty RTK signaling antagonist 4 gene; *BAK1*: BCL2 antagonist/killer 1 gene; *BMP7*: bone morphogenetic protein 7 gene; *TGFBR3*: transforming growth factor beta receptor 3 gene; *HOXD*: homeobox D cluster genes; OR: odds ratio; CI: confidence interval; TGCTs: testicular germ cell tumors

from the control group were found for both the TGCT group (T allele,  $P = 0.00004$ ; TT genotype,  $P = 0.00006$ ) and the TM group (T allele,  $P = 0.04$ ).

For *HOXD* (rs17198432), significant associations were demonstrated only with TGCTs (A allele,  $P = 0.0001$ ; AA genotype,  $P = 0.001$ ). For *BAK1* (rs210138), significant

**Table 2: Allele and genotype frequencies of KIT ligand gene (rs995030, rs1508595), sprouty RTK signaling antagonist 4 gene (rs4624820, rs6897876), BCL2 antagonist/killer 1 gene (rs210138), bone morphogenetic protein 7 gene (rs388286), transforming growth factor beta receptor 3 gene (rs12082710), and homeobox D cluster genes (rs17198432) in testicular microlithiasis patients**

Alleles and genotypes	Allele and genotype frequencies (absolute value/frequency)		P	OR (95% CI)
	TM (n=137)	Control (n=153)		
<i>SPRY4</i> (rs4624820)				
A	163/0.595	167/0.546	0.23 <sup>a</sup>	1.22 (0.88–1.70)
G	111/0.405	139/0.454		0.82 (0.59–1.14)
AA	50/0.365	44/0.288	0.37 <sup>b</sup>	1.42 (0.87–2.33)
AG	63/0.460	79/0.516		0.80 (0.50–1.27)
GG	24/0.175	30/0.196		0.87 (0.48–1.58)
<i>SPRY4</i> (rs6897876)				
C	139/0.507	174/0.569	0.14 <sup>a</sup>	0.78 (0.56–1.08)
T	135/0.493	132/0.431		1.28 (0.92–1.78)
CC	39/0.285	51/0.333	0.31 <sup>b</sup>	0.80 (0.48–1.31)
CT	61/0.445	72/0.471		0.90 (0.57–1.43)
TT	37/0.270	30/0.196		1.52 (0.88–2.63)
<i>KITLG</i> (rs995030)				
A	53/0.193	46/0.239	0.19 <sup>a</sup>	0.77 (0.51–1.14)
G	221/0.807	148/0.761		1.31 (0.88–1.95)
AA	7/0.051	3/0.020	0.01 <sup>b*</sup>	2.69 (0.68–10.62)
AG	39/0.285	67/0.438		0.51 (0.31–0.83)
GG	91/0.664	83/0.542		1.67 (0.04–2.69)
<i>KITLG</i> (rs1508595)				
A	61/0.223	102/0.333	0.003 <sup>a*</sup>	0.57 (0.40–0.83)
G	213/0.777	204/0.667		1.75 (1.20–2.53)
AA	12/0.088	20/0.131	0.01 <sup>b*</sup>	0.64 (0.30–1.36)
AG	37/0.270	62/0.405		0.54 (0.33–0.89)
GG	88/0.642	71/0.464		2.07 (1.29–3.33)
<i>BAK1</i> (rs210138)				
A	213/0.777	256/0.837	0.07 <sup>a</sup>	0.68 (0.45–1.03)
G	61/0.223	50/0.163		1.47 (0.97–2.22)
AA	76/0.555	103/0.673	0.12 <sup>b</sup>	0.60 (0.38–0.97)
AG	61/0.445	50/0.327		1.65 (1.03–2.66)
GG	0/0	0/0		1.12 (0.02–56.65)
<i>BMP7</i> (rs388286)				
C	169/0.617	214/0.699	0.04 <sup>a*</sup>	0.69 (0.49–0.98)
T	105/0.383	92/0.301		1.45 (1.02–2.04)
CC	43/0.314	69/0.451	0.05 <sup>b</sup>	0.56 (0.34–0.90)
CT	83/0.606	76/0.497		1.56 (0.98–2.48)
TT	11/0.080	8/0.052		1.58 (0.62–4.06)
<i>TGFBR3</i> (rs12082710)				
C	111/0.405	129/0.422	0.69 <sup>a</sup>	0.93 (0.67–1.30)
T	163/0.595	177/0.578		1.07 (0.77–1.49)
CC	23/0.168	32/0.209	0.59 <sup>b</sup>	0.76 (0.42–1.38)
CT	65/0.474	65/0.425		1.22 (0.77–1.94)
TT	49/0.358	56/0.366		0.96 (0.60–1.56)
<i>HOXD</i> (rs17198432)				
A	32/0.117	25/0.082	0.16 <sup>a</sup>	1.49 (0.86–2.58)
C	242/0.883	281/0.918		0.67 (0.39–1.17)
AA	0/0	2/0.013	0.05 <sup>b</sup>	0.22 (0.01–4.63)
AC	32/0.234	21/0.137		1.92 (1.04–3.52)
CC	105/0.766	130/0.850		0.58 (0.32–1.05)

