Coordinated Cancer Germline Antigen Promoter and Global DNA Hypomethylation in Ovarian Cancer: Association with the BORIS/CTCF Expression Ratio and Advanced Stage

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Abstract

**Purpose:** Cancer germline (CG) antigens are frequently expressed and hypomethylated in epithelial ovarian cancer (EOC), but the relationship of this phenomenon to global DNA hypomethylation is unknown. In addition, the potential mechanisms leading to DNA hypomethylation, and its clinical-pathologic significance in EOC, have not been determined.

**Experimental Design:** We used quantitative mRNA expression and DNA methylation analyses to determine the relationship between expression and methylation of X-linked (MAGE-A1, NY-ESO-1, XAGE-1) and autosomal (BORIS, SOHLH2) CG genes, global DNA methylation (5mdC levels, LINE-1, Alu, and Sat-α methylation), and clinical-pathology, using 75 EOC samples. In addition, we examined the association between these parameters and a number of mechanisms proposed to contribute to DNA hypomethylation in cancer.

**Results:** CG genes were coordinately expressed in EOC and this was associated with promoter DNA hypomethylation. Hypomethylation of CG promoters was highly correlated and strongly associated with LINE-1 and Alu methylation, moderately with 5mdC levels, and rarely with Sat-α methylation. BORIS and LINE-1 hypomethylation, and BORIS expression, were associated with advanced stage. GADD45A expression, MTHFR genotype, DNMT3B isoform expression, and BORIS mRNA expression did not associate with methylation parameters. In contrast, the BORIS/CTCF expression ratio was associated with DNA hypomethylation, and furthermore correlated with advanced stage and decreased survival.

**Conclusions:** DNA hypomethylation coordinately affects CG antigen gene promoters and specific repetitive DNA elements in EOC, and correlates with advanced stage disease. The BORIS/CTCF mRNA expression ratio is closely associated with DNA hypomethylation and confers poor prognosis in EOC. *Clin Cancer Res; 17(8); 2170–80. ©2011 AACR.*

Introduction

Epithelial ovarian cancer (EOC) is the fourth leading cause of cancer death in U.S. women, and more than 80% of patients are diagnosed with advanced disease (1, 2). Although initially responsive to chemotherapy, women diagnosed with advanced disease frequently relapse, resulting in a poor survival rate (1). Therapeutic options for patients with recurrent EOC are limited, and novel interventions are urgently needed.

Cancer germline (CG) (or cancer-testis) antigens have received significant interest as cancer vaccine targets due to their restricted expression in normal tissues with frequent expression in cancer, high immunogenicity, and roles in oncogenesis (3, 4). CG antigen vaccines have shown encouraging results in clinical trials, particularly those targeting MAGE-A3 or NY-ESO-1 (3, 5, 6). The limitations to this approach include the frequently low or heterogeneous expression of CG antigens in human tumors (7). Successful clinical development of CG antigen vaccines will benefit from a greater understanding of the molecular mechanisms and clinicopathology associated with their expression in cancer.

In normal somatic tissues, CG genes are repressed by epigenetic mechanisms including DNA methylation, recruitment of methylated DNA binding proteins, and repressive histone modifications (4). These epigenetic marks are lost or reduced in cancer cells that express these genes and, in particular, promoter DNA hypomethylation plays a key role in inducing CG antigen gene expression (4, 8, 9). How CG gene expression and methylation relate
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Translational Relevance

Cancer germline (CG) antigens are immunogenic targets of anticancer vaccines, which are undergoing clinical evaluation in epithelial ovarian cancer (EOC) and other malignancies. Here we show that CG antigen genes of distinct families and chromosomal localization are coordinately expressed in EOC and that expression often, but not always, coincides with promoter DNA hypomethylation. CG promoter hypomethylation is highly coordinated and is associated with global DNA hypomethylation in EOC, including the hypomethylation of LINE-1 and Alu repetitive elements. DNA hypomethylation is also associated with advanced clinical stage. Notably, DNA hypomethylation is associated with an increased mRNA expression ratio of the paralogous epigenomic regulators BORIS and CTCF. The BORIS/CTCF expression ratio is also associated with increased stage and decreased overall and progression-free survival in EOC. In summary, DNA hypomethylation is a coordinated event in EOC that is associated with the BORIS/CTCF expression ratio and advanced stage.

