Evaluation of Epigenetic Drug Targeting of Heterogenous Tumor Cell Fractions Using Potential Biomarkers of Response in Ovarian Cancer

Anand Kamal Singh, Nishi Chandra, and Sharmila A. Bapat

Abstract

Purpose: Resolution of aberrant epigenetic changes leading to altered gene expression during transformation and tumor progression is pertinent for mechanistic understanding of disrupted pathways in cancer. Such changes provide for biomarkers that can be applied in drug screening and improved disease management.

Experimental Design: Genome-wide profiling and analyses of promoter DNA methylation, histone modifications, and gene expression of an in vitro progression model of serous ovarian adenocarcinoma were carried out. Similar in silico analyses and comparison of methylation and gene expression of early- and late-grade ovarian cancer samples in The Cancer Genome Atlas assigned a clinical relevance to our study. Candidate biomarkers were evaluated for epigenetic drug treatments in experimental animal models on a background of differing tumor cell responses arising from intratumor heterogeneity.

Introduction

Genetic and epigenetic changes disrupt gene expression and molecular behavior of normal cells, driving them toward transformation (1, 2). Methylation of cytosine residues in CpG islands within gene promoter regions directly regulates transcription by inhibiting binding of specific transcription factors to the DNA, while indirect regulation through recruitment of repressive chromatin remodeling methyl-CpG-binding proteins is reported (3–7). Besides DNA methylation, histone modifications, particularly trimethylation of histone 3 tails at lysines 4, 9, or 27 [H3K4me3 (K4), H3K9me3 (K9), H3K27me3 (K27), respectively], are extensively studied in the context of transcriptional regulation (8–11). Definitive roles for histone methyltransferases and histone acetyltransferases as "writer" and histone demethylases and deacetylases as "eraser" molecules in tumor initiation and progression further confirm the involvement of methylated histones in cancer (12–14).

Epigenetic alterations being less rigid than genetic changes hold the promise of possible treatment avenue in different tumor types by rendering tumor cells responsive to drugs through reversal of aberrant "epigenetic marks" (15). Ovarian cancer, the most lethal among gynecologic malignancies, is associated with late diagnosis, rapidly advancing disease, and frequent, aggressive post-therapeutic recurrence (16, 17). Altered CpG methylation, identified as an early event in epithelial ovarian cancer pathogenesis (18–22), complements histone methylations in modulating biologic functions in the disease (23, 24). In the current study, we studied these epigenetic mechanisms in correlation with altered gene expression in an in vitro progression model of serous epithelial ovarian cancer (25). Furthermore, to explore the clinical relevance of our findings, we concurrently analyzed methylation and expression data of serous ovarian tumors available with The Cancer Genome Atlas (TCGA; ref. 26; http://tcga-data.nci.nih.gov/tcga/dataAccessMatrix.htm). These consolidated efforts identified a subset of epigenetic biomarkers associated with ovarian cancer transformation and progression. We further evaluated the suitability of these biomarkers in predicting in vivo tumor responses to four epigenetic drugs through resolution of discrete cellular fractions within xenografts (27, 28). Together, our findings present a novel and comprehensive approach for the
Translational Relevance

Epigenetic gene dysregulation is associated with tumor formation and progression to a malignant stage. In the current study, altered DNA and histone methylations along with expression profiles of an in vitro progression model of serous ovarian cancer were compared with those in tumors to derive a panel of candidate biomarkers. Three of these markers, viz., PTGIS, MEST, and RXRγ, were further profiled across heterogeneous cell fractions in tumors to predict possible tumor recurrence following treatment with either 5-Aza-dC, trichostatin A, curcumin, or CBB1007. Such evaluation of drug efficiencies has different long-term regenerative implications, as it not only assigns predictive potential to candidate biomarkers in response to treatment but also indicates the tumor cell population likely to be refractory to treatment.

Materials and Methods

Cells, culture, and xenograft generation

A4, a serous ovarian adenocarcinoma cell line established in our laboratory (25), was derived as a nontumorigenic single-cell clone isolated from patient tumor ascites that underwent spontaneous transformation. These paired cells considered as representative of pretransformed and transformed cellular spontaneous transformation. These paired cells considered as representative of pretransformed and transformed cellular states (A4-P and A4-T, respectively; ref. 29), were maintained in MEM medium + 5% FBS + 1% nonessential amino acids and grown at 37°C, 5% CO2 in humidified atmosphere. P3KH26/PKH67 (Sigma) labeling of cells is described earlier (27). A total of 2.5 × 10⁶ A4-T cells were used for generating subcutaneous xenografts in 8-week-old NOD/SCID mice (Female, NOD.CB17-Prkdcscid) maintained under sterile air-flow conditions. All experimental animal procedures were done in accordance with NCCS Institutional Animal Ethics Committee clearances, laws, and policies. Animals were monitored every alternate day after cell injection. Harvested tumors were measured and processed for digestion and other studies. Tumor volume = length × (width²)/2 cm³ (30).