\*Statistically significant ( $P < 0.05$ ) differences; <sup>a</sup>The two-sided Fisher's test; <sup>b</sup>Chi-square test. *KITLG*: KIT ligand gene; *SPRY4*: sprouty RTK signaling antagonist 4 gene; *BAK1*: BCL2 antagonist/killer 1 gene; *BMP7*: bone morphogenetic protein 7 gene; *TGFBR3*: transforming growth factor beta receptor 3 gene; *HOXD*: homeobox D cluster genes; TM: testicular microlithiasis; OR: odds ratio; CI: confidence interval

differences were also found for the G allele ( $P = 0.03$ ) and the GG genotype ( $P = 0.01$ ) in the TGCT group compared with the control. However, there were no remarkable differences in

*TGFBR3* (rs12082710) and *SPRY4* (rs4624820, rs6897876) allele and genotype frequencies between the TGCT/TM groups and the control.

*KITLG* and *BMP7* genotypes were remarkably different from those in the control for both TGCT and TM groups (Table 1 and 2, respectively). The *P* value in the TM group was higher than that in the TGCT group but was still significant.

Thus, we have determined that some genotypes, *i.e.*, *KITLG*, *BMP7*, *HOXD*, and *BAK1*, correlate with a high risk of TGCTs development (Table 1). According to GWASs, *KITLG*, *BAK*, and *SPRY4* genes are associated with TGCTs, and *BMP7*, *TGFBR3*, and *HOXD* genes are associated with the development of TDS.<sup>5,8</sup> The results of our study indicate that *KITLG* and *BMP7* are also associated with TM. Evidently, some of these genes are common in TGCTs, TM, and TDS (Figure 2).

## DISCUSSION

Recently, several GWASs have revealed a number of genetic variations with important roles in TGCT development, including variations in *KITLG*, *SPRY4*, *BAK1*, Doublesex- and mab-3-related transcription factor 1 gene (*DMRT1*), telomerase reverse transcriptase gene (*TERT*), activating transcription factor 7 interacting protein gene (*ATF7IP*), hematopoietic prostaglandin D synthase gene (*HPGDS*), mitotic arrest deficient 1 like 1 gene (*MAD1L1*), ring finger and WD repeat domain 3 gene (*RFWD3*), testis expressed 14, intercellular bridge forming factor gene (*TEX14*), and protein phosphatase, Mg<sup>2+</sup>/Mn<sup>2+</sup> dependent 1E gene (*PPM1E*).<sup>8-12</sup> Some TGCT-associated genes are functionally related to the early stages of human gametogenesis (*KITLG*, *BMP7*, deleted in azoospermia-like gene [*DAZL*], PR/SET domain 14 gene [*PRDM14*]) or embryonic cell differentiation and gonad development (*DMRT1*, *HPGDS*). There are also genes that control the processes of DNA repair (minichromosome maintenance complex component 3 associated protein gene [*MCM3AP*], RAD51 paralog C gene [*RAD51C*], ring finger and WD repeat domain 3 gene [*RFWD3*]), chromosome segregation, microtubule assembly (mitotic arrest deficient 1 like 1 gene [*MAD1L1*], testis expressed 14, intercellular bridge forming factor gene [*TEX14*]), and tumorigenesis (*KITLG*, *BAK1*, *PRDM14*).

The strongest association with TGCTs was identified for the *KITLG* gene, which is located on 12q22 and encodes a KIT ligand. The signaling pathway KIT/KITLG (Kit receptor signaling pathway) is critical for the migration and survival of primordial germ cells (PGCs), and its deregulation leads to the appearance of ectopically migrated PGCs. The

