Published in final edited form as:
*Clin Cancer Res*. 2011 April 15; 17(8): 2170–2180. doi:10.1158/1078-0432.CCR-10-2315.

# 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 clinicopathological 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 clinicopathology, 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.

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**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.

### Keywords

DNA methylation; DNA hypomethylation; cancer germline antigens; cancer testis antigens; ovarian cancer

## INTRODUCTION

EOC is the fourth leading cause of cancer death in U.S. women, and greater 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.

CG (a.k.a. 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 to the overall epigenetic status of tumors has been the topic of a limited number of investigations (10–12). While 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 (a.k.a. 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 to normal ovary (7,23). The initial aim of the current 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. 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 IRB approved protocols, as described previously (7,23,24). Pathology specimens were reviewed at RPCI, and tumors were classified according to WHO criteria (25). Supplementary Table S1 lists the tumor samples and clinicopathology. 72/75 samples (96%) were EOC (including primary peritoneal), while the remaining three samples included one granulosa, one immature teratoma, and one primitive neuroectodermal tumor (PNET).

### Quantitative reverse transcriptase PCR (qRT-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 5–7, and were designed to amplify the originally reported transcript (26). These primers amplify 4/6 of the recently reported *BORIS* transcript sub-families (13/23 total isoforms) (27). *CTCF* and *GADD45A* primers were designed using Primer 3 (sequences available upon request) (28). Samples were run in triplicate, and data were normalized to *GAPDH*.

### Western blot analyses

Frozen tissues were crushed using a mortar and pestle pre-chilled with liquid nitrogen. Powdered extracts were then lysed on ice using RIPA buffer and sonicated with a Bioruptor (Diagenode). 30 μg protein extracts were separated using NuPAGE 4–12% 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 HRP-conjugated secondary antibodies (GE Healthcare), and incubated with ECL reagent (Perkin Elmer). BORIS and CTCF protein expression were determined by standard densitometry analysis, after normalization to β-Actin. All immunoreactive BORIS bands were specific, based on experiments using competitor peptide (Abcam) (data not shown), and were added together to determine total BORIS expression.

### DNA methylation analyses

5-methyl-deoxycytidine (5mdC) levels were determined by liquid chromatography-mass spectrometry (LC-MS) (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 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 was done on duplicate samples, and assays were repeated at least twice.

### NY-ESO-1 immunohistochemistry staining (IHC)

IHC staining of NY-ESO-1 was done as described previously (24).

### MTHFR genotyping

*MTHFR* genotype analysis was done as described previously (34).

### 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).

### Statistical analyses

Kendall's tau was used to test associations between molecular and clinicopathological 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 1999–2009, 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 using the false discovery rate (FDR) under 0.05, assuming that tests are independent or positively correlated (36). Based on a total of 404 tests, the largest P-value to be significant with the FDR control was 0.0144. In select instances, other statistical analyses including linear regression, Spearman's, Pearson's, Mann-Whitney, and unpaired T-tests with Welch's correction, were conducted using GraphPad Prism.

## RESULTS

### CG antigen gene expression and promoter DNA hypomethylation

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. 62/75 tumor samples 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 was available and was 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* vs. *XAGE-1* mRNA expression is shown in Fig. 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 to normal ovary (Supplementary Fig. S1) (7, 23). A summary of CG promoter methylation in EOC is shown in Supplemental Table 3. 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* vs. *XAGE-1* promoter methylation is shown in Fig. 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 either 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 Fig. 2B, which plots the methylation levels of the 20 highest vs. 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 four distinct parameters: 5mdC levels using LC-MS (29), and *LINE-1* (7), *Alu (Alu Sx)* (31), and *Sat-α* (30) methylation 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*, 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 to other markers of global DNA methylation (Fig. 3A). Linear regression of *Alu* vs. *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 (5/5 CG genes tested) (Fig. 3C). Fig. 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 3/5 (2/5 after FDR correction) or 1/5 CG genes, respectively (Fig. 3C). Based on this result, we examined the region ±2kB from the predicted transcriptional start site (TSS) of each CG gene for the presence of *LINE-1*, *Alu*, and *Sat-α* elements. Interestingly, each CG promoter contains one 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 above 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 prior 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 using PCR amplification of genomic DNA followed by RFLP, as described previously (34). This analysis revealed that of 70 EOC tumors analyzed, 31 (41%) contain 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 using 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, *DNMT3B3Δ5*, using qRT-PCR (17). Analysis of 36 EOC samples revealed no significant difference in *DNMT3B3Δ5* expression between *LINE-1* hypomethylated and hypermethylated EOC (data not shown).

