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Role of promoter hypermethylation in Cisplatin treatment response of male germ cell tumors

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Abstract

Background: Male germ cell tumor (GCT) is a highly curable malignancy, which exhibits exquisite sensitivity to cisplatin treatment. The genetic pathway(s) that determine the chemotherapy sensitivity in GCT remain largely unknown.

Results: We studied epigenetic changes in relation to cisplatin response by examining promoter hypermethylation in a cohort of resistant and sensitive GCTs. Here, we show that promoter hypermethylation of RASSF1A and HIC1 genes is associated with resistance. The promoter hypermethylation and/or the down-regulated expression of MGMT is seen in the majority of tumors. We hypothesize that these epigenetic alterations affecting MGMT play a major role in the exquisite sensitivity to cisplatin, characteristic of GCTs. We also demonstrate that cisplatin treatment induce de novo promoter hypermethylation in vivo. In addition, we show that the acquired cisplatin resistance in vitro alters the expression of specific genes and the highly resistant cells fail to reactivate gene expression after treatment to demethylating and histone deacetylase inhibiting agents.

Conclusions: Our findings suggest that promoter hypermethylation of RASSF1A and HIC1 genes play a role in resistance of GCT, while the transcriptional inactivation of MGMT by epigenetic alterations confer exquisite sensitivity to cisplatin. These results also implicate defects in epigenetic pathways that regulate gene transcription in cisplatin resistant GCT.

Background

Adult male germ cell tumors (GCTs) are considered to be a model system for a curable malignancy because of their exquisite sensitivity to cisplatin (CDDP)-based combination (cisplatin, etoposide, with or without bleomycin) chemotherapy. Histologically, GCTs present as a germ cell (GC)-like undifferentiated seminoma (SGCT) or a differentiated nonseminoma (NSGCT). NSGCTs display complex differentiation patterns that include embryonal, extra-embryonal, and somatic tissue types [1]. Furthermore, embryonal lineage teratomas differentiate into various somatic cell types that may undergo malignant transformation to epithelial, mesenchymal, neurogenic, or hematologic tumors [2]. Seminomas are exquisitely sensitive to radiation therapy while NSGCTs are highly sensitive to treatment with CDDP-based chemotherapy. Despite this sensitivity to chemotherapy, 20–30% of metastatic tumors are refractory to initial treatment, requiring salvage therapy and accounting for high mortalitiy. Such patients are treated with high dose and experimental chemotherapy protocols [3]. The underlying molecular basis of this exquisite drug responsiveness of GCT remains to be fully understood [4].

Little is known about the genetic basis of chemotherapy response in GCT. Studies have previously identified that TP53 mutations and gene amplification may play a role in GCT resistance [5,6]. It has also been recently shown that microsatellite instability is associated with the treatment resistance in GCT [7]. An epigenetic alteration by promoter hypermethylation that plays a role in inactivating tumor suppressor genes in a wide-variety of cancers also has been shown to occur in GCT [8-10]. We previously showed the absence of promoter hypermethylation in SGCT and acquisition of unique patterns of promoter hypermethylation in NSGCT [8]. However, the role of such epigenetic changes in GCT resistance and sensitivity remains unknown.

In the present study, we evaluated the status of hypermethylation in 22 gene promoters in 39 resistant and 31 sensitive NSGCTs. We found that *RASSF1A* and *HIC1* promoter hypermethylation was associated with highly resistant tumors. Evidence was also obtained suggesting that promoter hypermethylation is induced against the initial CDDP treatment and that this hypermethylation plays a crucial role in further treatment response. We show that changes in the patterns of gene expression occur during the *in vitro* acquisition of a highly refractory tumor to CDDP, which irreversibly affects the response to demethylating and histone deacetylase inhibiting agents.

Results

Promoter hypermethylation in relation to chemotherapy resistance and sensitivity

Based on our previous observations in GCT, we studied 22 gene promoters for hypermethylation in 70 NSGCTs derived from 60 patients [8]. Promoter hypermethylation was found in nine of 22 genes examined. One or more genes were methylated in 41 (59%) tumors. The frequency of hypermethylation for each of the genes was: *RASSF1A* (35.7%), *HIC1* (31.9%), *BRCA1* (26.1%), *APC* (24.3%), *MGMT* (20%), *RARB* (5.7%), *FHIT* (5.7%), *FANCF* (5.7%), and *ECAD* (4.3%). This frequency was similar to our previously published observations on unselected patients with NSGCTs [8].

The frequency of overall promoter hypermethylation (one or more of the 22 genes methylated) was similar in the sensitive (18 of 29 patients; 62%) and resistant (21 of 31 patients; 68%) tumors. However, the frequency of promoter hypermethylation of individual genes differed between sensitive and resistant tumors. *RASSF1A* (52% in resistant vs. 28% in sensitive) and *HIC1* (47% in resistant vs. 24% in sensitive) genes showed higher frequency of promoter hypermethylation in resistant tumors (Table 2, Fig. 1). These differences were not statistically significant due to small number of tumors studied. However, the differences were more pronounced when the sensitive and highly resistant tumors were compared (discussed below). On the other hand, the sensitive tumors exhibited higher frequency of promoter hypermethylation compared to resistant tumors in *MGMT* (31% vs. 13%) and *RARB* (14% vs. 0%; P = 0.05) (Table 2, Fig. 1). Other genes that exhibited frequent hypermethylation showed no significant differences (*APC*, 24% vs. 29%; *BRCA1*, 31% vs. 30%) between the sensitive and resistant groups. These data, thus, suggest that promoter hypermethylation of *RASSF1A* and *HIC1* is associated with chemotherapy resistance phenotype, while promoter hypermethylation of *MGMT* and *RARB* genes is commonly seen in sensitivetumors.

