Molecular Cancer

Research

Characteristic promoter hypermethylation signatures in male germ cell tumors

Sanjay Koul[1], Jane Houldsworth[2], Mahesh M Mansukhani[1], Alessia Donadio[3], James M McKiernan[4], Victor E Reuter[5], George J Bosl[3], Raju S Chaganti[3] and Vundavalli V Murty*[1,6]

Address: [1]Department of Pathology, College of Physicians & Surgeons of Columbia University, 630 West 168th Street, New York, NY, 10032, USA, [2]Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA, [3]Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA, [4]Department of Urology, College of Physicians & Surgeons of Columbia University, 630 West 168th Street, New York, NY, 10032, USA, [5]Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA and [6]Institute for Cancer Genetics, College of Physicians & Surgeons of Columbia University, 630 West 168th Street, New York, NY, 10032, USA

E-mail: Sanjay Koul - sk1276@columbia.edu; Jane Houldsworth - houldswj@mskcc.org; Mahesh M Mansukhani - mm322@columbia.edu; Alessia Donadio - aldonadio@hotmail.com; James M McKiernan - jmm23@columbia.edu; Victor E Reuter - reuterv@mskcc.org; George J Bosl - boslg@mskcc.org; Raju S Chaganti - chagantr@mskcc.org; Vundavalli V Murty* - vvm2@columbia.edu
*Corresponding author

Abstract

Background: Human male germ cell tumors (GCTs) arise from undifferentiated primordial germ cells (PGCs), a stage in which extensive methylation reprogramming occurs. GCTs exhibit pluripotentality and are highly sensitive to cisplatin therapy. The molecular basis of germ cell (GC) transformation, differentiation, and exquisite treatment response is poorly understood.

Results: To assess the role and mechanism of promoter hypermethylation, we analyzed CpG islands of 21 gene promoters by methylation-specific PCR in seminomatous (SGCT) and nonseminomatous (NSGCT) GCTs. We found 60% of the NSGCTs demonstrating methylation in one or more gene promoters whereas SGCTs showed a near-absence of methylation, therefore identifying distinct methylation patterns in the two major histologies of GCT. DNA repair genes *MGMT*, *RASSF1A*, and *BRCA1*, and a transcriptional repressor gene *HIC1*, were frequently methylated in the NSGCTs. The promoter hypermethylation was associated with gene silencing in most methylated genes, and reactivation of gene expression occured upon treatment with 5-Aza-2' deoxycytidine in GCT cell lines.

Conclusions: Our results, therefore, suggest a potential role for epigenetic modification of critical tumor suppressor genes in pathways relevant to GC transformation, differentiation, and treatment response.

Background

Promoter methylation has been well recognized as an important epigenetic change in the development of cancer [1]. Normally, CpG islands in the promoter regions of a number of genes are present in an unmethylated state [2]. Aberrant methylation of CpG islands in promoters is characteristic of several genes in cancer leading to loss of gene expression. A nonrandom pattern of promoter hypermethylation has been noted in specific genes in specific tumor types, although some genes are commonly methylated in diverse tumors [3,4]. The extent of aberrant promoter hypermethylation and its association with loss of gene function in cancer suggests that CpG island methylation is an important mechanism in inactivating tumor suppressor genes (TSGs).

Germ cell tumors (GCTs) are the most common cancer in men between the ages 20–40 with an incidence of 4.2 cases per 100,000 [5]. GCTs arise by transformation of spermatogonial lineage cells and display pluripotentiality for embryonal and extra-embryonal lineage differentiation [6]. Histologically, they may present as undifferentiated germ cell (GC)-like seminomas (SGCTs) or highly differentiated nonseminomas (NSGCTs). NSGCTs display complex differentiation patterns that include embryonal, extra-embryonal, and somatic tissue types [6]. Teratomas with somatic differentiation can undergo additional malignant transformation with characteristics of epithelial, mesenchymal, neurogenic, or hematologic tumors [7]. While the majority of GCTs exhibit exquisite sensitivity to cisplatin-based chemotherapy, a small proportion of metastatic tumors remain resistant. Therefore, male GCTs comprise a unique model system to investigate the biology and genetics of GC transformation, differentiation, and chemotherapy resistance/sensitivity [6].

During the life span of a normal GC, extensive methylation reprogramming occurs [6]. However, the role of epigenetic changes in GCT etiology and biology are not well studied. To investigate such a role, we evaluated the status of promoter hypermethylation of 21 genes in GCT specimens and cell lines. We found an absence of promoter hypermethylation in SGCT and acquisition of unique patterns of promoter hypermethylation in NSGCT. We also showed that the hypermethylation leads to loss of expression in most of the genes and reactivate upon treatment with demethylating drug 5-Aza-2' deoxycytidine.

