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Original Article

Promoter methylation of candidate genes associated with familial testicular cancer

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Abstract: Recent genomic studies have identified risk SNPs in or near eight genes associated with testicular germ cell tumors (TGCT). Mouse models suggest a role for *Dnd1* epigenetics in TGCT susceptibility, and we have recently reported that transgenerational inheritance of epigenetic events may be associated with familial TGCT risk. We now investigate whether aberrant promoter methylation of selected candidate genes is associated with familial TGCT risk. Pyrosequencing assays were designed to evaluate CpG methylation in the promoters of selected genes in peripheral blood DNA from 153 TGCT affecteds and 116 healthy male relatives from 101 multiple-case families. Wilcoxon rank-sum tests and logistic regression models were used to investigate associations between promoter methylation and TGCT. We also quantified gene product expression of these genes, using quantitative PCR. We observed increased *PDE11A*, *SPRY4* and *BAK1* promoter methylation, and decreased *KITLG* promoter methylation, in familial TGCT cases *versus* healthy male family controls. A significant upward risk trend was observed for *PDE11A* when comparing the middle and highest tertiles of methylation to the lowest [odds ratio (OR) =1.55, 95% confidence intervals (CI) 0.82-2.93, and 1.94, 95% CI 1.03-3.66], respectively; P_{trend}=0.042). A significant inverse association was observed for *KITLG* when comparing the middle and lowest tertiles to the highest (OR=2.15, 95% CI 1.12-4.11, and 2.15, 95% CI 1.12-4.14, respectively; P_{trend}=0.031). There was a weak inverse correlation between promoter methylation and *KITLG* expression. Our results suggest that familial TGCT susceptibility may be associated with promoter methylation of previously-identified TGCT risk-modifying genes. Larger studies are warranted.

Keywords: Promoter methylation, testicular germ cell tumors, familial testicular cancer, epidemiology, candidate gene

Introduction

Testicular germ cell tumor (TGCT) is the most commonly-diagnosed cancer among young men in industrialized countries. Until recently, little was known of its genetic susceptibility, despite numerous epidemiological studies suggesting a strong genetic basis [1-4]. Genome-wide linkage analysis in familial TGCT found no significant evidence of genetic linkage, and suggested that multiple common, low-penetrance loci likely contributed to TGCT susceptibility [5]. Candidate gene studies identified two loci of interest, the Y -chromosome *gr/gr* deletion [6] and *PDE11A* gene variants [7]. In addition, recent genome-wide association studies (GWAS) have uncovered candidate TGCT susceptibility loci in six gene regions: *KITLG*, *SPRY4*, *BAK1*, *TERT-CLPTM1L*, *ATF7IP*, and *DMRT1* [8-10]. A candidate gene study of single nucleotide polymorphisms (SNPs) in *BAK1, DMRT1, TERT-CLPTM1L,* and *KITLG* gene regions showed that these risk variants predispose to both familial and bilateral TGCT [11]. The GWAS risk SNPs together only accounted for 11% of the risk to brothers and 16% of the risk to sons of TGCT cases [10], suggesting that the majority of TGCT risk loci are still unidentified.

Notably, most of the identified TGCT susceptibility loci are located in genes involved in biologically-plausible pathways. Three loci are involved in the KITLG-KIT signaling pathway that regulates the survival, proliferation and migration of germ cells [12]: *KITLG* encodes the ligand that activates the receptor tyrosine kinase KIT;

SPRY4 encodes an inhibitor of the mitogen-activated protein kinase pathway, which is activated by the KITLG-KIT pathway [13]; and *BAK1* is a pro-apoptotic protein, expression of which is repressed by the KITLG-KIT pathway [14]. *PDE11A* is highly-expressed in endocrine steroidogenic tissues, especially the testis, and its loss causes infertility (a known human TGCT risk factor [15]) in mice [16, 17]. The most recently-identified loci [10] in the *TERT-CLPTM1L*, *ATF7IP*, and *DMRT1* gene regions are involved in telomere maintenance (*TERT-CLPTM1L* and *ATF7IP*) and sex determination (*DMRT1*).

DNA methylation is an epigenetic modification that can be heritable without changing the DNA sequence. DNA methylation regulates gene transcription by targeting CpG islands found in the 5' regulatory and promoter regions of genes [18]. Hypermethylation of these islands has been described in carcinogenesis, and is a well-documented mechanism by which tumor suppressor genes are silenced [19]. Our previous study of global LINE-1 methylation among multiple-case testicular cancer family members suggested that inheritance of LINE-1 methylation levels may be associated with familial TGCT risk [20]. Interestingly, transgenerational epigenetic interactions have been shown to control susceptibility to TGCTs in mice with a *Dead-end homologue 1* (*Dnd1*) mutation [21]. *Dnd1* is expressed in the fetal murine testis during the critical period when TGCTs are believed to develop. Although germline mutations in *DND1* have been excluded as contributing significantly to human TGCT risk [22], the epigenetics of *DND1* have not been described.

Aberrant DNA methylation may provide an alternate genetic mechanism for familial TGCT susceptibility. The promoter regions of candidate genes may be regulated through epigenetic silencing, and could show a unique profile in high-risk families. Motivated by our prior work [20], the exciting results of recent GWAS studies [8-11], and our inability to confirm a classical Mendelian mode of inheritance for familial TGCT [15], we undertook an exploratory, pilot study aimed at investigating the association between promoter methylation of 5 candidate genes involved in normal testicular embryologic development and/or male infertility (*PDE11A*, *KITLG*, *SPRY4*, and *BAK1*, as well as the *DND1* candidate gene from the mouse model) and TGCT risk among individuals from 101 multiple-case testicular cancer families. These experiments were designed prior to the identification of *TERT-CLPTM1L*, *ATF7IP*, and *DMRT1* as genomic regions of interest in TGCT, hence their omission from the current analysis. In addition, we examined expression of the protein products of these candidate genes to determine if promoter methylation levels correlated with gene expression.

