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Hypermethylation of genes in testicular embryonal carcinomas

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Background: Testicular embryonal carcinoma (EC) is a major subtype of non-seminomatous germ cell tumours in males. Embryonal carcinomas are pluripotent, undifferentiated germ cell tumours believed to originate from primordial germ cells. Epigenetic changes during testicular EC tumorigenesis require better elucidation.

Methods: To identify epigenetic changes during testicular neoplastic transformation, we profiled DNA methylation of six ECs. These samples represent different stages (stage I and stage III) of divergent invasiveness. Non-cancerous testicular tissues were included. Expression of a number of hypermethylated genes were examined by quantitative RT-PCR and immunohistochemistry (IHC).

Results: A total of 1167 tumour-hypermethylated differentially methylated regions (DMRs) were identified across the genome. Among them, 40 genes/ncRNAs were found to have hypermethylated promoters. Quantitative RT-PCR confirmed downregulation of 8 out of 9 of the genes. Among the confirmed genes, five were sex-linked genes, including X-linked genes *STAG2*, *SPANXD/E* and *MIR1184*, and Y-linked genes *RBMY1A1/1B/1D* and *FAM197Y2P*. *RBMY1A* is a testis-specific gene for spermatogenesis. *RNF168* and *USP13* are potential tumour suppressors. Expression of RBMY1A was lost in EC and seminoma as documented in the Protein Atlas. We confirmed downregulation of *USP13* in EC by IHC.

Conclusions: Our genome-wide analysis of testicular EC identified methylation changes in several previously unknown genes. This may provide insight of crosstalk between normal germ cell development and carcinogenesis.

Testicular tumour is the most common malignancy in young men between ages of 20 and 39 years. Over 90% are testicular germ cell tumours (TGCTs) that originate from germ cells. Testicular germ cell tumours (TGCTs) are heterogeneous neoplasms that arise relatively earlier than other carcinomas (Bahrami *et al*, 2007; McIver *et al*, 2013; Looijenga, 2014).

In clinical practice, TGCTs are classified into two histopathological categories, namely seminoma and non-seminoma. Non-seminoma consists of mixed types of embryonal carcinoma (EC), teratoma, yolk sac tumour, choriocarcinoma and sometimes seminoma (Crundwell, 2004). Among them, EC is the most common non-seminomatous germ cell tumour (NSGCT). It is present in ~77% of mixed NSGCTs. Pure forms of NSGCT are relatively uncommon, and ~72% of pure NSGCT are EC (Bahrami *et al*, 2007).

Different from seminoma, EC arises at an earlier age, ~10 years earlier than the average for seminoma. Comparison of the histological types shows a higher incidence of inguinal metastasis for NSGCT (4.9%) than seminoma (0.5%; Daugaard *et al*, 2006). As EC is an aggressive tumour, vascular invasion to parenchymal vessels and rete testis invasion are frequently detected in stage I and II NSGCT. About two-third of cases develop retroperitoneal

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lymph node or distant metastases (Hamid and Umbas, 2009; Yilmaz *et al*, 2013). Because of these features, our current focus is on pure EC only, whereas seminoma and other subtypes in mixed NSGCT are excluded. It is critical to use relatively pure form of EC for methylation analysis, as the epigenetic variation among heterogeneous NSGCTs complicates the interpretation of experimental data.

Many risk factors have been well documented to increase the chance of having TGCT. Cryptorchidism increases the risk of developing TGCT due to failure of normal descent of testes (Lip *et al*, 2013). Patients with congenital disorders such as Klinefelter's syndrome or Down syndrome have a higher risk of TGCT because of the abnormal sexual development (Dieckmann *et al*, 1997; Swerdlow *et al*, 2005). Positive family history is also a risk factor, indicative of genetic causes of TGCT (Nordsborg *et al*, 2011). Data from molecular profiling and marker expression suggest many similarities between TGCT development and normal embryogenesis (for examples, the expression of pluripotency marker OCT3/4) (Jones *et al*, 2004; Hatada *et al*, 2008; Almstrup *et al*, 2010; Kristensen *et al*, 2013). Recent methylation studies indicated that epigenetic factors may have an essential role in the genesis of germ cell neoplasia (Cheung *et al*, 2010; Mirabello *et al*, 2012; Chen *et al*, 2014). In addition, environmental factors may contribute to risk independent of genetic susceptibility (Smiraglia *et al*, 2002; Kristensen *et al*, 2008). However, the responsible epigenetic changes for development of germ cell neoplasms remain to be elucidated.

