Characteristic promoter hypermethylation signatures in male germ cell tumors

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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 pluripotentiality 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 occurred 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 reactivates 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, p14ARF, 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 APAFI) 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, APAFI, 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
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

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**Figure 1**

**Analysis of promoter methylation in GCT.** Frequency of CpG methylation in nonseminomatous and seminomatous histologies.
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
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 BRCA1 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 variety 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-

### 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.
70–80% of patients who present metastatic disease are
type [27]. More than 90% of newly diagnosed GCTs and
of death [25,26]. It has been suggested that the high-levels
methylation, on the other hand, associates with high-risk
sensitivity to alkylating agents in cancer [24,25]. Lack of
Epigenetic gene silencing of the
observations).
MSP analysis. Therefore, these data indicate that other ep-
genetic and/or genetic changes may be involved in regu-
lating 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-med-
ated 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
cells that did not exhibit methylation by
methylation analysis. These data thus sug-
gest that global demethylation may not only influence the
down-regulation of methylated genes but also unmethylated
genes. Such a phenomenon has previously been reported
[31,32].

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

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.

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 pheno-
type [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 re-
sistance to the cisplatin curative regimen leading to high
mortality in this group. The molecular basis of this exqui-
site 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 ap-
opoptosis due to failure of repair of DNA adducts formed
by alkylating agents. Lack of MGMT expression in the major-
ity of GCTs suggests a potential role for this protein in lack
of repair of cisplatin-induced DNA damage that may re-
sult 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 hu-
man 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 hy-
permethylation 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
promoter genes 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 sug-
gest 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].
ing seminomatous and nonseminomatous GCTs. Promoter methylation was frequently seen in DNA repair genes \textit{MGMT}, \textit{RASSF1A}, and \textit{BRCA1}, and a transcriptional repressor gene \textit{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 \textit{MGMT} gene expression in GCTs of all histologic subsets. Given the importance of the \textit{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 \textit{MGMT}, \textit{RARB} and \textit{RASSF1A} genes. Further characterization of the exact mechanisms involved in epigenetic gene silencing, especially in the \textit{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 \textit{SssI} 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 \textit{RARB}, \textit{TIMP3}, \textit{CDKN2A}, \textit{p14ARF}, \textit{MGMT}, \textit{DAPK}, \textit{CDH1}, \textit{GSTP1}, \textit{APC} promoter 1A, \textit{RB1}, \textit{MLH1}, \textit{TP73}, \textit{BRCA1}, \textit{FHIT}, and \textit{HIC1} have been described previously [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]:

| Gene   | Methylation-specific Primers |
|--------|------------------------------|
| \textit{BTG1} | 5'-GTCGTTCGTTTTTACGTTTTT-3' |
| \textit{NEDD1} | 5'-GGATATTTTTAGTTTACGCGG-3' |
| \textit{NEDD1} | 5'-CGACCCCCCTATATATTACTACG-3' |
| \textit{NEDD1} | 5'-TGGATATTTTTAGTTTACGCGG-3' |
| \textit{NEDD1} | 5'-CAACCCCCCTATATATTACTACG-3' |
| \textit{APAF1} | 5'-GCCTGCCAGTATTTATGTAATAA-3' |
| \textit{APAF1} | 5'-CAAACCGAGCAACCCCGAA-3' |
| \textit{APAF1} | 5'-GGTGTGTGTGTGTGTATGTAATAA-3' |
| \textit{APAF1} | 5'-CACAAACCAACCCCAAA-3' |
| \textit{NME1} | 5'-GTTCGTGCGTGTAAGTATGTTG-3' |
| \textit{NME1} | 5'-CCACCGACAAAAACGAATCCA-3' |
| \textit{NME1} | 5'-GTTCGTGCGTGTAAGTATGTTG-3' |
| \textit{NME2} | 5'-TTTCGTCGCGTCGGGTC-3' |
| \textit{NME2} | 5'-GGTGTGTGTGTGTATGTAATAA-3' |
| \textit{NME2} | 5'-CACACAAAACTACGAAAAATCCAC-3' |
| \textit{NME2} | 5'-GTTCGTGCGTGTAAGTATGTTG-3' |
| \textit{NME2} | 5'-CACACAAAACTACGAAAAATCCA-3' |
| \textit{RASSF1A} | 5'-ACGCGTTGCGTATCGCGCCG-3' |
| \textit{RASSF1A} | 5'-CCCGCGACGACTACGCTACC-3' |
| \textit{RASSF1A} | 5'-ATGTGTGTGTATGTAATGTTG-3' |
| \textit{RASSF1A} | 5'-CCACCAACACTACACTACCC-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:

\[
\begin{align*}
\text{MGMT-F} & \quad 5'\text{-GCACGAAATAAAGCTCCTGG-3'} \quad (124–143 \text{ bp}) \\
\text{MGMT-R} & \quad 5'\text{-AGGGCTGCTAATTGCTGGTA-3'} \quad (380–399 \text{ bp}) \\
\text{MLH1-F} & \quad 5'\text{-CTGGACGAGACAGTGTTGA-3'} \quad (52–71 \text{ bp}) \\
\text{MLH1-R} & \quad 5'\text{-CTCACCTCGAAGGCCATAGG-3'} \quad (308–327 \text{ bp}) \\
\text{APC-F} & \quad 5'\text{-AAGCCGGAAGGATCTGTAT-3'} \quad (329–348 \text{ bp}) \\
\text{APC-R} & \quad 5'\text{-TCCAATTGCCITCTGCAT-3'} \quad (588–607 \text{ bp}) \\
\text{RARB-F} & \quad 5'\text{-AATTCAGTGAACTGGCCACC-3'} \quad (770–789 \text{ bp}) \\
\text{RARB-R} & \quad 5'\text{-GGCAAAGGTGAACACAAGGT-3'} \quad (1010–1029 \text{ bp}) \\
\text{CDH1-F} & \quad 5'\text{-CTCGACACCCCGATTCAAAGT-3'} \quad (335–354 \text{ bp}) \\
\text{CDH1-R} & \quad 5'\text{-CTGGACGAGACAGTGTTGA-3'} \quad (615–634 \text{ bp}) \\
\text{TIMP3-F} & \quad 5'\text{-CTTCCGAGAGTCTCTGTGC-3'} \quad (1440–1450 \text{ bp}) \\
\text{TIMP3-R} & \quad 5'\text{-GGCGTAGTGTTTGGACTGGT-3'} \quad (1713–1732 \text{ bp}) \\
\text{BRCA1-F} & \quad 5'\text{-TCAGCTTGACACAGGTTTG-3'} \quad (676–695 \text{ bp}) \\
\text{BRCA1-R} & \quad 5'\text{-GGTTGTATCCGCTGCTTTGT-3'} \quad (896–915 \text{ bp}) \\
\end{align*}
\]

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 tests. 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.

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