Abstract

Infiltration of tumors with effector T cells is positively associated with therapeutic efficacy and patient survival. However, the mechanisms underlying effector T-cell trafficking to the tumor microenvironment remain poorly understood in patients with colon cancer. The polycomb repressive complex 2 (PRC2) is involved in cancer progression, but the regulation of tumor immunity by epigenetic mechanisms has yet to be investigated. In this study, we examined the relationship between the repressive PRC2 machinery and effector T-cell trafficking. We found that PRC2 components and demethylase JMJ3-mediated histone H3 lysine 27 trimethylation (H3K27me3) repress the expression and subsequent production of Th1-type chemokines CXCL9 and CXCL10, mediators of effector T-cell trafficking. Moreover, the expression levels of PRC2 components, including EZH2, SUZ12, and EED, were inversely associated with those of CD4, CD8, and Th1-type chemokines in human colon cancer tissue, and this expression pattern was significantly associated with patient survival. Collectively, our findings reveal that PRC2-mediated epigenetic silencing is not only a crucial oncogenic mechanism, but also a key circuit controlling tumor immunosuppression. Therefore, targeting epigenetic programs may have significant implications for improving the efficacy of current cancer immunotherapies relying on effective T-cell-mediated immunity at the tumor site.

Introduction

Effector T cells are indispensible for protective tumor immunity and efficacy of cancer treatment (1–3). In colon cancer, the presence of CD8+ T cells and its cytotoxic antitumor molecules is a parameter for improved patient survival and tumors without signs of metastasis (4). Th1-type chemokines CXCL9 and CXCL10 mediate the trafficking of the main antitumor immune cells, including Th1 and CD8+ T cells, into the tumor microenvironment. However, it is not well understood how Th1-type chemokine expression is controlled in the tumor and subsequently how it affects effector T-cell trafficking into the human cancer microenvironment.

Epigenetic regulation, including histone modifications, can mediate gene repression (5). One epigenetic repression machinery involves the polycomb repressive complex 2 (PRC2), which trimethylates histone 3 lysine 27 (H3K27me3; refs. 6, 7). H3K27me3 is a repressive histone mark associated with facultative heterochromatin that functions to recruit regulatory proteins to repress gene transcription (7). PRC2 component, enhancer of zeste homolog 2 (EZH2), is highly expressed in multiple cancers (8, 9). Its role in cancer cell proliferation, invasiveness, and differentiation has been widely studied (8, 9). However, it is not known whether EZH2 and the other PRC2 proteins are involved in the regulation of human T-cell tumor trafficking, and, in turn, tumor immunity. Cancer epigenetic studies suggest that an abnormal evolution of repressive epigenetic marks including histone modification may directly contribute to cancer development and progression (10, 11). However, the nature of cancer epigenetic repression is involved in the control of cancer immunity remains unanswered. Given the relevance of effector T cells and their homing in antitumor immunity (1, 2, 4), we hypothesized that cancer epigenetic repression PRC2 machinery represses Th1-type chemokines in colon cancer, and in turn, alters effector T-cell tumor migration and effective antitumor immunity. The validation of this hypothesis will lead to a notion that cancer epigenetic repression-mediated Th1-type chemokine repression is a novel immune evasive mechanism and the repressive machinery may be a target for novel cancer immunotherapy.
Thus, in the current work, we tested this hypothesis in the context of human colon cancer at the molecular and clinical level.

Materials and Methods

Human subjects and colon cancer tissues
Patients diagnosed with colon carcinomas were recruited in the study. All usage of human subjects in this study was approved by local Institutional Review Boards. Six to 9 fresh colon tissues were collected from patients with colon cancer and ulcerative colitis. Primary colon cancer cells, colon epithelial cells, and all the in vitro functional assays were performed with single cells from fresh colon cancer and colitis tissues as previously described (12). In addition, patients with colorectal carcinoma were evaluated from datasets in Oncomine.org.

Cell culture
Primary colon cancer cell lines (C1) were established from fresh colon cancer tissue. Routine short tandem repeat analysis is done to determine the uniqueness of the primary line (12). DLD-1 and SW480 cell lines (ATCC) were used in the experiments. Single colon epithelial cells were made from fresh colon tissues from patients with colon cancer and ulcerative colitis. Colon cancer cells were treated with recombinant IFNγ (R&D Systems), DZNep (Sigma), GSK126 (GlaxoSmithKline), and GSK-J4 (GlaxoSmithKline), for different time points and concentrations.