Table 1: Histologic and phase characteristics of sensitive and resistant NSGCTs

	Sensitive (N = 31)	Resistant (N = 39)
Histology		
Teratoma	14	15
Embryonal carcinoma	6	2
Yolk sac tumor	3	6
Mixed tumor/malignant transformation	8	16
Phase at tissue collection		
Primary untreated (P)	1	5
Metastatic untreated (M1)	12	4
One regimen of chemotherapy (C1)	18	14
Two regimens of chemotherapy (C2)	-	8
Three or more regimens of chemotherapy (C3)	-	8

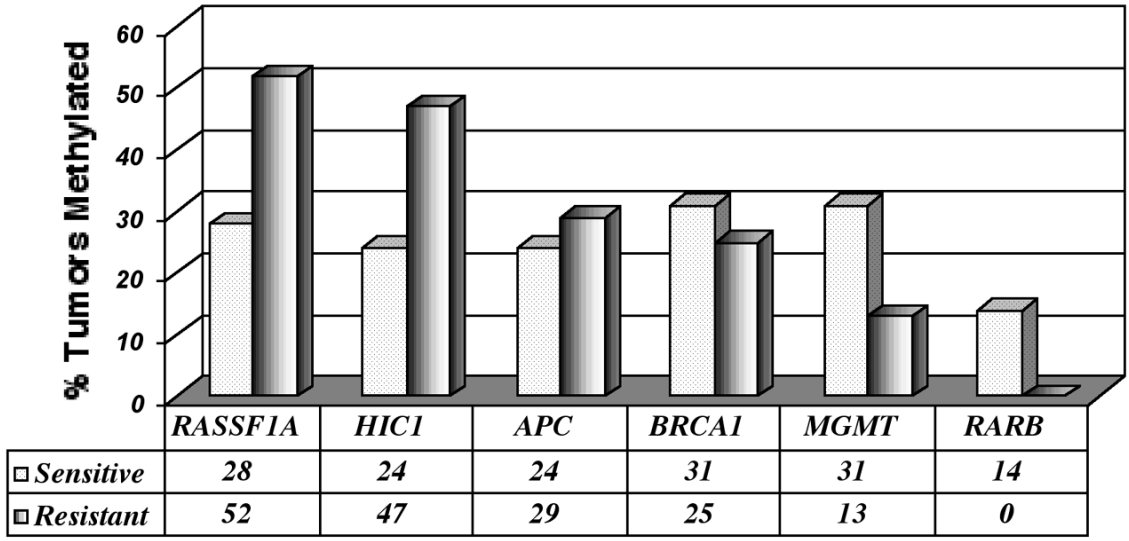

Figure 1
Promoter hypermethylation in patients with sensitive and resistant GCTs in response to cisplatin combination chemotherapy. *RASSF1A* and *HIC1* genes showed frequent methylation in resistant tumors, while MGMT and RARB promoters were more commonly methylated in sensitive tumors.

Table 2: Frequency of promoter methylation of individual genes in sensitive and resistant NSGCT

Gene	Sensitive[1] (N = 29) (%)	Resistant[2] (N = 31) (%)	P-value
APC	7 (24)	9 (29)	0.77
BRCA1[3]	9 (31)	9 (30)	1.0
ECAD	1 (3)	2 (6)	1.0
FANCF	2 (7)	2 (6)	1.0
FHIT	2 (7)	2 (6)	1.0
HIC1[3]	7 (24)	14 (47)	0.10
MGMT	9 (31)	4 (13)	0.12
RARB	4 (14)	0	0.05
RASSF1A	8 (28)	16 (52)	0.07

[1] Consists of tumors, with or without retroperitoneal lymph node, sensitive for one cycle of chemotherapy [2] Consists of tumors, with or without retroperitoneal lymph node, required of two or more cycles of chemotherapy, all resistant tumors, and all patients died of disease [3] Only 30 resistant tumors studied for methylation status

CDDP treatment induces de novo promoter hypermethylation in vivo

To assess the effect of CDDP-treatment on promoter hypermethylation, we examined tumor tissues that were collected at different phases of resistance (Table 1). The frequency of hypermethylation at different phases was: P, 16.7%; M1, 37.5%; C1, 75%; C2, 62.5%, and C3, 62.5%. Tumors from patients who underwent one or more regimens of chemotherapy (C1, C2, or C3 phases) exhibited a significantly (P = 0.001) higher (34 of 48 patients; 71%) frequency of promoter hypermethylation compared to those from untreated (P and M1) (7 of 22 tumors; 32%) patients after adjusting for sensitive/resistance status. The frequency of promoter hypermethylation was also significantly higher in tumors from treated patients when sensitive and resistant groups were analyzed separately (P ≤ 0.02). The differences in overall promoter hypermethylation between untreated tumors (P/M1; 32%) and C1 tumors (75%) were highly significant (P = 0.004), while the differences between untreated tumors and C2/C3

Table 3: Promoter hypermethylation in various phases of treatment in NSGCT

Gene	Phase[1]				
				p-value[3]	
	P/M1 (N = 22)	C1 (N = 32)	C2/C3 (N = 16)	P/M1 vs C1	P/M1 vs C2/C3
APC	2 (9.1)	8 (25)	7 (43.8)	0.25	0.03
BRCA1[2]	4 (18.2)	12 (37.5)	2 (13.3)	0.22	1.0
HIC1[2]	3 (13.6)	11 (34.4)	8 (53.5)	0.17	0.09
MGMT	0	11 (34.4)	3 (18.8)	0.003[4]	
RARB	0	4 (12.5)	0	0.14[4]	
RASSF1A	5 (22.7)	10 (31.3)	10 (62.5)	0.72	0.09