Methylated DNA immunoprecipitation, chromatin immunoprecipitation, promoter array, transcriptome analyses, and correlation between methylation and expression data

Methylated DNA immunoprecipitation (MeDIP)/chromatin immunoprecipitation (ChIP) was performed with fragmented (300–1,000 bp) genomic DNA from sonicated A4-P or A4-T cells. For immunoprecipitation, 4 mg of sonicated DNA was incubated for 12 hours at 4°C with anti-5-methylcytosine/K4/K9/K27 monoclonal antibody; standard protocols were followed for further enrichment and hybridization (31). Genome-wide promoter methylation profiling was performed using Agilent Human Promoter CpG 244 k Array (G4425A). Agilent Human Promoter CoC 244 k (G4489A) was used for histone methylation (31). MeDIP-chip (promoter DNA methylation) or ChIP-on-ChIP (histone methylation) data analysis was performed with Agilent genomic workbench. Preprocessing and initial analysis was done by applying the Whitehead neighborhood error model algorithm in Agilent DNA analytics. Data from each array were subjected to Median Blanks subtraction, inter-array median normalization, and dye-bias median normalization. Probe distribution on arrays to identify regions of increased/probe signals/peaks were extracted and collapsed with Gene Set Enrichment Analysis (32) to obtain negatively and positively enriched probes (enrichment ratios, <−1 and >+1, respectively; P < 0.05). For DNA methylation datasets, negatively enriched probes were considered as hypomethylated, while positively enriched probes were considered as hypermethylated genes.

Gene expression profiles of A4-P and A4-T cells submitted earlier as GSE18054 were subjected to univariate analysis for class comparison as described earlier (P < 0.01; 33). TCGA ovarian cancer samples were segregated into two groups, wherein group 1 comprised of grade 1 and 2 samples (n = 6 and 69, respectively) that were comparable with A4P, while group 2 consisting of grade 3 samples (n = 484) were compared with A4T. Datasets of these tumors were subjected to class comparison with univariate test for probes with P < 0.01; analysis was done using BRB array tools for collation and median deviation analyses); Perl programming was further used to identify hypomethylated and hypermethylated genes (β values <0.3 and >0.7, respectively; ref. 34). A flowchart of the outcome of this derivation is represented in Supplementary Fig. S1. Correlation between DNA methylation and gene expression was visualized as Starburst plots. Briefly, P values of differential genes for methylation and expression in three different datasets, viz., A4, group 1 TCGA, and group 2 TCGA, were determined by Bonferroni correction before applying a false discovery rate (FDR) adjustment across all probes for pairwise comparison (FDR adjusted to P < 0.05; the Benjamini and Hochberg method was used; ref. 35).

Bisulfite genomic sequencing

Bisulfite modification of 500 ng to 1 μg genomic DNA from A4P and A4T cells was achieved using the EpiTect Bisulfite Kit (Qiagen). Primers for bisulfite genomic sequencing (BGS) were designed around differentially enriched CpG islands identified through MeDIP using Methprimer software (36). PCR products generated from amplification of bisulfate-modified DNA were purified by using the QiaQuick Gel Extraction Kit (Qiagen) and cloned into pGEMT easy vector (Promega). Blue-white screening and selection of at least 10 clones followed transformation; clones were sequenced by the Big Dye Terminator method and analyzed for CpG methylation using BiQ Analyzer software (37). Methylation index (MI) was calculated as

\[
MI = \frac{\text{Number of methylated CpGs} \times 100}{\text{Total number of CpGs}}
\]

Drug dosage and administration

Standard MTT assays were performed to identify IC₅₀ doses of 5Aza-dC, TSA, curcumin, and CBB1007 (5, 3, 3, and 7 μmol/L, respectively, at 48 hours) for A4T cells; these drug concentrations were used in the in vitro functional assays. For in vivo studies, drugs were administered 15 days after initiation of A4 subcutaneous xenografts at tumor site in NOD/SCID mice (n = 3 for each treatment and control) at a final drug concentration of 5, 3, 3, and 7 mg/kg of body weight (5Aza-dC, TSA, curcumin, and CBB1007, respectively).
respectively). Drug regime comprised of administration on three alternate days followed by 1 week recovery before tumors were harvested for analysis (detailed regimes described in Supplementary Fig. 5A-D).

RNA extraction, cDNA preparation, RT-PCR, quantitative PCR, ChIP-qPCR
RNA extraction, cDNA preparation, and amplification from cells and tumors were performed as described earlier (29). Amplified products were run on 1.5% Agarose gel; β-actin was used as the internal control in all reactions. Each gene expression was normalized with corresponding β-actin expression in the sample and fold changes estimated versus control samples. Gel images were captured on a SYNGENE gel doc system at 3-ms UV exposure and converted into JPEG format without post processing. Denaturation analysis was carried out with gel analyses software GENETOOL. qPCR analyses with specific gene primers were carried out with Step one plus in 96-well plate format using SYBR Green Mix (Life Technologies). Changes in threshold cycle (Ct) values were calculated as \( \Delta C_t = C_t \) (test) – C_t (control); fold change was calculated as: fold change = \( 2^{-\Delta\Delta C_t} \). Actin expression was used for normalization; nontemplate controls accounted for possible contaminating DNA in reaction mixtures. ChIP-PCRs were performed as per standard protocols using K4/K2/K27 antibodies and primers flanking enriched probes; specific primer sequences for all amplifications can be provided on request.

