Identification of coexistence of DNA methylation and H3K27me3 specifically in cancer cells as a promising target for epigenetic therapy

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Abstract

Alterations of epigenetic modifications are promising targets for cancer therapy, and several epigenetic drugs are now being clinically utilized. At the same time, individual epigenetic modifications have physiological functions in normal cells, and cancer cell specificity is considered difficult to achieve using a drug against a single epigenetic modification. To overcome this limitation, a combination of epigenetic modifications specifically or preferentially present in cancer cells is a candidate target. In this study, we aimed to demonstrate (i) the presence of a cancer cell-specific combination of epigenetic modifications by focusing on DNA methylation and trimethylation of histone H3 lysine 27 (H3K27me3) and (ii) the therapeutic efficacy of a combination of DNA demethylation and EZH2 inhibition. Analyses of DNA methylation and H3K27me3 in human colon, breast and prostate cancer cell lines revealed that 24.7 ± 4.1% of DNA methylated genes had both DNA methylation and H3K27me3 (dual modification) in cancer cells, while it was 11.8 ± 7.1% in normal cells. Combined treatment with a DNA demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC) and an EZH2 inhibitor, GSK126, induced marked re-expression of genes with the dual modification, including known tumor-suppressor genes such as IGFBP7 and SFRP1, and showed an additive inhibitory effect on growth of cancer cells in vitro. Finally, an in vivo combined treatment with 5-aza-dC and GSK126 inhibited growth of xenograft tumors more efficiently than a single treatment with 5-aza-dC. These results showed that the dual modification exists specifically in cancer cells and is a promising target for cancer cell-specific epigenetic therapy.

Introduction

Epigenetic alterations, including aberrant DNA methylation and alterations in histone modifications, are frequently present in human cancers (1,2), and are promising targets for cancer therapy (3,4). Currently, DNA demethylating agents, 5-azacytidine (azacitidine) and 5-aza-2′-deoxycytidine (decitabine; 5-aza-dC), are clinically being utilized for patients with myelodysplastic syndromes (5–7), and histone deacetylase (HDAC) inhibitors, vorinostat and romidepsin, are utilized for patients with cutaneous T-cell lymphomas (8). Not only in hematological malignancies but also in solid tumors, multiple trials of these epigenetic drugs have been conducted, and the efficacy has been shown, at least in non-small cell lung cancers (9). In addition, inhibitors of various histone methyltransferases, such as DOT1L (EPZ004777), EZH2 (E11, EPZ-6438 and GSK126) and G9a (BIX-01294), have been developed, and their efficacies have been demonstrated in preclinical studies (10–14).

Among the various epigenetic modifications, DNA methylation and trimethylation of histone H3 lysine 27 (H3K27me3) have critical roles in carcinogenesis. DNA methylation of promoter CpG islands (CGIs) is involved in the repression of tumor-suppressor genes, such as BRCA1, CDKN2A (p16) and RASSF1A genes (15–17). H3K27me3 is involved in the repression of tumor-suppressor genes, such as CDH1 (E-cadherin) and DKK1 (18,19), independently of DNA methylation (20). At the same time,
individual epigenetic modifications, including DNA methylation and H3K27me3, have physiological functions in normal cells (21,22), such as repression of transposable elements and genes required for embryonic development and cellular differentiation (23,24). Therefore, cancer cell specificity is considered difficult to achieve using a drug against a single epigenetic modification.

To overcome this limitation, a combination of epigenetic modifications specifically or preferentially present in cancer cells is a potential target. As a candidate for such a combination, we here focused on DNA methylation and H3K27me3 because of their functional crosstalk during carcinogenesis. Namely, H3K27me3 in normal cells functions as a premark of aberrant DNA methylation induction in cancer cells and also in normal-appearing tissues exposed to chronic inflammation (25–30). Switching of repression by H3K27me3 to that by DNA methylation is frequently observed for various genes during carcinogenesis (31). Since DNA methylation and H3K27me3 exist in a mutually exclusive manner in embryonic stem cells and normal cells (32,33), a failure in switching may generate a cancer cell-specific combination of epigenetic modifications, DNA methylation and H3K27me3. Indeed, it was recently reported that a combination of DNA methylation and H3K27me3 is specifically present in cancer cells (34).