[1] See Table 1 for definition of phase [2] Only 15 tumors studied in C2/C3 phase [3] Adjusted for sensitive/resistant status [4] Model cannot be fit due to sparse data; Fisher's exact p-value given

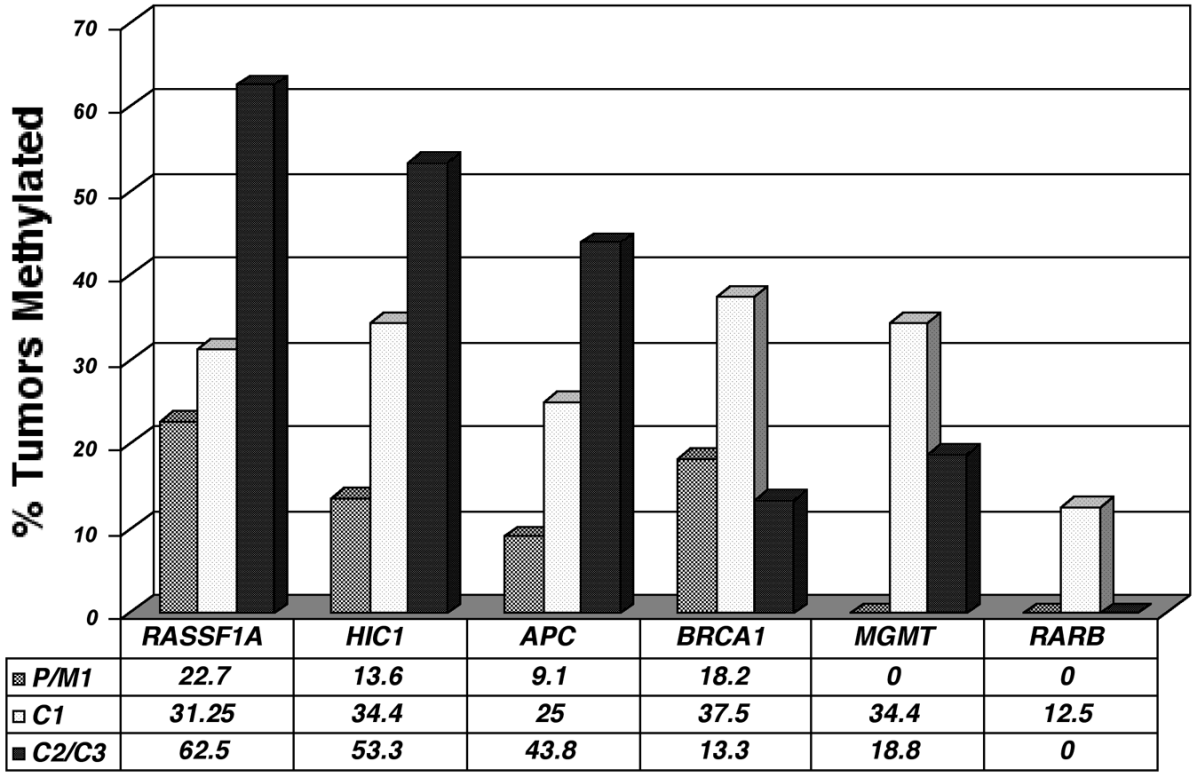

Figure 2
Comparisons of promoter hypermethylation frequencies in different phases of treated patients with GCTs. Phase definitions are shown in Table 1. P, untreated primary tumor; M1, untreated metastatic tumor; C1, one regimen of chemotherapy; C2/C3, two or more regimens of chemotherapy. Promoter methylation of *RASSF1A*, *HIC1*, and *APC* genes was significantly high in resistant tumors. The *MGMT*, *BRCA1*, and *RARB* genes show higher frequency of promoter methylation in tumors exposed to one cycle of chemotherapy.

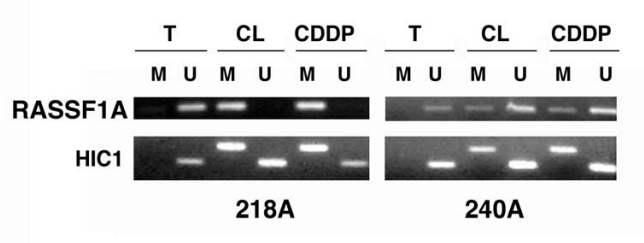

Figure 3
Promoter methylation changes *in vitro* in GCT cell lines. Appearance of *de novo* promoter hypermethylation in cell lines. T, tumor; CL, cell line; CDDP, cisplatin-treated cell line. M, methylated DNA; U, unmethylated DNA; Cell lines examined are shown below. Note absence of methylation in primary tumor in both the cell lines and appearance of novel methylated allele in cell line DNA. Note both alleles of *RASSF1A* are methylated in 218A cell line.

tumors (62.5%) were less pronounced (P = 0.09). These data, thus, suggest that the higher frequency of promoter hypermethylation seen in treated tumors may be due in response to CDDP treatment. This de novo increase in promoter hypermethylation was evident in most analyzed genes. Comparison among tumors that were not treated (P/M1), those that were treated with one cycle of chemotherapy (C1) and those that were treated with two or more cycles (C2/C3) showed notable differences for *APC*, *MGMT*, *HIC1*, *RARB* and *RASSF1A* (Table 3). Promoter hypermethylation of *RASSF1A*, *HIC1*, and *APC* genes was higher in the treated tumors, with highly resistant (C2/C3) tumors exhibiting the highest incidence (Fig. 2). However, this trend was different for *BRCA1*, *MGMT*, and *RARB* genes. These genes exhibited either no methylation or low frequency of hypermethylation in untreated tumors, while the C1 tumors had the highest incidence of promoter hypermethylation (Fig. 2). However, this was decreased or absent in highly resistant (C2/C3) tumors. These data, thus, strongly suggest that promoter hypermethylation was induced in response to the first exposure to CDDP and the tumors harboring promoter hypermethylation responded differently to further treatment in a gene specific manner. Thus, our data indicate that the tumors with promoter hypermethylation of *RASSF1A*, *HIC1*, and *APC* resulted in failure to respond to further treatment, while the tumors harboring promoter hypermethylation of *MGMT*, *RARB*, and *BRCA1* responded favorably.