Finally, we sought to determine the relationship between global DNA methylation and EOC clinicopathology.

Materials and Methods

Human tissue samples

Normal ovary (n = 10) or ovarian or primary peritoneal tumor samples (n = 75) were obtained from patients undergoing surgical resection at Roswell Park Cancer Institute (RPCI) under Institutional Review Board–approved protocols, as described previously (7, 23, 24). Pathology specimens were reviewed at RPCI, and tumors were classified according to World Health Organization criteria (25). Supplementary Table S1 lists the tumor samples and clinicopathology. Of 75 samples, 72 (96%) were EOC (including primary peritoneal), whereas the remaining 3 samples included 1 granulosa, 1 immature teratoma, and 1 primitive neuroectodermal tumor.

Quantitative reverse transcriptase PCR

Quantitative reverse transcriptase PCR (qRT-PCR) was done as described previously (9). MAGE-1, XAGE-1, NY-ESO-1, and BORIS primers were reported previously (7, 9, 23). BORIS primers overlap exons 5 to 7, and were designed to amplify the originally reported transcript (26). These primers amplify 4 of 6 of the recently reported BORIS transcript subfamilies (13 of 23 total isoforms; ref. 27). CTCF and GADD45A primers were designed by Primer 3 (sequences available on request; ref. 28). Samples were run in triplicate, and data were normalized to GAPDH.

Western blot analyses

Frozen tissues were crushed by using a mortar and pestle prechilled with liquid nitrogen. Powdered extracts were then lysed on ice by using radioimmunoprecipitation assay buffer and sonicated with a Bioruptor (Diagenode). Protein extracts (30 μg) were separated using NuPAGE 4% to 12% Bis-Tris Gels (Invitrogen). Western blots were probed with anti-human BORIS (Abcam), anti-human CTCF (Abcam), or anti-human β-actin (Santa Cruz). Blots were then probed with horseradish peroxidase–conjugated secondary antibodies (GE Healthcare), and incubated with enhanced chemiluminescence reagent (Perkin Elmer). BORIS and CTCF protein expressions were determined by standard densitometry analysis, after normalization to β-actin. All immunoreactive BORIS bands were specific, based on experiments by using competitor peptide (Abcam; data not shown), and were added together to determine total BORIS expression.

DNA methylation analyses

5-Methyl-deoxycytidine (5mC) levels were determined by liquid chromatography–mass spectrometry (LC-MS; ref. 29). Sodium bisulfite pyrosequencing was used to determine methylation of LINE-1, Alu (Alu Sx), and SAT-α, as described previously (7, 30, 31). Pyrosequencing was also used to determine methylation of BORIS, MAGE-A1, XAGE-1, NY-ESO-1, and SOHLH2 promoters, as described.

to the overall epigenetic status of tumors has been the topic of a limited number of investigations (10–12). Although this work suggests that the epigenetic activation of CG genes is associated with global DNA hypomethylation, this model has not been adequately addressed due to the restricted number of CG genes investigated (chiefly one gene, MAGE-A1), the limited number of primary tumors studied, the qualitative methods used to analyze DNA methylation, and the fact that distinct measures of global methylation status (e.g., different classes of repetitive elements) have not been investigated (10–12). In addition, the potential mechanisms accounting for either CG gene hypomethylation and/or global DNA hypomethylation remain unresolved. In this context, mechanisms that have been proposed to contribute to DNA hypomethylation include GADD45A expression (13, 14), methylenetetrahydrofolate reductase (MTHFR) genotype (10), DNMT3B isoform expression (15–17), and BORIS (or CTCFL) expression (18, 19).

DNA methylation changes play a key role in the pathogenesis of EOC (20, 21). These changes include Cpg island hypermethylation of tumor suppressor genes, reduced 5-methylcytosine (5mC) levels, and hypomethylation of microsatellite repeat sequences including Sat2 and Sat-α (20–22). In addition, we have previously reported that specific CG antigen promoters, and the LINE-1 repetitive element, are hypomethylated in EOC as compared with normal ovary (7, 23). The initial aim of this study was to clarify the relationship between epigenetic regulation of CG antigen genes and global DNA hypomethylation in EOC. Second, we examined the relevance of mechanisms proposed to contribute to DNA hypomethylation in cancer.