Apoptosis assays, FACS staining, and resolution of various tumor cell fractions
Annexin V–FITC–based apoptosis was assayed as described earlier (38) and acquisitions were made on FACSCanto II; Diva software (Rector Dickinson) was used for data analysis. For analysis of heterogeneous populations, unlabeled tumor cells were used as controls for gating total dye quenching events, while freshly labeled cells were used as positive controls. Propidium iodide (PI), Hoechst–PyroninY staining was carried out as described (28).

Clonogenecity (adherent/nonadherent/soft agar colony), spheroid formation, wound healing assays, and immunostaining
A total of 5 × 10^3 sorted tumor cells were added per well in 96-well culture plates. After incubation at 37°C for 14 days, cells were washed twice with PBS and stained with 0.05% crystal violet in 20% methanol. For soft agar assays, 5 × 10^3 sorted tumor cells were suspended in 0.5% low melting agarose (Sigma) in 2 × MEM, plated above a layer of 1% agarose in 35 mm dish, incubated at standard conditions for 3 weeks, colonies stained with 0.005% crystal violet. Adherent and nonadherent colonies were photographed and counted with ImageJ software. For generating tumor spheroids, 5 × 10^3 sorted tumor cells were plated per well in 24-well ultra-low attachment plates in MEM + 1% FBS. Developing spheroids were counted at days 6, 9, and 12 (20 × inverted phase contrast microscope). For wound healing/cell migration, 96-well plates were seeded with 1,000 sorted tumor cells/well, media changed every alternate day, and cells allowed to grow until 90% confluency. Wound was inflicted with a pipette tip, two washes with 1 × PBS followed to remove floating cells and media (lacking serum) were added. Migration was monitored for 72 hours and images captured on Olympus IX71 microscope and analyzed by Tscratch Software. For immunostaining, adherent cells/spheroids were washed with 1 × PBS, fixed with 4% paraformaldehyde, membranes permeabilized with 0.01% Triton X-100. Following blocking with 5% goat serum in PBS, primary antibody (Sigma) was added (30 minutes, ambient temperature), followed by secondary Alexa 488-labeled antibody (Invitrogen) for 20 minutes; Hoechst (Sigma-Aldrich) was used for nuclear counterstaining and confocal images were captured (63 × Carl Zeiss).

Statistical analyses
Unless mentioned otherwise, all experiments were done in triplicates and data represented as mean ± SD (Sigma Stat software). Paired t-test was performed to determine significant differences between the groups.

Results
Genome-wide DNA methylation and expression analyses of the progression model and correlation with corresponding datasets and tumor TCGA groups
Whitehead prearray neighborhood model–based analysis (threshold at \( P < 0.05 \)) of the A4 progression promoter methylation data associated 13,786 and 2,253 genes with A4P and A4T, respectively. Differential enrichment analysis (\( +1 \leq \text{enrichment ratios} \leq 1 \)) further revealed 2,395 positively and 1,159 negatively enriched (hyper- and hypomethylated) genes in A4-P cells, and 257 hyper- and 559 hypomethylated genes in A4-T cells. This correlates with 137 genes that undergo promoter hypermethylation and 152 genes undergoing promoter hypermethylation during progression from a pretransformed to transformed state (Fig. 1A, i; Supplementary Fig. S2A). Similar class comparison of gene expression data revealed 1,764 differentially expressed genes (957 up- and 807 down-regulated, respectively) during this progression (Supplementary Fig. S2B). Correlations between methylation and expression identified 76 hypomethylated–upregulated genes, and 31 hypermethylated–downregulated genes during A4 progression (Fig. 1A, ii; Supplementary Table S1).

To probe for clinical relevance of these genes during disease progression, we similarly analyzed DNA methylation and expression datasets of 539 TCGA ovarian adenocarcinoma samples that were segregated as group 1 (grades 1 and 2) and group 2 (grade 3; Materials and Methods). This identified 5,776 hypo- and 621 hypermethylated genes for group 1 tumors of which 1,536 and 155 were up- and downregulated, respectively, at the expression level; while the 4,294 hypo- and 147 hypermethylated genes in group 2 tumors, 2,028 and 54 genes were up- and downregulated, respectively (Fig. 1B, i and ii; Supplementary Fig. S2C). Higher negative enrichment suggests promoter hypomethylation rather than hypermethylation as being significant in ovarian tumor progression. Overlapping the A4 and TCGA datasets further identified a common association of 5 hypo- and 3 hypermethylated genes with pretransformed/early grade, and 15 hypo- and 2 hypermethylated genes with transformation and malignant high-grade disease [Fig. 1C (i and ii) and 1D (ii); Supplementary Fig. S1]. Functional annotation of these genes assigned cell component morphogenesis, regulation of cell proliferation, and/or apoptosis with neuronal development as being important during transformation and tumor progression (Supplementary Fig. S2D). Of these strongly associated genes, we had earlier identified MAL, MEST,
Figure 1.
Identification of differentially methylated genes in SeOvCa progression A, heatmap representing differentially methylated genes in the A4 progression model (i); starburst plots correlating DNA methylation and expression of differentially methylated genes in A4 progression (76 hypomethylated—upregulated; 31 hypermethylated—downregulated, ii); B, heatmap representing TCGA DNA methylation data, samples were segregated grade-wise into two groups (group 1, grades 1 and 2, \( n = 75 \), left; group 2, grade 3, \( n = 484 \), right; i); B, starburst plots correlating DNA methylation and expression of differentially methylated genes in group 1 and 2 TCGA samples (ii); C, Venn diagram representing hypo- and hypermethylated genes common to comparative stages between the A4 model and TCGA samples (i, ii), D, heatmap representation of differentially methylated genes specific to (i), A4-P cells and group 1 tumors (ii), A4-T cells and group 2 tumors.
Validation of differentially methylated genes in the progression model