Results

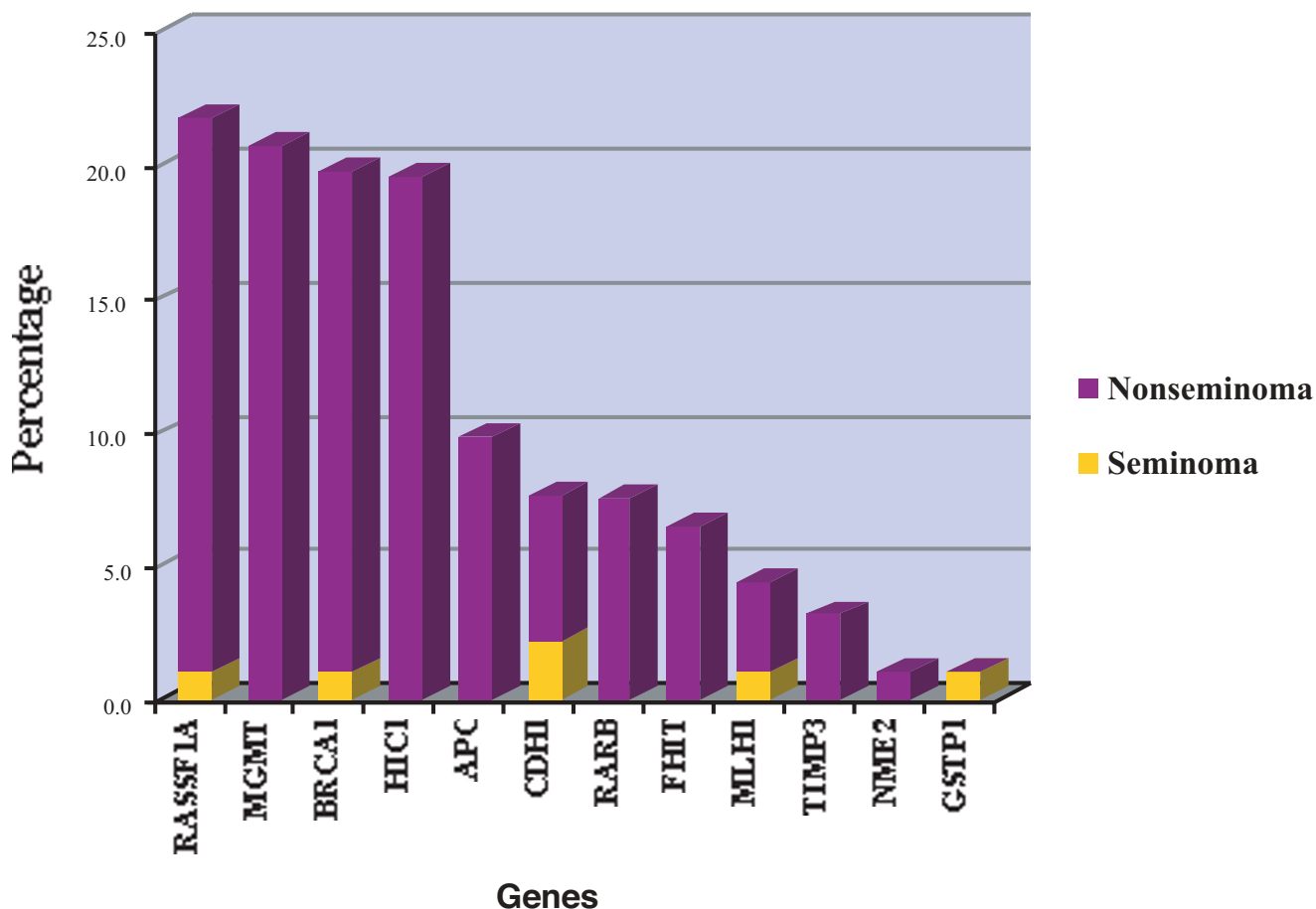## Promoter hypermethylation is common in NSGCT and rare in SGCT

We assessed 92 GCT DNAs representing all histologic subsets of NSGCT, and SGCT, and four normal testes for methylation status of CpG islands of 21 gene promoters by methylation-specific PCR (MSP) (Fig. 1). Of these, 15

genes (*MGMT, RASSF1A, APC, RARB, CDH1, MLH1, TIMP3, GSTP1, DAPK, CDKN2A, p14^{ARF}, BRCA1, FHIT, TP73,* and *HIC1*) had previously been shown to be commonly methylated in various solid tumor types [4]. Six additional genes (*RB1, NME1, NME2, BTG1, NEDD1,* and *APAF1*) were studied because of their possible involvement in genetic alterations in GCT as indicated by LOH studies [8–10]. The *RB1* gene at 13q14.2 showed frequent loss of heterozygosity (LOH) in GCT [9]. The *NME1* and *NME2* genes mapped to 17q21.3 also were affected by LOH and exhibited loss of expression in teratoma [9]. The *BTG1, APAF1,* and *NEDD1* genes mapped to the 12q22 common-deletion region, and thus considered as candidate TSGs in GCT [8,10,11].

Promoter hypermethylation was not found in normal testes for any of the tested genes except *CDH1*. *CDH1* exhibited methylation in two of the four normal testes analyzed. However, promoter hypermethylation was detected in 43 of the 92 (46.7%) GCTs studied with an individual gene frequency of: *RASSF1A*, 21.7%; *MGMT*, 20.7%; *BRCA1*, 19.8%, *HIC1*, 19.6%; *APC*, 9.8%; *RARB*, 7.6%; *CDH1*, 7.6%; *FHIT*, 6.5%, *MLH1*, 4.3%; *TIMP3*, 3.3%; *GSTP1*, 1.1%; and *NME2*, 1.1% (Fig. 1). The remaining nine genes did not show methylation. Hypermethylation of one or more genes was found only in 5 of 29 (17.2%) SGCTs but in 38 of 63 (60.3%) NSGCTs (Fig. 1). Four of the five SGCTs that exhibited promoter hypermethylation were methylated at a single locus and one tumor at two loci, whereas 27 of the 38 NSGCTs exhibited two or more methylated loci. Promoter hypermethylation was seen in all histologic subsets of NSGCT, with yolk sac tumor (YST) exhibiting a higher frequency of methylation compared to other histologies (Fig. 2 & Fig. 3).