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previously (7, 23, 32, 33). The location of the pyrosequencing primers, CpG sites analyzed, CpG island location and characteristics, and additional gene information is given in Supplementary Table S2. LC-MS and pyrosequencing were done on duplicate samples, and assays were repeated at least twice.

**NY-ESO-1 immunohistochemistry staining**

Immunohistochemistry (IHC) staining of NY-ESO-1 was done as described previously (17).

**MTHFR genotyping**

MTHFR genotype analysis was done as described previously (24).

**DNMT3B isoform analysis**

RT-PCR was used to measure the expression of DNMT3B splice variants as described previously (15). qRT-PCR was used to measure the expression of the DNMT3B3Δ5 variant as described previously (17).

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**Statistical analyses**

Kendall’s tau was used to test associations between molecular and clinicopathologic parameters (35). Categorical data (NY-ESO-1 IHC, stage, and grade) were transformed into ordered data according to standard methods. EOC histology was transformed into ordered data in ascending order from best to worst prognosis, based on our experience at RPCI from 1999 to 2009, using the following formula: endometrioid = 0, mucinous = 1, serous = 2, mixed = 3, clear cell = 4, carcinosarcoma = 5. Non-EOC tumors were excluded from histology association analyses. MTHFR genotype was transformed into ordered data from highest to lowest functioning enzyme activity, using the following formula: C = 1, C/T = 2, T = 3. For tests of survival, Kaplan–Meier curves, the log-rank test, and/or the Cox proportional hazard model was used. For all comparisons, the significance level was set at 0.05. To account for multiple comparisons, we also carried out the multiple comparison error control by using the false discovery rate (FDR) less than 0.05, assuming that tests are

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Figure 1. CG antigen gene expression and promoter DNA methylation in EOC. A, Kendall’s tau analysis of CG antigen mRNA (BORIS, MAGE-A1, NY-ESO-1, XAGE-1) and protein (NY-ESO-1) expressions in EOC samples. mRNA expression and protein expression were determined as described in Materials and Methods. Each box lists the correlation coefficient (top; minus sign indicates a negative correlation), P value (middle; underlined), and number of samples (bottom). Shaded boxes indicate significant correlations (P < 0.05). Shaded boxes containing asterisks indicate correlations that remain significant after FDR correction (P < 0.0144). B, MAGE-A1 versus XAGE-1 mRNA expression in EOC. C, Kendall’s tau analysis of CG antigen promoter methylation (BORIS, MAGE-A1, NY-ESO-1, SOHLH2, XAGE-1). Promoter methylation was determined as described in Materials and Methods. Box information and labeling is described in (A). D, BORIS versus XAGE-1 promoter DNA methylation in EOC. A linear regression line is shown ( r² = 0.2576, P < 0.0001).
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To determine the relationship between the expression of different CG antigen genes in EOC, we measured the expression of representative X-linked (MAGE-A1, NY-ESO-1, XAGE-1) and autosomal (BORIS and SOHLH2) CG genes, using qRT-PCR. Of the 75 tumor samples, 62 yielded high-quality RNA suitable for analysis (data not shown). SOHLH2 was not expressed at detectable levels in these samples, and thus correlation testing was not performed for this gene (data not shown). For NY-ESO-1, IHC data were available and were also used in correlation analyses (24). A summary of CG antigen mRNA expression in EOC is shown in Supplementary Table S3. Kendall’s tau analysis revealed that the expression of different CG antigen mRNAs is often directly correlated (Fig. 1A). NY-ESO-1 mRNA and IHC expression is also directly correlated (but not significantly after FDR correction), suggesting that the expression of the NY-ESO-1 protein is partially under transcriptional control (Fig. 1A). The only mRNA pair that does not correlate is BORIS and NY-ESO-1 (Fig. 1A). For illustrative purposes, a plot of MAGE-A1 versus XAGE-1 mRNA expression is shown in Figure 1B.