BGS-based validation of the signature genes along with differentially methylated CYC1 and POCK (P < 0.05) affirmed demethylation of CYC1, POCK, MAL, and MEST (P ≤ 0.01; 2- to 4-fold decrease in MI; Fig. 2A and B) and methylation of PAPSS2 and PTGIS (P ≤ 0.05; >1.5 fold increase in MI) during progression. Although methylation status of the Cl CpG (−247 to −700 bp) island in FBN1 promoter could not be validated, two other CpG islands, viz., Cii (−731 to −1051) and Cii (−1,287 to −1,645) were indeed differentially methylated (Fig. 2A). Associations of differential promoter methylation and expression of these genes were also affirmed; thus, hypomethylated CYC1, MAL, MEST, and POCK were expressed, while hypermethylated FBN1 and PTGIS were repressed (P ≤ 0.01; 2- to 10-fold increased/decreased expression respectively; Fig. 2C, i and ii). Effectively, convincing correlations between CpG methylation and expression during progression were identified.

Genome-wide histone methylation analyses and correlation with gene expression during progression identifies additional epigenetic markers

Genome-wide histone methylation profiles (K4,K9,K27) during A4 progression established through ChIp-on-chip (CoC) were analyzed to identify gene promoters differentially enriched with histone marks based on probe specificity and enrichment ratios (P ≤ 0.05; ER > 1). Transformation was associated with maximum enrichment of monovalent K4 followed by K27 and K9 methylation; bivalent K4–K9, K9–K27, K4–K27, and trivalent K4–K9–K27 marks were also significantly enriched (Fig. 3A and Supplementary Table S2). Functional association of specific histone marks with gene expression was identified, whereby K4 enriched genes were expressed at high levels, those with K9 or K27 marks at low levels, bivalent K4–K9 or K4–K27 marks at moderate to high levels, while genes carrying bivalent K9–K27 (22) or trivalent K4–K9–K27 (39) marks were repressed (Fig. 3B and Supplementary Table S3).

To validate the exclusive functional effects of histone methylation on gene expression distinct from those of DNA methylation, 23 differentially regulated genes that did not exhibit methylated promoters but exclusively harbored histone marks in the progression model as well as TCGA cohort (Supplementary Table S4) were profiled for differential expression (Fig. 3C and Supplementary Fig. S3A). Six of these genes did not follow the histone code in both datasets. K4-associated upregulated genes included HDAC2, SINE4, MB1D1, RRM2, UTX, and PAX2 was downregulated by K9, NCR4 by K9–K27, and ASC2 by K4–K9–K27 marks. Differential expression of HOXA1 and EMD were not indicated in tumor grade progression, while anticorespective differential expression of RXXy, IL4, NCR2, UTX, WNT8B, SMARCA2, HOXB7 between the two models under study, viz., A4 and TCGA, tumor grade progression indicated discordance between in vitro and clinical data as is often reported.

Further functional validation of such associations between histone marks and gene expression in the progression model revealed a variance from the CoC-based identification (Fig. 3D and E). Of the K4-targeted genes, MB1D1 and HDAC2 affirmed the association, while in SINE3, RRM2, and UTX, promoter activation and gene expression is additionally associated with reduced K9 and/or K27 marks in transformed cells. Likewise, both monovalent K9 gene promoters, viz., HOXA1 and PAX2, additionally acquired K27 marks and lost K4 marks to correlate with decreased expression. Of the K27-associated genes, WNT8b exhibited an additional K9 mark leading to bivalent repressive regulation; RXXy promoter acquired K27 with loss of K4 and K9 marks, while contrary to CoC prediction the Il4 promoter lost K4 and K27 but acquired a K9 mark. Similarly, multivalent marks were affirmed through specific histone affinities and expression for EMX (K4–K9), CY2B61 (K4–K27), and ASCL2 (K4–K9–K27) promoters; a weak association of (K9–K27) was evident at the NCR2 promoter (Fig. 3D). Cell function–based annotation of these genes identified altered metabolism, transcriptional regulation, and cellular biosynthesis during transformation (Supplementary Fig. S3B).