Since we previously showed that yolk sac tumor (YST) exhibit higher frequency of hypermethylation compared to other histologic types among the genes tested [8], we included histology as an additional covariate in the above analyses in an attempt to account for histological differences in promoter hypermethylation. We found that the differences in overall promoter hypermethylation between untreated and treated tumors were no longer significant when adjusted for histology (data not shown). The small number of observations in each histological group, however, prevents us from making any meaningful conclusions from this analysis. Although the data is indicative of histologic differences in promoter methylation, further analysis of gene specific promoter hypermethylation on a larger panel of tumors is needed to satisfactorily address this issue.

No effect of CDDP treatment in vitro *on promoter methylation*

Since we showed CDDP treatment induces promoter hypermethylation in tumors *in vivo*, we wanted to test whether a similar phenomenon occurs *in vitro*. To investigate this, we exposed four NSGCT cell lines to two different concentrations of CDDP for various time periods as described in the methods. The specimens from which the 169A, 218A, and 240A cell lines derived were also included in the panel of tumors studied for hypermethylation. In addition, two independent clones derived from 833K-E and 240A as D1 and D4 resistant cells (see materials and methods) were also examined. We did not detect changes in promoter hypermethylation in 18 (*APC*, *GSTP1*, *BRCA1*, *DAPK*, *p16*, *p14*, *MGMT*, *APAF1*, *RASSF1A*, *HIC1*, *RB*, *TIMP3*, *FANCF*, *RARB*, *CDH1*, *TP53*, *FHIT*, and *MLH1*) genes examined. These results clearly indicate that CDDP treatment *in vitro*, within the tested concentrations, does not cause promoter hypermethylation. However, we found methylation of *BRCA1*, *RASSF1A*, or *HIC1*gene promoters in three cell lines but not in their corresponding primary tumors (Fig. 3). The genes methylated in cultured tumor cells were the same genes that were also frequently methylated in NSGCT patients. The gene promoters that did not exhibit frequent methylation in primary tumors were not methylated in cultured tumor cells.

Loss of activation of gene expression to inhibitors of methylation and histone deacetylation in acquired CDDP resistance in vitro

To further examine the effect of CDDP treatment *in vitro*, we then studied expression of *MGMT*, *HIC1*, and *FANCF*, the genes that were either promoter hypermethylated or down regulated in GCT, in D1 and D4 clones derived from the cell lines 833K-E and 240A.

MGMT is a DNA repair enzyme that protects cells against the effects of alkylating agents by removing adducts formed at the O^6 position of guanine in DNA [11]. Tumor sensitivity to alkylating agents has been shown to depend on *MGMT* expression [12]. Resistant tumors are generally

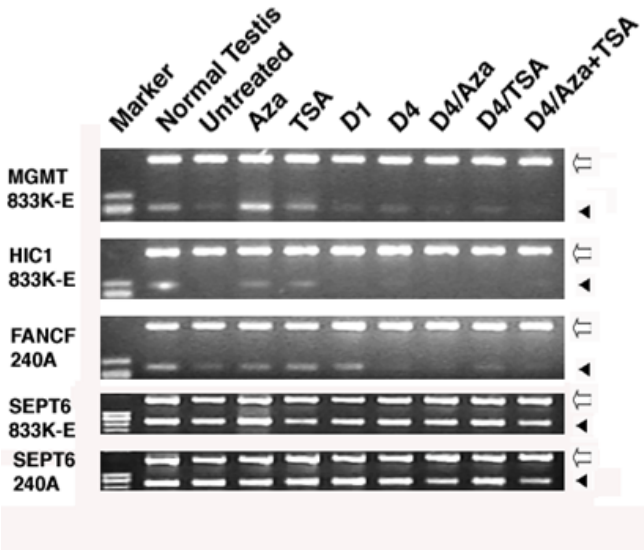

Figure 4
RT-PCR analysis of gene expression in relation to CDDP-induced resistance in GCT cell lines. Aza, 5-Aza-2'-deoxycytidine; TSA, trichostatin; D1, low refractory CDDP resistant cells; D4, high refractory CDDP resistant cells; *SEPT6*, septin 6. Actin (empty arrow) was used as an internal control. Filled arrowhead indicates the specific gene. Septin 6 gene was used as another control.

shown to express high levels of the *MGMT* gene [12]. To examine the role of *MGMT* in in vitro-acquired CDDP resistance of GCT, we studied mRNA levels in 833K-E and 240A cell lines that harbored methylated promoters. Both showed low levels of detectable mRNA by RT-PCR. However, only the 833K-E showed reactivation of expression upon treatment with 5-Aza-C or TSA, while these agents had no effect on the 240A cell line. The levels of *MGMT* mRNA in D1 and D4 refractory cells were either remained at the levels similar to untreated cells in D1 cells or were slightly increased in D4 cells in both the cell lines (Fig. 4). The latter is consistent with the role of *MGMT* in efficiently repairing DNA adducts in resistant cells and this could be due to partial demethylation of the promoters in highly resistant D4 cells in these cell lines. Concordant with demethylation of promoter in D4 cells, these cells fail to up-regulate gene expression after 5-Aza-C or TSA treatment (Fig. 4).