Real-time reverse transcriptase PCR
RNA was isolated from the cells by TRIzol (Ambion) and converted to cDNA using reverse transcriptase PCR (cloned AMV reverse transcriptase, Invitrogen). The mRNA was then quantified by real-time RT-PCR using StepOnePlus (Applied Biosystems). Specific primers are included in the Supplementary Information (Supplementary Table S1). Fast SYBR Green Master Mix (Applied Biosystems) was used to detect ΔCt method with normalization to GAPDH. Unless otherwise noted, fold change was calculated according to the comparative Ct method with normalization to GAPDH. Unless otherwise noted, fold change with normalization to control is shown in the figures.

Lentiviral transduction and transfection
The lentiviral vectors, pGIPZ or pGreen, encoding gene-specific shRNAs were used to transduce colon cancer cells to establish stable cell knockdowns. Lentiviral shRNAs (Supplementary Table S2) were from the Vector Core at the University of Michigan or provided by Arul Chinnaiyan (University of Michigan, Ann Arbor, MI; ref. 8). The lentiviral transduction efficiency was confirmed by GEP, which was coexpressed by the lentiviral vector. The knockdown efficiency was assessed by Western blotting and real-time PCR. For transfections, Eugene HD (Promega) was used to transfect colon cancer cells with PKH3 (empty vector) and pcMV HA IMiD3 (Addgene; #24167) according to the manufacturer’s protocol. The overexpression was confirmed by Western blotting.

ELISA
The protein levels of CXCL9 and CXCL10 were detected by ELISA (R&D Systems) from the supernatants of treated colon cancer cells or single cells from fresh colon cancer and colitis tissue.

Western blot analysis
Western blotting was performed with specific antibodies against Histone H3 (9715, Cell Signaling Technology), β-actin (A5441, Sigma), EZH2 (612667, BD Biosciences), SUZ12 (46264, Santa Cruz Biotechnology), EED (28701, Santa Cruz Biotechnology), and H3K27me3 (07449, Millipore). Signals were detected by ECL reagents (GE Healthcare).

T-cell migration assays
In vitro migration assay was performed in a Transwell system with a polycarbonate membrane of 6.5 mm diameter with a 3 μm pore size as described (13, 14). Activated T cells were treated with anti-human CXCR3 or isotype, and added to the top chamber. Supernatant from the cultured colon cancer cells was added to the bottom chamber. After incubation at 37°C for 12 hours, the phenotype and number of T cells in the top and bottom chambers were determined by FACS (LSRII, BD Biosciences).

Chromatin immunoprecipitation
Chromatin immunoprecipitation (ChIP) was performed and as previously described (12, 15). Crosslinking was performed with 1% formaldehyde or 1% paraformaldehyde for 10 minutes. Sonication was performed with the Misonix 4000 water bath sonication unit at 15% amplitude for 10 minutes. Protein/DNA complex was precipitated with specific antibodies against H3K27me3 (6002, Abcam) and IgG control (Millipore). ChIP-enriched chromatin was used for RT-PCR with SYBR Green Master Mix, normalizing to input. Specific primers are listed in supplementary information (Supplementary Table S1; ref. 16).

Statistical analysis
Dependent on data distribution and experimental design, paired or unpaired Student’s t test and Mann–Whitney U tests were used. Correlation coefficients (Spearman correlation) denoted by r, together with a P value, were computed to measure correlation between different genes. Survival functions were estimated by Kaplan–Meier methods using genes classified as high or low based on mean or median expression values. Data was censored at the last follow-up for patients who were disease-free or alive at the time of analysis. All analyses were done using SAS 9.3 software. P < 0.05 was considered significant.

