DNA methylation profiles delineate epigenetic heterogeneity in seminoma and non-seminoma

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BACKGROUND: It remains important to understand the biology and identify biomarkers for less studied cancers like testicular cancer. The purpose of this study was to determine the methylation frequency of several cancer-related genes in different histological types of testicular cancer and normal testis tissues (NT).

METHODS: DNA was isolated from 43 seminomas (SEs), 14 non-SEs (NSEs) and 23 NT, and was assayed for promoter methylation status of 15 genes by quantitative methylation-specific PCR. The methylation status was evaluated for an association with cancer, and between SEs and NSEs.

RESULTS: We found differential methylation pattern in SEs and NSEs. MGMT, VGF, ER-β and FKBP4 were predominately methylated in NSEs compared with SEs. APC and hMLH1 are shown to be significantly more methylated in both subtypes in comparison with NT. When combining APC, hMLH1, ER-β and FKBP4, it is possible to identify 86% of the NSEs, whereas only 7% of the SEs.

CONCLUSIONS: Our results indicate that the methylation profile of cancer-associated genes in testicular cancer correlates with histological types and show cancer-specific pattern for certain genes. Further methylation analysis, in a larger cohort is needed to elucidate their role in testicular cancer development and potential for therapy, early detection and disease monitoring.

Keywords: testicular cancer; DNA methylation; epigenetics; biomarker; seminoma; non-seminoma

Testicular cancer is the most commonly diagnosed malignancy among young men aged 15 to 40 years, and its incidence has doubled in the past 40 years (Chia et al, 2010). An annual increase of 3–6% is reported for Caucasian populations. In the United States, approximately 8500 newly diagnosed testicular cancer cases and 350 deaths were expected in 2010 (Jemal et al, 2010). Testicular germ cell tumours (TGCTs) represent over 95% of the testicular cancers and histopathologically are classified into two major groups of seminomas (SE) or non-SEs (NSE), frequently occurring as mixed tumours (Holmes et al, 2008). Histologically, SE resembles primordial germ cells/gonocytes, whereas NSE shows somatic, primitive embryonal or extra-embryonal differentiation (Horwich et al, 2006). Carcinoma in situ or intra-tubular germ cell neoplasia unclassified are believed to be the origin of both SE and NSE (Rajpert-De Meyts, 2006). They have a primordial germ cell/gonocyte origin, and it is important to emphasise that epigenetic reprogramming is known to occur during germ cell development.

The treatment of testicular cancer includes orchectomy, and according to the metastatic condition surveillance, or adjuvant retroperitoneal surgery, radiation and chemotherapy are offered. The decision whether to choose the adjuvant treatment regimen is based on clinical parameters, leading to around 25% development of metastasis in patients on surveillance or unnecessary adjuvant treatments in 20% of the patients. Genetic understanding of each tumour will enable to better tailor the treatment.

The importance of epigenetic alterations has long been demonstrated in carcinogenesis. Several studies have shown that methylation-associated silencing inactivates certain tumour suppressor genes (TSGs) as effectively as mutations and is one of the cancer-predisposing hits described in Knudson’s two hit hypothesis. Promoter methylation, the most studied epigenetic alteration, is also increasingly recognised as a major mechanism of gene inactivation during TGCT progression (Manton et al, 2005; Ellinger et al, 2009). CG dinucleotide-rich regions, also known as CpG islands, in or near the proximal promoter regions of genes are targets for DNA methylation, leading to effective transcriptional silencing (Herman, 1999). In normal cells, CpG methylation is an important mechanism for regulating gene expression, whereas in cancer cells, aberrant promoter methylation (hypermethylation) can lead to abnormal gene silencing, including repression of TSGs. Another relevant aspect is that environmental and endogenous conditions can influence the epigenetic processes. The exposure to
certain environmental risk factors may be related to the onset of cancer or to participate in carcinogenesis (Brait et al, 2009). There are many evidences that endogenous factors (such as oestrogens or androgens inhibitors) exposure may lead to cancer (Godmann et al, 2009), and endogenous factors (like hormonal stimuli) induce methylation of promoter region of certain genes (Kutanzi et al, 2010). Even though TGCT has high survival rates due to good responses to therapy, significant consequences of multimodality therapies exist with regard to general health, secondary late malignancies, reproduction and economic productivity (Sokoloff et al, 2007). A great need for understanding TGCT biology still exists to help curb its increasing incidence and potentially adopt effective prevention strategies.