Materials and methods

Study population

Families with two or more cases of TGCT or a single family member with bilateral TGCT were recruited as part of the NCI Clinical Genetics Branch Familial TGCT study; details are described elsewhere [23]. Family members ≥age 12 years were invited to participate in the study if they were first-degree relatives of a case, spouses of a case who had participating children, non-first-degree blood relatives who provided a genetic link between cases, or blood relatives with cancer. All participants completed detailed questionnaires and provided blood samples; a subset of participants underwent a detailed medical evaluation at the NIH Clinical Center. Blood was collected between 2003 and 2006 and stored at the NCI/CGB Biorepository. The majority of patients underwent blood collection 5 or more years after they were diagnosed with TGCT (median time between diagnosis and blood draw was 5 years). The parent study was reviewed and approved by the NCI Institutional Review Board (NCI Protocol 02-C-0178; NCT00039598), and all participants provided written informed consent.

DNA extraction and bisulfite treatment

Genomic DNA was extracted from fresh whole blood by standard methods. For bisulfite conversion, 500ng of DNA was treated using the EZ DNA Methylation-Gold™ kit (Zymo Research Corp., Orange, CA) according to the manufacturer's recommendations. The final elution was in 20 μl of M-Elution Buffer.

PCR amplification and pyrosequencing

The genomic sequence (using published GenBank sequences) of each gene was searched with an online search engine (www.cpgislands.com) to identify CpG islands in the promoter

region (Supplementary Table S1). The transcription start site was based on Ensembl transcripts for each gene (Supplementary Table S1). DNA methylation (%5-methylcytosine [5-mC]) of consecutive CpG sites was quantified using pyrosequencing of the bisulfite-treated DNA by EpigenDx Laboratory Service (Worcester, MA). Gene-specific primers, assay details, and cycling conditions are given in Supplementary Table S1. Each of the newly-designed primer sets was tested for PCR bias in a mixing experiment using *in vitro* methylated and non-methylated DNA. For each gene, PCR was performed using 10X PCR buffer, 3.0 mM $MgCl_2$, 200 μM of each dNTP, 0.2 μM each of forward and reverse primers, HotStar DNA polymerase (Qiagen, Valencia, CA) 1.25 U, and 10 ng of bisulfite converted DNA per 50 μl reaction. The PCR was performed with one of the primers biotinylated to convert the PCR product to single-stranded DNA templates. The PCR products (each 10 μl) were sequenced by Pyrosequencing PSQ96 HS System (PSQ H96A, Qiagen Pyrosequencing) following the manufacturer's instructions, according to the established method [24]. Built-in controls were used to verify bisulphite conversion efficiency. The methylation status of each CpG site in the promoter region of each gene was individually analyzed as a T/C SNP using QCpG software (PSQ H96A, Qiagen Pyrosequencing). The relative 5-mC content was expressed as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (5-mC/[5-mC + unmethylated cytosine] = %5-mC). The median coefficient of variation (CV) among 28 blinded replicate samples for each gene was below 12%.

Gene expression

RNA was extracted from cryopreserved lymphocytes, and cDNA was created using the High-capacity cDNA Reverse Transcription Kit from Applied Biosystems (PN 4368814), following the manufacturer's protocol with 20ng of total RNA per reaction. The following TaqMan Gene Expression assay IDs from Applied Biosystems were used for each gene: Hs00241497_m1 for *KITLG*; Hs00184027_m1 for *PDE11A*; Hs00832876_g1 for *BAK1*; Hs01935412_s1 for *SPRY4*; and Hs00832091_s1 for *DND1*. The Pre-Amplification master mix from Applied Biosystems (PN 4391128) was used to pre-amplify all of the assays. A 0.2X dilution pool of all of the 20X assays from Applied Biosystems was created, and 1.25uL of the cDNA was mixed with 2.5uL of pre-amp master mix, and 1.25uL of the 0.2x assay pool. The following cycling conditions were used: 95°C for 10 min, then 14 cycles of 95°C for 15 sec and 60°C for 4 min. Samples were diluted 1:5 with 1X TE and loaded into the 48x48 dynamic array from Fluidigm (PN BMK-M-48.48) according to the manufacturer's protocol. A delta delta Ct analysis was performed using GAPDH as the endogenous control. To increase precision, each sample was tested 4 times, and the 4-test mean was used in statistical analyses (average fold change). The median CV for the fold change in expression among replicate samples was 5.8%.

Statistical analysis

The percent methylation between TGCT patients and controls (unaffected male relatives) was analyzed as a continuous variable using a non-parametric Wilcoxon rank-sum test and linear regression models. We used unconditional logistic regression models to estimate the odds ratio (OR) and 95% confidence intervals (CI) for the strength of the association between TGCT risk and promoter methylation, adjusting for potential confounders (*e.g.*, age). For this analysis, methylation levels were categorized in tertiles or dichotomized at the median, based on the distribution in the healthy male family controls. Analyses were done for each individual CpG site in each gene's promoter region and for the average methylation across the entire promoter CpG island (combined methylation level). We computed the variance of the OR estimates using a robust variance estimator to adjust for the correlations between participants from the same family. Spearman rank correlations and general linear models were used to investigate the strength of the associations between %5-mC and age and TGCT type, and between %5-mC and gene expression (average fold change in expression). Statistical significance refers to a $P \leq 0.05$ or an OR with 95% CI that excludes 1.0. All analyses were carried out using SAS software version 9.1 (SAS Institute, Cary, NC). The Basic Local Alignment Search Tool (BLAST) was used to compare the *SPRY4* nucleotide sequence to mammalian sequence databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The 1000 Genomes data (June 2011 release; http://www.1000genomes.org/home) was used to determine minor allele frequencies (MAF).