To identify epigenetic alterations during testicular tumorigenesis, we profiled the DNA methylome of six ECs and two non-cancerous normal testes. The neoplastic samples represent stage I and stage III and exhibited different degrees of invasiveness. A set of hypermethylated genes was found in aggressive ECs. These genes, including several sex-linked genes, may have a central role in the regulation of energy metabolism and spermatogenesis and tumour suppression.

MATERIALS AND METHODS

Tumour specimens. We obtained genomic DNA directly extracted from pathologically confirmed ECs provided by Oncomatrix (San Marcos, CA, USA). Non-cancerous testicular tissues from healthy donors were obtained from Biochain (Hayward, CA, USA). Matched RNA samples from the same specimen, if available, were also obtained. Additional normal/tumour RNA samples were included in the real-time quantitative PCR (qPCR) analysis. Tumour staging was based on the pathologic report for testicular tumour by TNM classification. Tissue arrays for testicular cancer progression were purchased from US Biomax (TE2081; Rockville, MD, USA).

MeDIP and DNA-tiling hybridisation. Methylated DNA immunoprecipitation (MeDIP) was performed as we previously described (Cheung *et al*, 2010). Genomic DNA (5 μg) of tumour/control samples were sonicated on ice to generate random fragments of 100–500 bp in size. Fragmented DNA was subsequently heated at 95 °C, snap-cooled on ice and then incubated with mouse anti-5-methylcytidine monoclonal antibody (anti-5mC, Eurogenetec, Seraing, Belgium) in IP buffer containing 10 mM sodium phosphate (pH 7.0), 140 mM sodium chloride and 0.05% Triton X-100 for 2 h at 4 °C with gentle shaking. Magnetic