Several genetic alterations have been shown in TGCT’s. For example, abnormalities in the short arm of chromosome 12 (Mostert et al, 1998), as well as loss on chromosomes 1, 3, 5, 9, 11, 12q, 13q, 17p and 18q (Mathew et al, 1994; Lothe et al, 1995; al-Jehani et al, 1995; Honorio et al, 2003; Oosterhuis and Looijenga, 2005) have been reported. But major TSGs having a role in TGCTs are yet to be identified (Honorio Looijenga, 2005) have been reported. But major TSGs having a role in TGCTs are yet to be identified (Honorio et al, 2003). One of the most intriguing questions in the biology of TGCTs is how such distinct histological tumour subtypes (SE and NSE) can arise from the same cell type (cell of origin). Both subtypes exhibit similar cyogenetic abnormalities (Lutzker and Barnard, 1998). So far, molecular alterations that could distinct SEs from NSEs have not been clear yet. A pioneer study, performed in 1991, has shown that hypermethylation was abundant in NSEs, but not in SEs (Peloutok, 1991). In 2002, Smiraglia et al (2002) demonstrated significant epigenetic differences between SEs and NSEs by a global methylation approach. In addition, studies of the X chromosome have demonstrated little or no methylation in SEs, and increased methylation in NSEs, particularly in more highly differentiated NSEs (Looijenga et al, 1997). Netto et al (2008) analysed global methylation status by immunohistochemistry and concluded that SEs cells generally retain the lack of methylation that occurs due to normal developmental erasure of methylation marks, whereas NSEs do not, thus showing more methylation. This was independently shown as well (Wermann et al, 2010).

In the present study, we used a candidate gene approach to investigate the methylation profile of 57 primary TGCT (of which 43 were SEs and 14 were NSEs) and 23 normal testis (NT) by quantitative methylation-specific PCR (QMSP). QMSP has been successfully used in other tumour models and has the benefit of providing accurate and precise data regarding the level of methylation in the various tumours. Six of the genes we evaluated, including ARF, APC, MGMT, RAR-β2, CCNA1 and hMLH1, were previously shown to be aberrantly methylated in TGCT (Muller et al, 2000; Koul et al, 2002; Honorio et al, 2003; Olszow et al, 2005; Lind et al, 2007). The remaining nine genes we have studied has been found to be methylated in other cancer types, but not yet tested in TGCT; these genes include AIM1, PGP9.5, S100A2, ER-α, ER-β, MCM, VGF, FKBP4 and SSBP2. Among the later genes, four genes (MCAM, VGF, FKBP4 and SSBP2) were recently discovered by our group, using a pharmacological unmasking strategy in different cancer types (MCAM, VGF, FKBP4 and SSBP2) (Hoque et al, 2008).

**Materials and Methods**

**Study cohort**
The TGCT and NT samples were retrospectively collected from the Johns Hopkins Medical Institutions tissue archive. To be included in the cohort, an eligible patient had to have a confirmed diagnosis of TGCT (or normal tissue) and a sufficient amount of archived tumour (or normal) material to allow for DNA extraction (tissue preserved in sectioned blocks; > 50% of tumour cells). A total of 75% (43 out of 57) of our TGCT samples were SEs and 25% (14 out of 57) were NSEs. We also analysed 23 NT from archived samples (tissue removed on castration procedures). Thus, a total of 80 samples (57 tumours and 23 normals) were tested for methylation pattern by QMSP. Demographic and clinical information was obtained from the computerised tumour registry at the Johns Hopkins Healthcare System. The tumours were classified according to the WHO Classification of Tumours, Pathology and Genetics of the Urinary System and Male Genital Organs (Looijenga, 2009). Patient characteristics included in this study are summarised in Table 1. Approval for research on human subjects was obtained from the Johns Hopkins University Institutional Review Boards. This study qualified for exemption under the US Department of Health and Human Services policy for protection of human subjects (45 CFR 46.101(b)) (IRB 03-11-12-06e).

**Gene selection**
A total of 15 genes were selected for promoter methylation status analysis. Among these genes, six were previously assessed on testicular tissues (ARF, CCNA1, MGMT, MLH1, APC and RAR-β2; Muller et al, 2000; Koul et al, 2002; Honorio et al, 2003; Olszow et al, 2005; Lind et al, 2007), five were not yet tested in this tissue type (AIM1, PGP9.5, S100, ER-α and ER-β; Fiegl et al, 2004; Brait et al, 2008; Carvalho et al, 2008), and four were recently identified genes by our pharmacological unmasking strategy in different cancer types (MCAM, VGF, FKBP4 and SSBP2) (Hoque et al, 2008).

**DNA extraction**
After initial patient de-identification, all original histological slides from the TGCT or normal specimens were reviewed by a pathologist to reconfirm the diagnosis. A representative block was retrieved for DNA extraction. Microdissected NT samples from autopsy material were used for controls. Histological slides from the formalin-fixed, paraffin-embedded tissue were prepared.