Interestingly, progression was associated with increased DNA hypomethylation and activating histone marks that suggests a high transcriptional turnover during transformation. An important functional correlation relevant in this context emerged as activation of the transcriptional repressor machinery (SIN3A, MB1D1, HDAC2) through association with a K4 mark. Other novel observations include repression of differentiation and immunoresponsive genes (HOXA1, WNT8b, RXXy, IL4) that could alter the kinetics of tumor regeneration during progression by generating differentiation arrested progenitors and apoptosis-resistant tumor cells. In an earlier proteomics-based study, we identified RXXy downregulation as a key feature in acquisition of resistance to apoptosis during transformation (29). Importantly, the present finding suggesting this repression to be mediated by a K27 mark assigns dysregulation at the epigenetic level, and provides an opportunity to reseed tumor cells with epigenetic drugs. Collectively, this data assigns importance to acquired DNA and histone methylation in regulating cellular pathways during disease progression.

Epigenetic drugs restrict ovarian cancer growth with each drug exhibiting differential targeting of discrete tumor cell populations

Exposure of the progression model to four epigenetic drugs, including 5-Aza-dC, trichostatin A (TSA), curcumin, and a novel LSD1 inhibitor, CB1007 (LSD1i; Merck-Millipore), strikingly showed more rapid and pronounced apoptotic effects in A4T than A4P cells (Fig. 4A and Supplementary Fig. S4A). All drugs were cytotoxic; curcumin and TSA aggressively induced late apoptosis (Annexin V+/PI+) as compared with 5-Aza-dC and LSD1i that induced a higher frequency of early apoptosis (Annexin V−/PI−) indicating a latency in drug action. We also evaluated residual regenerative potential by evaluating tumor spheroid forming capability of A4T cells following each treatment (Supplementary Fig. S4B). This indicated that while all drugs effectively targeted this self-renewal capability, TSA and curcumin were more efficient than 5-Aza-dC and CB1007. Furthermore, assessment of in vivo drug effects on A4T xenografts mice subjected to defined regimes indicated significant tumor regression with each of these drugs (Supplementary Fig. S4C).

Toward a mechanistic understanding of drug cytotoxicity, we delineated the specific cellular targets of each drug vis-à-vis discrete cellular subsets resolved through flow cytometry based on the
Figure 2.
Validation of differentially methylated genes during SeOvCa progression. A, BGS of hypomethylated (CYC1, POGK, MAL, MEST) and hypermethylated (PTGIS, FBN1, PAPSS2) genes in A4P and A4T cells. Predicted CpG islands in gene promoters are indicated above the BGS profile; methylated and unmethylated CpGs are indicated as black and white circles, respectively; CpG islands marked with an asterisk (*) were validated through BGS; transcriptional start site (TSS) is indicated as an arrow;Ci, i–iii represent CpG islands 1, 2, 3, respectively; methylation index is indicated in numbers above each BGS profile; B, graphical representation of MI of differentially methylated genes validated through BGS; C, mRNA expression analysis through qRT-PCR of hypomethylated-upregulated and hypermethylated-downregulated genes; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001.

Singh et al.

Clinical Cancer Research

5156 Clin Cancer Res; 21(22) November 15, 2015

Published OnlineFirst June 30, 2015; DOI: 10.1158/1078-0432.CCR-15-0505
cancer stem cell (CSC) hierarchy, genetically unstable populations (aneuploidy) within xenografts and differential cell cycling as described earlier (28). Briefly, label-chase of vital membrane dyes (PKH26/PKH67) resolves the CSC hierarchy as three cell subsets with a decreasing order of regenerative potential, viz., PKHhi cells (quiescent CSCs), PKHlo (progenitors), and PKHneg (host and differentiated tumor cells; Supplementary Fig. S4D, Level 1). Another parameter defined is genetic instability, established through DNA content profiling with propidium iodide (PI) staining that demarcates euploid and aneuploid fractions within the xenograft (Supplementary Fig. S4D, Level 2). PI staining also quantifies cells in basic cell-cycle phases (G0–G1, S, and G2–M); supplementing this with combinatorial Hoechst–Pyronin Y staining (DNA and RNA content-based analysis) further resolves G0 from G1 fractions (Supplementary Fig. S4D, Level 3).

All four epigenetic drugs significantly target the PKHneg fraction (tumor bulk); this accounts for the tumor regression in response to drugs exposure (Fig. 4B, left). Concurrent enrichment of regenerative PKHlo and PKHhi cells however suggests that these residual progenitors and CSCs may generate recurrent disease. Ploidy analyses revealed 5-Aza-dC and TSA treatments to effectively target the aneuploid fraction, while enhanced aneuploidy was evident after CBB1007 treatment (Fig. 4B, central panels). In comparing the effects on tumor cell-cycle kinetics, 5-Aza-dC treatment exhibited elevated G0 levels (slower cell-cycle progression with increased differentiation/dormancy), while CBB1007 had opposite effects of reduced G0 with rapid cell cycling (Fig. 4B, right). Enhanced S-phase with cell cycling was observed following TSA or curcumin treatments. Importantly, such analysis that reveals a more incisive understanding of drug effects and cellular targets indicated 5-Aza-dC to be most effective in restricting emergence of genetically unstable cells that are a likely source of drug resistance. We further tested the regenerative and invasive capabilities of the residual tumor cell fractions post 5-Aza-dC treatment that affirmed effective elimination of regenerative aneuploid cells in the residual tumors. While PKHhi CSCs exhibited highest functional capabilities as assayed for formation of tumor spheroids, adherent, and nonadherent colonies and cell migration, PKHhi progenitors were not found lacking.
and surprisingly few PKH\textsuperscript{lo}\textsuperscript{hi} cells also acquired these capabilities (Fig. 4C, i–iv; Supplementary Fig. S5). Together, this suggests the reestablishment of a proliferative hierarchy that is refractory to 5-Aza-dC.