Table 1. Characteristics of study subjects and the average promoter methylation level for each candidate gene.

Variables	No. (%)	*KITLG*		*PDE11A*		*SPRY4*		*BAK1*		*DND1*	
		Mean level†	SD	Mean level	SD	Mean level	SD	Mean level	SD	Mean level	SD
Family, healthy	116 (43.1)	2.68	1.0	13.53	3.9	1.94	0.7	1.73	0.7	82.95	2.6
TGCT patients	153 (56.9)	2.39	0.8	14.41	4.7	2.09	0.8	1.94	1.0	82.74	2.4
P		0.028		0.082		0.24		0.12		0.16	
Age group*											
13-35	90 (33.5)	2.8	1.4	13.36	3.6	1.93	0.6	1.68	0.8	82.66	2.5
36-50	93 (34.6)	2.33	0.6	13.0	3.6	1.74	0.5	1.87	0.7	83.19	2.6
51-94	86 (31.9)	2.71	0.9	13.87	4.2	2.04	0.8	1.76	0.7	83.2	2.6
P		0.26		0.69		0.23		0.64		0.57	
TGCT histological type‡											
Seminoma	74 (48.4)	2.29	0.7	13.99	3.7	2.07	0.8	1.93	1.0	82.38	2.6
Non-seminoma	75 (49.0)	2.47	0.7	14.77	5.6	2.1	0.7	1.78	0.7	83.03	2.2
P		0.37		0.71		0.49		0.37		0.17	

No, number of subjects (percent of subjects); SD, standard deviation; †, the average % methylation across the entire promoter CpG island; * % methylation for each age group is estimated for controls only; ‡4 cases were not otherwise specified.

Results

Table 1 shows the characteristics of the 116 healthy male family controls and 153 TGCT cases. The mean age of the healthy male family controls was 45 (standard deviation [SD] 19.8; range 13 to 94) and the mean age of the TGCT cases was 41 (SD 11; range 17 to 79). There were no significant correlations between age or TGCT histology and promoter methylation levels for any of the 5 genes (**Table 1**).

Promoter methylation associations with TGCT

KITLG

There were 24 CpG sites in the promoter region of *KITLG*. We observed lower methylation levels at most individual CpG sites in the promoter region of *KITLG* in the familial TGCT cases (average % methylation: 2.4) compared with the healthy male family controls (average % methylation: 2.7). There were significant differences between methylation in TGCT cases and controls at 4 CpG sites and for the average methylation across the entire promoter region (**Figure 1** and Supplementary Table S2). With methylation categorized into tertiles based on the control distribution, low methylation (first tertile of methylation) was associated with an increased risk of TGCT at each of these 4 CpG sites and across the promoter region (ORs ranged from 1.8 to 2.2; **Figure 2** and Supplementary Table S3).

PDE11A

There were 7 CpG sites in the promoter region of *PDE11A*. Methylation levels were higher in the TGCT cases (average % methylation: 14.4) compared with the male family controls (average % methylation: 13.5) at all CpG sites; levels were significantly different at one CpG site (**Figure 1** and Supplementary Table S2). High methylation (top tertile of methylation) was significantly associated with an increased risk of TGCT at 2 CpG sites in the promoter region (ORs = 1.9, 95% CI 1.02-3.7, and 2.3, 95% CI 1.2-4.3; **Figure 2** and Supplementary Table S3). There was an increased risk of TGCT associated with high methylation (top tertile of methylation) for the average methylation across the promoter region (OR 1.94, 95% CI 1.03-3.65; P_{trend}= 0.04).

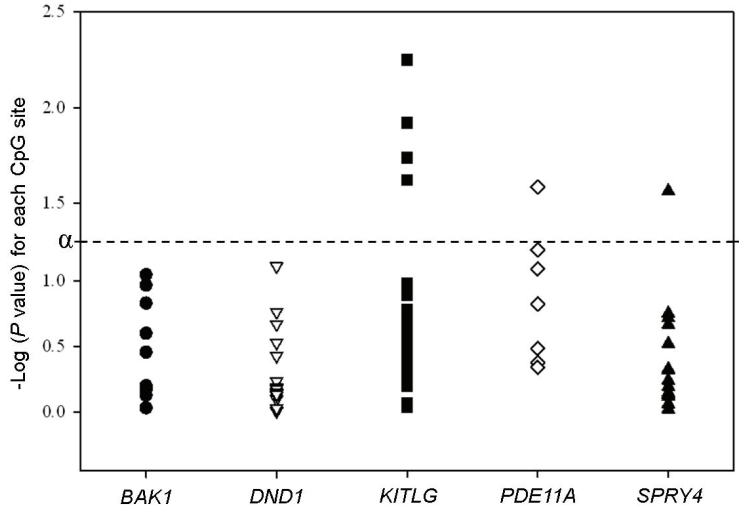

Figure 1. Plot of Wilcoxon rank sum *P* values for each individual CpG site in the promoter region of the five genes. *P* values are from a Wilcoxon rank sum test comparing the % methylation at each individual CpG site in the promoter CpG island of each gene among TGCT cases and their healthy male relatives. α = 0.05; dashed line represents an extension of α.

The sequence and variant status of *PDE11A* in 90 of our TGCT cases has been previously published [7]. We have incorporated this sequence information to determine if TGCT cases without a known *PDE11A* inactivating variant [7] have increased promoter methylation. There were no statistically significant differences between promoter methylation levels in cases with and without a sequence variant (*P* values >0.2). However, if we exclude the 20 cases with a known sequence variant, the remaining cases did have a slightly higher TGCT risk associated with high methylation (top tertile of methylation) at most CpG sites and for the average methylation across the promoter region (OR 2.01, 95% CI 1.04-3.90) compared to the results including all cases (OR 1.94, 95% CI 1.03-3.65), and an additional CpG site became significantly associated with risk.