In this study, we will first confirm the cancer cell specificity of the combination of DNA methylation and H3K27me3. Then, to reveal the potential of this combination as a target for cancer cell-specific epigenetic therapy, we will show whether or not a combination of DNA demethylation and EZH2 inhibition is effective for (i) re-expression of genes with both DNA methylation and H3K27me3 and (ii) inhibition of cancer cell growth in vitro and in vivo.

Materials and methods

Cell lines and drug treatment

Human prostate cancer cell lines (Du145 and PC3), breast cancer cell lines (MCF7 and MDA-MB-231), colon cancer cell lines (HCT116 and RKO), a normal prostate epithelial cell line (RWPE1) and normal human colon epithelial cells (FHC) were purchased from the American Type Culture Collection (Rockville, MD). Normal human mammary epithelial cells were purchased from Cambrex (East Rutherford, NJ).

PC3 and MCF7 were seeded on day 0, and were treated (i) with 5-aza-2′-deoxycytidine and/or entinostat (ChemScene, Maplewood, NJ) and (ii) with 5-aza-2′-deoxycytidine and/or GSK126 (Active Biochem, Monmouth Junction, NJ) for 4 days. Cell numbers were counted on day 5, and the cells were harvested. For 5-aza-2′-deoxycytidine and GSK126, drug concentrations used in the combined treatment were determined based on the inhibitory effect on DNA methylation (5-aza-2′-deoxycytidine) or H3K27me3 (GSK126) (Supplementary Figs. 1A and 2A available at Carcinogenesis Online). The selected doses of 5-aza-2′-deoxycytidine showed inhibitory effects on cell growth similar to the neighboring doses (Supplementary Fig. 1B available at Carcinogenesis Online). The selected dose of GSK126 showed a mild inhibitory effect on cell growth (Supplementary Fig. 2B available at Carcinogenesis Online). The selected dose of entinostat showed a mild inhibitory effect on cell growth (Supplementary Fig. 3 available at Carcinogenesis Online).

Genomic DNA was extracted by the standard phenol/chloroform method, and was quantified using a Quant-it Picogreen dsDNA Assay Kit (Life Technologies, Carlsbad, CA). Total RNA was extracted from cancer cell lines and their normal counterpart cells using ISOGEN (Nippon Gene, Tokyo, Japan).

Analysis of DNA methylation

Genome-wide analysis of DNA methylation was performed using an Infinium HumanMethylation450 BeadChip array (Illumina, San Diego, CA) as described previously (35). The DNA methylation level of an individual probe (CpG site) was obtained as the β value that ranged from 0 (unmethylated) to 1 (fully methylated). A total of 482 421 CpG sites were assembled into 296 494 genomic blocks, collections of CpG sites which were classified by their locations from transcription start sites (TSSs) (TSS500 (regions between 200 bp upstream and 1500 bp upstream from TSS), TSS200 (a 200-bp upstream region from TSS), 5′-UTR, the 1st exon, the gene body, 3′-UTR and an intergenic region) and their relative location against a CGI (N Shelf, N Shore, CGI, S Shore, S Shelf and non-CGI (35)). Among the 296 494 genomic blocks, 61 422 were located in CGIs and 7384 of them were located in TSS200 (TSS200 CGIs). Individual TSS200 CGIs contained 1–14 (average 3.5 ± 1.8) CpG sites, whose DNA methylation levels can be detected by a BeadChip array. The DNA methylation level of an individual TSS200 CGI (individual gene) was evaluated using the mean β value of all the CpG sites within an individual TSS200 CGI. Genes with β values of 0.9 or more and those of 0.2 or less were defined as methylated and unmethylated genes, respectively. Genes with β values of 0.2–0.9 were considered as partially methylated genes.