We next used pyrosequencing to determine the relationship between the promoter methylation of different CG antigen genes. In agreement with our earlier studies, we observe that CG gene promoters are hypomethylated in EOC, as compared with normal ovary (Supplementary Fig. S1; refs. 7, 23). A summary of CG promoter methylation in EOC is shown in Supplementary Table S3. Note that additional tumor samples were available for DNA methylation analysis, relative to qRT-PCR. Methylation of CG gene promoters, including X-linked and autosomal genes, consistently show a significant direct correlation (Fig. 1C). For illustrative purposes, a plot of BORIS versus XAGE-1 promoter methylation is shown in Figure 1D. Linear regression analysis confirmed a significant relationship between methylation of this gene pair (Fig. 1D).

We next determined the relationship between promoter methylation and CG gene expression in EOC. In contrast with the association between the expression or the methylation status of different CG genes, the association between the mRNA expression and promoter methylation of individual CG genes is less consistent (Fig. 2A). For BORIS, there is a significant inverse correlation, after FDR correction (Fig. 2A). This relationship is also illustrated in Figure 2B, which plots the methylation levels of the 20 highest versus lowest BORIS mRNA expressing samples. For NY-ESO-1, there is an indirect correlation between promoter methylation and IHC expression, which is not significant after FDR correction (Fig. 2A). Overall, the data suggest that promoter DNA methylation partially, but not entirely, accounts for CG antigen gene expression status in EOC.

**CG antigen gene regulation and global DNA methylation**

To determine global DNA methylation, we measured 4 distinct parameters: 5mdC levels by using LC-MS (29), and LINE-1 (7), Alu (Alu Sx; ref. 31), and Sat-α (30) methylation by using pyrosequencing. Each of these parameters can be altered in cancer (37). Although global DNA hypomethylation is known to occur in EOC, the relationship between different global parameters is unknown. A summary of global methylation levels in EOC is shown in Supplementary Table S3. Kendall’s tau revealed a highly significant direct association between 5mdC levels, LINE-1 methylation, and Alu methylation (Fig. 3A). In contrast, Sat-α methylation correlates only with LINE-1, suggesting divergence in
the regulation of microsatellites (tandem repeats) compared with other markers of global DNA methylation (Fig. 3A). Linear regression of Alu versus LINE-1 methylation in EOC verified a significant direct relationship between these parameters (Fig. 3B). LINE-1 appears to be a useful overall marker for global DNA methylation status in EOC, as it significantly associates with all other global methylation measures.

Kendall’s tau revealed that the methylation of both X-linked and autosomal CG gene promoters is associated with LINE-1 and Alu methylation (5 of 5 CG genes tested; Fig. 3C). Figure 3D illustrates this relationship for NY-ESO-1 and LINE-1 methylation. In contrast to LINE-1 and Alu, 5mdC and Sat-α methylation correlate with 5 of 5 (2 of 5 after FDR correction) or 1 of 5 CG genes, respectively (Fig. 3C). On the basis of this result, we examined the region ±2 kb from the predicted transcriptional start site of each CG gene for the presence of LINE-1, Alu, and Sat-α elements. Interestingly, each CG promoter contains 1 or more LINE-1 or Alu elements, but no Sat-α sequences, suggesting a mechanistic link between methylation of CG gene promoters and retrotransposons (Supplementary Table S2). In contrast to CG promoter methylation, CG antigen mRNA expression is not significantly correlated with global methylation (data not shown). These data again suggest that mechanisms in addition to DNA methylation may influence CG antigen gene expression in cancer (4).

**DNA hypomethylation correlates with the BORIS/CTCF expression ratio**

The data presented earlier suggest that a shared mechanism promotes CG antigen promoter and global DNA hypomethylation in EOC. We therefore investigated a number of potential mechanisms that could account for coordinated DNA hypomethylation. Throughout these studies, we used LINE-1 as a hallmark of DNA hypomethylation in EOC, as it correlates with both global and CG antigen promoter hypomethylation (Fig. 3).

Recent data suggest that GADD45A is involved in DNA demethylation (13, 14). We thus used qRT-PCR to determine GADD45A expression in EOC (data not shown).
Kendall’s tau indicated that GADD45A expression directly correlates with LINE-1 methylation (correlation coefficient $= 0.202; P = 0.02, n = 60$), but no other methylation parameters (data not shown). The LINE-1 correlation is in the opposite direction as expected if GADD45A contributes to DNA hypomethylation, and thus was not examined further.