BORIS is a paralog of the imprinting regulator CTCF (39). Prior 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 and 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 using qRT-PCR. As expected, *CTCF* is expressed at higher levels than CG antigen genes, consistent with its widespread expression in human tissues (Supplementary Table S3) (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, while BORIS was expressed as multiple bands, consistent with a recent report (27). Quantification of the protein expression vs. 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* of each CG gene promoter, as well as all three repetitive elements. This analysis revealed that each of these genes contain two or more consensus CTCF binding

*http://insulatordb.uthsx.edu)

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. Clinicopathological 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, Fig. 5B and C diagrams *BORIS* and *LINE-1* methylation, and Fig. 6A diagrams the *BORIS/CTCF* ratio, as a function of disease stage.

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 was 27.1 months 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 was 17.0 months 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 5-methylcytosine (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 5mdC, *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, 5mdC, *LINE-1*, and *Alu* are strongly associated, while *Sat-α* methylation correlates only with *LINE-1*. *LINE-1* and *Alu* elements are non-LTR 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 5mdC 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 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 two 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). While it appears likely that cell/tissue context is a key determinant of the response to BORIS overexpression, the present 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 clinicopathological 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 as well as its utility of as a biomarker in EOC.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Drs. Michael Higgins and Jennifer Black (RPCI) for critical reading of the manuscript, and Dr. Valentina Bollati (Università degli Studi di Milano) for kind assistance with the *Sat-α* methylation assay.

**Funding Sources:** NIH RO1CA11674 (ARK); Cancer Vaccine Collaborative Grant from the Cancer Research Institute/Ludwig Institute for Cancer Research (KO); Ovarian Cancer Research Fund (KO, ARK); NIH 5T32CA009072 (AWR); NIH CA16056 (Roswell Park Cancer Institute).

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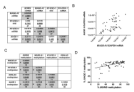

**Fig. 1.**

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) expression in EOC samples. mRNA expression and protein expression were determined as described in the *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* vs. *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 the *Materials and Methods*. Box information and labeling is described in *A*. *D*, *BORIS* vs. *XAGE-1* promoter DNA methylation in EOC. A linear regression line is shown ($r^2=0.2576$, $P<0.0001$).

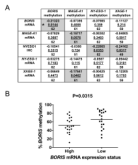

**Fig. 2.**

Kendall's tau analysis of CG antigen expression vs. promoter methylation. *A*, Gene expression and DNA methylation were determined as described in the *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 (P<0.05) or borderline (P<0.06) correlations. Shaded boxes containing asterisks indicate correlations that remain significant after FDR correction (P<0.0144). *B*, Plot of *BORIS* methylation in the 20 tumors with highest *BORIS* mRNA expression (Mean *BORIS/GAPDH* copy number = $1.618 \times 10^{-2}$) vs. the 20 tumors with lowest *BORIS* mRNA expression (Mean *BORIS/GAPDH* copy number = $7.353 \times 10^{-5}$). The Mann Whitney test P value is shown.

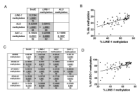

**Fig. 3.**

CG antigen gene methylation and global DNA methylation in EOC. *A*, Kendall's tau analysis of global DNA methylation (5mdC, *LINE-1*, *Alu*, *Sat-α*). Methylation was determined as described in the *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*, *LINE-1* vs. *Alu* methylation in EOC. A linear regression line is shown ($r^2$=0.3964, P<0.0001). *C*, Kendall's tau analysis of global DNA methylation and CG antigen promoter methylation. Box information and labeling is described in *A*. D, *LINE-1* vs. *NY-ESO-1* promoter methylation in EOC. A linear regression line is shown ($r^2$=0.2563, P<0.0001).