Gene-specific analysis of DNA methylation was performed by quantitative methylation-specific PCR and bisulfite sequencing. Quantitative methylation-specific PCR was performed using primers specific to methylated or unmethylated DNA (Supplementary Table 1 available at Carcinogenesis Online) and DNA methylation levels were calculated as [number of methylated molecules/number of the total DNA molecules (methylated molecules + unmethylated molecules) × 100]. Bisulfite sequencing was performed using universal primers for methylated and unmethylated DNA sequences (Supplementary Table 2 available at Carcinogenesis Online). The PCR product was cloned into pGEM-T Easy vector (Promega, Madison, WI), and sequenced using a DYE/terminator cycle sequencing kit (GE Healthcare, Buckinghamshire, UK) and an ABI PRISM 310 sequencer (PE Biosystems, Foster City, CA).

Analysis of H3K27me3

Chromatin immunoprecipitation for H3K27me3 was performed as described previously (29). Briefly, 30 μg of chromatin extracted from cross-linked cells was immunoprecipitated using 2 μg of antibody against H3K27me3 (07–449, Millipore, Billerica, MA). Immunoprecipitated chromatin was treated with RNaseA and proteinase K, and DNA was recovered by phenol/chloroform extraction and isopropanol precipitation. The precipitated DNA was dissolved in 30 μl of 1× TE (10mM Tris–HCl, pH 8.0, 1mM ethylenediaminetetraacetic acid).

Genome-wide analysis of H3K27me3 was performed using a human CGI oligonucleotide microarray (Agilent technologies, Santa Clara, CA) as described previously (29). H3K27me3 levels (Bound signal/Input signal) of individual genes were evaluated using genomic blocks used for DNA methylation analysis (by a BeadChip array). CGI microarray probes located within 1000 bp from any BeadChip array probes in a genomic block were assigned to the genomic block (Supplementary Fig. 4 available at Carcinogenesis Online). The position of a CGI microarray probe was defined by the center position of a probe. The H3K27me3 level of an individual genomic block was evaluated using the mean H3K27me3 level of all the probes assigned to a genomic block. Genomic blocks whose H3K27me3 levels were 1.5 or more were defined as those with H3K27me3.

Analysis of H3K27me3 levels of individual genes was performed by ChiP–quantitative PCR as described previously (29) using primers listed in Supplementary Table 3 available at Carcinogenesis Online. Analysis of DNA methylation of chromatin immunoprecipitated DNA was performed using 19 of 30 μl of immunoprecipitated DNA.

Analysis of gene expression

Genome-wide analysis of gene expression was performed using a SurePrint G3 Human GE Microarray 8-60K v2 (Agilent Technologies). From 200 ng
of total RNA, Cy3-labeled cRNA was synthesized using a Low Input Quick Amp Labeling Kit (Agilent Technologies) and 600 ng of labeled cRNA was fragmented and hybridized to a microarray. The microarray was scanned with an Agilent G2565BA microarray scanner (Agilent Technologies). The scanned data were processed using Feature Extraction Ver.10.7 software (Agilent Technologies), and analyzed using GeneSpring Ver.12.5 software (Agilent Technologies). The signal intensity of each probe was normalized so that the 75th percentile of signal intensity of all the probes would be 1.0. Mean signal intensity of all the probes within a gene was used as its expression level, and genes with signal intensities of 0.5 or more were considered to be expressed. Analysis of gene expression levels of individual genes was performed by quantitative RT-PCR as described previously (29) using primers listed in Supplementary Table 4 is available at Carcinogenesis Online.

Immunofluorescence

Cells were fixed with 4% formaldehyde and permeabilized by 1% Triton X-100 in 1× phosphate-buffered saline (PBS) (−). The cells were incubated in blocking buffer (1% bovine serum albumin in 1× PBS (−)), and then incubated with rabbit polyclonal antibody against H3K27me3 (1:1000; 07-449; Millipore) and mouse monoclonal antibody against histone H3 (1:1000; 300–34783; Wako, Tokyo, Japan). After washing with 1× PBS (−), cells were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:1000, Life Technologies) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:1000, Life Technologies). After washing with 1× PBS (−), coverslips were mounted using ProLong Gold antifade reagent with DAPI (Life Technologies). Fluorescence of stained cells was detected using a BZ-9000 microscope system (Keyence, Osaka, Japan).