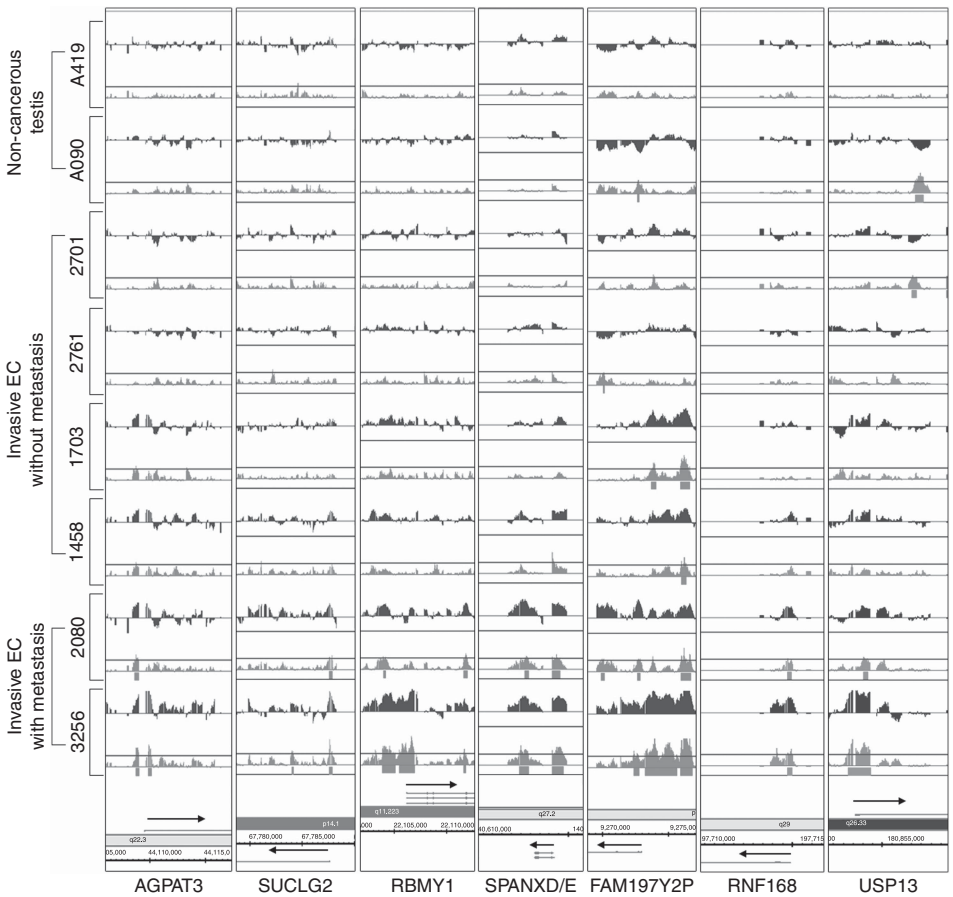

Figure 1. Genomic representation of differentially methylated genes. Hypermethylation is observed at the promoters of seven representative genes (*AGPAT3*, *SUCLG2*, *RBMY1*, *SPANXD/E*, *FAM197Y2P*, *RNF168* and *USP13*) in metastatic and non-metastatic ECs. Orientation of transcription for each gene is indicated by arrows. Blue peaks represent probe signal, whereas red peaks represent *P*-value (cutoff = 0.01).

beads conjugated with sheep anti-mouse IgG (Invitrogen, Grand Island, NY, USA) were added to the IP mixture and incubated for another 2 h. After incubation, the beads were washed three times in IP buffer and then digested by 80 μg of proteinase-K for 3 h at 50 °C. DNA was extracted by phenol/chloroform and ethanol precipitation. Following MeDIP, DNA was amplified using Whole Genome Amplification Kit (Sigma-Aldrich, St Louis, MO, USA), biotin-labelled and hybridised to Human Tiling Array 2.0R Chips (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instruction. After overnight hybridisation at 45 °C, chips were washed and stained on the Affymetrix Fluidic Station 450 and scanned on GeneChip Scanner GCS3000 (Affymetrix). Technical replicate of MeDIP-chip procedure was performed using the same DNA sample.

Analysis of microarray data. Raw data of tiling array (.CEL files) were submitted for quantile normalisation using the Tiling Analysis Software (TAS; Affymetrix). Pairwise comparisons were performed to compare tumour samples of each stage with normal testicular tissues in TAS. Differentially methylated regions (DMRs) were generated using parameters as we previously described (Cheung *et al*, 2010). To identify differentially methylated genes, promoter annotations (+500 to −1500 bp relative to TSS) were retrieved from Refseq in UCSC Genome Browser, and mapped to DMRs using web-based tool TileMapper (Cheung *et al*, 2013). To visualise genomic features of differentially expressed genes, CEL and BED (which contains DMR information) files were loaded in Integrated Genome Browser. Array data were deposited at GEO (Series GSE66784).

Real-time qPCR analysis. Total RNA samples were converted to cDNA using SuperScript III (Invitrogen). Diluted cDNA was mixed with gene-specific primers and SYBR Green master mix for qPCR analysis in Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Grand Island, NY, USA). Relative gene expression level was normalised by 18S rRNA. All RT-qPCR experiments were replicated three times and represented as mean ± s.d. The list of the primer sequences is shown in Supplementary Table 2.

Immunohistochemistry. Tissue array was deparaffinized in xylene (3 min, 2 ×) and rehydrated in serial ethanol (100–50%). Antigen retrieval was performed by heating the slides in sodium citrate solution (10 mM, pH 6.0, 0.05% Tween-20) using a microwave oven. Endogenous peroxidase was blocked by Dako REAL Peroxidase-Blocking Solution (Dako North America, Carpinteria, CA, USA). Slides were blocked with 10% goat serum before incubation with antibodies. Anti-USP13 (1 : 100 dilution) antibody was purchased from Sigma-Aldrich (HPA004827) and incubated with tissues at 4 °C overnight. HRP-labelled secondary antibody conjugated in polymer (Dako North America) was incubated with tissues at room temperature for 1 h. After thorough washing, slides were stained with DAB substrates and counter-stained with haematoxylin and viewed under light microscope (Leica, Wetzlar, Germany). The staining intensity was averaged by two duplicated tissue spots and scored as negative (<10%), weak (10–25%), moderate (25–75%) and strong (>75%), according to the common guidelines (Gremel *et al*, 2014).

Statistics. One-way ANOVA was used to compare the difference in gene expression between normal testicular tissues and ECs. Fisher's exact test was used to compare the difference between normal and different subtypes of TGCT. $P<0.05$ is considered statistically significant.