A

| | *BORIS* methylation | *MAGE-A1* methylation | *NY-ESO-1* methylation | *XAGE-1* methylation | 5mdC | *LINE-1* methylation | *ALU* methylation | *SAT-α* methylation |
|---|---|---|---|---|---|---|---|---|
| *BORIS/CTCF* mRNA | -0.4274 | -0.12113 | -0.16341 | -0.22619 | -0.02406 | -0.31364 | -0.28057 | -0.02815 |
| | * <.0001 | 0.169 | 0.0605 | * 0.0116 | 0.7842 | * 0.0003 | * 0.003 | 0.7701 |
| | 62 | 61 | 62 | 59 | 61 | 62 | 55 | 52 |

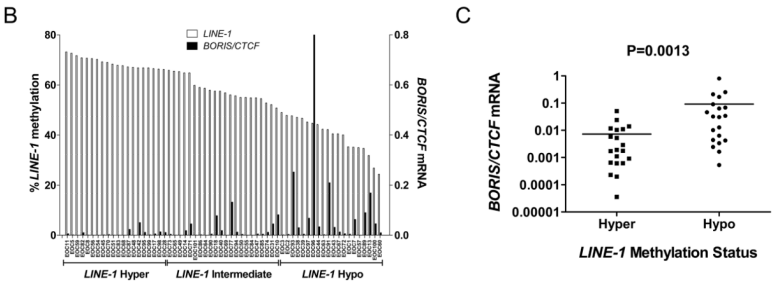


**Fig 4.**

Association between *BORIS/CTCF* mRNA expression ratio and DNA methylation in EOC. *A, BORIS* and *CTCF* mRNA expression were determined as described in the *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 vs. *BORIS/CTCF* mRNA ratio across 62 analyzed EOC samples. Samples are plotted in descending order of *LINE-1* methylation, and are assigned into three groups based on *LINE-1* methylation status. C, *BORIS/CTCF* mRNA expression in the *LINE-1* hypermethylated (n=20) vs. *LINE-1* hypomethylated (n=20) groups, as demarcated in *B*. The Mann Whitney test P value is shown.

A

| | *BORIS* mRNA | *BORIS/CTCF* mRNA | *BORIS* methylation | *MAGE-A1* methylation | *LINE-1* methylation |
|---|---|---|---|---|---|
| Age | -0.05762<br>0.5115<br>62 | 0.04377<br>0.6181<br>62 | -0.04355<br>0.5809<br>76 | -0.17098<br>0.0318<br>75 | -0.07092<br>0.3694<br>76 |
| Stage | 0.26016<br>* 0.0113<br>61 | 0.27615<br>* 0.0074<br>61 | -0.25033<br>* 0.0069<br>73 | -0.08421<br>0.3678<br>72 | -0.25683<br>* 0.0057<br>73 |
| Histology | -0.05292<br>0.6097<br>59 | 0.06216<br>0.5443<br>60 | -0.20787<br>0.0249<br>72 | -0.2138<br>0.0223<br>71 | -0.08144<br>0.3803<br>72 |

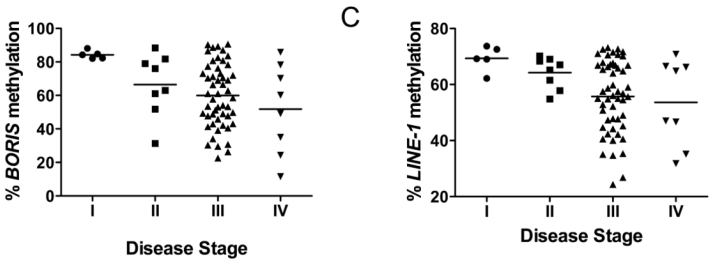


**Fig. 5.**

Molecular parameters and EOC clinicopathology. *A*, Kendall's tau analysis of molecular parameters (CG antigen expression and promoter methylation, global DNA methylation, and *BORIS/CTCF* mRNA ratio) and clinicopathological variables (age, stage, grade, histology, and first-line chemotherapy response) was performed as described in the *Materials and Methods*. Only clinicopathological 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 vs. disease stage. Mean bars are shown. *C*, *LINE-1* methylation vs. disease stage. Mean bars are shown.