SPRY4

There were 16 CpG sites in the promoter region of *SPRY4*. Methylation levels were slightly higher in TGCT cases (average % methylation: 2.1) compared with the male family controls (average % methylation: 1.9) at most promoter CpG sites. There was a significant difference between methylation levels in TGCT cases and controls at 1 CpG site (**Figure 1**; Supplementary Table S2), with high methylation (top tertile of methylation) associated with an increased risk of TGCT (OR 1.99, 95% CI 1.06-3.76; P_{trend}= 0.03; **Figure 2**; Supplementary Table S3).

In addition, we found a single nucleotide polymorphism (SNP) in the *SPRY4* promoter region of one multiple-case family, a heterozygous A>G change (genome build 37.2, Chr 5: 141,704,544) downstream of CpG number 6 (genome build 37.2, Chr 5: 141,704,546). This *SPRY4* variant was observed in 3 brothers: 2 were TGCT cases and one was unaffected. The unaffected father of these brothers was also included in our study and did not carry this *SPRY4* variant. This SNP has been previously identified in the 1000 Genomes study, and reported in dbSNP (rs146162871 in the 5' UTR). Using data from 1000 Genomes for 1094 individuals (June 2011 release), the MAF of the variant G allele was 0.7% in all populations, 0% in persons of European ancestry; only 8 individuals of African ancestry carried the variant G allele (7/8 were AG heterozygotes). Using BLAST, the A allele was highly-conserved among other mammalian species (rhesus macaque, chimpanzee, mouse, rat, rabbit, and cat).

BAK1

There were 10 CpG sites in the promoter of *BAK1*. Most CpG sites in the promoter region had slightly higher methylation levels in TGCT cases (average % methylation: 1.9) compared with the male family controls (average % methylation: 1.7), but none of these differences were statistically significant (**Figure 1**; Supplementary Table S2). However, high methylation (top tertile of methylation) for the combined methylation level across the promoter region was associated with an increased risk of TGCT (OR 1.86, 95% CI 1.01-3.42; P_{trend}= 0.04; Supplementary Table S3).

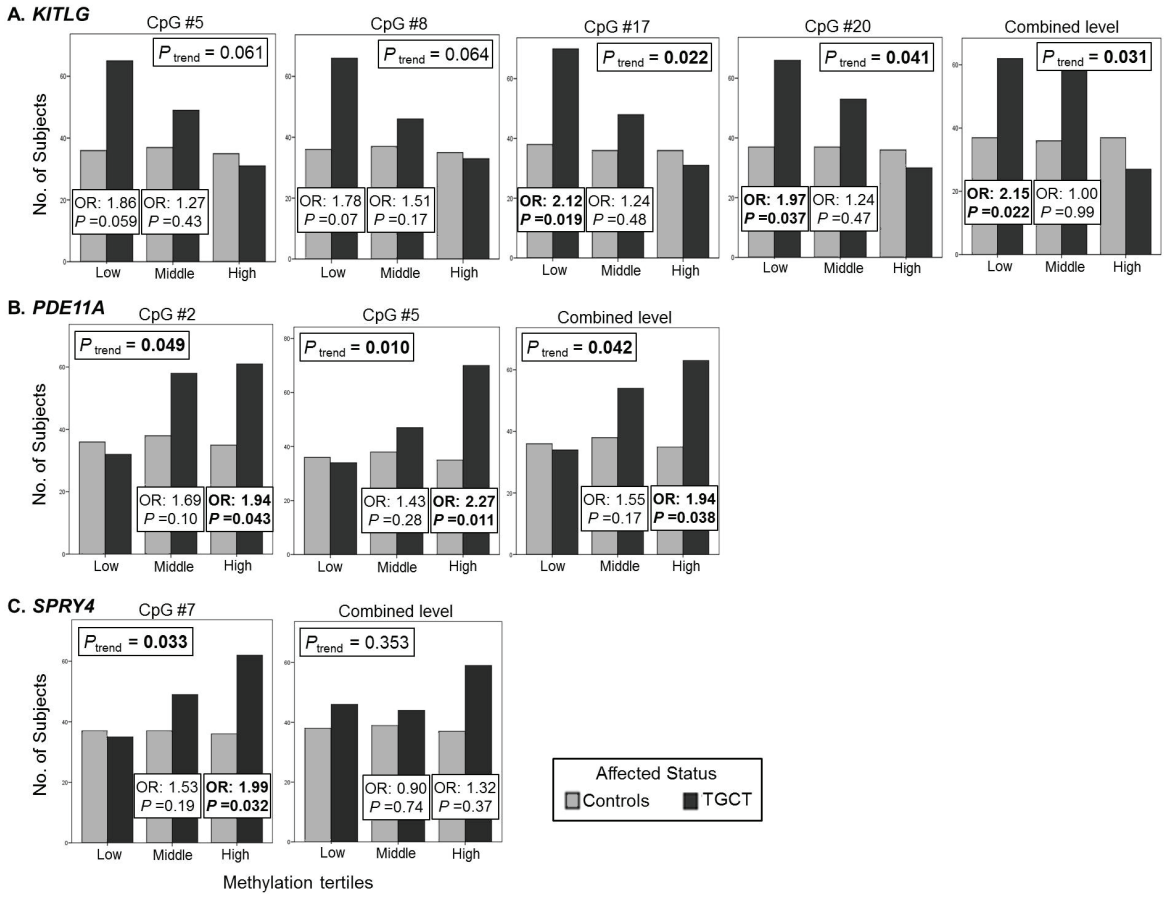

Figure 2. Graphs illustrating the number of individuals in each methylation tertile (low, middle, high methylation) and odds ratios (OR) for the association between TGCT risk and promoter methylation levels for *KITLG* (A.), *PDE11A* (B.), and *SPRY4* (C.). Only the average methylation across the promoter region and the CpG sites that were significantly associated with risk of TGCT are shown. For *PDE11A* and *SPRY4*, ORs are for the middle and highest tertiles of methylation compared to low methylation; and, for *KITLG*, ORs are for having middle or low methylation compared to having high methylation. Logistic regression models were adjusted for age. Methylation levels for each promoter region, and for each individual CpG site, were categorized into tertiles, based on the distribution in the controls. Significant ORs ($P < 0.05$) are bolded.