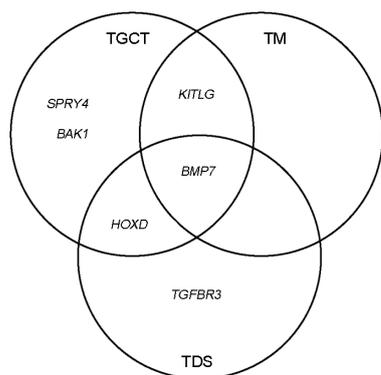
abnormal development of postmigratory PGCs (gonocytes) can become a source of intratubular germ cell neoplasia and eventually TGCTs.<sup>13</sup> The role of the KIT/KITLG signaling pathway in the development of both familial and sporadic TGCTs was confirmed by an association with *SPRY*, *BAK1*, and *PDE11A* genes, which are functionally related to this pathway.<sup>11</sup> In our investigation, we demonstrated significant differences between the TGCT and control groups in the alleles and genotypes of *KITLG*, *BAK1*, *BMP7*, and *HOXD*. This confirms the importance of these genes for TGCT development. Ferlin *et al.*<sup>14</sup> previously demonstrated the association of the *KITLG* gene with TGCT development, particularly testicular seminoma. However, to the best of our knowledge, this study is the first to present an association between TGCTs and *BMP7* and *HOXD*. According to the GWAS data, *BMP7* (rs388286) and *HOXD* (rs17198432) are genetic factors unrelated to TGCTs but related to TDS development.<sup>5</sup>

Interestingly, the *HOXD* locus (rs17198432 alleles and genotypes) is associated with TGCT development. *HOXD* genes are located in a cluster on chromosome 2 (locus 2q31.1) in the region between the ATP synthase membrane subunit C locus 3 gene (*ATP5G3*) and Lunapark, ER junction formation actor gene (*KIAA1715*) (<https://www.genecards.org>; <https://genome.ucsc.edu>). The *ATP5G3* gene encodes a subunit of mitochondrial ATP synthase, which catalyzes ATP synthesis using an electrochemical proton gradient across the inner membrane during oxidative phosphorylation.<sup>15</sup> The *KIAA1715* gene plays a specific role in the formation and interactions of the endoplasmic reticulum and also presumably participates in the development of limbs and the central nervous system in mammals. The role of these genes in tumorigenesis and TDS development is unclear. However, there is a homeotic *HOXD* gene cluster located distally in the telomere direction. This gene cluster, as well as other homologues of homeobox A cluster genes (*HOXA*; 7p14), homeobox B cluster genes (*HOXB*; 17q21), and homeobox C cluster genes (*HOXC*; 12q13), encodes a highly conserved family of transcription factors, which play an important role in the morphogenesis of multicellular organisms.<sup>16</sup> Deletions of *HOXD* genes or their 5'-regions lead to severe abnormalities of limbs and gonads; point mutations in the *HOXD13* gene result in synpolydactyly and brachydactyly.<sup>16</sup> The homeotic *HOXD* gene cluster could also be related to reproductive problems and germ cell tumors; however, the exact mechanism behind this remains unknown.

TM, together with testicular atrophy, cryptorchidism, and male subfertility/infertility, is considered to be a clinical condition associated with a high risk of developing TGCTs.<sup>17</sup> It was proposed that TM, as well as TGCTs, commonly results from TDS.<sup>3</sup> Several reports have described cases of the simultaneous presence of TM and TGCTs in patients and have noted the increased prevalence of TM in men with familial TGCTs.<sup>18-20</sup>

The detection of common genetic factors associated with the pathogenesis of these disorders may indicate a connection between them and probable common mechanisms behind their development. Among the genes reported to be associated with TDS (according to GWASs), namely, *BMP7*, *TGFBR3*, *HOXD*, and *KITLG*, only *KITLG* and *BMP7* demonstrated a significant association with TM in the present study. Thus, we have confirmed the importance of the KIT/KITLG signaling pathway, not only for TGCTs but also for TM development.

Another gene associated with both TM and TGCTs is *BMP7*. This gene encodes a growth factor related to ligands of the bone morphogenetic protein (BMP) family, which is involved in the TGF- $\beta$  signaling pathway and participates in tissue formation during embryonic development.<sup>21</sup> By binding with TGF- $\beta$  receptors, ligands



**Figure 2:** A Venn diagram showing genes (*KITLG*, *BAK*, *SPRY4*, *BMP7*, *TGFBR3*, and *HOXD*) involved in the development of testicular germ cell tumors (TGCTs), testicular microlithiasis (TM), and testicular dysgenesis syndrome (TDS). *SPRY4*: sprouty RTK signaling antagonist 4 gene; *KITLG*: KIT ligand gene; *BAK1*: BCL2 antagonist/killer 1 gene; *TGFBR3*: transforming growth factor beta receptor 3 gene; *BMP7*: bone morphogenetic protein 7 gene; *HOXD*: homeobox D cluster genes.