RESULTS

Hypermethylation in ECs. We attempted to identify epigenetic alterations that occurred during TGCT tumorigenesis. The current focus is on EC, a subtype of the most common germ cell tumour in both mixed and pure NSGCTs. Furthermore, ECs are highly aggressive in nature, with both local and distant metastasis. To examine genomic 'hotspots' associated with tumour progression, we analysed the global DNA methylation of six ECs representing non-metastatic tumours without local invasion (stage I, pT1), non-metastatic tumours with vascular/lymphatic invasion (stage I, pT2) and tumours with distant metastasis and vascular/lymphatic invasion (stage III, pT2). Non-cancerous testicular tissues were included as controls (Supplementary Table 1). Genome-wide methylation analysis revealed distinct methylation profiles between cancerous *versus* non-cancerous tissues. We were specifically interested in the DMRs identified from ECs. The majority of these DMRs are hypermethylation. A total of 1167 hypermethylated DMRs were enriched in the ECs. To gain knowledge of the regulatory relationship of the DMRs on genes (including non-coding RNAs), we mapped the hypermethylated DMRs to Refseq promoters (Figure 1). A total of 40 genes/ncRNAs were identified (Table 1). The other DMRs (~97%) were mapped to gene bodies or intergenic regions, consistent with our previous finding in other testicular cancer cells (Cheung *et al*, 2010). The function of such DMRs remains to be elucidated. For the 40 genes/ncRNAs with hypermethylated promoters, 10 were found in pathways of metabolism including TCA cycle and mitochondrial protein import (by REACTOME). Altered energy metabolism in cancer cells is well established. Although only 40 genes/ncRNAs were identified in our study, 27 (67.5%) were implicated in cancer, as identified by Ingenuity Pathway Analysis (Table 2; Supplementary Figure 1).

DNA hypermethylation at promoters is usually associated with transcriptional repression. To investigate whether the identified hypermethylated genes/ncRNAs are associated with reduced transcription, the relative mRNA expression of nine randomly selected genes/ncRNAs (*AGPAT3*, *MIR1184*, *SUCLG2*, *RBMY1*, *SPANXD*, *RNF168*, *USP13*, *FAM197Y2P* and *STAG2*) was examined by RT-qPCR. Although we had limited sample size,

Table 1. Hypermethylated genes in EC

Chr	Genes
chr1	*OR2T8*
chr2	*TRIM43B, INO80B*
chr3	*FAM157A, SUCLG2, USP13, NPHS2, ASB3, CACNA2D3, C1orf182, RNF168, ADCY5, LOC729375, LOC100128640*
chr4	*INPP1, SMEK2, UGT2B28, C1orf87*
chr7	*AOAH, SPACA1, RNF144B, LOC285733, MIR592, CYP3A5*
chr17	*MAPK7, CDK5RAP3*
chr19	*PIP5K1C, BTBD2, ZNF699,*
chr21	*DSCR3, AGPAT3, ABCG1*
chr22	*ACO2, PHF5A, SAMM50*
chrX	*STAG2, SPANXD/SPANXE, MIR1184-1,2,3*
chrY	*RBMY1B/RBMY1A1/RBMY1D, FAM197Y2P*

Table 2. Gene ontology analysis of the hypermethylated genes on diseases and biofunctions

Categories	# of genes	*P*-value
Cancer	27	2.06E − 02
Cancer, gastrointestinal disease, hepatic system disease	15	1.38E − 02
Hereditary disorder, neurological disease, psychological disorders, skeletal and muscular disorders	4	2.38E − 02
Infectious disease	4	2.52E − 02
Cancer, neurological disease	3	3.58E − 02

8 out of 9 genes (except *STAG2*) showed reduced expression in tumours of both metastatic and non-metastatic ECs (Figure 2), suggesting promoter methylation may hinder transcription of these genes.