Integration of biomarker–drug associations along with resolution of tumor subpopulations is useful in prediction of long-term epigenetic drug efficacies

Functional assays associated all treatments with residual regenerative potential despite significant tumor regression. Toward an improved understanding, we queried the possibility of associating the epigenetic biomarkers as possible predictors of drug resistance/refractory behavior in specific tumor cell subpopulation(s). The entire biomarker panel was thus profiled for altered expressions following drug treatment \textit{in vitro} and \textit{in vivo} to establish possible associations based on known mechanism of action (Fig. 5A). 5-Aza-dC, TSA, and CBB1007 treatments for DNA demethylation, histone deacetylase, and LSD1 inhibition, respectively, would mediate derepression, hence upregulated genes (fold-change ≥ 1.5) would qualify as optimal targets. Such an association established \textit{PTGIS} as a marker for 5-Aza-dC as well as TSA (aberrant promoter and histone methylation); while a ~2.5 fold-change suggested \textit{RXXR} (aberrant K27 histone methylation) as a marker for CBB1007 treatment (Supplementary Fig. S6A). Contrarily, evaluating curcumin for its role in inhibiting gene repression (through histone acetylation alone despite its range of other effects; fold change ≤ 0.5), identified four putative markers, viz., \textit{MEST}, \textit{SIN3A}, \textit{RRM2}, and \textit{UTX}. Earlier reports of \textit{MEST} as an ovarian cancer biomarker (aberrant demethylated promoter and loss of imprinting; ref. 39) led to its selection in further detailed evaluation of drug action.

Thus, the frequency of tumor cells expressing \textit{PTGIS} is enhanced following exposure to 5-Aza-dC as well as TSA, that for \textit{MEST} is decreased on curcumin treatment, and \textit{RXXR} expression is elevated following CBB1007 exposure [Figs. 5A and 6A (i) and B (ii); Supplementary Fig. S6B]. Such profiling further introduced a complexity of molecular heterogeneity within the xenograft by identifying distinct fractions in which the marker is expressed (marker\textsuperscript{pos}) and those lacking marker.
expression (marker^{neg}). Toward complete understanding and assessment of drug efficacy vis-a-vis molecular as well as cellular tumor heterogeneity, we further delineated drug responses across the tumor regenerative hierarchy, genetic instability, and differentially cycling cell populations not only over the entire xenograft, but also within the marker^{pos} and marker^{neg} fractions.

1. **PTGIS** as a candidate marker for drugs targeting aberrant promoter and histone methylation: 5-Aza-dC and TSA treatments led to significantly higher enrichment of CSCs and progenitors (PKH^{hi} and PKH^{lo}, respectively) in the PTGIS^{pos} over the PTGIS^{neg} fraction. A major part of the PTGIS^{pos} fraction which constitutes most of the differentiated tumor bulk (PKH^{neg}) controls was drastically reduced (Fig. 5C, i). Enhanced PTGIS expression was also evident in spheroids generated from residual PKH^{neg}, PKH^{lo}, and PKH^{hi} cells; Supplementary Fig. S4C). Unfortunately, this suggests that other intrinsic drug-resistant mechanisms may possibly shadow the tumor suppressor effects of PTGIS. Indeed, cell-cycle analysis further revealed that while PTGIS^{pos} CSCs reenter a cycling phase following both treatments, PTGIS^{neg} CSCs remain quiescent (Fig. 5B, ii and Supplementary Fig. S4D, i). 5-Aza-dC treatment, however, restricts PKH^{lo} progenitor growth (G_{1–S} growth arrest within PTGIS^{neg} fraction and quiescence in PTGIS^{pos} fraction, while no effects of TSA on progenitor cell cycling were evident. In evaluating the effects on emergence of genetic instability, PTGIS^{pos} euploid fractions were enhanced following on 5-Aza-dC/TSA treatments, while aneuploid fractions were diminished in the PTGIS^{neg} fraction (Fig. 5D-i). Thereby, although both treatments effectively restrict genetic instability, cycling aneuploid cells in the PTGIS^{pos} fraction following TSA treatment (Fig. 5C-ii) may further repopulate the tumor. 5-Aza-dC on the other hand, appears to effectively restrict frequency and cycling of aneuploid populations in either PTGIS^{pos} or PTGIS^{neg} fractions.