The *HIC1* gene showed hypermethylated promoters in both 833K-E and 240A cell lines. Analysis of mRNA showed that only 240A cell line exhibited a detectable level of expression, while it was absent in the 833K-E cell line. *HIC1* was reactivated after treatment with 5-Aza-C or TSA in 833K-E, while the treatment had no effect on 240A cell line (Fig. 4). However, both the D1 and D4 clones derived from these cell lines showed complete lack of expression of *HIC1* and failed to respond to either 5-Aza-C or TSA (Fig. 4).

The *FANCF* gene belongs to the family of six Fanconi anemia proteins that facilitate mono-ubiquitinylation of FANCD2, which plays a role in a large multimeric protein complex required for DNA repair [13]. Acquired CDDP resistance in ovarian carcinoma correlates with subtle methylation/demethylation of *FANCF* promoter leading to the suggestion that demethylation of this gene causes CDDP resistance [14]. Here, we examined the *FANCF* gene expression in the development of in vitro CDDP resistance. The *FANCF* promoter was not methylated in both 833K-E and 240A cells and low levels of mRNA expression were found in both. However, both cell lines showed an up-regulated expression of *FANCF* mRNA after 5-Aza-C or TSA treatment. Although the levels of *FANCF* expression in D1 cells of 833K-E remain at the levels in untreated cells, the D1 cells of 240A showed an elevated level of mRNA (Fig. 4). However, the D4 resistant clones from both cell lines showed a decrease in expression compared to untreated cells and lost the ability to respond to 5-Aza-C or TSA (Fig. 4). Analysis of a control gene did not affect the pattern of expression in relation to *in vitro* CDDP resistance in these cells (Fig. 4).

Taken together, the results obtained from all three genes studied here indicate that changes in gene expression occur in the development of low to high refractory CDDP resistance. Highly resistant clones fail to respond to demethylating or histone deacetylase inhibiting agents in activating gene expression suggesting that irreversible changes occur in a pathway that control gene transcription.

MGMT *is partially methylated and down regulated in most GCTs*

We showed earlier promoter hypermethylation of *MGMT* in 21% NSGCT and complete lack of or down-regulated gene expression by RT-PCR in 96% of tumors [8]. To examine whether the down-regulated RNA levels reflected in decreased protein, we performed an immunohistochemical analysis of MGMT on a tissue array containing 18 SGCTs and 18 NSGCTs. The MGMT expression was absent in 33 (91.7%) tumors. The remaining 3 tumors (two yolk sac tumor and one immature teratoma) were weakly positive compared to the controls (data not shown). Thus, the combined data on analyses of RT-PCR from our previous study and the levels of protein reported here showed down-regulated levels of MGMT expression in all histologic types of GCT. The cells from GCT have been reported to exhibit reduced efficiency in the removal of CDDP-induced adducts [15,16]. Concordantly, more

than 85% of metastatic GCT can be cured with chemotherapy [3]. Since GCTs exhibit either promoter hypermethylation or down-regulated gene expression of the *MGMT* gene in most tumors, we suspected that this gene may play a role in CDDP sensitivity. Our previous data suggest that down-regulated expression of *MGMT* occurs by other mechanisms, in addition to complete promoter hypermethylation. We previously ruled out mutational inactivation of this gene in GCT [8]. The MSP method only detects complete hypermethylation of CpG islands and partial methylation will not be identified by this method. Since the down regulated *MGMT* expression can be reactivated after cellular exposure to 5-aza-C in unmethylated GCT cell lines, we reasoned that partial methylation might exist. To test this, we sequenced 98 CpG methylation sites spanning the promoter of *MGMT* in genomic DNA from two normal testes, one methylated cell line, seven NSGCTs and five SGCTs which have unmethylated promoters by MSP (Fig. 5). Normal testes showed a few random methylated CpGs in a small number of clones. The MSP positive Tera-2 cell line had dense methylation of the entire *MGMT* promoter. All seven NSGCTs and five SGCTs showed partial methylation of the promoter region at specific CpG residues (Fig. 5). Three (T-228A, T-186B, and T-288A) of the seven NSGCTs studied were also classified as resistant GCTs. The frequency of methylated residues varied within clones derived from the same tumor and between tumors. The present data, thus, suggest, but does not prove, that partial methylation of *MGMT* promoter accounts for down-regulated gene expression in GCT. Overall, these results indicate a role of *MGMT* promoter hypermethylation state, either complete or partial, in determining the exquisite sensitivity to CDDP in GCT.

Discussion

The molecular mechanisms that determine the curability of GCT to CDDP-based combination chemotherapy are unclear [17-20]. Understanding the genetic basis of this exquisite sensitivity could lead to the development of a more effective treatment for resistant tumors. A number of genetic mechanisms for CDDP resistance, such as enhanced adduct repair, drug inactivation, or tolerance to DNA damage, have been proposed [21].

We and others previously reported that epigenetic alterations in the promoters of specific genes occur in NSGCT [8-10,22]. We also showed that promoter hypermethylation was associated with gene repression in NSGCT and this down regulated expression is reactivated upon demethylation suggesting a potential role for epigenetic changes in GCT biology [8]. These results prompted us to examine the possible involvement of epigenetic changes in chemo-sensitivity and resistance in GCT. To achieve this, we investigated epigenetic changes in resistant and sensitive NSGCTs and found a high incidence of promoter hypermethylation of *RASSF1A* and *HIC1* in resistant tumors, while promoter hypermethylation of *MGMT* and *RARB* genes was associated with sensitive tumors.

RASSF1A has shown to be epigenetically inactivated in a wide variety of tumor types suggesting a major role for this gene in cancer [23]. In the present study, we demonstrated that a higher frequency of resistant tumors carry promoter methylation compared to sensitive GCT, suggesting that *RASSF1A* hypermethylation is associated with the resistance phenotype. *RASSF1A* represents a long isoform of human *RASSF1* gene, which encodes a diacylglycerol (DAG)-binding domain at the NH_2 terminus, a RAS-association domain at COOH terminus, which interacts with the XPA protein. *RASSF1A* gene functions as a negative regulator of cell growth [23].