Western blotting

Proteins in total cell lysate were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and were transferred to a polyvinylidene difluoride membrane (Millipore). H3K27me3, histone H3 and EZH2 were detected using rabbit polyclonal antibody against H3K27me3 (1:1000; 07-449; Millipore), rabbit polyclonal antibody against histone H3 (1:5000; ab1791; Abcam, Cambridge, UK) and mouse polyclonal antibody against EZH2 (1:1000; 31475; Cell Signaling Technology, Danvers, MA), respectively. Protein bands were quantified by using ImageJ 1.47v software.

Xenograft tumor formation assay in nude mice

PC3 cells (1.5×10^6 cells) were inoculated subcutaneously into 6-week-old male nude mice (BALB/cAnCrl-nu/nu; CLEA Japan, Tokyo, Japan). 5-Aza-dC (20 or 100 mg/kg) and/or GSK126 (10 or 15 mg/kg) were intraperitoneally administered three times per week. The length and width of tumors were measured using calipers and the tumor volume was calculated as [length × width^2 × 0.5]. After 8 weeks, tumors were collected for the measurement of tumor weights, and total blood was collected for the analysis of the number of leukocytes, erythrocytes and platelets. All the animal experiments were approved by the Committee for Ethics in Animal Experimentation at the National Cancer Center.

Statistical analysis

The differences in H3K27me3 levels were evaluated by the Mann–Whitney U-test. The differences in cell growth were evaluated by the Student’s t-test.

Gene ontology analysis

Gene ontology analysis was performed by DAVID bioinformatics resources (36,37) as described previously (29). The enrichment of genes in a biological process was analyzed by comparing a fraction of genes with an ontology among genes with gain (or loss) of H3K27me3 in cancer cells with that among the genes with TSS200 CGIs.

Results

Increase of genes with both DNA methylation and H3K27me3 in cancer cells

DNA methylation status was compared between cancer cell lines and their normal counterpart cells in the colon, mammary glands, and prostate for 61 422 genomic blocks with CGIs. Bimodal distribution of DNA methylation levels was observed in both cancer cell lines and normal cells, but the number of methylated blocks was larger in cancer cell lines than in normal cells (Figure 1A). When the analysis was limited to 7384 TSS200 CGIs, the six cancer cell lines had methylation of 238–969 genes while normal cells had that of only 55–75 genes (Supplementary Table 5 is available at Carcinogenesis Online).

Then, H3K27me3 status was compared between cancer cell lines and normal cells. The numbers of genomic blocks (Figure 1B) and TSS200 CGIs (Supplementary Table 5 is available at Carcinogenesis Online) with H3K27me3 were similar between cancer (743–1165 TSS200 CGIs) and normal cells (576–973 TSS200 CGIs). When the changes of H3K27me3 status were analyzed, 331–645 and 254–554 TSS200 CGIs showed gain and loss, respectively, of H3K27me3 in cancer cells (Supplementary Table 6 is available at Carcinogenesis Online). Genes with gain of H3K27me3 tended to have gene functions related to cell communication and cell–cell signaling. In contrast, genes with loss of H3K27me3 tended to have gene functions related to development (Supplementary Table 7 is available at Carcinogenesis Online).

Overlap between genes with DNA methylation and those with H3K27me3 was then analyzed. In cancer cell lines, 24.7±4.1% of methylated genes (49–248 genes) had both DNA methylation and H3K27me3 (dual modification). On the other hand, in normal cells, only 11.8±7.1% of DNA methylated genes (4–12 genes) had the dual modification (Figure 1C). Among the genes with the dual modification, known tumor-suppressor genes, such as IGFBP7 and SFRP1 (38–40), were present (Supplementary Table 8 is available at Carcinogenesis Online). These results showed that the fraction of genes with the dual modification was increased in cancer cells compared with their normal counterpart cells.