Epigenetic changes in sex-linked genes. From the list of hypermethylated genes, we noticed several sex-linked genes/ncRNAs that were epigenetically changed, including X-linked genes *STAG2*, *SPANXD/E* and *MIR1184*, and Y-linked genes *RBMY1A1/1B/1D* and *FAM197Y2P* (Table 1). Interestingly, the X-linked *SPANXD/E* is localised to a previously identified susceptibility locus on chromosome Xq27 for TGCT and prostate cancer (Rapley *et al*, 2000; Kouprina *et al*, 2007). Consistent with this, the *SPANXD* transcript was expressed in normal testis but not detected in our EC samples (Figure 2). Although it is sex linked, *SPANXD* is not testis specific. Another sex-linked gene, *RBMY1A1* is a testis-specific RNA-binding protein and is important for spermatogenesis. Like *SPANXD*, the *RBMY1A1* transcript is lost in both metastatic and non-metastatic EC, as measured by RT-qPCR (Figure 2). As the result obtained from RT-qPCR represents the average mRNA level in the whole testis, we asked whether RBMY1A1 is restricted to germ cells. Immunohistochemistry (IHC) data from Protein Atlas indicated strong expression of RBMY1A1 protein in spermatogonia, spermatocytes and spermatids, but not in Sertoli cells or Leydig cells of somatic origin, confirming the germ cell specificity of this gene (Figure 3A and D). Antibody detected very few cells expressing RBMY1A1 in ECs and seminomas (Figure 3B, C, E and F). The IHC data confirmed the downregulation of RBMY1A1 in ECs and seminomas (Figure 3; Uhlen *et al*, 2005). As the majority of type-2 TGCTs originate from germ cells, it suggests that epigenetic silencing of sex-linked genes might have a crucial role in suppressing tumour initiation or its progression. The identification of DNA methylation of these sex-linked genes suggests that epigenetic alterations associated with testicular transformation may involve genes critical for germ cell development.

Downregulation of USP13 in EC. *USP13* is another downregulated candidate gene as confirmed by RT-qPCR. Unlike *RBMY1A*, *USP13* is not testis specific. It may act as a tumour suppressor by stabilizing PTEN (Zhang *et al*, 2013). To further confirm our result in an independent cohort of TGCT patients, we performed IHC using tissue microarray. The array covers 46 seminomas, 8 yolk sac tumours, 16 ECs, 13 benign tumours (mature teratoma, tuberculosis and atrophy), 13 adjacent normal tissues and 4 normal tissues (Table 3). Each case is duplicated.