Hic1encoding a zinc finger transcription factor acts as a tumor suppressor gene [24]. *HIC1* is silenced by promoter hypermethylation in several types of human cancer [25]. We found a higher frequency of resistant tumors harboring *HIC1* promoter hypermethylation.

On the other hand, we showed promoter hypermethylation of *MGMT* and *RARB* genes associated with CDDP sensitivity. *MGMT* gene encodes O(6)-methylguanine-DNA methyltransferase and plays an important role in removing DNA adducts formed by alkylating agents [11]. Epigenetic silencing of *MGMT* has been shown to confer enhanced sensitivity on cancer cell to alkylating agents, while the lack of methylation and high-levels of protein expression contribute to drug-resistance phenotype [12,26,27]. We showed here that either complete or partial methylation of *MGMT* occurs in a majority of GCT. These data suggest that the complete promoter methylation of *MGMT* plays a role in favorable response to CDDP treatment. However, the demonstration here of partial methylation in most GCTs provides a possible mechanism for down-regulated expression of *MGMT*, which is commonly seen in this tumor. These results, thus, support the view that the epigenetic alteration in *MGMT* may be a factor in the exquisite sensitivity of GCT to CDDP. Such a model provides opportunities to alter MGMT pathway and chemosensitize relapsed tumor to CDDP.

Retinoids control gene transcription by activating retinoic acid receptors (RAR$\propto$, β and γ) and retinoid X receptors (RXR$\propto$, β and γ). Expression of these receptors regulates organogenesis, organ homeostasis, cell growth, and differentiation and death [28]. It is well established that changes in expression of RARs play a major role in cancer development and response of tumor cells to treatment of all-trans retinoic acid (ATRA). A number of premalignant lesions and cancers have been shown to exhibit a loss of

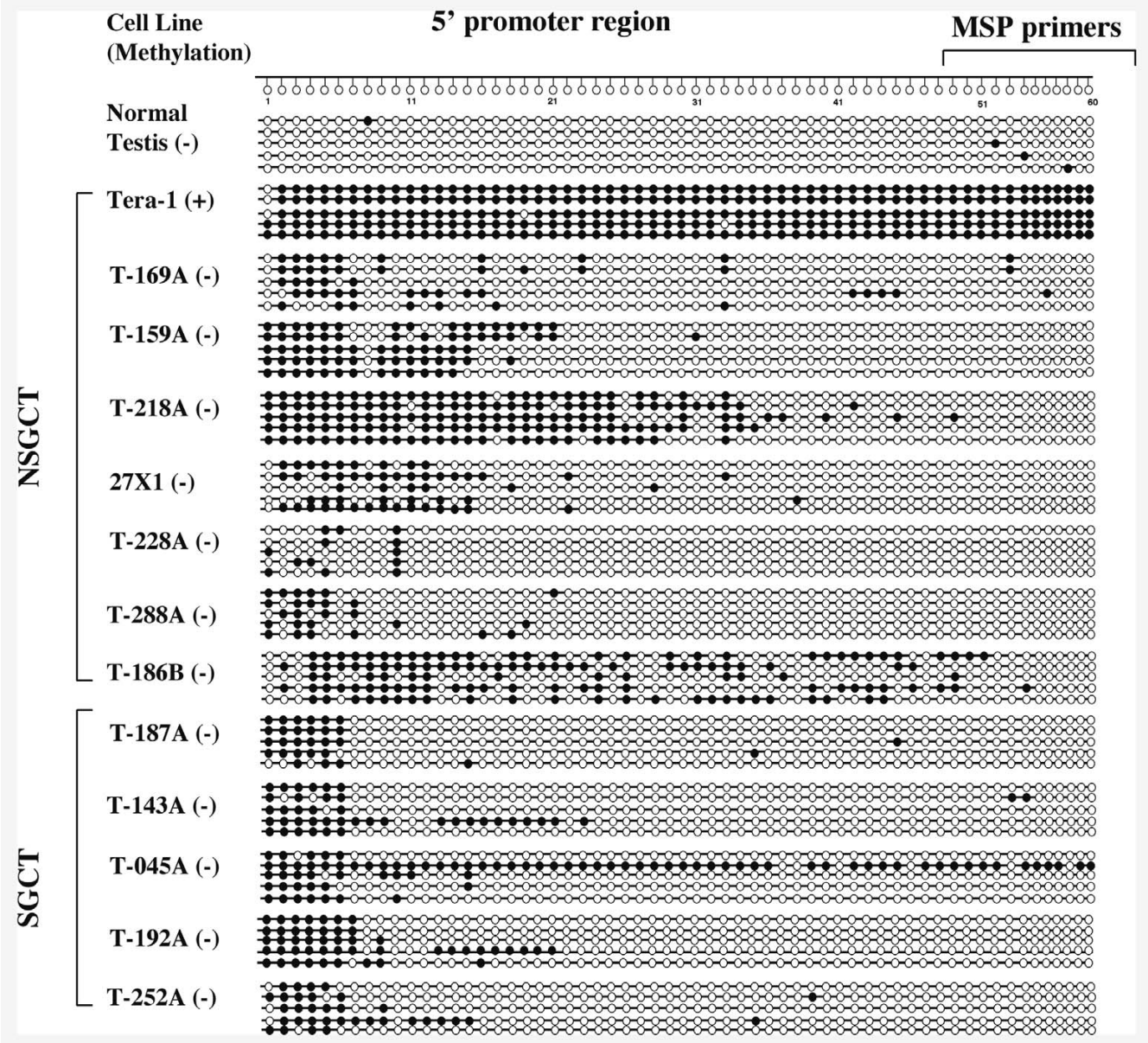

Figure 5

Analysis of promoter methylation by bisulphite sequencing in GCT. CpG methylation status in 5 independent clones sequenced is shown for each tumor. Shown on top by vertical lollypops connected to horizontal line are CpGs examined on the 5' promoter region. Site of MSP primer is indicated. Shown below is the CpG sequence information for each tumor. Tumor numbers are shown on left. Methylation status is indicated in parenthesis next to the tumor. Filled circles indicate methylated CpG sites and empty circles indicate unmethylated CpGs. NSGCT, nonseminomatous germ cell tumor; SGCT, seminomatous germ cell tumor.