The existence of DNA methylation and H3K27me3 on the same DNA molecules

The existence of DNA methylation and H3K27me3 on the same DNA molecules was analyzed in two cancer cell lines, PC3 and MCF7, by bisulfite sequencing of chromatin immunoprecipitated DNA (32,41). First, higher H3K27me3 levels were confirmed in the genes with the dual modification, CNN3, SFRP1 and SLCE6A15, than in housekeeping genes, EEF1A1 and GAPDH (Figure 2A). Then, the DNA methylation status of the DNA molecules immunoprecipitated by anti-H3K27me3 antibody was analyzed. All the DNA molecules sequenced were densely methylated at promoter CGIs of these genes (Figure 2B). The result showed that DNA methylation and H3K27me3 coexisted on the same DNA molecules.

High H3K27me3 levels in normal cells for genes with the dual modification

DNA methylation and H3K27me3 status in normal counterpart cells were analyzed for genes with the dual modification and genes with only DNA methylation. H3K27me3 was present in normal cells for 54.6±14.4% (20–150 genes) of genes with the dual modification in cancer cell lines and for 28.1±7.3% (34–266 genes) of genes with only DNA methylation (Supplementary Table 9 is available at Carcinogenesis Online). When the H3K27me3 level was analyzed, it was significantly higher in genes with the dual modification than in genes with only DNA methylation (Figure 3). Neither DNA methylation nor H3K27me3 was present in normal cells for 29.9±17.4% (4–104 genes) of genes with the dual modification in cancer cell lines and for 37.0±16.8% (20–363 genes) of genes with only DNA methylation (Supplementary Table 10 is available at Carcinogenesis Online).
Table 9 is available at Carcinogenesis Online). These results showed that genes with the dual modification frequently had high H3K27me3 levels in normal counterpart cells.

The repressive effect of the dual modification on gene expression

To analyze the effect of the dual modification on gene expression, the expression levels of the genes with the dual modification were compared with (i) those of genes with only DNA methylation, (ii) those of genes with only H3K27me3 and (iii) those of genes without DNA methylation or H3K27me3. The genes with the dual modification had the lowest expression levels, along with the genes with only DNA methylation, in all the four cancer cell lines (Figure 4A). Characteristics of the genes with the dual modification and the resultant gene silencing were then examined. To this end, from the genes with the dual modification, we isolated genes that had neither DNA methylation nor H3K27me3 and were expressed in normal counterpart cells (Supplementary Table 9 is available at Carcinogenesis Online). This group of genes, except for SLC6A12 (in MCF7) and SPSB4 (in MDA-MB-231), was repressed to almost undetectable levels in cancer cell lines (Figure 4B). Well-established tumor-suppressor genes, such as IGFBP7 and SFRP1, were present among this group of genes. These results showed that the dual modification was involved in the repression of genes expressed in normal cells, including tumor-suppressor genes.

Efficient re-expression by combination of DNA demethylation and EZH2 inhibition

Re-expression of genes with the dual modification by a combined treatment with a DNA demethylating agent, 5-aza-dC and an EZH2 inhibitor, GSK126, was attempted (Figure 5A). Genes with the dual modification, such as IGFBP7 and SFRP1 and SLC6A15, were re-expressed more efficiently by the combined treatment than by a single treatment with 5-aza-dC or GSK126 (Figure 5B and C; Supplementary Figure 5 is available at Carcinogenesis Online). In contrast, the effect of combined treatment was not observed...
Figure 2. The coexistence of DNA methylation and H3K27me3 on the same DNA molecules. (A) H3K27me3 levels of genes with the dual modification. H3K27me3 levels were higher in the genes with the dual modification, SFRP1, SLC6A15 and CNN3, than in housekeeping genes, EEF1A1 and GAPDH. (B) Bisulfite sequencing of DNA molecules immunoprecipitated by anti-H3K27me3 antibody. All the DNA molecules were densely methylated for SFRP1 (PC3 and MCF7), SLC6A15 (PC3) and CNN3 (MCF7). Closed circle, methylated CpG site; open circle, unmethylated CpG site; vertical bar, CpG site; and arrow, TSS.