| Characteristic | Number of patients (%) |
|---------------|------------------------|
| **Hystologic diagnosis** |                      |
| Seminoma       | Non-seminoma           | Normal             |
| Total samples  | 43 (100)               | 14 (100)           | 23 (100)             |
| Site           |                        |                    |
| Right          | 25 (58.1)              | 10 (71.4)          | 5 (21.7)             |
| Left           | 18 (41.9)              | 4 (28.6)           | 3 (13.1)             |
| Unknown        | 0 (0)                  | 0 (0)              | 15 (65.2)            |
| Age (years)    |                        |                    |
| Median age (range) | 34 (22 – 62)   | 27.5 (23 – 38)     | 55 (20 – 81)         |
| Race           |                        |                    |
| Caucasian      | 35 (81.4)              | 10 (71.4)          | 18 (78.3)            |
| African American| 4 (9.3)                 | 2 (14.2)           | 5 (21.7)             |
| Others         | 4 (9.3)                | 3 (21.4)           | 0 (0)                |
| **Stage**      |                        |                    |
| I              | 40 (93.0)              | 6 (42.9)           |                     |
| II             | 3 (7.0)                | 5 (35.7)           |                     |
| III            | 0 (0)                  | 3 (21.4)           |                     |

*NA = Not applicable.*

Table 1 Demographic and clinical characteristics of testicular cancer patients (N = 57, seminomas = 43, non-seminomas = 14) and normals (N = 24)
Slides were microdissected to obtain >50% neoplastic cells. DNA was extracted using standard protocols as previously described (Brait et al, 2008). Briefly, DNA was obtained by digestion with 50 μg ml⁻¹ proteinase K (Roche, Indianapolis, IN, USA) in the presence of 1% SDS at 48 °C for 2 days, followed by phenol/ chloroform extraction and ethanol precipitation, and finally dissolved in 20 μl of LoTE (2.5 mmol l⁻¹ EDTA and 10 mmol l⁻¹ Tris-HCL) and stored at −20 °C until used.

Sodium bisulphite treatment

DNA extracted from tumour or normal tissue was subjected to bisulphite treatment with the EpiTect Bisulphite kit (QIAGEN, Valencia, CA, USA), according to the manufacturer’s instructions. Treated DNA was stored at −80 °C until used.

Methylation analysis

Bisulphite-modified DNA was used as a template for fluorescence-based real-time PCR, as previously described (Brait et al, 2009). Amplification reactions were carried out in triplicate in a final volume of 20 μl containing 1 μl of bisulphite-modified DNA, 600 nm concentrations of forward and reverse primers, 200 nM probe, 0.6 U of platinum Taq polymerase (Invitrogen, Frederick, MD, USA), 200 μM concentrations each of dATP, dCTP, dGTP and dTTP, and 6.7 mM MgCl₂. Primers and probes were designed to specifically amplify the promoters of the 15 genes of interest and the promoter of a reference gene, β-actin; primer and probe sequences, and annealing temperatures are provided in Supplementary Table 1. Amplifications were carried out using the following profile: 95 °C for 3 min, followed by 50 cycles at 95 °C for 15 s and 60 °C for 1 min. Amplification reactions were carried out in 384-well plates in a 7900HT sequence detector (Applied Biosystems, Foster City, CA, USA) and were analysed by a sequence detector system (SDS 2.3; Applied Biosystems). Each plate included studied DNA samples, positive (in-vitro methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls and multiple water blanks. Leukocyte DNA from a healthy individual was methylated in vitro with excess SssI methyltransferase (New England Biolabs Inc., Beverly, MA, USA) to generate completely methylated DNA, and serial dilutions (90–0.009 ng) of this DNA (New England Biolabs Inc., Beverly, MA, USA) and were analysed by a sequence detector system (SDS 2.3; Applied Biosystems). Each plate included studied DNA samples, positive (in-vitro methylated leukocyte DNA) and negative (normal leukocyte DNA or DNA from a known unmethylated cell line) controls and multiple water blanks. Leukocyte DNA from a healthy individual was methylated in vitro with excess SssI methyltransferase (New England Biolabs Inc., Beverly, MA, USA) to generate completely methylated DNA, and serial dilutions (90–0.009 ng) of this DNA were used to construct a calibration curve for each plate. All samples were within the assay’s range of sensitivity and reproducibility, based on the amplification of the internal reference standard (threshold cycle (CT) value for β-actin of 40). The relative level of methylated DNA for each gene in each sample was determined as a ratio of methylation-specific PCR-amplified gene to β-actin (reference gene), and then multiplied by 1000 for easier tabulation (average value of triplicates of the gene of interest divided by the average value of triplicates of β-actin × 1000).

This measure represents the relative level of methylation in a particular sample and was used for direct comparison of samples. For a tumour sample to be considered methylated at a specific gene, it had to meet two specific criteria. Amplification must have been reproducible at least two times within the triplicate run, and the β-actin-normalised mean methylation value must have fallen within the range of the serial standard curve dilutions. The lack of DNA contamination was verified by the absence of amplification of a distilled water negative control for each QMSP run.

Statistical analysis

The primary objective in this study was to describe the methylation patterns of 15 well-established or putative TSGs in NT and two TGCT types, SE and NSE.

Gene methylation was treated as a continuous variable. Univariate and multivariable logistic regression models were constructed sequentially to examine the association of gene methylation with the disease status (tumour vs normal). Odds ratios (OR) were estimated with 95% confidence intervals (CI), which quantified the strength of the association and its uncertainty. The final model was selected by using the backward selection method in multivariable logistic regression.