Relationship between promoter hypermethylation and gene expression

To examine the biological role of promoter hypermethylation in GCT, we assessed the levels of gene expression by semi-quantitative RT-PCR in 23 tumors (15 NSGCTs and 8 SGCTs) with known methylation status. Eight genes (MGMT, RASSF1A, BRCA1, APC, RARB, CDH1, MLH1, and TIMP3) that exhibited methylation in >3% of the cases were examined (Fig. 4, Table 1). Levels of expression of each gene were assessed by comparing with the respective control values, obtained from the averages calculated from 2 to 4 normal testes, after normalization against ACTB. All tumors with promoter hypermethylation of the MGMT and MLH1 genes exhibited an absence or down-regulated expression of the respective gene, while 8 of 10 cases with RASSF1A methylation and 3 of 5 tumors with RARB methylation showed down-regulated expression (Table 1). The other four genes (BRCA1, APC, CDH1, and TIMP3) did not show a consistent pattern of correlation between methylation and loss of gene expression (Table
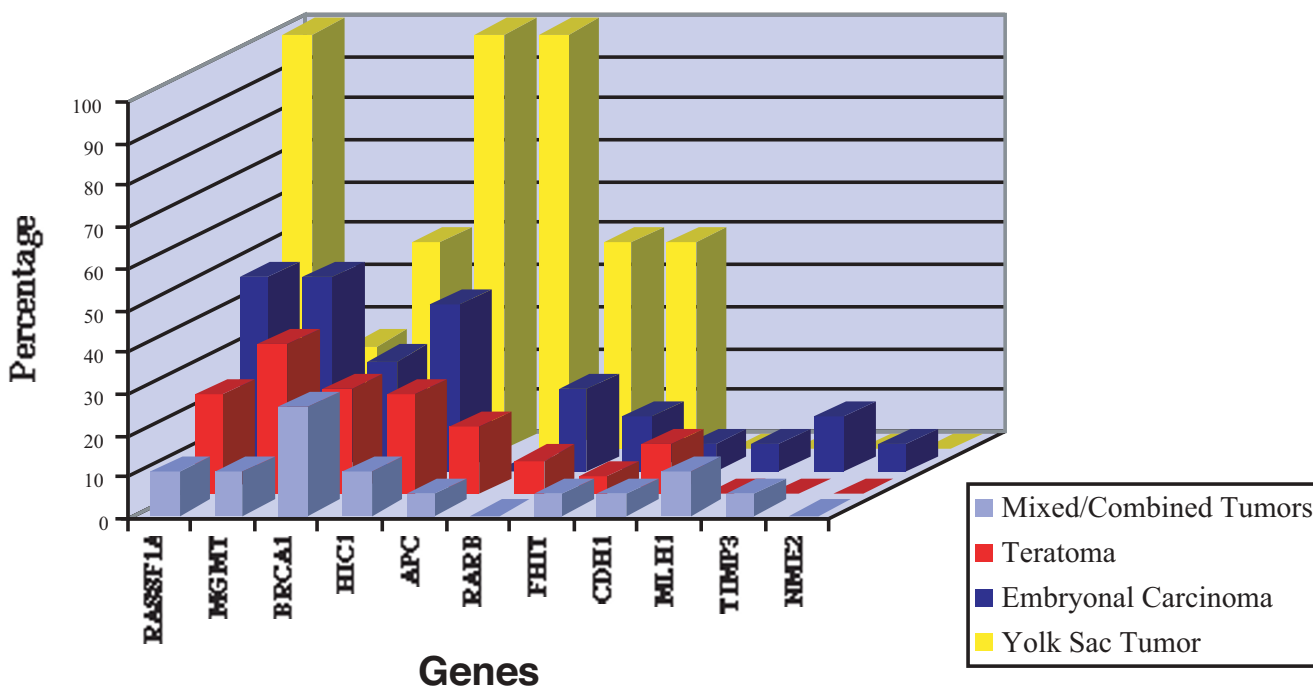
Figure 1
Analysis of promoter methylation in GCT. Frequency of CpG methylation in nonseminomatous and seminomatous histologies.

1). Of note, the MGMT gene exhibited down-regulated expression in 22 of the 23 (95.7%) tumors, including all the tumors that showed promoter methylation. No consistent down-regulation of expression of the other genes that lacked promoter methylation was detected in the same panel of specimens. These data, thus, showed loss of MGMT expression in majority of GCTs of all histologic subsets.
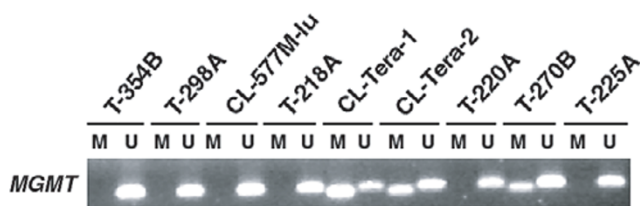
Demethylation reactivates the gene expression
To further examine the role of promoter methylation in gene inactivation, we treated five NSGCT cell lines (2102E-R, 833K-E, Tera-1, Tera-2, and 218A) with 5-Aza-2' deoxycytidine and analyzed the expression of MGMT, RASSF1A, RARB, and BRCA1 genes. The MGMT gene exhibited promoter hypermethylation in four of the cell lines, which upon treatment with 5-Aza-2' deoxycytidine showed reactivation of expression in three (2102E-R, 833K-E, and Tera-2). The cell line Tera-1 did not reactivate

expression after azacytidine treatment (Fig. 5). One cell line (T-218A) showed no promoter hypermethylation of MGMT by MSP and no detectable levels of mRNA expression by RT-PCR but expression was reactivated after 5-Aza-2' deoxycytidine treatment. Two of the five cell lines showed methylation in the RARB gene, one of which (Tera-2) showed detectable levels of gene expression in untreated cells (Fig. 5). The other four cell lines including the one with promoter hypermethylation (2102E-R) exhibited no detectable levels of RARB expression. Azacytidine treatment activated gene expression in all the five cell lines, whether or not promoter had detectable methylation (Fig. 5). The RASSF1A gene was methylated in 3 of the 5 cell lines studied. While one of the methylated cell lines showed expression in untreated cells, all other cell lines did not show detectable levels of mRNA. Azacytidine treatment reactivated expression of RASSF1A in one of each of the two methylated and two unmethylated cell lines. The BRCA1 gene showed promoter methylation in

Figure 2
Frequency of CpG methylation in different histologic subsets of nonseminoma.