DND1

There were 15 CpG sites in the promoter of *DND1*. Across the promoter region, there were no significant differences between methylation levels in TGCT cases (average % methylation: 82.7) and male family controls (average % methylation: 82.9; **Figure 1** and Supplementary Table S2).

Combining the effects of average promoter methylation in these candidate genes considered 2 or 3 at a time did not have an additive effect on TGCT risk, with most risk estimates increasing only slightly (data not shown). The strongest combined effect was observed among those having high methylation (top tertile) of *PDE11A, SPRY4*, and *BAK1* compared with having low methylation of these genes (OR 4.77, 95% CI 1.5-15.8), and there was a strong upward risk trend with increasing number of these genes with high methylation (P_{trend}= 0.007). Stratified analyses by tumor type showed similar associations between combined methylation level across the promoter region and TGCT risk for both the seminoma and non-seminoma tumors (Supplemental Table S4). The associations were only significant for cases with seminoma tumors and lower promoter methylation of *KITLG* and higher promoter methylation of *BAK1*. There was also a significant association between lower promoter methylation of *DND1* and risk of

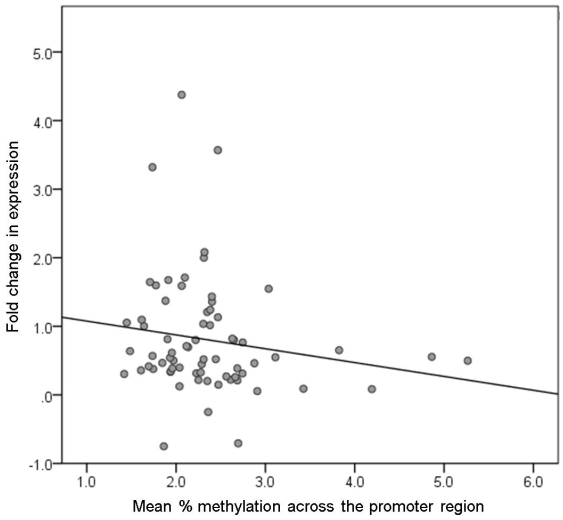

Figure 3. Gene expression and mean % promoter methylation of *KITLG*. The scatterplot shows the fold change in expression of *KITLG* and the mean % methylation across the promoter region in TGCT cases only.

seminoma tumors. The strong biologically plausibility of *DND1* being a TGCT risk modifier warrants a careful look at this gene. However, since we have not corrected our exploratory analyses for false discovery due to multiple testing, the statistical significance here must be regarded with caution.

Gene expression and promoter methylation

There were 109 TGCT cases and 10 healthy male family members with cryopreserved lymphocytes available for RNA extraction and expression analysis. For *KITLG*, there was an inverse correlation between gene expression and average methylation across the promoter region in our TGCT cases (r = -0.21, *P* = 0.080; **Figure 3**). For the 4 individual CpG sites that had methylation levels significantly associated with TGCT, methylation levels at 3 were inversely correlated with gene expression in TGCT cases, but none were individually statistically significant (*P* >0.1). Methylation levels at 3 other CpG sites in the promoter region of *KITLG* showed a significant inverse correlation with gene expression (r = -0.26 to -0.28; *P* = 0.02).

PDE11A failed to amplify in any of the samples tested, suggesting very low or no expression in our samples. For *SPRY4*, there was no correlation between the individual CpG site significantly associated with TGCT risk and gene expression (r = -0.017, *P* = 0.88). Methylation levels across the promoter regions of *BAK1* and *DND1* were not associated with gene expression (r = 0.02, *P* = 0.81; and r = -0.11, *P* = 0.24, respectively).

Discussion

Our data suggest that increased promoter methylation of *PDE11A*, *SPRY4* and *BAK1*, and decreased promoter methylation of *KITLG* in primary lymphocytes, are associated with risk of familial TGCT. *PDE11A*, *SPRY4* and *BAK1* genes may be inactivated and *KITLG* potentially activated by these changes in promoter methylation in TGCT cases. Although, the absolute differences in methylation levels between cases and controls were small, they were statistically significant. The strongest associations were observed between *KITLG* (low methylation) and *PDE11A* (high methylation) promoter methylation changes and increased TGCT risk. These differences are consistent with the known activating and inactivating effects, respectively, of the cancer risk alleles in these two genes (see below). In general, however, we did not observe strong associations between promoter methylation and gene expression in lymphocytes; there may be a tissue-specific effect of aberrant DNA methylation, with blood cell assays underestimating this tissue-specific effect.

Our methylation findings fit with prior observations and hypotheses regarding how each gene/pathway may modify TGCT risk. The KIT pathway has been suggested to be constitutively activated in human TGCTs as a result of gain-of-function mutations in the *KIT* oncogene and/or overexpression of KIT [25]. In mice, germline heterozygous deletions of *KITLG* cause an increase in TGCT incidence [25]. The decreased *KITLG* promoter methylation we observe in TGCT cases may also activate the KIT pathway. In addition, the significant association between lower promoter methylation and only seminoma tumors fits with previous studies that have observed over-expression of KIT predominantly in seminoma tumors [26, 27]. The weak inverse correlation we observed between promoter methylation and KITLG expression suggests that decreased promoter methylation may increase KITLG expression in lymphocytes only modestly. The GWAS SNPs in *KITLG* previously associated

with TGCTs were not correlated with expression changes in lymphoblastoid cell lines [8].