2. **MEST** as a candidate marker for drugs targeting hypomethylation and/or LOI. A majority of MEST^{pos} cells throughout the regenerative hierarchy were eliminated following curcumin treatment that reflected on decreased MEST expression in the entire tumor. MEST^{pos} CSCs were arrested in G_{1–S} phase, however, MEST^{neg} CSCs and progenitors were significantly enriched and in a cycling state (Fig. 6A, ii–v), while other cells were in G_{0} phase (Supplementary Fig. S6D, ii).
3. RXRγ as a candidate marker for drugs targeting histone methylation. CBB1007 treatment led to upregulated RXRγ expression in A4T cells as well as xenografts (Fig. 6B, i). This enriched expression resulted from drastically reduced RXRγ<sup>neg</sup> differentiated cells and concurrent increase in RXRγ<sup>pos</sup> CSCs and progenitors. Despite their enrichment, a significant fraction of RXRγ<sup>pos</sup> progenitors was in G<sub>0</sub> (Fig. 6B, ii and Supplementary Fig. S6D, iii). The entire RXRγ<sup>pos</sup> fraction as well as RXRγ<sup>neg</sup> CSCs and progenitors appeared to be cycling that possibly triggers genetic instability (Fig. 6B-iii; 6B-iv). Most aneuploid cells were, however, arrested in the G<sub>1</sub>–S phase (Fig. 6B, v).

**Discussion**

The stealth of epithelial ovarian cancer progression to an aggressive, drug-resistant disease has rendered it to be a "silent killer." In the current study, we identified epigenetic dysregulation of cellular functions that complement aberrant gene and protein expression patterns in our experimental model that recapitulates disease progression (25, 29, 31, 40). Questioning the clinical relevance of these epigenetic changes with tumors in the TCGA database revealed a small number of biomarker genes potentially associated with transformation (refs. 41–44; Box 1, level I). Thereby, reversal of the expression patterns of these genes could aid in predicting tumor cell responses to epigenetic drugs during ovarian cancer treatment (Box 1, level II).

Predictive biomarkers represent specific biologic characteristics that demarcate patient subpopulation(s) likely to benefit from a given therapy (45). Several studies demonstrating stabilization of expression patterns in response to epigenetic drug treatments remain limited due to lack of complete evaluation/prediction of long-term clinical responses (46, 47). Importantly, intratumoral heterogeneity and minimal residual disease (MRD) are almost never considered in such studies and can become major deterrents in achieving drug efficacy (27, 28, 48). This realization led us to critically dissect out drug responses not only in terms of reversal of putative epigenetic biomarker expression patterns, but also evaluate functional associations with specific targeting of discrete tumor cell fractions as resolved through differential regenerative potential and genetic instability. Further examining biomarker associations, stability of their expression, cell cycling, and regenerative potential in residual tumor fractions established important read-outs toward a complete and efficient prediction of drug efficacy (Box1 and Levels III and IV). Thus, profiling of PTGIS following 5Aza-dC and TSA treatments predicted residual cycling PTGIS<sup>pos</sup> CSCs as being likely to regenerate a drug-resistant hierarchy. Similarly, residual MEST<sup>pos</sup> CSCs and all progenitors possibly contribute to curcumin refractoriness, while CBB1007 treatment was ineffective in eliminating RXRγ<sup>pos</sup> CSCs and progenitors. Thus, reversal of biomarker expression by itself is not sufficiently predictive due to crosstalks with other drug resistance mechanisms and detailed functional assays and resolution intratumor heterogeneity are essential in validating the accuracy of such predictions. Stabilization of the cell cycle to restrict aneuploidy is a novel, important effect of 5Aza-dC. Its combination with platinum/taxol (that resensitize cycling tumor cells toward apoptosis; refs. 49, 50) and also including a drug that targets the
Box 1. Identification of Potential Biomarkers of Response in Epithelial Ovarian Cancer

I. Biomarker identification and validation (Figs. 1, 2, and 3 and Supplementary Figs. S1–S3; Supplementary Tables S1–S4)

<table>
<thead>
<tr>
<th>DNA methylation</th>
<th>6 genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypomethylated-upregulated: CYCL, AML, MEST, POGK</td>
<td></td>
</tr>
<tr>
<td>Hypermethylated-downregulated: FBN1, PTGIS</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Histone methylation</th>
<th>14 genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>K4 MBD1, HDAC2, SINEA, RRM2, UTX</td>
<td></td>
</tr>
<tr>
<td>K9 HDX1, PAX2</td>
<td></td>
</tr>
<tr>
<td>K27 IL4, WNT8B, RXR</td>
<td></td>
</tr>
<tr>
<td>K4-K9 EMD</td>
<td></td>
</tr>
<tr>
<td>K4-K27 CYP26B</td>
<td></td>
</tr>
<tr>
<td>K9-K27 NCR2</td>
<td></td>
</tr>
<tr>
<td>K4-K9-K27 ASCL2</td>
<td></td>
</tr>
</tbody>
</table>