Results

Inverse correlation exists between Th1-type chemokines and PR22 in colon cancer
Th1-type chemokines CXCL9 and CXCL10 mediate effector CD8+ T-cell tumor trafficking. CD8+ T-cell tumor infiltration is associated with improved cancer patient survival. Th1-type chemokines are correlated with effector T-cell density in some human tumors, including colon cancer, and positively associated with colon cancer patient survival (17, 18). However, it is poorly understood how Th1-type chemokine expression is controlled in human colon cancer. We found that the levels of CXCL9 and CXCL10 mRNA (Fig. 1A) and protein (Fig. 1B) were higher in colitis colon compared with colon cancer tissue. The chemokines CXCL9 and CXCL10 can be stimulated by IFNγ. When we treated single epithelial cells from the microenvironments of colon cancer, adjacent colon tissue, and colitis with IFNγ, the levels of CXCL9 and CXCL10 were higher in adjacent tissues (Fig. 1C) and colitis tissues (Fig. 1D) than colon cancer tissues. Patients with ulcerative colitis are at increased risk for developing colorectal cancer. The
data suggest that Th1-type chemokine expression in colon cancer may evolve and become repressed when going from inflammatory tissue to cancer.

The PRC2 complex (including EZH2, embryonic ectoderm development (EED), and suppressor of zeste 12 homolog (SUZ12)) represses gene transcription through methylation of H3K27 (6, 19). We hypothesized that the PRC2 complex repressed Th1-type chemokines in colon cancer. To test this hypothesis, we analyzed a colon cancer tissue microarray from Oncomine.org (20) for potential correlations between PRC2 and Th1-type chemokines, CXCL9 and CXCL10. In support of our hypothesis, we found significant negative correlations between the PRC2 complex components and CXCL9 and CXCL10 (Fig. 1E–I). The data suggests that the high levels of PRC2 may control and repress Th1-type chemokine expression in colon cancer.

PRC2 represses Th1-type chemokine expression in colon cancer

Given the inverse relationship between PRC2 and Th1-type chemokines (Fig. 1), we investigated whether PRC2 machinery represses Th1-type chemokine expression in colon cancer. We initially examined the potential effect of 3-Deazaneplanocin A (DZNep), a pharmacologic PRC2 inhibitor (19), on Th1-type chemokine expression in human colon cancer cells. In response to IFNγ treatment, we observed that treatment with DZNep led to higher levels of CXCL9 and CXCL10 expression in a primary colon cancer cell line (C1; Fig. 2A and B), DLD-1 (Fig. 2C and D), and SW480 (Fig. 2E and F) colon cancer cells. As expected, DZNep
reduced the expression of EZH2, SUZ12, and EED (Supplementary Fig. S1A). However, the effect of DZNep was specific to chemokines as other IFNγ-associated genes, including IFNγ receptor (IFNGR2; Supplementary Fig. S1B) and HLA-B (Supplementary Fig. S1C) was not affected.

We next genetically knocked down EZH2 expression with lentivirus-based shRNA for EZH2 (shEZH2) in primary colon cancer C1 cells. shEZH2 specifically reduced the expression of EZH2 and SUZ12 (Supplementary Fig. S1D), and resulted in elevated Th1 type chemokine mRNA (Fig. 2G and H) release in response to IFNγ stimulation (Fig. 2G and H). In addition, specific knockdown of EZH2 with shSUZ12, importantly, removed H3K27me3 on these areas (Fig. 3A–C) and increased Th1-type chemokine expression (Supplementary Fig. S1F). GAPDH and HOXB1 were used as a negative and positive control, respectively (Fig. 3D). Thus, H3K27me3 may be involved in the Th1-type chemokine gene silencing in colon cancer.

GSK126 is a highly selective, potent small-molecule inhibitor of EZH2 methyltransferase activity (21). GSK126 treatment (5 μmol/L) abolished the global level of H3K27me3 without inhibiting EZH2 (Supplementary Fig. S2B). GSK126 treatment led to reduced CXCL9 and CXCL10 expression in primary colon cancer C1 cells (Fig. 3E and F). Thus, H3K27me3-specific methyltransferase and demethylase regulate Th1-type chemokine repression in colon cancer cells.

PRC2 affects T-cell migration toward colon cancer

Next, we examined whether the PRC2 complex can affect T-cell migration via controlling CXCL9 and CXCL10 expression. We...
collected supernatants from colon cancer cells transfected with shEZH2 and control lentiviral vectors. We showed that CD4⁺ (Fig. 4A) and CD8⁺ (Fig. 4B) T cells more efficiently migrated towards shEZH2 supernatant than control. The migration was blocked by monoclonal antibody (mAb) against CXCR3, the receptor for CXCL9 and CXCL10 on T cells (Fig. 4A and B). Thus, the PRC2 component, EZH2, controls T-cell trafficking toward colon cancer-derived CXCL9 and CXCL10.