expression of *RARB* due to promoter hypermethylation [28]. In the present study, we found *RARB* promoter hypermethylation only in sensitive NSGCTs. The data, therefore, suggest that *RARB* down-regulation may favor response to CDDP treatment. The mechanisms regulated by *RARB* responsive genes in GCT require further studies to understand the role of this gene in chemotherapy response.

Previous studies indicated that tumor cells exposed to anticancer agents induce DNA hypermethylation resulting in the silencing of genes that play role in drug metabolism and resistance [29]. Human tumor cells exposed to high concentrations of CDDP *in vitro* induces alterations in 5-methyl Cytosine (5-mCyt) [30]. Similarly, *in vivo* exposure of bone marrow cells to cytosine arabinoside (araC) alone or a combination of hydroxyurea, VP-16 and araC also result in a several-fold increase of 5-mCyt content in leukemic blasts ([30]. Thus, the exposure of tumor cells to cytotoxic chemotherapy agents *in vitro* and *in vivo* causes an induction of DNA hypermethylation. In the present study, we examined whether CDDP treatment *in vivo* causes such a hypermethylation in GCT by studying specific gene promoters. Our results suggest that initial CDDP treatment in tumors induces promoter hypermethylation of certain gene promoters such as *MGMT*, *RARB*, and *BRCA1* (Fig. 2). This induction of methylation in these genes hypersensitize the tumor to further treatment, while tumors that had promoter hypermethylation of *RASSF1A*, *HIC1*, and *APC* are selected upon further treatment to develop drug resistance (Figs. 1 and 2). Such a model of CDDP-induced non-random hypermethylation can predict response to further treatment and allows specific gene targeted therapeutic approaches for resistant GCTs. Hypermethylation of *RASSF1A*, *HIC1*, and *APC* genes provides a plausible mechanism for the propensity of these tumors to CDDP resistance, and demethylation could result in restoration of hypersensitivity. Well documented evidence in certain tumor types suggest that drug resistance disrupts general mechanisms of chemosensitivity by targeting mutations and gene amplifications [31]. Here, we demonstrate that epigenetic alterations in specific genes also play a role in chemosensitivity to CDDP in GCT. The present data, thus, suggest that specific gene promoter hypermethylation induced by drugs may serve as prognostic indicator of treatment response in NSGCT. In view of the biological relevance of DNA methylation, CDDP-induced hypermethylation shown here in GCTs will have clinical significance in drug-response phenotypes.

CDDP-induced promoter hypermethylation in tumor cells might set in motion a cascade of ectopic gene expression events that might release tumor from normal homeostatic controls. These changes include deamination of 5-methyl cytosine in CpG causing genetic instability (i.e., mutations), transducing epigenetic changes into genetic alterations, or inactivation of methylated genes. To test the latter possibility, we tested gene expression in four different clones from two highly resistant cell lines. We could not reactivate the gene expression by exposure to the demethylating agent 5-Aza-C or histone deacetylase inhibitor TSA, implying that a common epigenetic and/or genetic mechanisms that regulate transcriptional activation of hypermethylated genes was affected in highly resistant cells rather than simple methylation changes in specific gene promoters.

The cytotoxic effectiveness of CDDP against tumor cell is believed to be mediated through the formation of DNA adducts, which inhibit DNA replication and transcription [32,33]. Cisplatin primarily forms intra-strand GpG cross-links, which are removed by neucleotide excision repair (NER) [34]. Highly regulated steps involving a number of proteins coordinate the NER in human cells. One hypothesis to explain the hypersensitivity of GCT to CDDP is that there is a deficiency in one or more components of this repair machinery [35]. Recently, it has been shown that elevated testis-specific high-mobility group (ts-HMG) DNA-binding proteins may enhance sensitivity to CDDP [34]. Our results suggest a potential molecular mechanism of CDDP-induced transcriptional inactivation of genes prone to hypermethylation. The CDDP exposure may cause genetic damage that might sequester essential proteins from their designated function such as elements of DNA repair pathways. The results presented here support the notion that epigenetic mechanisms play a role in CDDP-response in a gene specific manner. As cellular response to CDDP treatment in GCT is believed to be a complex process, future studies to address this issue need to examine both epigenetic and genetic alterations.

Conclusions

Our studies provide evidence that the *RASSF1A* and *HIC1* inactivation by promoter hypermethylation play a role in NSGCT resistance and may serve as markers for the identification of resistant tumors. The epigenetic alterations in *MGMT* may be an important factor in conferring the exquisite sensitivity of GCT to CDDP. Although the molecular mechanisms of GCT resistance are unclear currently, our findings of epigenetic alterations in the *RASSF1A*, *HIC1*, *MGMT*, and *RARB* genes may serve as prognostic indicators of CDDP-related treatment response and provide molecular targets of therapy to chemo-sensitize the resistant tumors. In view of the biological relevance of DNA methylation, CDDP-induced hypermethylation shown here in GCTs will have clinical significance in drug-response phenotypes and provides opportunities to modulate pathways controlled by these genes.