Correlation in methylation between genes was also examined so as to aid in the selection of genes that independently contributed to distinguishing tumour from normal samples (or the two subtypes: SEs and NSEs). Receiver-operating characteristic (ROC) analyses were conducted to evaluate the marker validity to differentiate tumour and normal. Sensitivity, specificity and area under the curve (AUC) were estimated along with 95% CI. Correlated ROC curves were compared using DeLong’s method, which is a non-parametric approach (DeLong et al, 1988).

On the basis of the correlations among the 15 genes obtained using a non-parametric approach along with the individual gene performance, the genes shown to be independent of each other by Spearman correlation test, were selected to be included in a logistic regression model. This model was used to assess the probability of tumour. The genes explored further for panel performance were those with P < 0.10 in the final model. Multi-variable logistic regression was performed with genes of interest, as well as patient characteristics (age, race and site), entered the model simultaneously and were eliminated by backwards selection. An association was considered statistically significant with P-value < 0.05. All P-values reported are two-sided.

The optimal cutoff was determined as the point at which it simultaneously maximised sensitivity and specificity. We used cancer cases and NT samples for generating ROC curves for individual genes, and the cut point of methylation values were established with the values that optimally differentiate the two sample groups. Representative ROC curves are available in Supplementary Figure 1.

RESULTS

Patients with TGCT were aged between 22 and 62 years (median = 33). Age range of controls was 20 to 81 (median = 55). Patients with SE were aged between 22 and 62 years (median = 34), and for NSE, were between 23 and 38 years (median = 27.5). Control subjects were significantly older compared with patients with overall TGCT (P < 0.05, Wilcoxon non-parametric test). The samples include 46 stage-I TGCT and 11 ≥ stage-II TGCT. Thirty-five TGCT analysed were from right site and 22 from left site. The majority of the samples were obtained from Caucasian. Patient characteristics included in this study are summarised in Table 1.

Overall methylation frequency in TGCTs

A total of 15 genes (AIM1, ARF, CCNA1, MGMT, hMLH1, PGP9.5, S100A2, APC, RAR-β2, ER-α, ER-β, MCAM, VGF, FKBP4 and SSBP2) were analysed for promoter methylation using QMSP in 57 tumour samples, comprising 43 SEs and 14 NSEs, and 23 NT samples. The observed frequencies of each gene using optimal cutoffs that maximised specificity and sensitivity are summarised in Table 2.

VGF show a significant cancer-specific methylation (P = 0.008, by Fisher’s exact test). A total of 14 out of 57 TGCT showed methylation of the promoter region of VGF, whereas no methylation was observed in NT. hMLH1 was significantly methylated in TGCT (39% (22 out of 57)) in comparison with normal (4% (1 out of 23)); P = 0.002, by Fisher’s exact test).

Methylation in SE and NT

A total of 15 genes (AIM1, ARF, CCNA1, MGMT, hMLH1, PGP9.5, S100A2, APC, RAR-β2, E-Rz, ER-β, MCAM, VGF, FKBP4 and
SSBP2 was analysed for promoter methylation in 23 NT and 43 SE cases by QMSP. Results for all genes in all SE cases and controls (NTs) are presented in Table 3a. Individual gene sensitivity and specificity was determined on the basis of an empiric cutoff values.

| Genes    | Number of tumors with methylation/total number of tumors (%, 95% CI) | Number of tumors with methylation/total number of tumors (%, 95% CI) |
|----------|---------------------------------------------------------------|---------------------------------------------------------------|
| AIM1     | 44,817                                                       | 3/57 (5.1, 1.1 – 14.6)                                      |
| ARF      | 0.3                                                          | 2/57 (3.5, 0.4 – 12.1)                                      |
| CCNA1    | 8.173                                                        | 5/57 (8.8, 2.9 – 19.3)                                      |
| MCAM     | 1073                                                         | 23/57 (40.4, 27.6 – 54.2)                                   |
| MGMT     | 0.193                                                        | 8/57 (14.6, 3.3 – 25.8)                                     |
| MLH1     | 0.096                                                        | 22/57 (38.6, 26.0 – 52.4)                                   |
| PGP9     | 177.09                                                      | 2/57 (3.5, 0.4 – 12.1)                                      |
| S100A2   | 858.15                                                      | 23/57 (40.4, 27.6 – 54.2)                                   |
| SSBP2    | 623.99                                                      | 8/57 (14.6, 3.3 – 25.8)                                     |
| APC      | 19.077                                                      | 7/57 (12.3, 5.1 – 23.7)                                     |
| RAR-β2   | 14.273                                                      | 1/57 (1.8, 0.4 – 9.4)                                       |
| VGF      | 0.023                                                        | 14/57 (24.6, 14.1 – 37.8)                                   |
| ER-α     | 72.072                                                      | 6/57 (10.5, 4.0 – 21.5)                                     |
| ER-β     | 10.145                                                      | 1/57 (2.1, 0.1 – 12.3)                                      |
| FKB4P4   | 35.001                                                      | 7/57 (12.5, 5.1 – 23.7)                                     |