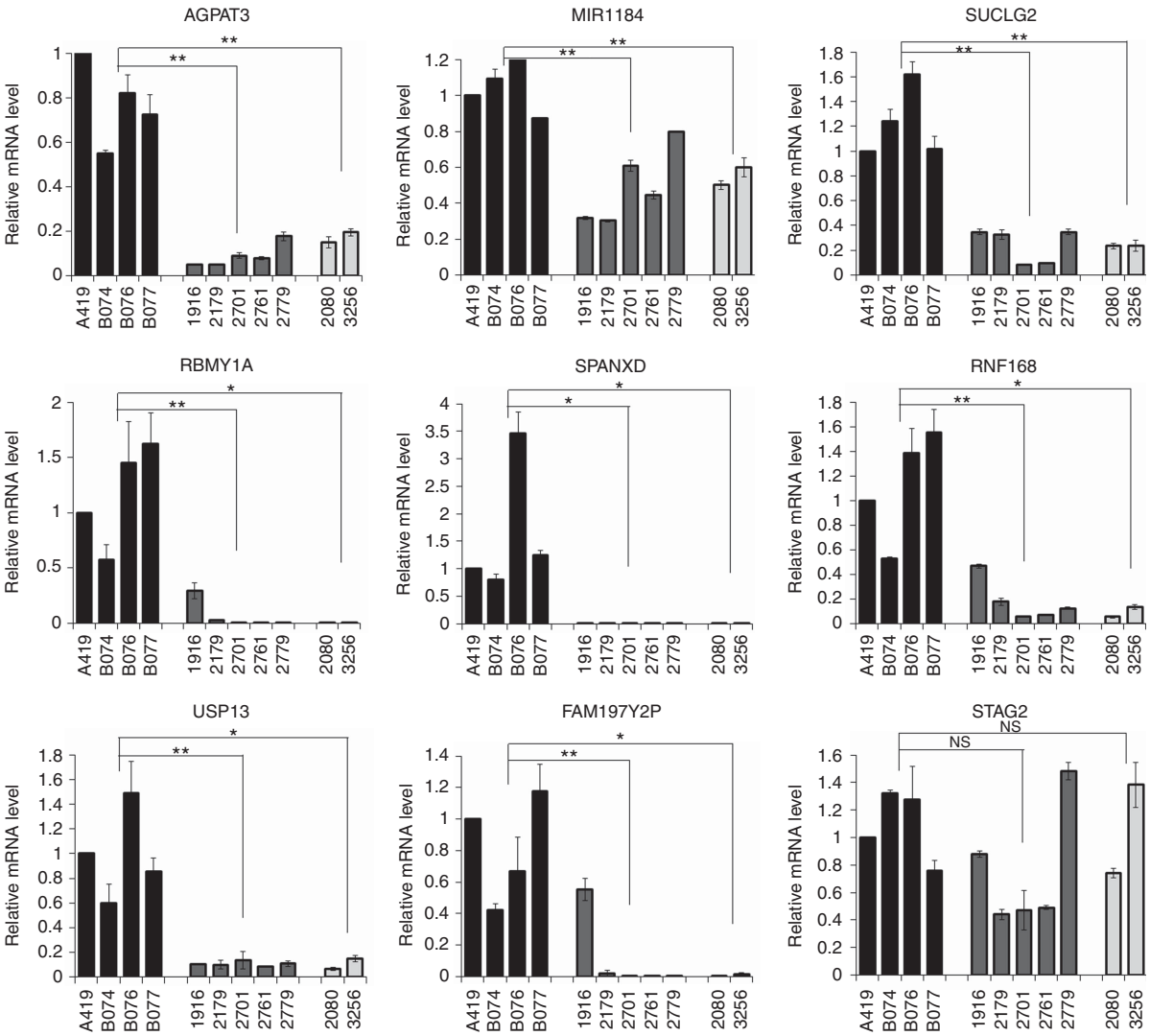

Figure 2. Relative expression of nine hypermethylated genes/ncRNAs. Gene expression is measured by quantitative RT-PCR and normalised by 18S rRNA. Values represent mean ± s.d. Non-cancerous testicular tissues: A419, B074, B076 and B077; non-metastatic EC: 1916, 2179, 2701, 2761 and 2779; metastatic EC: 2080 and 3256. *$P<0.05$, **$P<0.01$; NS = not significant (one-way ANOVA).

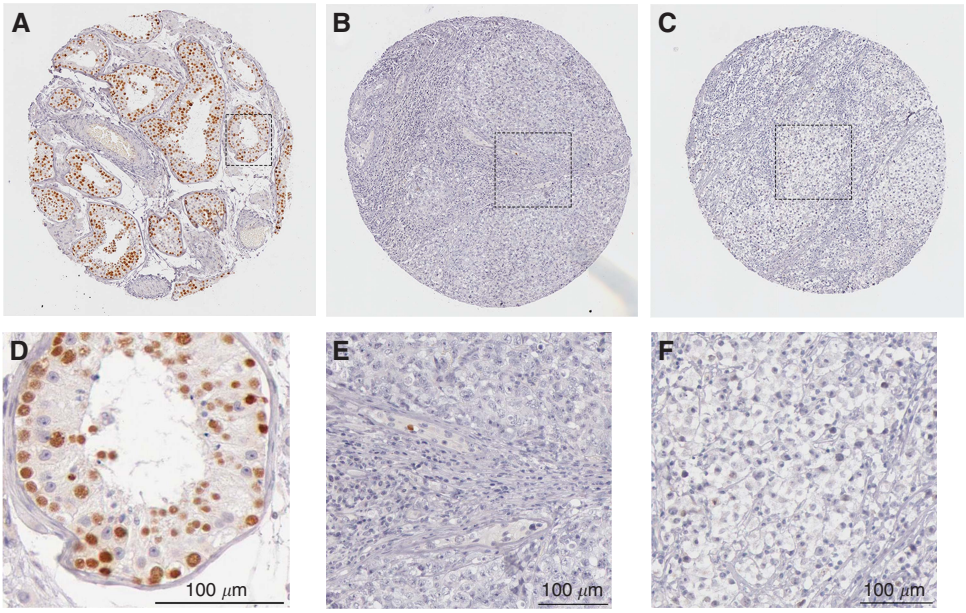

Figure 3. Immunohistochemistry of RBMY1A on testicular tissue, EC and seminoma. RBMY1A protein is detected by a rabbit polyclonal antibody (Sigma-Aldrich, HPA001534). (**A**) Non-cancerous testicular tubules. (**B**) EC. (**C**) Seminoma. (**D–F**) Magnified view from **A–C**. Pictures were adopted from The Human Protein Atlas (http://www.proteinatlas.org). Scale bar, 100 μm.