Figure 3
Methylation-specific PCR of *MGMT* gene. U, unmethylated; M, methylated; Tumor (T) and cell line (CL) numbers are indicated on the top.

2 of 5 cell lines. Irrespective of whether the promoter is methylated or not, the BRCA1 gene was expressed in all cell lines and the treatment of azacytidine had little or no effect on levels of gene expression (Fig. 5)

Discussion
Epigenetic mechanisms of gene silencing are increasingly being recognized to affect a number of molecular pathways in human cancer [12]. The extent and the nature of such epigenetic modifications in GCTs are currently poorly understood. Here we show that hypermethylation is common in NSGCT and rare in SGCT. Several studies have shown that both NSGCTs and SGCTs exhibit similar genetic alterations, including isochromosome for the

short arm of chromosome 12, i(12p) [6]. Thus epigenetic alterations such as those detected in the current study is one distinct molecular change that distinguishes these two histologic subsets. The rare CpG hypermethylation seen in five SGCT patients may be due to the existence of a minor NSGCT component that might have escaped the histologic diagnosis. A unique feature of GCTs is their origin from germ cells at a stage in development where they undergo epigenetic reprogramming [6,13]. The absence of this epigenetic modification in SGCTs is consistent with their GC-like nature as previously noted [6,14]. On the other hand, the extensive promoter hypermethylation seen in NSGCTs suggests a mechanistic role in their potential for embryonal and extra-embryonal lineage differentiation [6,14]. Establishment of DNA methylation in the mammalian genome is controlled by at least three DNA methyltransferases (DNMTs), *DNMT1*, *DNMT3a* and *DNMT3b* [15]. The role of these DNMTs in differential de novo methylation in SGCT vs. NSGCT remains to be elucidated.

The overall higher frequency of promoter methylation seen in NSGCTs is noticeably evident in DNA repair genes *RASSF1A*, *BRCA1*, and *MGMT*, and the hypermethylated in cancer 1 (*HIC1*) gene, which encodes a transcription factor. These genes map to sites already known to be genetically altered in GCT specimens. The 3p21.3 region to

Table 1: Relation between promoter hypermethylation by MSP and gene expression by RT-PCR.

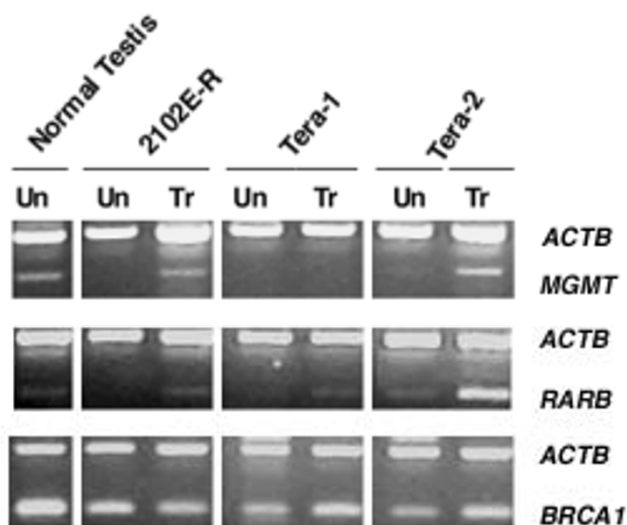| Gene	No. Studied (% down-regulated)	Methylation status and expression levels			
		Methylated		Unmethylated	
		No.	Lack of or down-regulated expression (%)	No.	Lack of or down-regulated expression (%)
MGMT	23 (95.7)	8	8 (100)	15	14 (93.3)
RASSF1A	22 (50.0)	10	8 (80)	12	3 (25)
RARB	22 (40.9)	5	3 (60)	17	6 (35.3)
MLH1	21 (33.3)	3	3 (100)	18	4 (22.2)
BRCA1	16 (18.8)	6	2 (33.3)	10	1 (10)
APC	23 (26.1)	2	- (0)	21	6 (28.6)
CDH1	20 (20.0)	2	1 (50)	18	3 (16.7)
TIMP3	20 (15.0)	3	1 (33.3)	17	2 (11.8)

Figure 4
Analysis of gene expression of *MGMT* gene in GCT. Multiplex RT-PCR analysis. Tumor (T) and cell line (CL) numbers are indicated on the top. Actin was used as an internal control.

which *RASSF1A* maps undergoes deletions in many solid tumor types, including GCTs [9]. *RASSF1A* encodes a splice variant of human RAS effector homologue, which interacts with the XPA protein and functions as a negative regulator of cell growth [16,17]. *RASSF1A* has been shown to be inactivated by promoter methylation in a variety of tumor types [16–19]. The 17q21 and 17p13 regions, to which *BRCA1* and *HIC1* map, respectively, also have been characterized by high frequency of LOH in GCT [9]. The *BRCA*1 gene plays critical roles in DNA repair and recombination, cell cycle checkpoint control, and transcription and has been shown to be hypermethylated in breast-ovarian cancer [4]. The *HIC1* gene is also often hypermethylated in many human cancers [20–22]. The DNA repair gene *MGMT* encodes O(6)-methylguanine-DNA methyltransferase and this enzyme effectively removes DNA adducts formed by alkylating agents [23]. Epigenetic inactivation of the *MGMT* gene was reported in a wide va-

riety of cancers [24,25]. Also, a low frequency of methylation of the *APC*, *RARB*, and *FHIT* genes was detected in NSGCTs. Thus, the frequent hypermethylation in the *MGMT*, *BRCA1*, and *RASSF1A*, and *HIC1* define the methylation profile in NSGCT. These data suggest that promoter hypermethylation leading to gene silencing may affect key pathways in germ cell tumorigenesis.