Table 3a Seminomas and normal testicular samples: (a) promoter methylation frequency in seminomas and normal testicular samples

| Genes    | Sensitivity, % (95% CI) | Specificity, % (95% CI) | AUC (95% CI) |
|----------|-------------------------|-------------------------|--------------|
| MCAM, APC| 0.649371                | 76.7 (61.4 – 88.3)       | 78.3 (56.3 – 92.5) | 0.81 (0.70 – 0.92) |
| MCAM, APC, MLH1 | 0.620704 | 79.1 (64.0 – 90.0) | 78.3 (56.3 – 92.5) | 0.84 (0.74 – 0.94) |
| MCAM, APC, MLH1, AIM1 | 0.601593 | 81.4 (66.6 – 91.6) | 78.3 (56.3 – 92.5) | 0.85 (0.75 – 0.95) |
| MCAM, APC, MLH1, AIM1, PGP9.5 | 0.590917 | 81.4 (66.6 – 91.6) | 78.3 (56.3 – 92.5) | 0.86 (0.76 – 0.95) |

Table 3b Seminomas and normal testicular samples: (b) analysis of gene panels, based on logistic regression model, considering optimal cutoff for differentiating both groups

Methylation in NSE and NT

The frequency of promoter methylation, including the cutoff value at each gene included in this panel is listed in Tables 4a. Briefly the methylation frequency were: AIM1 21%, ARF 0%, CCNA1 14%, MCAM 71%, MGMT 50%, MLH1 71%, PGP9.5 14%, S100A2 57%, SSBP2 57%, APC 50%, RAR-β2 21%, VGF 50%, ER-α 36%, ER-β 64% each and FKB4P4 36%.

On the basis of the correlations among all the 15 genes obtained using a non-parametric approach along with the individual gene performance, six genes (APC, VGF, MGMT, hMLH1, ER-β and FKB4P4) were shown to be independent of each other and each of them shows the strongest predictive potential representing the corresponding cluster, and thus, were selected to be included in a multivariable logistic regression model that modelled the probability of tumour. However, issues arose with such small sample size, that for MGMT, APC and FKB4P4, the maximum likelihood estimates are not possible, as there is no variation in methylation values for NT samples (e.g., all normal samples had zero methylation values for each of these three genes). Thus, only hMLH1, APC and ER-β were considered in the multivariable logistic model. It turned out that hMLH1 remained statistically significant in this final model (P = 0.044), which indicated its independent effect in predicting tumour. Performance of hMLH1 combined with APC and ER-β was explored as well. The significance of hMLH1 was examined with adjustment for the current available patient characteristics (age, race and site), using the logistic regression model. The independent effect of the genes of interest was evaluated with adjustment for age only. Results indicated that association of hMLH1 hypermethylation with by maximising sensitivity and specificity, and the AUC values were calculated for each gene. At least one TSG locus was methylated in 37 out of 43 SE cases (86%), and a total of 11 out of 43 (25.6%) tumour samples showed methylation at three or more of the loci. By a non-parametric statistical approach (DeLong’s method), we determined the correlation of methylation events among all the 15 genes. By Spearman correlation test, eight genes (AIM1, CCNA1, MCAM, PGP9.5, hMLH1, ARF, VGF and APC) showed to be independent of each other and were selected to be included in a logistic regression model, which predict the probability of tumour. Patient characteristics (age, race and site) were included in the multi-variable logistic regression model, together with the previously mentioned eight genes. Smoking status and alcohol consumption were not considered in this analysis, as there were too many unknowns reported. Backward selection was used and the significance level of staying in the final model was set at P < 0.10. Among all eight genes, only APC and MCAM remained statistically significant and stayed in the final model (P = 0.057 and P = 0.035, respectively) after controlling for patient characteristics (age, race and site). We then grouped the independent genes in different combinations (AIM1, CCNA1, MCAM, PGP9.5, hMLH1, ARF, VGF and APC), considering optimal cutoffs that separated both groups, and established the maximum possible specificity at 78%, and each combination had a slight different sensitivity, with no significant improvement as shown in Table 3b.
methylated gene was observed methylated in 12 out of 14 (86%) of the samples with 100% specificity for NSEs.

### Methylation levels and frequency across sample types

When dividing the groups into normal, SEs and NSEs, one would expect to see a differential methylation of the analysed genes, as described.