The MTHFR C667T polymorphism leads to an Ala—Val substitution that renders lower enzyme activity, decreasing the intracellular pool of S-adenosylmethionine available for DNA methylation (34, 38). A previous study of human glioblastoma reported a link between the MTHFR 667T allele, global DNA hypomethylation, and MAGE-A1 expression (10). We determined MTHFR allelic status in EOC by PCR amplification of genomic DNA followed by RFLP, as described previously (34). This analysis revealed that of the 70 EOC tumors analyzed, 40 (57%) contained the C allele, 31 (41%) contain both C and T alleles, and 8 (11%) contain the T allele (data not shown). Kendall’s tau revealed that MTHFR genotype does not associate with LINE-1 or other methylation parameters (data not shown).

Enzymatically deficient DNMT3B isoforms resulting from mRNA splice variants have been linked to DNA hypomethylation (15–17). To test their involvement in hypomethylation in EOC, we analyzed 44 tumors, including 21 with high LINE-1 methylation (mean = 70.13% methylation, SD = 2.15) and 23 with low LINE-1 methylation (mean = 40.33, SD = 8.17). We profiled samples for DNMT3B 5’ and 3’ splice variants by RT-PCR (15). Hypomethylated EOC samples tended to show higher expression of full-length DNMT3B1; however, there were no clear differences in the expression of DNMT3B isoforms (data not shown). We also measured the expression of a recently discovered DNMT3B splice variant, DNMT3B3AS5, using qRT-PCR (17). Analysis of 36 EOC samples revealed no significant difference in DNMT3B3AS5 expression between LINE-1 hypomethylated and hypermethylated EOC (data not shown).

BORIS is a paralog of the imprinting regulator CTCF (39). Previous work suggests that BORIS may contribute to activation and hypomethylation of CG antigen genes (19, 40). Nevertheless, in our dataset BORIS expression does not correlate with methylation of other CG genes or with global DNA methylation (Fig. 2; data not shown). Because BORIS and CTCF may play opposing roles in epigenetic regulation (39), we hypothesized that the ratio of BORIS to CTCF expression, rather than BORIS expression alone, may associate with DNA hypomethylation in EOC. To test this, we determined the expression of CTCF in EOC samples by qRT-PCR. As expected, CTCF is expressed at higher levels

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Association between BORIS/CTCF mRNA expression ratio and DNA methylation in EOC. A, BORIS and CTCF mRNA expressions were determined as described in Materials and Methods. Kendall’s tau analysis of the BORIS/CTCF mRNA expression with CG antigen promoter methylation and global DNA methylation parameters is shown. Each box lists the correlation coefficient (top; minus sign indicates a negative correlation), $P$ value (middle; underlined), and number of samples (bottom). Shaded boxes indicate significant ($P < 0.05$) or borderline ($P < 0.07$) correlations. Shaded boxes containing asterisks indicate correlations that remain significant after FDR correction ($P < 0.0144$). B, LINE-1 methylation versus BORIS/CTCF mRNA ratio across 62 analyzed EOC samples. Samples are plotted in descending order of LINE-1 methylation, and are assigned into 3 groups on the basis of LINE-1 methylation status. C, BORIS/CTCF mRNA expression in the LINE-1 hypermethylated ($n = 20$) versus LINE-1 hypomethylated ($n = 20$) groups, as demarcated in (B). The Mann–Whitney test $P$ value is shown.
than CG antigen genes, consistent with its widespread expression in human tissues (Supplementary Table S3; ref. 41). Similar to BORIS, CTCF expression does not correlate with CG antigen promoter or global DNA methylation (data not shown). Remarkably though, the BORIS/CTCF mRNA expression ratio significantly and indirectly correlates with multiple DNA methylation parameters (Fig. 4A). This association is also apparent when LINE-1 methylation is plotted against BORIS/CTCF mRNA expression over the panel of tumors (Fig. 4B), or when the 20 tumors with the highest LINE-1 methylation are plotted against the 20 tumors with the lowest LINE-1 methylation (Fig. 4C).