Aberrant promoter methylation changes that occur in cancer are associated with transcriptional repression and loss of function of the gene by interrupting the binding of proteins involved in transcription activator complex [12]. Our gene expression analysis by RT-PCR demonstrated that all tumors that showed methylation of *MGMT* and *MLH1* also showed down-regulated expression, while *RASSF1A* and *RARB* genes showed down-regulation of mRNA levels in most of the methylated tumors. Thus in these cases, promoter hypermethylation is one mecha-

Figure 5
Analysis of gene expression after treatment of GCT cell lines with the demethylating drug 5-Aza-2' deoxycytidine. Un, untreated; Tr, treated with Azacytidine. Actin was used as an internal control. Cell lines are indicated on the top.

nism whereby gene expression can be deregulated in GCTs. On the other hand, methylation of *BRCA1*, *APC*, *CDH1*, and *TIMP3* genes did not correlate with expression levels. Interestingly, *MGMT* gene was also down regulated in 14 of the 15 tumors that did not exhibit methylation by MSP analysis. Therefore, these data indicate that other epigenetic and/or genetic changes may be involved in regulating the expression of *MGMT* in GCT. The MSP method detects only methylation of full-length CpG islands and cannot identify partial methylation of the promoters. Thus, role of partial methylation in down-regulating *MGMT* cannot be ruled out. Other epigenetic mechanisms involving defects in chromatin modification factors such as the association of methyl-CpG binding proteins, acetylation and methylation of histone proteins are also becoming known [15]. The role of these chromatin-mediated components in inactivating the *MGMT* gene remains to be examined in GCT. To determine whether the down-regulated expression of the *MGMT* gene is due to genetic mutations, we examined the entire coding region in 30 GCTs and found no inactivating mutations (unpublished observations).

Epigenetic gene silencing of the *MGMT* confers enhanced sensitivity to alkylating agents in cancer [24,25]. Lack of methylation, on the other hand, associates with high-risk of death [25,26]. It has been suggested that the high-levels of *MGMT* proteins contribute to a drug-resistant phenotype [27]. More than 90% of newly diagnosed GCTs and 70–80% of patients who present metastatic disease are

cured with cisplatin-based chemotherapy [28]. However, 20–30% of the patients with metastatic disease exhibit resistance to the cisplatin curative regimen leading to high mortality in this group. The molecular basis of this exquisite chemotherapy sensitivity of GCT and resistance is poorly understood. We have previously shown that subsets of resistant tumors exhibit *TP53* gene mutations and chromosomal amplifications [6]. However, the role of *MGMT* in GCT sensitivity or resistance to chemotherapy is not known. Our current observation that undetectable levels of *MGMT* gene expression in >95% of GCTs appears to suggest that the lack of the O(6)-methylguanine-DNA methyltransferase enzyme may direct cells to undergo apoptosis due to failure of repair of DNA adducts formed by alkylating agents. Lack of *MGMT* expression in the majority of GCTs suggests a potential role for this protein in lack of repair of cisplatin-induced DNA damage that may result in exquisite sensitivity in this tumor. It has been shown that engineered over-expression of wild-type p53 *in vitro* causes inhibition of *MGMT* transcription in human tumor cells [29]. Abundant over-expression of wild-type p53, owing to their stage of origin, is a characteristic feature of GCTs [30]. A possibility also exists that the *MGMT* expression may, in general, be down regulated in tumors arising from embryonic-type cells. To examine this, we analyzed 22 cases of Wilms' tumor but found no decreased levels of the *MGMT* gene expression (data not shown). These data, therefore, rule out the possibility that not all tumors arising from embryonic-type cells show down-regulated expression of *MGMT*.

Transcriptional silencing of genes resulting from DNA hypermethylation of CpG islands is reversed by treatment of the hypo-methylating agent 5-aza-2'-deoxycytidine in a dose and duration-dependent manner. Since a number of gene promoters were hypermethylated and showed down-regulated mRNA in GCT, we wanted to test whether hypomethylation reactivates the gene expression in these tumors. We found that azacytidine treatment resulted in reactivation of gene expression in almost all cell lines that showed promoter methylation of *MGMT*, *RASSF1A* and *RARB* genes, with the exception of the cell line Tera-1. In addition, a number of genes that showed no evidence of full-length CpG methylation was also reactivated upon azacytidine treatment. This was most evident for the *RARB* gene, where all five cell lines showed reactivation whether or not the promoter was methylated. These data thus suggest that global demethylation may not only influence the expression of methylated genes but also unmethylated genes. Such a phenomenon has previously been reported [31,32].

Conclusions
The data presented here show that promoter hypermethylation is an important molecular signature differentiat-

ing seminomatous and nonseminomatous GCTs. Promoter methylation was frequently seen in DNA repair genes *MGMT*, *RASSF1A*, and *BRCA1*, and a transcriptional repressor gene *HIC1*. Promoter methylation of most genes resulted in transcriptional repression. The data also suggest that multiple mechanisms, in addition to the promoter methylation, may play a role in silencing of *MGMT* gene expression in GCTs of all histologic subsets. Given the importance of the MGMT protein in treatment response to alkylating agents, this molecular switch may play a critical role in sensitivity to cisplatin-based therapy in GCTs. Demethylation of the promoters reactivated the gene expression in *MGMT*, *RARB* and *RASSF1A* genes. Further characterization of the exact mechanisms involved in epigenetic gene silencing, especially in the *MGMT* gene, may provide important clues in understanding the pathways relevant to GCT biology.