### Table 4a

| Genes  | Cutoff | Number of tumors with methylation/ total number of tumors (%) | Number of tumors with methylation/ total number of tumors (%) |
|--------|--------|---------------------------------------------------------------|---------------------------------------------------------------|
| AIM1   | 44.817 | 3/14 (21.4, 4.7 – 50.8)                                       | 1/23 (4.3, 0.1 – 22.0)                                        |
| ARF    | 0.3    | 2/14 (0.0, 0.6 – 6.9)                                          | 2/23 (8.7, 1.1 – 28.0)                                        |
| CCNA1  | 8.173  | 8/14 (57.1, 28.9 – 82.3)                                       | 4/43 (9.3, 2.6 – 22.1)                                        |
| MGMT   | 0.193  | 1/4 (0.0, 0.0 – 14.3)                                          | 1/23 (0.0, 0.0 – 14.3)                                        |
| MLH1   | 0.359  | 1/14 (0.0, 0.0 – 35.7)                                         | 1/43 (0.0, 0.0 – 35.7)                                        |
| PGF    | 177.09 | 2/14 (14.3, 1.8 – 42.8)                                        | 0/43 (0.0, 0.0 – 8.2)                                         |
| APC    | 19.077 | 2/14 (14.3, 1.8 – 42.8)                                        | 0/43 (0.0, 0.0 – 8.2)                                         |
| RARβ2  | 14.273 | 3/14 (21.4, 4.7 – 50.8)                                        | 0/23 (0.0, 0.0 – 4.7)                                         |
| VGF    | 0.023  | 1/4 (0.0, 0.0 – 35.7)                                          | 0/23 (0.0, 0.0 – 4.7)                                         |
| ER-α   | 72.072 | 5/14 (35.7, 12.8 – 64.9)                                       | 0/23 (0.0, 0.0 – 14.3)                                        |
| ER-β   | 10.145 | 9/14 (64.3, 35.1 – 87.2)                                       | 7/23 (30.3, 13.2 – 52.9)                                      |
| FKBP4  | 35.001 | 1/4 (0.0, 0.0 – 35.7)                                          | 0/23 (0.0, 0.0 – 4.7)                                         |

Abbreviation: CI = confidence interval.

### Table 4b

| Combined genes | Cutoff based on predictive probability of tumor (%) | Sensitivity (%) | Specificity (%) | AUC (95% CI)    |
|----------------|-----------------------------------------------|----------------|----------------|----------------|
| MLH1, APC and ER-β| 0.225668                                              | 85.7 (57.2–98.2) | 82.6 (61.2–95.1) | 0.89 (0.75 – 1.00) |

Abbreviations: AUC = area under the curve; CI = confidence interval. *Predictive probabilities were obtained from the logistic regression models.

### Table 5a

| Genes  | Cutoff | Number of tumors with methylation/ total number of tumors (%) | Number of tumors with methylation/ total number of tumors (%) |
|--------|--------|---------------------------------------------------------------|---------------------------------------------------------------|
| AIM1   | 44.817 | 3/14 (21.4, 4.7 – 50.8)                                       | 1/23 (4.3, 0.1 – 22.0)                                        |
| ARF    | 0.3    | 2/14 (0.0, 0.6 – 6.9)                                          | 2/23 (8.7, 1.1 – 28.0)                                        |
| CCNA1  | 8.173  | 8/14 (57.1, 28.9 – 82.3)                                       | 4/43 (9.3, 2.6 – 22.1)                                        |
| MGMT   | 0.193  | 1/4 (0.0, 0.0 – 14.3)                                          | 1/23 (0.0, 0.0 – 14.3)                                        |
| MLH1   | 0.359  | 1/14 (0.0, 0.0 – 35.7)                                         | 1/43 (0.0, 0.0 – 35.7)                                        |
| PGF    | 177.09 | 2/14 (14.3, 1.8 – 42.8)                                        | 0/43 (0.0, 0.0 – 8.2)                                         |
| APC    | 19.077 | 2/14 (14.3, 1.8 – 42.8)                                        | 0/43 (0.0, 0.0 – 8.2)                                         |
| RARβ2  | 14.273 | 3/14 (21.4, 4.7 – 50.8)                                        | 0/23 (0.0, 0.0 – 4.7)                                         |
| VGF    | 0.023  | 1/4 (0.0, 0.0 – 35.7)                                          | 0/23 (0.0, 0.0 – 4.7)                                         |
| ER-α   | 72.072 | 5/14 (35.7, 12.8 – 64.9)                                       | 0/23 (0.0, 0.0 – 14.3)                                        |
| ER-β   | 10.145 | 9/14 (64.3, 35.1 – 87.2)                                       | 7/23 (30.3, 13.2 – 52.9)                                      |
| FKBP4  | 35.001 | 1/4 (0.0, 0.0 – 35.7)                                          | 0/23 (0.0, 0.0 – 4.7)                                         |

Abbreviation: CI = confidence interval.

### Table 5b

| Combined genes | Cutoff based on predictive probability of tumor (%) | Sensitivity (%) | Specificity (%) | AUC (95% CI)    |
|----------------|-----------------------------------------------|----------------|----------------|----------------|
| APC            | 0.3644                                      | 78.6 (49.2–95.3) | 90.7 (77.9–97.4) | 0.87 (0.74–0.99) |
| APC, MLH1, ER-β and FKBP4 | 0.124726                      | 85.7 (57.2–98.2) | 93.0 (80.9–98.5) | 0.90 (0.81–1.00) |

Abbreviations: AUC = area under the curve; CI = confidence interval. *Predictive probabilities were obtained from the logistic regression models.
genes analysed throughout the testicular tissues are shown in Figure 1.