II. Epigenetic drug screening (Figs. 4 and 5A; Supplementary Figs. S4 and S5)

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Assay</th>
<th>5-Aza-dC</th>
<th>TSA</th>
<th>Curcumin</th>
<th>CBB1007</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell cytotoxicity</td>
<td>(i) MTT</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>(ii) Tumor regression</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td></td>
<td>(iii) Apoptosis (Annexin V)</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Posttreatment regenerative potential</td>
<td>(i) PKH-based hierarchy</td>
<td>△ CSCs</td>
<td>△ CSCs</td>
<td>△ CSCs</td>
<td>△ CSCs</td>
</tr>
<tr>
<td></td>
<td>(ii) Spheroid formation</td>
<td>△ (1.5-fold)</td>
<td>△ (3-fold)</td>
<td>△ (2-fold)</td>
<td>△ (1.2-fold)</td>
</tr>
<tr>
<td>Genetic instability</td>
<td>DNA content (aneuploidy)</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
<td>▼</td>
</tr>
<tr>
<td>Cell-cycle kinetics of residual cells</td>
<td>DNA and RNA content</td>
<td>△ GO</td>
<td>△ S</td>
<td>△ S</td>
<td>△ G2–M</td>
</tr>
</tbody>
</table>

III. Modulation of biomarker expression following treatment (Fig. 5A and Supplementary Figs. S4 and S6A)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Biomarker</th>
<th>Cultured cells</th>
<th>Tumor cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Aza-dC</td>
<td>PTGIS</td>
<td>39-fold increase</td>
<td>10-fold increase</td>
</tr>
<tr>
<td>TSA</td>
<td>PTGIS</td>
<td>24-fold increase</td>
<td>8.25-fold increase</td>
</tr>
<tr>
<td>Curcumin</td>
<td>MEST</td>
<td>7.66-fold decrease</td>
<td>4-fold decrease</td>
</tr>
<tr>
<td>CBB1007</td>
<td>RXRγ</td>
<td>10-fold increase</td>
<td>2.7-fold increase</td>
</tr>
</tbody>
</table>

IV. Efficacy of drug treatment with respect to potential biomarker expression (Fig. 6B–D and Supplementary Figs. S6B and S6D)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Biomarker</th>
<th>Effects following treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-Aza-dC</td>
<td>PTGIS</td>
<td>(i) Diminished frequency of differentiated and aneuploid cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Drug resistance from self-renewing PTGISpos CSCs and progenitors</td>
</tr>
<tr>
<td>TSA</td>
<td>PTGIS</td>
<td>(i) Diminished frequency of differentiated and aneuploid cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Drug resistance from self-renewing residual PTGISpos CSCs and progenitors</td>
</tr>
<tr>
<td>Curcumin</td>
<td>MEST</td>
<td>(i) Diminished frequency of MESTpos cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Enrichment of self-renewing MESTpos CSCs and progenitors</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(iii) Induced cycling of MESTpos aneuploid fractions</td>
</tr>
<tr>
<td>CBB1007</td>
<td>RXRγ</td>
<td>(i) Diminished frequency of RXRγpos differentiated fractions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(ii) Drug resistance from self-renewing residual RXRγpos CSCs and aneuploid cells</td>
</tr>
</tbody>
</table>

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

Publicly available TCGA and GEO datasets were used in this study and are referenced.

Authors’ Contributions

Conception and design: S.A. Bapat
Development of methodology: A.K. Singh, S.A. Bapat

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A.K. Singh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.K. Singh, N. Chandra
Writing, review, and/or revision of the manuscript: A.K. Singh, S.A. Bapat
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.A. Bapat
Study supervision: S.A. Bapat

Acknowledgments

The authors thank the Experimental Animal and Confocal Facilities at NCCS for providing research support. Technical assistance by Ms. Rutika Naik and Mr. Avinash Mali is gratefully acknowledged.

Grant Support

This research is funded by grants to S.A. Bapat from the Department of Biotechnology, Government of India, New Delhi (BT/PR/11465/MED/30/...
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ovarian cancer identified by methylome and expression profiling. Onco-
gene 2012;31:4567–76.

osteosarcoma cells to trail-induced apoptosis. Cell Physiol Biochem

Colorectal cancer DNA methylation marker panel validated with high

45. Italiano A. Prognostic or predictive? It’s time to get back to definitions! J

epigenetic states and acquired drug resistance in cancer. Nat Rev Cancer
2014;14:747–53.

47. Ying D, Batra J, Singh AK, Bapat S, Judith CA. Transforming the future of

48. Easwaran H, Tsai HC, Baylin SB. Cancer epigenetics: tumor heterogeneity,

pharmacodynamic study of decitabine in combination with carboplatin in
patients with recurrent, platinum-resistant, epithelial ovarian cancer. Can-

A randomised, phase II trial of the DNA-hypomethylating agent 5-aza-2’-
deoxycytidine (decitabine) in combination with carboplatin vs carbopla-
tin alone in patients with recurrent, partially platinum-sensitive ovarian
Evaluation of Epigenetic Drug Targeting of Heterogenous Tumor Cell Fractions Using Potential Biomarkers of Response in Ovarian Cancer

Anand Kamal Singh, Nishi Chandra and Sharmila A. Bapat


Updated version
Access the most recent version of this article at:

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2015/07/01/1078-0432.CCR-15-0505.DC1

Cited articles
This article cites 49 articles, 11 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/21/22/5151.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.