Methods
Tumor tissues and cell lines
A total of 92 GCT tumor tissues consisting of 83 primary tumors and nine cell lines were used in this study. The tumor biopsies were ascertained from patients evaluated at Memorial Sloan-Kettering Cancer Center (MSKCC) as described previously [11] after appropriate institutional review board approval. Frozen tumor tissues or cell pellets were utilized for DNA and/or RNA isolation by standard methods. Histologically, 29 of these tumors were SGCTs, 44 NSGCTs, and 19 mixed or combined tumors. Nine cell lines derived from GCT have been previously described [8]. DNA and RNA isolated from four normal testes were used as controls.

Methylation Specific PCR (MSP)
Genomic DNA was treated with sodium bisulphite as previously described [33]. Placental DNA treated in vitro with *Sss*I methyltransferase (New England Biolabs, Beverly, MA) and similarly treated normal lymphocyte DNA were used as controls for methylated and unmethylated templates, respectively. The primers used for methylated and unmethylated-specific PCR for genes *RARB*, *TIMP3*, *CDKN2A*, *p14^{ARF}*, *MGMT*, *DAPK*, *CDH1*, *GSTP1*, *APC* promoter 1A, *RB1*, *MLH1*, *TP73*, *BRCA1*, *FHIT*, and *HIC1* have been described previously [http://pathology2.jhu.edu/pancreas/prim0425.htm#MSP]; [34–37]. For additional genes, we designed the following gene-specific primers for methylated (MF and MR) and unmethylated (UF and UR) sequences according to Herman et al [33]:

BTG1-MF 5'-GTCGTTCGTTTTTTACGTTTTT-3'

BTG1-MR 5'-CGACCCGAATATAAAAAAAATAC-3'

BTG1-UF 5'-GTTGTTTGTTTTTTATGTTTTTTTTT-3'

BTG1-UR 5'-CAACCCAAATATAAAAAAAATACA-3'

NEDD1-MF 5'-GGATATTTTTTAGTTTAGCGCG-3'

NEDD1-MR 5'-CGACCCCCTATTATATTACTACG-3'

NEDD1-UF 5'-TGGATATTTTTTAGTTTAGTGTG-3'

NEDD1-UR 5'-CAACCCCCTATTATATTACTACA-3'

APAF1-MF 5'-GCGCGTTCGTTTATGTAAATA-3'

APAF1-MR 5'-CAAACCGACGAAACCCGAA-3'

APAF1-UF 5'-GGTGTGTGTTTGTTTATGTAAATA-3'

APAF1-UR 5'-CACAAACCAACAAAACCCAAA-3'

NME1-MF 5'-GTTTCGTGCGTGTAAGTGTTG-3'

NME1-MR 5'-CCACCGACAAAAACGAATCCA-3'

NME1-UF 5'-GTTTTGTGTGTGTAAGTGTTGT-3'

NME1-UR 5'-CCACCAACAAAAACAAATCCAC-3'

NME2-MF 5'-TTTTCGGTCGCGTCGGGTC-3'

NME2-MR 5'-GCGCGAAACCTACGAAAAATC-3'

NME2-UF 5'-GTTTTTTGGTTGTGTTGGGTTG-3'

NME2-UR 5'-CACACAAAACCTACAAAAAATCA-3'

RASSF1A-MF 5'-ACGCGTTGCGTATCGCGCG-3'

RASSF1A-MR 5'-CCGCGACGACTACGCTACC-3'

RASSF1A-UF 5'-ATGTGTTGTGTATTGTGTGGGG-3'

RASSF1A-UR 5'-CCACAACAACTACACTACCCC-3'

PCR products were run on 2% agarose gels and visualized after ethidium bromide staining. Purified MSP products were sequenced in representative specimens by direct sequencing to confirm the methylation scored on agarose gels.

Semi-quantitative analysis of mRNA expression
To assess gene expression, total RNA isolated from normal testes, the cell lines, and tumor tissues, and polyA+ RNA of testis obtained from Clontech (Palo Alto, CA) was reverse transcribed using random primers and the Pro-STAR first strand RT-PCR kit (Stratagene, La Jolla, CA). A semi-quantitative analysis of gene expression was performed

using 26 to 28 cycles of multiplex RT-PCR with β-actin (*ACTB*) as control and gene specific primers spanning at least 2 exons, except in *RASSF1A*. For the latter, we used single PCR with primers and conditions as previously described [16]. The gene primers used and their positions in respective cDNAs were:

MGMT-F 5'-GCACGAAATAAAGCTCCTGG-3' (124–143 bp)

MGMT-R 5'-AGGGCTGCTAATTGCTGGTA-3' (380–399 bp)

MLH1-F 5'-CTGGACGAGACAGTGGTGAA-3' (52–71 bp)

MLH1-R 5'-CTCACCTCGAAAGCCATAGG-3' (308–327 bp)

APC-F 5'-AAGCCGGGAAGGATCTGTAT-3' (329–348 bp)

APC-R 5'-TCCAATTGCCTTCTGGTCAT-3' (588–607 bp)

RARB-F 5'-AATTCAGTGAACTGGCCACC-3' (770–789 bp)

RARB-R 5'-GGCAAAGGTGAACACAAGGT-3' (1010–1029 bp)

CDH1-F 5'-CTCGACACCCGATTCAAAGT-3' (335–354 bp)

CDH1-R 5'-TGGGCCTTTTTCATTTTCTG-3' (615–634 bp)

TIMP3-F 5'-CTTCCGAGAGTCTCTGTGGC-3' (1440–1450 bp)

TIMP3-R 5'-GGCGTAGTGTTTGGACTGGT-3' (1713–1732 bp)

BRCA1-F 5'-TCAGCTTGACACAGGTTTGG-3' (676–695 bp)

BRCA1-R 5'-GGTTGTATCCGCTGCTTTGT-3' (896–915 bp)

The PCR products were run on 1.5% agarose gels, visualized by ethidium bromide staining and quantitated using the Kodak Digital Image Analysis System (Kodak, New Haven, CT). A tumor was considered to have lost expression when the gene showed complete lack of expression or at least 50% reduction from the normalized values obtained from the average calculated utilizing 2 to 4 normal testes. The effect of methylation on gene expression was similarly assessed on total RNA isolated from cell lines treated with the demethylating agent 5-Aza-2' deoxycytidine (Sigma) for five days at a concentration of 2–5 μM.