**Association between DNA methylation changes and clinicopathological factors**

There were no apparent correlations between any of the gene tested with any clinicopathological parameters, including age, site, race and disease stages, perhaps due to the limited number of sample size (data not shown). As shown before, we found differential methylation patterns in two different histological types (SEs and NSEs). Methylation values were compared as continuous variables, as well as dichotomised, and no correlation was observed in either analysis.

**DISCUSSION**

In this study, we attempted to define a set of methylation markers that would allow for an accurate discrimination among the two histological types.
most common types of TGCT (SE and NSE); as each type displays dissimilar clinical behaviour and successful pre-operative cytological or histological assessment is restricted. Through gene promoter methylation profiling with QMSP, six genes were found to be differentially methylated in the two tumour types. In particular, higher PGP9.5 methylation frequency was detected in SEs than NSEs, whereas high methylation frequency of MGMT, VGF, ER-β and FKBP4 were associated with NSE. Remarkably, both SE and NSE were methylated for APC and hMLH1. Netto et al (2008) showed evidence that SEs remain unmethylated, whereas the other histological types arise after de novo methylation, suggesting that they may arise in distinct periods of the development process of the germ cells, and that their genome methylation status may determine the degree of differentiation of the cells. Wermann et al (2010) also observed the same pattern of all SEs being unmethylated (as well as tumours of similar histology originating in other organs), whereas NSEs were consistently hypermethylated. Koul et al, (2002), using conventional MSP, analysed a panel of 21 genes and observed a near absence of methylation in SEs and a higher percentage in NSEs, suggesting a role for different panel of methylation-induced inactivation of TSGs in two common types of TGCT. Similar to our results, they also observed presence of methylation in the MGMT promoter in NSEs (21 vs 14% observed in this study) and absence in SEs. The other two genes that showed this same pattern were not evaluated by our study (BRCA1 and RASSF1A), and their panel did not include the other genes observed to be methylated in NSEs vs SEs (VGF, ER-β and FKBP4). Subtype-specific patterns of global methylation were also previously reported in TGCT (Smiraglia et al, 2002). Peltomaki (1991) observed hypomethylation in SEs, whereas NSEs were largely hypermethylated. The same observation was made in studies of the X chromosome.

**Figure 1** Continued.
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(Loojenga et al, 1997). In breast cancer, Lehmann et al (2002) observed that the presence of promoter methylation in DAPK was frequent in invasive lobular cancer (53%) and not frequent in another histological subtype (9% invasive ductal carcinoma). Other reports that include different epigenetic alterations in histological subtypes of the same cancer type include: presence of methylator phenotype in low-grade gliomas when compared with de novo glioblastomas (Laflaire et al, 2011), high frequency of SPN methylation in small cell lung cancer, whereas a rare frequency in non-small cell lung cancer (Osada et al, 2002). Consistent with epigenetic heterogeneity, there is a wide search for genetic alterations that would be able to distinguish SEs and NSEs. Coffey et al (2008) reported that Kit mutations are predominant in SEs and very rarely observed in NSEs. Major accentuated genetic differences are yet to be discovered.

Previous studies did indicate a role for hypermethylation of TSG promoters in the pathogenesis of SE. In our data, when comparing SEs versus NT samples, the genes that showed higher frequency of methylation in SE were PGP9.5, hMLH1 and ER-β, when compared with the observed frequency in normal samples. In combination, the strongest pair is APC and MCAM, showing 78.3% frequency in tumours and 23.3% in normals. With the same cutoff, adding hMLH1, the frequency in tumours was 79.1%, and adding both AIM1 and PGP9.5, or only one of them, it increases to 81.4%, with the same percentage in normals (23.3%). This panel containing four genes showed reasonable percentages to distinguish normal and tumour tissue. MCAM had not been previously evaluated in TGCT. In prostate cancer, other showed 85% methylation in tumours vs 0% in normal prostate (Hoque et al, 2008). To our knowledge, PGP9.5 and AIM1 had not been analysed in this tumour type. APC was previously observed with 10% of methylation in tumours and 0% in NT, but both positive tumours were NSE (Koul et al, 2002), and no APC methylation has been reported in S-se; it is important to emphasise that this study was performed using conventional MSP, a less sensitive technique than QMSP used in the present study (Hoque et al, 2004), which may explain the different percentages observed. In this same study, hMLH1 showed only 4% methylation in tumours and 0% in normals, occurring in both SEs and NSEs. In contrast to the comparison between NSEs and normals, there are two genes (MGMT and VGP) that stand out alone, both with a 0% frequency in normals and 50% frequency in SEs. VGP is a gene recently reported to be methylated in cancer by our group (Hoque et al, 2008) and had never been analysed in TGCT. MGMT had been reported to be frequently methylated in TGCT (Smith-Sorensen et al, 2002), showing 69% in NSEs. In 2004, Koul et al (2004) showed methylation in MGMT in 20% of NSEs analysed by conventional MSP, a less sensitive technique as mentioned earlier. Using empiric cutoffs, at least one of the three genes (hMLH1, APC and ER-β) was methylated in 13 out of 14 (93%) of NSE cases and 10 out of 23 (43%) of the NT samples. Previously reported methylation of APC and hMLH1 were 10% to less than 5%, respectively (Koul et al, 2002). There are no reported studies exploring methylation status of ER-β in TGCT. The downregulation of this gene was reported in S-se (Esposito et al, 2010); they detected decreased expression of ERβ protein in SEs when compared with NT, but they did not evaluate promoter methylation. This gene encodes a member of the family of oestrogen receptors and superfamily of nuclear receptor transcription factors. The gene product contains an N-terminal DNA-binding domain and C-terminal ligand-binding domain, and is localised to the nucleus, cytoplasm and mitochondria. It is able to interact with specific DNA sequences to activate transcription. Some isoforms dominantly inhibit the activity of other oestrogen receptor family members.