for genes with only DNA methylation (Figure 5B; Supplementary Figure 6 is available at Carcinogenesis Online) and genes with only H3K27me3 (Figure 5C; Supplementary Figure 7 is available at Carcinogenesis Online). Then, re-expression was confirmed in a genome-wide manner. Genes with the dual modification were re-expressed more efficiently by the combined treatment (Supplementary Figure 8 is available at Carcinogenesis Online). At the same time, even among the genes that had neither DNA methylation nor H3K27me3, 2-fold or more of upregulation was also observed (nonspecific changes) (Supplementary Figure 9 is available at Carcinogenesis Online).

The decrease of H3K27me3 level by a single treatment with GSK126 or by the combined treatment was confirmed in PC3 and MCF7 cell lines (Figure 5D–F; Supplementary Figure 2A is available at Carcinogenesis Online), but the EZH2 level was not changed (Figure 5E and F). These results showed that the combined treatment with a DNA demethylating agent and an EZH2 inhibitor was useful for re-expression of genes with the dual modification.

Additive inhibitory effect of the combination on cancer cell growth

First, the effect of the combined treatment with 5-aza-dC and GSK126 on growth of two cancer cell lines, PC3 and MCF7, was analyzed in vitro (Figure 6A and B). A single treatment with 5-aza-dC decreased growth of PC3 and MCF7 to 29.9 and 65.6%,
The combined treatment with 5-aza-dC and GSK126 inhibited growth of xenograft tumors (Figure 6C–E). In contrast, a single treatment with GSK126 had no inhibitory effect on growth of xenograft tumors, and that with 5-aza-dC could inhibit growth of six of eight xenografts. As for the side effects of the combined treatment, we did not observe significant changes of weights (Supplementary Figure 11A is available at Carcinogenesis Online). However, we observed a decrease in the numbers of leukocytes and erythrocytes and an increase in the number of platelets. The degree of these side effects was similar to those by a single treatment with 5-aza-dC (Supplementary Figure 11B is available at Carcinogenesis Online). These results showed that the combined treatment with 5-aza-dC and GSK126 inhibited growth of xenograft tumors more efficiently than a single treatment with 5-aza-dC.

Discussion

The combination of DNA demethylation and EZH2 inhibition had an additive inhibitory effect on cancer cell growth in vitro, and the efficacy was comparable with that by the combined treatment with 5-aza-dC and entinostat. The combined treatment also inhibited growth of xenograft tumors more efficiently than a single treatment with 5-aza-dC. This suggested that the cancer cell-specific dual modification is a promising target for cancer cell-specific epigenetic therapy. The effect of the combined treatment can be enhanced by (i) selecting DNA demethylating agents and EZH2 inhibitors suitable for combined treatment and (ii) optimizing the drug concentrations for the combination. Further to the increasing specificity for the dual modification, its reader proteins, if any, are ideal targets. The strategy of targeting a reader protein itself has been successful for histone acetylation, using inhibitors such as JQ1 and RVX-208, and was demonstrated to be effective in preclinical studies (42,43). Therefore, once reader proteins of the dual modification are identified, it might be possible to develop cancer cell-specific epigenetic therapy.

The presence of various physiological dual modifications, such as a combination of H3K4me3 and H3K27me3 (bivalent modification), has been reported (44). In this study, a cancer cell-specific combination of DNA methylation and H3K27me3 was used as a potential target for cancer cell-specific epigenetic therapy. However, genes with DNA methylation and H3K27me3 were also observed in normal cells although their number was extremely small, compared with that in cancer cells. In cancer cells, various epigenetic modifiers, such as DOT1L and EP300, are known to be dysregulated and their target modifications are altered (45,46). Therefore, the comparison of combinations of these epigenetic modifications between cancer cells and their normal counterpart cells might lead to identification of more combinations with cancer cell specificity.