Analysis of mutations
Single strand conformational polymorphism (SSCP) analysis was performed on all coding exons using primers flanking intronic sequences of the *MGMT* gene by standard methods.

Authors' contributions
Author 1 (SK) carried out the MSP and gene expression analysis. Author 2 (JH) coordinated the selection of tumors, and isolation of genomic DNA and RNA. Author 3 (MM) participated in the analysis of gene expression. Authors 4 and 5 (AD, JMM) have collected the clinical information. Author 6 (VER) participated in histologic diagnosis. Author 7 (GJB) was responsible for referring the patients and clinical information. Authors 8 and 9 (RSKC and VVVSM) have conceived and coordinated the study. All authors read and approved the final manuscript.

Acknowledgments

References
1. Baylin SB, Herman JG: **DNA hypermethylation in tumorigenesis: epigenetics joins genetics.** *Trends Genet* 2000, **16:**168-174
2. Ng HH, Bird A: **DNA methylation and chromatin modification.** *Curr Opin Genet Dev* 1999, **9:**158-163
3. Costello JF, Fruhwald MC, Smiraglia DJ, Rush LJ, Robertson GP, Gao X, Wright FA, Feramisco JD, Peltomaki P, Lang JC, Schuller DE, Yu L, Bloomfield CD, Caligiuri MA, Yates A, Nishikawa R, Huang HS, Petrelli NJ, Zhang X, O'Dorisio MS, Held WA, Cavenee WK, Plass C: **Aberrant CpG-island methylation has non-random and tumour-type-specific patterns.** *Nat Genet* 2000, **24:**132-138
4. Esteller M, Corn PG, Baylin SB, Herman JG: **A gene hypermethylation profile of human cancer.** *Cancer Res* 2001, **61:**3225-3229
5. Devesa SS, Blot WJ, Stone BJ, Miller BA, Tarone RE, Fraumeni JF Jr: **Recent cancer trends in the United States.** *J Natl Cancer Inst* 1995, **87:**175-182
6. Chaganti RS, Houldsworth J: **Genetics and biology of adult human male germ cell tumors.** *Cancer Res* 2000, **60:**1475-1482
7. Motzer RJ, Amsterdam A, Prieto V, Sheinfeld J, Murty VV, Mazumdar M, Bosl GJ, Chaganti RS, Reuter VE: **Teratoma with malignant transformation: diverse malignant histologies arising in men with germ cell tumors.** *J Urol* 1998, **159:**133-138
8. Bala S, Oliver H, Renault B, Montgomery K, Dutta S, Rao P, Houldsworth J, Kucherlapati R, Wang X, Chaganti RS, Murty VVVS: **Genetic analysis of the APAF1 gene in male germ cell tumors.** *Genes Chromosomes Cancer* 2000, **28:**258-268
9. Murty VVVS, Bosl GJ, Houldsworth J, Meyers M, Mukherjee AB, Reuter V, Chaganti RS: **Allelic loss and somatic differentiation in human male germ cell tumors.** *Oncogene* 1994, **9:**2245-2251
10. Murty VVVS, Montgomery K, Dutta S, Bala S, Renault B, Bosl GJ, Kucherlapati R, Chaganti RSK: **A 3-Mb high-resolution BAC/PAC contig of 12q22 encompassing the 830-kb consensus minimal deletion in male germ cell tumors.** *Genome Res* 1999, **9:**662-671
11. Murty VVVS, Renault B, Falk CT, Bosl GJ, Kucherlapati R, Chaganti RS: **Physical mapping of a commonly deleted region, the site of a candidate tumor suppressor gene, at 12q22 in human male germ cell tumors.** *Genomics* 1996, **35:**562-570
12. Jones PA, Baylin SB: **The fundamental role of epigenetic events in cancer.** *Nat Rev Genet* 2002, **3:**415-428

13. Reik W, Dean W, Walter J: **Epigenetic reprogramming in mammalian development.** *Science* 2001, **293**:1089-1093

14. Smiraglia DJ, Szymanska J, Kraggerud SM, Lothe RA, Peltomaki P, Plass C: **Distinct epigenetic phenotypes in seminomatous and non-seminomatous testicular germ cell tumors.** *Oncogene* 2002, **21**:3909-3916

15. Burgers WA, Fuks F, Kouzarides T: **DNA methyltransferases get connected to chromatin.** *Trends Genet* 2002, **18**:275-277

16. Burbee DG, Forgacs E, Zochbauer-Muller S, Shivakumar L, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gazdar AF, Lerman MI, Zabarovsky E, White M, Minna JD: **Epigenetic inactivation of RASSF1A in lung and breast cancers and malignant phenotype suppression.** *J Natl Cancer Inst* 2001, **93**:691-699

17. Dammann R, Yang G, Pfeifer GP: **Hypermethylation of the cpG island of Ras association domain family 1A (RASSF1A), a putative tumor suppressor gene from the 3p21.3 locus, occurs in a large percentage of human breast cancers.** *Cancer Res* 2001, **61**:3105-3109