Inactivating *PDE11A* sequence variants have been associated with risk of familial and bilateral TGCTs [7]; we observed increased *PDE11A* promoter methylation in TGCT cases, an alteration that is potentially inactivating. Incorporating *PDE11A* sequence variant data [7] into our analysis showed that TGCT cases without a *PDE11A* variant had a slightly higher risk of TGCT associated with high promoter methylation, suggesting there may be increased *PDE11A* inactivation in these cases without a known inactivating variant. Our failure to detect PDE11A expression in lymphocytes may be due to either very low expression levels that were below the qPCR detection threshold or true absence of expression in our samples, since the limited number of control lymphocytes compromised our ability to make an accurate case-control comparison. Thus, we cannot definitively conclude that *PDE11A* expression in our TGCT cases has been lost. Others have observed very low or no expression of *PDE11A* in leukocytes [28, 29]. The *PDE11A* mutations previously associated with familial TGCT risk were associated with significantly decreased *PDE11A* expression in affected tumor tissues [7]; however, we were only able to examine expression in lymphocytes.

SPRY4 and *BAK1* are also involved in the KITLG-KIT pathway. The associations observed in *SPRY4* and *BAK1* were weaker than those just described, but are consistent with potential inactivation of these genes through increased promoter methylation. *SPRY4* inhibits the protein kinase pathway that is activated by *KITLG-KIT* signaling, and *BAK1* expression in testicular germ cells has been shown to be repressed by *KITLG-KIT* signaling [13, 14]. Rapley et al. [8] found that the *BAK1* allele associated with an increased risk of testis cancer in their GWAS was also associated with a lower expression of *BAK1* in lymphoblastoid cell lines. The increased promoter methylation we observed may also reduce the expression of *BAK1* in TGCT cases, although we did not observe a change in *BAK1* expression in our primary lymphocyte samples.

SPRY4 SNPs previously associated with TGCT risk were not correlated with expression changes [8]. The *SPRY4* promoter SNP, rs146162871, has not been previously associated with disease, to our knowledge. It was found in one TGCT multi-case family: the proband and his affected brother both carried this promoter G variant allele, as did their unaffected brother who has not developed a TGCT. The common A allele is highly-conserved among mammalian species, and the 0% MAF observed in individuals of European ancestry, similar to our FTC families, are consistent with the possibility that the G variant allele may be a very rare TGCT risk allele.

Others have found that aberrant constitutional DNA methylation of the *BRCA1* [30], *MLH1* [31, 32] and *MSH2* [33, 34] cancer predisposition genes accounts for a significant proportion of patients with features of inherited cancers [35]. Recently, *BRCA1* promoter methylation in peripheral blood was strongly associated with breast cancer and also with the development of *BRCA1* methylated tumors with features similar to *BRCA1* mutated tumors [30]. In addition, they observed a mosaic pattern of *BRCA1* methylation, with levels ranging from 0.1-17%; methylation in normal tissues can be either complete or in a mosaic form [36]. The low levels of methylation in *KITLG*, *SPRY4*, and *BAK1* may be suggesting methylation mosaicism in these genes.

Conclusions

We have found that differences in promoter methylation of *KITLG, PDE11A*, *SPRY4* and *BAK1* in primary lymphocytes are associated with familial TGCT. Promoter methylation of these candidate genes may modify familial TGCT risk in a manner that is compatible with the influence that variants in these genes exert on TGCT risk. Finally, our data suggest an inverse correlation between *KITLG* promoter methylation and lymphocyte expression of *KITLG*. Although we observed only a relatively small effect of methylation on lymphocyte protein expression, there may be stronger tissue-specific effects in the testis that could more plausibly influence gene expression and possibly carcinogenesis. This is the first study to investigate promoter methylation of these genes, and our data provide new insight into the complexity of TGCT genetics, to which epigenetic effects may be contributing. However, this pilot study must be viewed as an hypothesis-generating experiment, the results of which must be confirmed in larger studies, perhaps including sporadic TGCT, before definitive conclusions can be reached.

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Supplemental Files

Supplementary Table 1. The gene-specific assay details, including primers purchased, region examined for CpG sites, and PCR cycling conditions.

Gene	Primer names	CpG coverage region; Ensembl reference sequence	PCR amplicon (base-pairs)	PCR conditions
KITLG	ADS1344 FS1 ADS1344 FS2	-332 to -155 from ATG; ENSG00000049130	243	95ºC 15 min; 45 x (95ºC 30 s; 53ºC 30 s; 72ºC 30 s); 72ºC 5 min
PDE11A	ADS1346 FS1 ADS1346 FS2	-743 to -618 from ATG; ENSG00000128655	176	95ºC 15 min; 45 x (95ºC 30 s; 53ºC 30 s; 72ºC 30 s); 72ºC 5 min
SPRY4	ADS997 FS1_re ADS997 FS2	-13 to +130 from TSS; ENST00000344120	226	95ºC 15 min; 45 x (95ºC 30 s; 45ºC 30 s; 72ºC 30 s); 72ºC 5 min
BAK1	ADS1345FS1 ADS1345FS2 ADS1345FS3	-97 to +99 from TSS; ENSG00000030110	253	95ºC 15 min; 45 x (95ºC 30 s; 53ºC 30 s; 72ºC 30 s); 72ºC 5 min
DND1	ADS1347FS1 ADS1347FS2	-254 to -82 from ATG; ENSG00000183403	256	95ºC 15 min; 45 x (95ºC 30 s; 56ºC 30 s; 72ºC 30 s); 72ºC 5 min

TSS = transcription start site

45 x = 45 cycles

Supplementary Table 2. Mean percent methylation (standard deviation) at each CpG site in the promoter region of each gene.