of the BMP family activate transcription factors of the SMAD family, which regulate the expression of specific target genes. Reproductive functions of BMP include the modulation of testosterone biosynthesis, embryonic cell maturation, sperm quality, and intactness of reproductive tissues.<sup>22</sup> The BMP/TGF $\beta$  signaling pathway is responsible for the maintenance of pluripotency and self-renewal of embryonic stem cells, and for embryonic development of PGCs, which are precursors of immature germ cells. Moreover, since it is a growth factor, BMP can promote tumorigenesis in adulthood by maintaining cell proliferation.<sup>23</sup>

Therefore, the KITLG and BMP7 ligands play important roles in PGC development and differentiation in human embryos, but their deregulation could lead to the activation of cell proliferation and consequently to TGCTs in adulthood. Our data suggest that there is an etiological relationship between TGCTs and TM via TDS, based on the common genetic factors behind their emergence. It was hypothesized that TM and TGCTs could be events secondary to a single common disorder called tubular degeneration, which could explain the correlation between TGCTs and TM.<sup>24</sup> In accordance with this hypothesis, TM is not a risk factor associated with a predisposition to testicular cancer development in itself, but rather a marker of tubular degeneration. Presumably, TM could be a marker only in those men who already have cryptorchidism, low sperm quality, or other risk factors for TGCTs.<sup>25</sup>

Another explanation of our results is that some patients with TM will develop TGCTs in the future. However, it is not possible to predict the risk of tumor formation because of the young age of the patients in our set. In addition, specific environmental factors also influence the process of tumor formation (tumorigenesis). Moreover, as those patients make up a relatively small proportion of the TM patients in our study, we observed a lower *P* value than that for the TGCT group (Table 1 and 2).

To obtain more robust *P* values and prevent false-positive results, the FDR test was used, which makes it possible to reject some

controversial results. The FDR test in the TGCT group confirmed the data for *BMP7* (rs388286), *KITLG* (rs1508595), *HOXD* (rs17198432), and *KITLG* (rs995030) alleles and genotypes, but none of the evaluated polymorphisms in the TM group fulfilled the requirements of the FDR test (Table 3). However, the low significance of the results could be explained by the sample set or by an indirect effect of these genes in the development of TDS. Therefore, these allelic and genotypic variants should not be overlooked as potential genetic risk factors associated with TDS.

In conclusion, the present study demonstrated that variants of certain genes related to the KIT/KITLG and BMP/TGF $\beta$  signaling pathways and important for the development of TGCTs are more common in TM patients than in fertile men. However, the lack of strong associations with other TDS-associated genes indicates that TM may not be unequivocally associated with TDS symptoms.

#### AUTHOR CONTRIBUTIONS

ISD performed DNA isolation and molecular analyses, interpreted results, and drafted the manuscript. EVI and AAT performed clinical examination of TGCT patients and collected biological samples from tumor patients. OYL, EAV, and ABO performed clinical examination of TM patients and collected biological samples from TM patients. DNG diagnosed of TM by ultrasound examination. OBL supervised the study. VVR and DSM performed statistical analysis and interpreted results. VBC overviewed results and finalized the manuscript. MVN designed the study, interpreted the results, and supervised the study. All authors read and approved the final manuscript.

#### COMPETING INTERESTS

All authors declared no competing interests.

Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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**Table 3: Benjamini–Hochberg procedure (false discovery rate test) for investigated alleles and genotypes**

Genes/SNPs	Rank	<i>P</i>	( <i>i</i> / <i>m</i> ) <i>Q</i>
(Alleles) TGCTs FDR			
<i>BMP7</i> (rs388286) <sup>a</sup>	1	0.00004	0.00625
<i>KITLG</i> (rs1508595) <sup>a</sup>	2	0.0001	0.0125
<i>HOXD</i> (rs17198432) <sup>a</sup>	3	0.0001	0.01875
<i>BAK1</i> (rs210138)	4	0.03	0.025
<i>KITLG</i> (rs995030)	5	0.05	0.03
<i>SPRY4</i> (rs4624820)	6	0.08	0.037
<i>SPRY4</i> (rs6897876)	7	0.15	0.043
<i>TGFBR3</i> (rs12082710)	8	0.88	0.05
(Genotypes) TGCTs FDR			
<i>BMP7</i> (rs388286) <sup>a</sup>	1	0.00006	0.00625
<i>KITLG</i> (rs995030) <sup>a</sup>	2	0.0005	0.0125
<i>KITLG</i> (rs1508595) <sup>a</sup>	3	0.0007	0.01875
<i>HOXD</i> (rs17198432) <sup>a</sup>	4	0.002	0.025
<i>BAK1</i> (rs210138) <sup>a</sup>	5	0.01	0.03
<i>SPRY4</i> (rs4624820)	6	0.18	0.037
<i>TGFBR3</i> (rs12082710)	7	0.33	0.043
<i>SPRY4</i> (rs6897876)	8	0.35	0.05