Methods

Tumor specimens and stratification of chemotherapy resistance and sensitivity

Tumor tissues were identified by retrospective review of GCT specimens obtained during diagnostic evaluation at the Memorial Sloan-Kettering Cancer Center, New York, between 1987 and 1999. Patients were identified based on known response and resistance to chemotherapy. A

total of 70 GCT specimens from 60 patients comprised the study cohort. The sensitive tumors consisted of 31 tissues obtained from 29 patients that were relapse-free for more than two years as a result of chemotherapy alone or in combination with surgery. The resistant panel comprised of 39 tumors from 31 patients, with or without retroperitoneal lymph node metastasis, who either did not respond to one or more cycles of CDDP-based chemotherapy or responded and then relapsed, or died of disease after any number of cycles of treatment. Table 1 summarizes the histologic and phase characteristics of sensitive and resistant patients. Additionally, 36 unselected GCTs evaluated at Columbia University Medical Center were also studied.

Cell lines and drug treatment

Four NSGCT cell lines, an established 833K-E and three cell lines (169A, 240A and 218A) described by us earlier, were grown in high-glucose DMEM medium containing 15% fetal bovine serum, L-glutamine, and penicillin-streptomycin [36]. Sub-cultured cells after 24 hr were treated with the specific drugs at different concentrations and time periods. Cells in logarithmic phase were exposed to CDDP at 0.5 μM and 1.0 μM concentrations for 2 h and 24 hr, at which time drug was removed, and fresh culture medium was added. The CDDP-treated cells were continued to grow for 2, 4, and 7 days to clonally expand. We have also derived CDDP-refractory cells from 833K-E and 240A cell lines by growing for 21 and 16 days, respectively. Two independent clones derived from each of these cell lines were designated as 833K-E/C10, 833K-E/C13, 240A/C4, and 240A/C10. After further expansion, these cells were designated as D1-resistant cells for one time point drug treatment. These D1 cells were further treated serially with increasing concentrations (1.5 μM to 4.5 μM) of CDDP and were grown in culture for more than 90 days. The final passage cells were designated as D4 cells for four time points of drug selection. Untreated and the CDDP-resistant cell lines were exposed to demethylating agent 5-Aza-2' deoxycytidine (5-Aza-C) (Sigma) for five days at a concentration of 2.5 μM and trichostatin (TSA) at 250 nM for the last 24 hours or a combination of both.

Methylation Specific PCR (MSP) and gene expression

Genomic DNA was treated with sodium bisulphite as previously described [8]. 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 have been either described previously [8] or are available from authors upon request. PCR products were run on 2% agarose gels and visualized after ethidium bromide staining.

Gene expression was assessed on total RNA isolated from four normal testes, a commercially purchased normal testis RNA (Clontech, Palo Alto, CA) and the cell lines described above. Reverse transcription was performed using random primers and the Pro-STAR first strand RT-PCR kit (Stratagene, La Jolla, CA). A semi-quantitative analysis of gene expression in replicate experiments was performed using 26 to 28 cycles of multiplex RT-PCR with β-actin (*ACTB*) as a control and gene specific primers spanning at least 2 exons whenever possible. The gene primers used have either been described previously [8] or are available from authors. 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).

Bisulphite sequencing

Bisulphite-treated DNA was amplified with primers designed to amplify both methylated and unmethylated DNA. Two sets of primers were designed to cover the entire promoter region of the *MGMT* gene. The first set of primers was MGMT-cl-F3 5'-AGGATTTGAGAAAAGTAAGAGAG-3' and MGMT-cl-R3 5'-ATTTAACAAACTAAAACACAAAACC-3', and the second set of primers was MGMT-cl-F4 5'-TTTTTTTGTTTTTTTTAGGTTTT-3' and MGMT-cl-R4 5'-CAAACACCAACCATAATAACCAA-3'. PCR products were sub-cloned into pCR2.1-TOPO (Invitrogen) and DNA isolated from 15 to 20 clones for each tumor was sequenced.

Tissue microarray and immunohistochemical analysis

A panel of 36 unselected formalin-fixed, paraffin-embedded tissue specimens from 18 NSGCTs and 18 SGCTs was used to construct a tissue array (Beecher Instruments, Silver Spring MD). Representative areas of the biopsy were chosen to construct a 14 × 8 tissue array. Four micron-thick sections on the array were immuno-stained following deparaffinization and antigen retrieval using citrate buffer at pH6.0. The primary antibody against MGMT was obtained from NeoMarkers (Fremont, CA). The antibodies were detected with the Envision plus (DAKO, Carpenteria, CA) system, using diaminobenzidine as a chromogen. Tumors were considered positive for MGMT when cells showed brown nuclear staining. Interstitial and intravascular lymphocytes, as well as spermatogonia of any residual seminiferous tubules were used as internal controls.

Statistical analyses

Comparisons for the analyses of sensitive vs. resistant tumors were done via Fisher's exact test. For the eight cases that contributed multiple specimens, the specimen with the greatest number of methylated genes was used. Exact logistic regression was used for the phase comparisons with the analyses adjusted for sensitive/resistant status

and histology where noted. Here multiple specimens from the same patient were included. None of the p-values were adjusted for multiple comparisons due to the exploratory nature of the analysis.

Authors' contributions

SK carried out the methylation, cloning, sequencing and gene expression analysis. JMM participated in selection of tumor specimens, isolation of DNA and RNA. GN participated in the analysis of gene expression. JH coordinated the selection of tumors, tissue culture, isolation of genomic DNA and RNA. JB performed statistical analysis. DLB participated in obtaining the follow up on patients. AMA and MM participated in the preparation of tissue array and gene expression analysis. VER participated in histologic diagnosis. GJB was responsible for referring the patients and clinical information. RSKC and VVVSM have conceived and coordinated the study. All authors read and approved the final manuscript.

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