To determine whether BORIS and CTCF mRNA expression correlate with expression of the corresponding proteins, we performed Western blot analysis of a representative group of 19 EOC samples (Supplementary Fig. S2). CTCF was expressed as a prominent band of the expected molecular weight, whereas BORIS was expressed as multiple bands, consistent with a recent report (27). Quantification of the protein expression versus mRNA expression revealed a significant direct correlation for each protein (Supplementary Fig. S2). To further explore the potential relevance of BORIS and CTCF for DNA methylation regulation, we conducted an in silico analysis (http://insulatordb.uthsc.edu) of each CG gene promoter, and all 3 repetitive elements. This analysis revealed that each of these genes contain 2 or more consensus CTCF binding sites (data not shown), further supporting a model wherein BORIS and CTCF may regulate global DNA methylation status in EOC.

**Relationship of molecular parameters to clinicopathology**

We next determined the relationship of the key molecular parameters (CG gene expression, CG promoter methylation, global DNA methylation, BORIS/CTCF mRNA ratio) to EOC clinicopathology. We used Kendal’s tau to determine association between molecular parameters and age, stage, grade, histology, and first-line chemotherapy response. Clinicopathologic parameters significantly associated with each other in the anticipated directions (data not shown). No molecular parameter significantly \((P < 0.05)\) correlated with tumor grade or chemotherapy response (data not shown). In contrast, one or more molecular parameters correlate with age, stage, and histology, before FDR correction (Fig. 5A). The most notable association was with tumor stage, in which BORIS expression and the BORIS/CTCF ratio were directly correlated, and BORIS and LINE-1 methylation were indirectly correlated (Fig. 5A). To further illustrate this point, Figure 5B and C diagrams BORIS and LINE-1 methylation, and

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**Figure 5.** Molecular parameters and EOC clinicopathology. A, Kendal’s tau analysis of molecular parameters (CG antigen expression and promoter methylation, global DNA methylation, and BORIS/CTCF mRNA ratio) and clinicopathologic variables (age, stage, grade, histology, and first-line chemotherapy response) was performed as described in Materials and Methods. Only clinicopathologic variables or molecular parameters that showed significant associations are shown. Each box lists the correlation coefficient (top; minus sign indicates a negative correlation), \(P\) value (middle; underlined), and number of samples (bottom). Shaded boxes indicate significant correlations \((P < 0.05)\). Shaded boxes containing asterisks indicate correlations that remain significant after FDR correction \((P < 0.0144)\). B, BORIS promoter methylation versus disease stage. Mean bars are shown. C, LINE-1 methylation versus disease stage. Mean bars are shown.
We used the log-rank test to test the association between molecular parameters and overall and progression-free survival in EOC. At the $P < 0.05$ level, BORIS mRNA expression, the BORIS/CTCF ratio, and Alu hypomethylation were each associated with decreased overall survival (Fig. 6B and data not shown). Median overall survival for patients with BORIS/CTCF expression above or below the median value were 27.1 and 45 months, respectively. For progression-free survival, the only molecular parameter that showed a significant correlation was the BORIS/CTCF ratio (Fig. 6C). This correlation was highly significant and met the FDR cutoff (Fig. 6C). Median progression-free survival for patients with BORIS/CTCF expression above or below the median value were 17.0 and 23.3 months, respectively. Additionally, multivariate analysis of the BORIS/CTCF mRNA ratio and progression-free survival, using the Cox proportional hazard model, indicated that the association remained significant after adjustment by age ($P = 0.044$).
Discussion

Here we report that a representative subset of CG antigen genes are coordinately expressed and coordinately hypomethylated in EOC. These data suggest that both the regulation of expression and of methylation of different CG genes is controlled by similar mechanisms. In some cases, there is also an inverse association between promoter hypomethylation and CG gene expression. However, this relationship is inconsistent, suggesting that additional factors beyond DNA methylation status are likely to influence CG antigen gene expression. A number of these mechanisms have been recently reviewed (4).