<sup>a</sup>Alleles and genotypes fulfilled the FDR-test. *KITLG*: KIT ligand gene; *SPRY4*: sprouty RTK signaling antagonist 4 gene; *BAK1*: BCL2 antagonist/killer 1 gene; *BMP7*: bone morphogenetic protein 7 gene; *TGFBR3*: transforming growth factor beta receptor 3 gene; *HOXD*: homeobox D cluster genes; TGCT: testicular germ cell tumors. *i*: the individual *P* value's rank; *m*: total number of tests; *Q*: the false discovery rate; FDR: false discovery rate; SNPs: single-nucleotide polymorphisms



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**Supplementary Table 1: Polymerase chain reaction conditions for genotyping of KIT ligand gene (rs995030, rs1508595), sprouty RTK signaling antagonist 4 gene (rs4624820, rs6897876), BCL2 antagonist/killer 1 gene (rs210138), bone morphogenetic protein 7 gene (rs388286), transforming growth factor beta receptor 3 gene (rs12082710), and homeobox D cluster genes (rs17198432)**

Gene/locus	Marker/UCSC position	Primer, f (forward), r (reverse)	T (°C)	Product length, bp
SPRY4 5q31.3	rs4624820	f-ccacatggcataaattccta	56	153
	chr5:141681788	r-tcactcatggcgtgtaaagg		
	rs6897876	f-tgcgggacaacttctctga	60	157
	chr5:141687639	r-tgccttggttccagttat		
BAK1 6p21.31	rs210138	f-ccttcacctccagtttcaaatc	56	151
	chr6:33542538	r-cctcgtgaagcttcctagc		
KITLG 12q21.32	rs995030	f-acaggctgcagttgtatct	56	159
	chr12:88890671	r-ttaaggactgcgggacttgg		
	rs1508595	f-atgtcatcattggccgcat	56	153
	chr12:88986016	r-gaggttatgaaaatgagcatct		
TGFB3 1p22.1	rs12082710	f-gtcctgggtaactttgagc	56	162
	chr1:92155337	r-gcaaagctgtacccttgcct		
BMP7 20q13.2	rs388286	f-cctcaggccagactctgatt	56	188
	chr20:55465424	r-ctggcctagggtactgttga		
HOXDx 2q31.1	rs17198432	f-tcccaaaatgtcagttgt	60	207
	chr2:176389061	r-gtgggaagttgcatccattc		

KITLG: KIT ligand gene; SPRY4: sprouty RTK signaling antagonist 4 gene; BAK1: BCL2 antagonist/killer 1 gene; BMP7: bone morphogenetic protein 7 gene; TGFB3: transforming growth factor beta receptor 3 gene; HOXD: homeobox D cluster genes; T: temperature of incubation; UCSC: <https://genome.ucsc.edu>

**Supplementary Table 2: Polymerase chain reaction-restriction fragment length polymorphism and enzymes with length of restriction fragments**

Gene	Marker	Enzyme/restriction site	Restriction fragment (bp)	T, (°C)
SPRY4	rs4624820	Hinfl (G <sup>^</sup> ANTC)	153; 92; 61	37
	rs6897876	HpaII (CC <sup>^</sup> GG)	157; 80; 77	37
BAK1	rs210138	BstMBI ( <sup>^</sup> GATC)	151; 90; 61	37
KITLG	rs995030	RsaI (GT <sup>^</sup> AC)	159; 80; 79	37
	rs1508595	BspACI (C <sup>^</sup> CGC)	153; 32; 121	37
TGFB3	rs12082710	HpySE526I (A <sup>^</sup> CGT)	162; 102; 60	37
BMP7	rs388286	Bse3DI (CGTTAC <sup>^</sup> NN)	188; 155; 33	37
HOXDx	rs17198432	XbaI (T <sup>^</sup> CTAGA)	207; 121; 86	37

KITLG: KIT ligand gene; SPRY4: sprouty RTK signaling antagonist 4 gene; BAK1: BCL2 antagonist/killer 1 gene; BMP7: bone morphogenetic protein 7 gene; TGFB3: transforming growth factor beta receptor 3 gene; HOXD: homeobox D cluster genes; PCR-RFLP: polymerase chain reaction-restriction fragment length polymorphism; T: temperature of incubation