18. Lo KW, Kwong J, Hui AB, Chan SY, To KF, Chan AS, Chow LS, Teo PM, Johnson PJ, Huang DP: **High frequency of promoter hypermethylation of RASSF1A in nasopharyngeal carcinoma.** *Cancer Res* 2001, **61**:3877-3881

19. Dreijerink K, Braga E, Kuzmin I, Geil L, Duh FM, Angeloni D, Zbar B, Lerman MI, Stanbridge EJ, Minna JD, Protopopov A, Li J, Kashuba V, Klein G, Zabarovsky ER: **The candidate tumor suppressor gene, RASSF1A, from human chromosome 3p21.3 is involved in kidney tumorigenesis.** *Proc Natl Acad Sci U S A* 2001, **98**:7504-7509

20. Strathdee G, Appleton K, Illand M, Millan DW, Sargent J, Paul J, Brown R: **Primary ovarian carcinomas display multiple methylator phenotypes involving known tumor suppressor genes.** *Am J Pathol* 2001, **158**:1121-1127

21. Wales MM, Biel MA, el Deiry W, Nelkin BD, Issa JP, Cavenee WK, Kuerbitz SJ, Baylin SB: **p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13.3.** *Nat Med* 1995, **1**:570-577

22. Issa JP, Zehnbauer BA, Kaufmann SH, Biel MA, Baylin SB: **HIC1 hypermethylation is a late event in hematopoietic neoplasms.** *Cancer Res* 1997, **57**:1678-1681

23. Teicher BA: **Antitumor alkylating agents.** *Cancer: Principles and practice of oncology (Edited by: De Vita VT Jr, Hellman S, Rosenberg SA) Philadelphia: Lippincott-Raven* 1997, **1**:405-418

24. Esteller M, Hamilton SR, Burger PC, Baylin SB, Herman JG: **Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia.** *Cancer Res* 1999, **59**:793-797

25. Esteller M, Garcia-Foncillas J, Andion E, Goodman SN, Hidalgo OF, Vanaclocha V, Baylin SB, Herman JG: **Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents.** *N Engl J Med* 2000, **343**:1350-1354

26. Esteller M, Gaidano G, Goodman SN, Zagonel V, Capello D, Botto B, Rossi D, Gloghini A, Vitolo U, Carbone A, Baylin SB, Herman JG: **Hypermethylation of the DNA repair gene O(6)-methylguanine DNA methyltransferase and survival of patients with diffuse large B-cell lymphoma.** *J Natl Cancer Inst* 2002, **94**:26-32

27. Christmann M, Pick M, Lage H, Schadendorf D, Kaina B: **Acquired resistance of melanoma cells to the antineoplastic agent fotemustine is caused by reactivation of the DNA repair gene MGMT.** *Int J Cancer* 2001, **92**:123-129

28. Bosl GJ, Motzer RJ: **Testicular germ-cell cancer.** *N Engl J Med* 1997, **337**:242-253

29. Srivenugopal KS, Shou J, Mullapudi SR, Lang FF Jr, Rao JS, Ali-Osman F: **Enforced expression of wild-type p53 curtails the transcription of the O(6)-methylguanine-DNA methyltransferase gene in human tumor cells and enhances their sensitivity to alkylating agents.** *Clin Cancer Res* 2001, **7**:1398-1409

30. Bartkova J, Bartek J, Lukas J, Vojtesek B, Staskova Z, Rejthar A, Kovarik J, Midgley CA, Lane DP: **p53 protein alterations in human testicular cancer including pre-invasive intratubular germ-cell neoplasia.** *Int J Cancer* 1991, **49**:196-202

31. Soengas MS, Capodieci P, Polsky D, Mora J, Esteller M, Opitz-Araya X, McCombie R, Herman JG, Gerald WL, Lazebnik YA, Cordon-Cardo C, Lowe SW: **Inactivation of the apoptosis effector Apaf-1 in malignant melanoma.** *Nature* 2001, **409**:207-211

32. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JC, Liang G, Jones PA: **Histone H3-Lysine 9 methylation is associated with aberrant gene silencing in cancer Ccells and is rapidly reversed by 5-Aza-2'-deoxycytidine.** *Cancer Res* 2002, **62**:6456-6461

33. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB: **Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands.** *Proc Natl Acad Sci U S A* 1996, **93**:9821-9826

34. Simpson DJ, Hibberts NA, McNicol AM, Clayton RN, Farrell WE: **Loss of pRb expression in pituitary adenomas is associated with methylation of the RB1 CpG island.** *Cancer Res* 2000, **60**:1211-1216

35. Corn PG, Kuerbitz SJ, van Noesel MM, Esteller M, Compitello N, Baylin SB, Herman JG: **Transcriptional silencing of the p73 gene in acute lymphoblastic leukemia and Burkitt's lymphoma is associated with 5' CpG island methylation.** *Cancer Res* 1999, **59**:3352-3356

36. Esteller M, Silva JM, Dominguez G, Bonilla F, Matias-Guiu X, Lerma E, Bussaglia E, Prat J, Harkes IC, Repasky EA, Gabrielson E, Schutte M, Baylin SB, Herman JG: **Promoter hypermethylation and BRCA1 inactivation in sporadic breast and ovarian tumors.** *J Natl Cancer Inst* 2000, **92**:564-569

37. Zochbauer-Muller S, Fong KM, Maitra A, Lam S, Geradts J, Ashfaq R, Virmani AK, Milchgrub S, Gazdar AF, Minna JD: **5' CpG island methylation of the FHIT gene is correlated with loss of gene expression in lung and breast cancer.** *Cancer Res* 2001, **61**:3581-3585