Table 3. Expression of USP13 in TGCTs

	Negative	Weak	Moderate	Strong	*n*	[a]*P*-value
Normal	0	3	11	3	17	
Benign	3	2	5	3	13	0.223
SE	9	13	22	2	46	0.052
YST	3	2	3	0	8	0.038
EC	4	8	4	0	16	0.004

Abbreviations: EC = embryonal carcinoma; SE = seminoma; TGCS = testicular germ cell tumour.
[a]*P*-value is calculated by Fisher's exact test by comparing each group with the normal.

To increase statistical power, we grouped the adjacent normal tissues ($n = 13$) and normal tissues ($n = 4$) together. The result indicated that USP13 was expressed in normal testis including germ cells, Sertoli cells and Leydig cells (Figure 4B). On the basis of the staining intensity, 82.4% of normal cases (14 out of 17) expressed moderate to strong USP13 protein, compared with only 25% of ECs (4 out of 16) stained at a moderate level ($P = 0.004$, Fisher's exact test; Figure 4A–C). For seminoma, USP13 expression was also downregulated but to a less extent than ECs—52% cases of seminomas were stained at moderate or strong level ($P = 0.052$, Fisher's exact test). Interestingly, we observed a subpopulation of cells in seminoma stained with USP13 at moderate to strong level (Figure 4D). The present data cannot distinguish any pathological difference between these USP13-strong and USP13-weak carcinomas. In conclusion, by our genomic approach, we identified several epigenetically changed genes and these were confirmed by expression in ECs.

DISCUSSION

In this report, we identified 40 hypermethylated genes in EC through DNA methylation profiling. Many of the genes (27 out of 40) are related to cancer biology. Notably, one-forth are metabolism-related genes including the TCA cycle. The data suggest that an epigenetic mechanism leads to altered metabolic pathways associated with TGCT.

We also identified new candidate genes that were not previously linked to TGCT. For instance, RNF168 is an E3 ubiquitin ligase involved in repair of DNA double-strand break. Mutation of this gene gives rise to Riddle syndrome (Stewart *et al*, 2009). In a mouse model of Riddle syndrome, Rnf168 deficiency causes defective spermatogenesis and increased cancer susceptibility due to genomic instability and immunodeficiency. Such data support Rnf168 as a tumour suppressor by cooperation with p53 (Bohgaki *et al*, 2011). In EC, diminished expression of *RNF168* is concordant with hypermethylation. It is possible that impaired spermatogenesis may be accompanied with testicular germ cell tumorigenesis. Genes that govern tumour suppression could be regulated by epigenetic changes. From this study, we also found another gene, *USP13* to be epigenetically changed. USP13 is a newly identified tumour suppressor protein that functions through deubiquitylation and stabilisation of PTEN protein (Zhang *et al*, 2013). USP13 expression was found significantly decreased in ECs, but to a less extent in seminomas. We also observed a subpopulation of seminoma expressed USP13. As seminoma is relatively pure, we do not know the function of USP13 in seminoma. The role of USP13 in suppression of TGCT remains to be determined. It may add an avenue to the well-established role of PTEN in the transition of benign carcinoma *in situ* (CIS) to invasive TGCT (Kimura *et al*, 2003; Di Vizio *et al*, 2005; Hennenlotter *et al*, 2011).

Sex-linked genes encoded in X- or Y-chromosome have been known to regulate sex determination and inheritance of sex-linked traits. Some directly govern spermatogenesis and male infertility, such as genes located within the azoospermia factor region in Y-chromosome (Sadeghi-Nejad and Farrokhi, 2007). From this study, we identified several hypermethylated sex-linked genes. These include three X-linked genes/ncRNA (*STAG2*, *SPANXD/E* and *MIR1184-1/2/3*) and two Y-linked genes (*RBMY1A1/1B/1D* and *FAM197Y2P*). One of the X-linked genes, *SPANXD/E*, belongs to the cancer/testis-associated *SPANX* gene family. Together with other testis-specific genes, they are required for initiating the molecular and morphological changes in male germ cells necessary for the development of mature spermatozoa (Westbrook *et al*, 2006).