Several known tumor-suppressor genes, such as IGFBP7 and SFRP1 (38–40), were among the genes with the dual modification. IGFBP7 is known to be involved in the inhibition of the BRAF-MEK-ERK signaling pathway and also in the induction of cellular senescence and apoptosis (40). Repression of IGFBP7 is critical for development of melanoma with BRAF V600E mutation (40). SFRP1 is known as a negative regulator of the WNT signaling pathway, and repression of SFRP1 leads to the activation of the WNT signaling pathway (47,48). The combination of DNA demethylation and EZH2 inhibition could induce re-expression of these genes. In addition to such an epigenetic effect, it is
known that treatment with GSK126 induces both cytostatic and cytotoxic responses in lymphoma cell lines (13). Therefore, the therapeutic effect of the combined treatment was considered to be not only through the epigenetic effect but also through cytostatic and cytotoxic responses.

Mechanistically, in embryonic stem cells and normal cells, DNA methylation and H3K27me3 exist in a mutually exclusive manner (32,33). This is considered to be due to the inhibitory effect of DNA methylation on PRC2 recruitment (49). In contrast, in immortalized and transformed cells, the inhibitory effect of DNA methylation on PRC2 recruitment is known to be disrupted (33), and such disruption during carcinogenesis might be a possible mechanism of the preferential existence of the dual modification in cancer cells.

SLC6A12 and SPSB4 were not repressed by the dual modification in cancer cells. As for the possible reasons, these genes might have been transcribed from TSSs different from the TSSs that had the dual modification. TSSs of a gene can vary from tissue to tissue depending upon genes (50), and a TSS in the database is not always accurate. It was also considered that only one allele of the gene had the dual modification in cancer cells and that the other allele was transcribed.

Combined treatment with 5-aza-dC and GSK126 induced marked re-expression of some genes with the dual modification, such as CNN3, IGFBP7, NID1 and SFRP1, while not for the other genes, such as CPNE8, NEFL and QRFP1. To induce gene expression after the removal of repressive modifications, the presence of transcription factors required for the expression of individual genes is important. Therefore, it was considered that re-expression by the combined treatment might be dependent on the presence of sufficient amounts of such transcription factors in an analyzed cancer cell line.
Figure 5. Efficient re-expression of genes with the dual modification by combination of DNA demethylation and EZH2 inhibition. (A) Experimental protocol of drug treatment. Two cancer cell lines, PC3 and MCF7, were treated with 5-aza-dC (0.2 μM for PC3 and 0.3 μM for MCF7) and/or GSK126 (6 μM) for 4 days, and harvested on day 5. (B), (C) Relative re-expression levels of genes with the dual modification and those with a single modification. The expression level of a gene in cells treated with the combination was normalized to that in cells treated with 5-aza-dC only (B) or to that in cells treated with GSK126 only (C). Genes with the dual modification were re-expressed more efficiently by the combined treatment than by a single treatment. (D) Immunofluorescence analysis of H3K27me3 in cells treated with 5-aza-dC and/or GSK126. The H3K27me3 level was decreased by a single treatment with GSK126 or by combined treatment with 5-aza-dC and GSK126. The scale bar represents 50 μm. (E), (F) Western blotting of H3K27me3 and EZH2 in cells treated with 5-aza-dC and/or GSK126. H3K27me3 levels were decreased by a single treatment with GSK126 and the combined treatment to 4.5–61.5% of those in untreated cells. EZH2 levels were not decreased by treatment with GSK126 as reported previously (13).
In conclusion, a combination of DNA methylation and H3K27me3, which exists specifically in cancer cells, was considered to be a promising target for cancer cell-specific epigenetic therapy.

Supplementary material
Supplementary Table 1–9 and Figures 1–11 can be found at http://carcin.oxfordjournals.org/

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References