CpG*	*KITLG*			*PDE11A*			*SPRY4*			*BAK1*			*DND1*		
	% methylation (SD)			% methylation (SD)			% methylation (SD)			% methylation (SD)			% methylation (SD)		
	Controls	TGCT	$P_{wilcoxon}$	Controls	TGCT	$P_{wilcoxon}$	Controls	TGCT	$P_{wilcoxon}$	Controls	TGCT	$P_{wilcoxon}$	Controls	TGCT	$P_{wilcoxon}$
1	3.1 (2.7)	2.7 (1.9)	0.861	17.8 (4.8)	19.0 (6.7)	0.150	0.7 (1.0)	0.9 (1.2)	0.177	1.5 (1.4)	2.0 (5.6)	0.649	69.8 (2.3)	69.5 (2.0)	0.214
2	2.4 (1.9)	2.1 (1.5)	0.129	21.9 (6.4)	24.0 (8.8)	**0.026**	1.2 (1.2)	1.3 (1.3)	0.575	3.3 (1.6)	3.1 (1.4)	0.932	75.6 (2.3)	75.2 (2.3)	0.584
3	1.5 (1.6)	1.6 (1.5)	0.635	15.7 (5.2)	16.8 (6.0)	0.081	1.0 (1.2)	1.0 (1.3)	0.715	2.0 (1.4)	2.0 (1.4)	0.745	72.7 (25.3)	69.4 (26.3)	0.297
4	5.5 (3.6)	5.0 (2.6)	0.276	11.5 (3.5)	12.1 (4.0)	0.328	1.8 (1.7)	1.7 (1.8)	0.743	2.0 (1.4)	2.2 (1.2)	0.252	95.7 (6.0)	96.6 (4.3)	0.653
5	2.5 (2.7)	1.8 (2.1)	**0.012**	9.2 (2.6)	9.8 (2.9)	0.058	4.0 (1.5)	4.0 (1.6)	0.765	2.5 (1.7)	2.6 (1.6)	0.632	62.7 (7.2)	63.4 (6.1)	0.988
6	2.3 (1.4)	2.2 (1.1)	0.590	12.7 (3.6)	13.1 (4.3)	0.420	0.9 (1.4)	1.2 (1.7)	0.302	1.4 (1.3)	1.6 (1.5)	0.090	95.2 (10)	95.6 (4.3)	0.373
7	3.1 (3.5)	2.5 (2.3)	0.510	5.9 (3.3)	6.1 (3.4)	0.458	2.0 (1.5)	2.3 (1.6)	**0.027**	1.3 (1.4)	1.5 (1.5)	0.350	59.9 (6.3)	60.2 (6.1)	0.798
8	2.3 (2.7)	1.5 (1.8)	**0.018**				3.1 (1.8)	3.1 (1.8)	0.481	2.5 (1.8)	2.7 (2.0)	0.675	93.6 (0.9)	93.5 (0.9)	0.707
9	2.6 (3.3)	2.1 (2.5)	0.447				1.0 (1.2)	1.2 (1.8)	0.733	0.4 (0.8)	0.5 (1.0)	0.148	98.9 (2.1)	98.9 (1.4)	0.936
10	3.1 (2.8)	2.8 (2.6)	0.307				2.7 (1.3)	2.7 (1.4)	0.869	0.7 (1.5)	1.0 (1.7)	0.108	87.7 (1.6)	87.8 (1.4)	0.671
11	1.0 (1.3)	0.9 (1.4)	0.241				2.7 (2.0)	2.8 (1.8)	0.642				91.5 (1.4)	91.5 (1.7)	0.714
12	0.6 (1.5)	0.4 (1.1)	0.570				0.8 (1.1)	1.1 (1.3)	0.192				79.8 (2.5)	79.8 (2.1)	0.751
13	4.6 (1.9)	4.2 (1.6)	0.106				2.3 (1.3)	2.4 (1.5)	0.769				87.1 (1.4)	86.9 (1.8)	0.077
14	5.7 (2.1)	5.3 (1.6)	0.195				2.1 (1.5)	2.3 (1.6)	0.472				91.1 (3.3)	90.7 (2.6)	0.173
15	1.9 (1.2)	1.7 (1.0)	0.214				1.3 (1.6)	1.5 (1.7)	0.216				81.9 (9.1)	81.4 (9.3)	0.732
16	2.0 (1.4)	1.9 (1.1)	0.924				3.4 (1.8)	3.5 (2.1)	0.955						
17	3.9 (2.2)	3.2 (2.0)	**0.006**												
18	2.7 (1.9)	2.4 (1.9)	0.354												
19	2.2 (1.3)	2.0 (1.1)	0.167												
20	2.5 (1.3)	2.2 (1.1)	**0.024**												
21	1.6 (1.3)	1.5 (1.2)	0.625												
22	1.9 (1.4)	1.6 (1.1)	0.125												
23	2.5 (1.3)	2.6 (1.2)	0.555												
24	2.5 (1.3)	2.4 (1.3)	0.419												

* CpGs were numbered in the order they occur in the promoter region of each gene.

Supplementary Table 3. Relative risk of TGCT associated with promoter methylation levels across the promoter region and for select individual CpG sites by gene.