A study using cancer cell lines provided the initial evidence for an association between CG antigen promoter hypomethylation and global DNA hypomethylation (11). This correlation has since been confirmed in primary tumors, including a large study of 5mC, LINE-1, and MAGE genes in gastric cancer (12); a small study of Sat2 and MAGE-A1 in glioblastoma (10); and a small study of 5mC, LINE-1, and NY-ESO-1 in micro-dissected EOC by our group (7). Here we have conducted a more comprehensive analysis of this association, involving (i) a large number of primary tumors, (ii) quantitative methods of DNA methylation analysis, (iii) multiple measures of global DNA methylation status, (iv) different families of CG genes, and (v) robust statistical analyses. Our data reveal that global DNA methylation, in particular LINE-1 and Alu, are closely associated with the methylation of both X-linked CG genes of distinct families, as well as autosomal CG antigens. These data strongly suggest that mechanisms leading to DNA hypomethylation in EOC affect a wide variety of genomic locations. Interestingly, each CG gene studied here contains a LINE-1 or Alu element in proximity to its promoter, suggesting that regulation at these sites could be mechanistically connected to epigenetic regulation of the proximal 5′ CpG island. Consistent with this idea is a report showing that conserved sequence motifs found in autosomal CG genes resemble the Alu consensus sequence (33).

Among the global DNA methylation parameters examined, 5mC, LINE-1, and Alu are strongly associated, whereas Sat-α methylation correlates only with LINE-1. LINE-1 and Alu elements are non–long terminal repeat retrotransposons estimated to comprise up to 30% of the human genome, including a large proportion of genomic CpG sites (10); thus, it is not surprising that their methylation parallels total 5mC levels. Microsatellites, including Sat-α, become hypomethylated in ovarian cancer (21, 22); however, our data suggest that these elements may be under differential methylation control from interspersed elements. This observation is consistent with a recent study that reported distinct changes in DNA methylation in tandem and interspersed repeats in cancer (42). Importantly, LINE-1 status, as it correlates with all other DNA methylation parameters, appears to be an optimal biomarker for global DNA methylation assessment in EOC.

The mechanisms accounting for global DNA hypomethylation in cancer have been the topic of much speculation (18, 43, 44). To address this question, we systematically explored these mechanisms by using our dataset. Interestingly, none of the previously hypothesized mechanisms, including BORIS expression, were associated with global DNA hypomethylation. Given this result, we hypothesized that the BORIS/CTCF ratio could impact global DNA methylation, due to the antagonistic effects of the 2 proteins (18, 19, 45). Remarkably, and in agreement, we observe a strong direct association between the BORIS/CTCF mRNA ratio and DNA hypomethylation. Preliminary data suggest that this expression ratio may be maintained at the protein level. Interestingly, each of the CG genes and repetitive DNA elements studied here contain CTCF binding sites, suggesting that BORIS and/or CTCF binding at these genes may influence DNA methylation. In agreement, a recent study of head and neck cancer observed that coordinated CG gene expression correlated with the presence of CTCF binding sites in the promoter regions of the analyzed genes (40).

Studies using normal and cancerous cell lines have reported inconsistent results with regards to the effect of BORIS overexpression on CG antigen expression and DNA methylation (4). Of note, we recently reported that full-length BORIS overexpression in different ovarian cell models does not alter CG antigen expression, CG promoter methylation, or global DNA methylation (46). Although it appears likely that cell/tissue context is a key determinant of the response to BORIS overexpression, this study additionally suggests that CTCF expression levels may be a critical parameter guiding cellular response to BORIS. Moreover, specific BORIS isoforms may have distinct functional involvement in DNA hypomethylation, and may need to be assessed individually (27).

Our study strongly supports the clinical relevance of DNA hypomethylation in EOC. In agreement with other work, the clinicopathologic factor that best correlated with CG antigen expression and DNA hypomethylation was tumor stage (47). The association of BORIS hypomethylation and mRNA expression with advanced tumor stage is consistent with an oncogenic function for this protein, as suggested by in vitro studies (40). In addition to CG antigen gene induction, the connection between global DNA hypomethylation and advanced disease could reflect altered gene expression caused by hypomethylation of retrotransposon genes, or could relate to the promotion of genomic instability (48–50). In addition to its connection with DNA hypomethylation, the BORIS/CTCF expression ratio showed a highly significant association with increased stage and decreased progression-free survival. Future studies will focus on understanding the functional significance of this ratio and its utility as a biomarker in EOC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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