Epigenetic inactivation of hMLH1 is found in a wide range of cancers. hMLH1 is a DNA mismatch repair (MMR) gene and is an essential component of the DNA MMR pathway, and is frequently mutated in hereditary non-polyposis colon cancer also known as Lynch syndrome. Activation of the MMR pathway may trigger DNA damage signalling, a process which induces cell cycle arrest and can lead to cell death in case of major DNA damages (for review, please see Jiricny (2006)). hMLH1 is the most prominent target of epigenetic silencing in the MMR pathway in sporadic tumours, comprising ovarian, head and neck, breast and colorectal cancer (Herman et al, 1998). However, hypermethylation of hMLH1 is often associated with other hypermethylated genes, which complicates mechanistic interpretation of associations with response to therapy in patients (Shen et al, 2007). Mechanistic investigations using in vitro system have shown that treatment of hMLH1-methylated colon cancer cell lines with the demethylating agent 5-azacytidine (5-aza-dC) restores hMLH1 expression and subsequently renders the cells MMR proficient (Herman et al, 1998).

Promoter methylation of hMLH1 has been associated with chemoresistance to cisplatin-based therapies in ovarian cancer more than a decade ago (Strathdee et al, 1999). In our study, both SEs and NSEs displayed methylation in this locus, 27% and 71%, respectively. It is well established that NSE and SE (all TGCT) are widely responsive to cisplatin-based therapy (Bosl and Motzer, 1997), so one wouldn’t expect the high presence of hMLH1 methylation in this tumour type. Olasz et al (2005) hypothesised that this epigenetic alteration could be linked to the chemoresistance in a small group of testicular tumours, but could not observe any association. On the other hand, Honecker et al (2009) investigated a larger sample and observed that low or low expression of hMLH1 (on the protein level) was significantly associated with cisplatin-resistant TGCTs, and the absence of expression was also correlated with presence of hMLH1 promoter methylation. Probably the mechanisms of resistance/sensitivity to this chemotherapeutic agent are different among the ovarian and TGCT. It is widely accepted that hMLH1 dysfunction induces microsatellite instability (MSI) in various cancers, and loss of hMLH1 expression was related to its promoter methylation. However, no correlation was observed between hMLH1 methylation and MSI in TGCTs (Olasz et al, 2005). In our study, hMLH1 methylation was detected in 22 out of 57 (39%) of TGCTs. However, we did not perform expression or MSI analysis in these samples; therefore, we are not able to correlate hMLH1 methylation with expression as a whole and MSI. Esteller et al (2000) reported that promoter methylation of MGMT results in enhanced sensitivity to alkylating agents in gliomas. In the present study, MGMT was highly methylated in NSEs, which could indicate that the epigenetic silencing of this gene may be also linked to the cisplatin-based chemotherapic response, but this data needs to be further explored in a well-defined cohort consisting of cisplatin responsive group and non-responsive group.

Silencing TSG by DNA promoter methylation is an important feature of cancer. It is known that cancer types may vary in their epigenetic profiles in a tissue-specific pattern (Esteller et al, 2001). We used a candidate gene approach to test 15 promoter regions in a testicular cohort; all the markers evaluated here have shown differential methylation between normal and cancer tissues in two histological subtypes of germ cell cancers (Hoque et al, 2009). Our data shows different patterns of methylation not only between tumours and normal, but also between the two histological subtypes of germ cell tumours. These distinct promoter methylation profiles of the two main subtypes of germ cell testicular cancer, SEs and NSEs, may shed a light on how they develop and differentiate from the same cell of origin. In conclusion, our study constitutes a comprehensive profile of hypermethylated genes in testicular tissues. Several of genes tested in this study were not evaluated for promoter methylation in this cancer types before, which may partially be due to rarity of the disease and limited number of samples. The observation of the higher proportion of promoter methylation of putative and established TSGs in NSEs when compared with SEs may enrich
our knowledge of this tumour type. Further study using larger cohorts is needed to evaluate the potential use of these methylation markers in the detection, prognosis and therapeutic outcome of this rare tumour.

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