Category§	Controls *n* (%)	TGCT Cases *n* (%)	OR† (95% CI)	P_{trend}
KITLG				
Combined levels‡				
1st (low)	36 (32.7)	62 (41.6)	**2.15 (1.12, 4.14)**	
2nd (middle)	37 (33.6)	60 (40.3)	**2.15 (1.12, 4.11)**	
3rd (high)	37 (33.6)	27 (18.1)	1.00 (ref)	**0.031**
CpG #5				
1st (low)	36 (33.3)	65 (44.8)	1.86 (0.98, 3.54)	
2nd (middle)	37 (34.3)	49 (33.8)	1.46 (0.76, 2.79)	
3rd (high)	35 (32.4)	31 (21.4)	1.00 (ref)	0.061
CpG #8				
1st (low)	36 (33.3)	66 (45.5)	1.78 (0.94, 3.37)	
2nd (middle)	37 (34.3)	46 (31.7)	1.18 (0.61, 2.27)	
3rd (high)	35 (32.4)	33 (22.8)	1.00 (ref)	0.065
CpG #17				
1st (low)	38 (34.6)	70 (46.9)	**2.12 (1.13, 3.98)**	
2nd (middle)	36 (32.7)	48 (32.2)	1.71 (0.89, 3.31)	
3rd (high)	36 (32.7)	31 (20.8)	1.00 (ref)	**0.022**
CpG #20				
1st (low)	37 (33.6)	66 (44.3)	**1.97 (1.04, 3.73)**	
2nd (middle)	37 (33.6)	53 (35.6)	1.59 (0.83, 3.04)	
3rd (high)	36 (32.7)	30 (20.1)	1.00 (ref)	**0.041**
PDE11A				
Combined levels				
1st (low)	36 (33.0)	34 (22.5)	1.00 (ref)	
2nd (middle)	38 (34.9)	54 (35.8)	1.55 (0.82, 2.93)	
3rd (high)	35 (32.1)	63 (41.7)	**1.94 (1.03, 3.65)**	**0.042**
CpG #2				
1st (low)	36 (33.0)	32 (21.2)	1.00 (ref)	
2nd (middle)	38 (34.9)	58 (38.4)	1.69 (0.90, 3.19)	
3rd (high)	35 (32.1)	61 (40.4)	**1.94 (1.02, 3.67)**	**0.049**
CpG #5				
1st (low)	36 (33.0)	34 (22.5)	1.00 (ref)	
2nd (middle)	38 (34.9)	47 (31.1)	1.43 (0.75, 2.73)	
3rd (high)	35 (32.1)	70 (46.4)	**2.27 (1.21, 4.27)**	**0.010**
SPRY4				
Combined levels				
1st (low)	38 (33.3)	46 (30.9)	1.00 (ref)	
2nd (middle)	39 (34.2)	44 (29.5)	0.90 (0.49, 1.67)	
3rd (high)	37 (32.5)	59 (39.6)	1.32 (0.72, 2.40)	0.353
CpG #7				

1st (low)	37 (33.6)	35 (23.9)	1.00 (ref)	
2nd (middle)	37 (34.6)	49 (33.6)	1.53 (0.81, 2.91)	
3rd (high)	36 (32.8)	62 (42.5)	**1.99 (1.06, 3.76)**	**0.033**
BAK1				
Combined levels				
1st (low)	38 (33.3)	38 (25.2)	1.00 (ref)	
2nd (middle)	39 (34.2)	46 (30.4)	1.24 (0.66, 2.32)	
3rd (high)	37 (32.5)	67 (44.4)	**1.86 (1.01, 3.42)**	**0.042**

n, number of subjects (percent of subjects);

† odds ratios (95% confidence intervals), adjusted for age;

§ categorized into tertiles based on the distribution in controls;

‡ for the average % methylation across the entire promoter CpG island.

Supplemental Table 4. Relative risk of TGCT by type associated with average promoter methylation.

Tumor type	Controls *n* (%)	TGCT cases *n* (%)	OR† (95% CI)	*P* value
Seminoma				
KITLG				
Low§	55 (50.0)	48 (68.6)	2.19 (1.16, 4.128)	
High	55 (50.0)	22 (31.4)	1.00 (ref)	**0.015**
PDE11A				
Low	55 (50.5)	30 (41.7)	1.00 (ref)	
High	54 (49.5)	42 (58.3)	1.42 (0.78, 2.59)	0.25
SPRY4				
Low	57 (50.0)	34 (47.2)	1.00 (ref)	
High	57 (50.0)	38 (52.8)	1.12 (0.62, 2.01)	0.73
BAK1				
Low	57 (50.0)	25 (34.7)	1.00 (ref)	
High	57 (50.0)	47 (65.3)	1.89 (1.03, 3.47)	**0.041**
DND1				
Low	57 (50.0)	50 (67.6)	2.08 (1.13, 3.83)	
High	57 (50.0)	24 (32.4)	1.00 (ref)	**0.019**
Non-seminoma				
KITLG				
Low	55 (50.0)	47 (62.7)	1.37 (0.73, 2.56)	
High	55 (50.0)	28 (37.3)	1.00 (ref)	0.33
PDE11A				
Low	55 (50.5)	28 (37.3)	1.00 (ref)	
High	54 (49.5)	47 (62.7)	1.68 (0.91, 3.12)	0.09
SPRY4				
Low	57 (50.0)	32 (43.8)	1.00 (ref)	
High	57 (50.0)	41 (56.2)	1.23 (0.67, 2.26)	0.50
BAK1				
Low	57 (50.0)	31 (41.3)	1.00 (ref)	
High	57 (50.0)	44 (58.7)	1.53 (0.83, 2.80)	0.18
DND1				
Low	57 (50.0)	38 (51.4)	0.93 (0.51, 1.71)	
High	57 (50.0)	36 (48.6)	1.00 (ref)	0.81

§ average % methylation across the entire promoter CpG island dichotomized at the median, based on the distribution in controls for each gene;

n, number of subjects (percent of subjects);

† odds ratios (95% confidence intervals), adjusted for age.