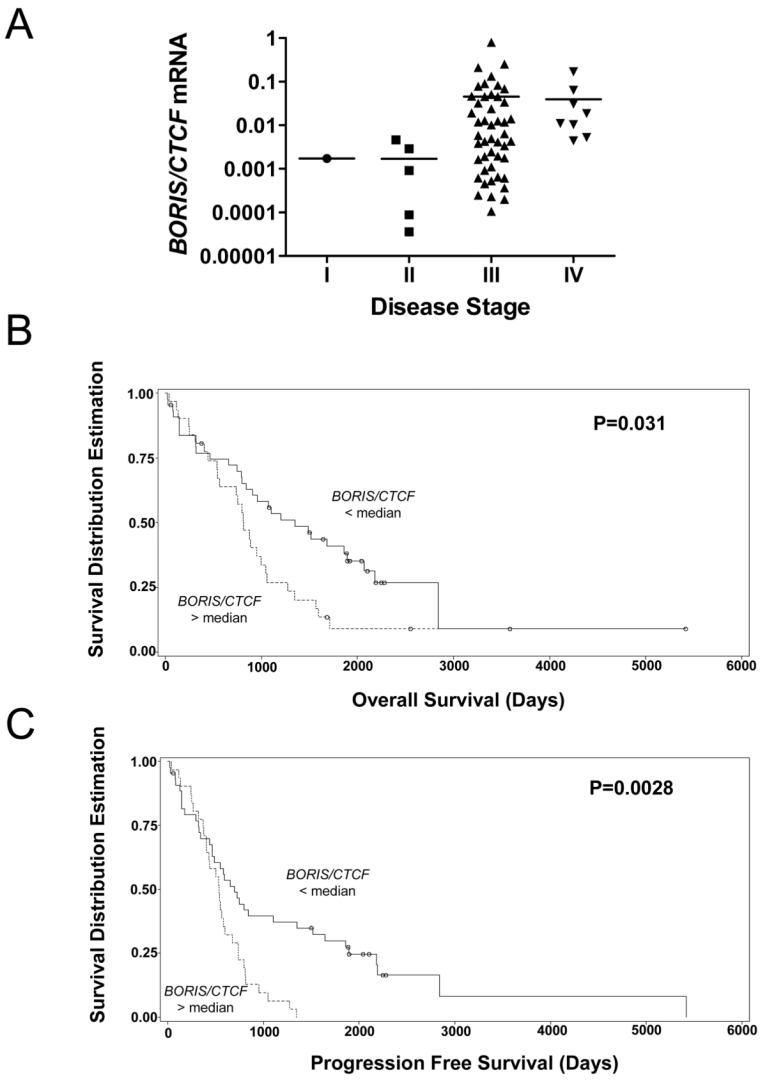


**Fig. 6.**

*BORIS/CTCF* mRNA ratio is associated with poor prognosis in EOC. *A*, BORIS/CTCF mRNA expression plotted against EOC disease stage. Mean bars are shown. *B*, Kaplan-Meier plot of overall survival as a function of *BORIS/CTCF* mRNA expression in EOC. Groups are separated based on the median *BORIS/CTCF* mRNA expression value (see Supplementary Table S3). Circles indicated censored data points. *C*, Kaplan-Meier plot of progression free survival as a function of *BORIS/CTCF* mRNA expression in EOC, plotted as described in panel *B*. In panels *B* and *C*, log-rank P-values are shown.