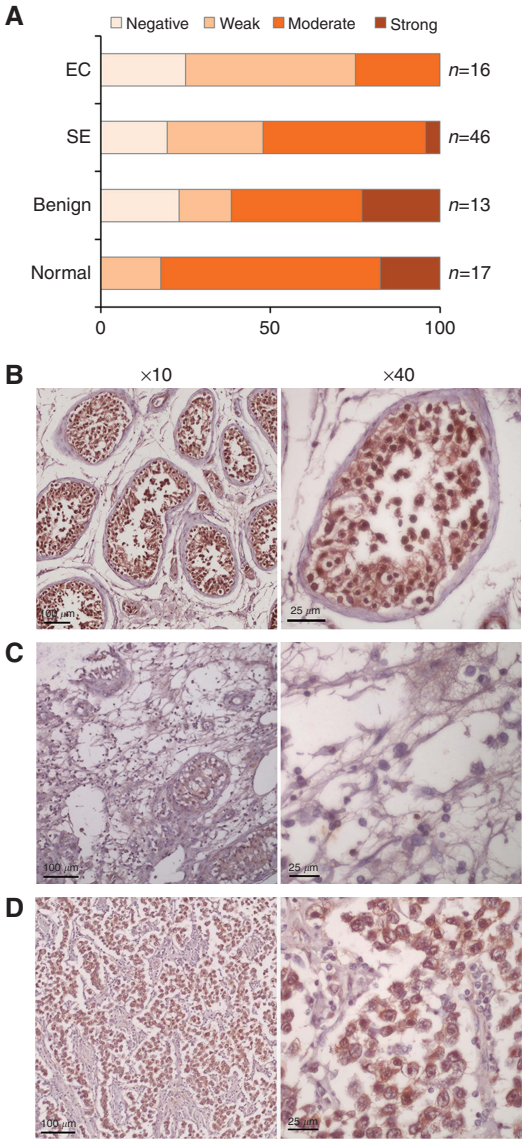

Figure 4. Immunohistochemistry of USP13 on testicular tissue, EC and seminoma. (**A**) Expression of USP13 protein in different subtypes of TGCT. Representative pictures of USP13 staining were shown in (**B**) for normal testis, (**C**) for EC and (**D**) for seminoma. EC = embryonal carcinoma; SE = seminoma. Scale bar, 100 μm (× 10) or 25 μm (× 40).

SPANXD/E encodes sperm proteins localised to the nucleus with a suggested role in spermatogenesis. However, the specific functions have not been well understood, although it is reported to be silenced in tumours (Zendman *et al*, 2003). Testis-specific, Y-linked genes have been proposed to have a pivotal role in the pathogenesis of TGCT, for instance, the *TSPY* gene (Li *et al*, 2007a, b; Akimoto *et al*, 2010). One of the Y-linked genes, *RBMY1A*, has been reported to be regulated by DNA methylation in urological prostate cancer (Dasari *et al*, 2002). *RBMY1A* encodes a testis-specific RNA-binding protein with unknown function in spermatogenesis and TGCT (Zeng *et al*, 2011; Alikhani *et al*, 2013). From the Protein Atlas, IHC analysis on normal testis, EC and seminoma tissues documented downregulation of this protein (Uhlen *et al*, 2005). Expression of *RBMY1A* is restricted to male germ cells, and disappears in ECs and seminomas. These data are consistent with those obtained in our qPCR analysis and implicate the regulatory role of methylation on *RBMY1A*, whereas the function of this protein and the potential utilisation as a marker in testicular tumorigenesis need to be further elucidated.

A limitation of the current study is the small number of pure EC samples available. Although mixed NSGCT represents 35–55% of all TGCTs, pure EC is relatively uncommon. A larger cohort of methylation profiling with bigger sample size should better define the critical genes dysregulated by epigenetic changes. Second, the difficulty to obtain fetal or even adult CIS impedes our ability to monitor the gene expression and epigenetic changes during the course of TGCT development. The spatiotemporal changes for the candidate genes identified through this study remain to be examined. Last, we did not observe a significant DNA methylation change for the three previously identified genes (*APOLD1*, *PCDH10* and *RGAG1*; Cheung *et al*, 2010). One possible reason is the heterogeneity of TGCT among different patients. A second reason is the limitation of ChIP-based method in detecting DNA methylation especially for whole-genome profiling. Bisulfite sequencing-based method may improve the accuracy and resolution of 5mC epigenome. In summary, our genome-wide analysis identified methylation changes in several previously unknown genes for testicular ECs.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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