Finally, we assessed the clinical relevance of this phenomenon by examining the association of chemokines and the PRC2 complex with patient survival. We first performed survival analysis on chemokynes by combining multiple datasets of colorectal carcinoma patients from oncomine.org. The patients were stratified in groups based on the mean expression level of each gene. When we divided patients with high or low expression of both CXCL9 and CXCL10, high CXCL9/CXCL10 associated with better patient overall survival (Supplementary Fig. S3A). As a confirmation, we also observed positive correlation with CD8 and CXCL9 and CXCL10 in different cohorts. It suggests that higher chemokines may lead to higher CD8⁺ T-cell tumor infiltration (Supplementary Fig. S3B). To examine the association between the 3 immune gene signature (CD8, CXCL9, and CXCL10) and the 3 PRC2 gene components (EZH2, EED, and SUZ12) with patient survival, we divided patients with high or low expression of these genes. We found that high CD8/CXCL9/CXCL10 (Fig. 4C) and low EZH2/SUZ12/EED (Fig. 4D) associated with better patient overall survival. We also analyzed the relationship between PRC2 complex component transcripts and CD4 and CD8 mRNA in patients with colorectal cancer. We found that CD4 and CD8 mRNA expression negatively correlated with the PRC2 complex components, EZH2, SUZ12, and EED mRNA (Fig. 4E-G). Altogether, the data suggests that PRC2 controls T-cell trafficking via repressing Th1-type chemokines and impacts colon cancer pathology.

Discussion

Epigenetic changes are biologically vital and often linked to cancer proliferation, progression, and metastasis (8, 9, 24, 25). Our studies have shown that the PRC2 components in colon cancer cells silence the tumor production of Th1-type chemokines, potentially restrain effector T-cell tumor infiltration, and in turn lessen anticancer immunity. Thus, PRC2 and H3K27me3-mediated Th1-type chemokine silencing is a novel immune evasion mechanism in human colon cancer. We propose a unifying mechanistic model of cancer in which epigenetic silencing plays both biologic and immunologic roles in supporting tumor progression.

Previous reports show that the expression of CXCR3 and CXCL10 correlates with metastasis in certain cancer including colon cancer (26–28). However, whether the expression of CXCR3 in the tumor cells leads to CXCL10 dependent metastasis is not fully elucidated. Nonetheless, our data shows that there is a positive correlation between CD8 and CXCL9 and CXCL10, and...
the expression of CD8, CXCL9, and CXCL10 is positively associated with colon cancer patient survival. In support of this observation, it has been shown that CD8^+ T cells and Th1-type chemokines positively predict colon cancer patient outcome (4, 29–31).

A critical question is what drives the epigenetic changes in the first place. Several reports suggest miRNA involvement of EZH2 regulation (particularly loss of miRNA101) in prostate cancer (8). Further studies will be needed to demonstrate the drivers of this repression in cancer cells versus normal epithelium and whether epigenetic changes are one of the earliest events in cancers, leading to immune evasion. We assume that this mechanism progressively evolves in cancer cells as colon inflammatory tissues express high levels of Th1-type chemokines. In contrast to irreversible genetic mutations, epigenetic alterations can be manipulated, making them a valuable target for therapy (32). To this end, it is crucial to understand the immune-associated epigenetic mechanisms in the human cancer and to dissect how different epigenetic modifiers differentially affect cancer immune signature gene expression. In this regard, we have noticed that GSK126, a compound acting as a direct histone methyltransferase (HMT) inhibitor and DZNep, a compound acting as an indirect HMT inhibitor, have shown certain differences in potencies in promoting cancer Th1-type chemokine expression. Perhaps additional mechanisms of DZNep, such as proteasomal degradation of PCR2 and TXNIP, which causes disruption of Th1-type chemokines, could explain this difference (33).

Current cancer immunotherapies (34–36) and classic therapies (1, 3) largely rely on efficient T-cell tumor trafficking and...
T-cell–mediated tumor immunity. Epigenetic reprogramming may unlock Th1-type chemokine repression, transform the tumor from poor T-cell infiltration to rich T-cell infiltration, and, ultimately, improve the effectiveness of any given cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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