VEGF/VEGFR-2 Upregulates EZH2 Expression in Lung Adenocarcinoma Cells and EZH2 Depletion Enhances the Response to Platinum-Based and VEGFR-2–Targeted Therapy

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Abstract

Purpose: To investigate the mechanisms of regulation and role associated with enhancer of zeste homolog 2 (EZH2) expression in lung cancer cells.

Experimental Design: We investigated the mechanisms of EZH2 expression associated with the VEGF/VEGFR-2 pathway. Furthermore, we sought to determine the role of EZH2 in response of lung adenocarcinoma to platinum-based chemotherapy, as well as the effect of EZH2 depletion on VEGFR-2–targeted therapy in lung adenocarcinoma cell lines. In addition, we characterized EZH2 expression in lung adenocarcinoma specimens and correlated it with patients’ clinical characteristics.

Results: In this study, we demonstrate that VEGF/VEGFR-2 activation induces expression of EZH2 through the upregulation of E2F3 and hypoxia-inducible factor-1α (HIF1α), and downregulated expression of miR-101. EZH2 depletion by treatment with 3-deazaneplanocin A and knockdown by siRNA decreased the expression of EZH2 and H3K27me3, increased PARP-C level, reduced cell proliferation and migration, and increased sensitivity of the cells to treatment with cisplatin and carboplatin. In addition, high EZH2 expression was associated with poor overall survival in patients who received platinum-based adjuvant therapy, but not in patients who did not receive this therapy. Furthermore, we demonstrated for the first time that the inhibition of EZH2 greatly increased the sensitivity of lung adenocarcinoma cells to the anti-VEGFR-2 drug AZD2171.

Conclusion: Our results suggest that the VEGF/VEGFR-2 pathway plays a role in regulation of EZH2 through the upregulation of E2F3 and hypoxia-inducible factor-1α (HIF1α), and downregulated expression of miR-101. EZH2 depletion decreases the malignant potential of lung adenocarcinoma and sensitivity of the cells to both platinum-based and VEGFR-2–targeted therapy. Clin Cancer Res; 20(14); 3849–61. ©2014 AACR.
Enhancer of zeste homolog 2 (EZH2) overexpression occurs in a wide variety of cancers. However, the mechanisms of regulation and role associated with EZH2 expression in lung cancer cells are unknown. In this study, we demonstrate for the first time that the VEGF/VEGFR-2 pathway is a novel regulator of EZH2 expression in malignant epithelial lung cancer cells through E2F3, hypoxia-inducible factor-1α, and miR-101. Our work elucidates the importance of EZH2 in lung adenocarcinoma pathogenesis, and identifies it as a potential target for overcoming resistance of tumors to platinum-based chemotherapy and VEGFR-2–targeted therapy. Our findings can be translated into significant clinical benefits such as reducing the doses used, which in turn can reduce the severity of side effects of these therapies.

In addition to its role in tumor cells, upregulation of EZH2 gene expression in endothelial cells is regulated by the VEGF/VEGFR-2 pathway at both the transcriptional and posttranscriptional levels (3, 8–10). At the transcriptional level, VEGF increases the expression of the transcription factor E2F, which directly enhances EZH2 expression (8, 9); this effect can be blocked by treatment with an anti-VEGFR-2 antibody (8). In endothelial cells, VEGF/VEGFR-2 activity downregulates expression of miR-101 and thus indirectly increases expression of EZH2 (9). In breast cancer cells, a hypoxic tumor microenvironment increases EZH2 expression via the action of hypoxia-inducible factor (HIF)-1α (11). In this context, we recently observed that VEGF regulates HIF1α expression levels in NSCLC cell lines overexpressing VEGFR-2 independently of hypoxia (13). This suggests the possibility that the VEGF/VEGFR-2 pathway may regulate tumor expression of EZH2 via HIF1α expression.

We investigated the ability of the VEGF/VEGFR-2 pathway to regulate the expression of EZH2 in lung adenocarcinoma cell lines and the biologic impact of EZH2 abrogation by pharmacologically induced and siRNA-mediated depletion of EZH2 on tumor cell proliferation, migration, and chemoresistance in response to both standard platinum-based chemotherapy and VEGFR-2–targeted therapy in lung adenocarcinoma cell lines. To further explore the role and function of EZH2 in lung cancer pathogenesis, we characterized EZH2 and miR-101 expression in lung adenocarcinoma specimens and correlated it with clinical characteristics of patients. Our studies provide evidence of how EZH2 expression is deregulated, its important role of EZH2 in lung cancer pathogenesis, and the possibility of making it a therapeutic target and the clinicopathologic consequences for patients of its deregulation in lung adenocarcinoma.

Materials and Methods

Cell lines and tumor specimens
Lung adenocarcinoma cell lines were provided by Drs. Adi Gazdar and John Minna (The University of Texas Southwestern Medical Center) and authenticated using DNA fingerprinting (14). The cell lines were cultured in RPMI 1640 (Cellgro; Mediatech, Inc.) containing 10% FBS and antibiotics (Sigma-Aldrich) at 37°C in 5% CO2 in a cell culture incubator.

 Archived frozen and formalin-fixed, paraﬁn-embedded tumor specimens obtained from patients with NSCLC who underwent surgical resection with curative intent were collected from the Lung Cancer Specialized Program of Research Excellence tissue bank at The University of Texas MD Anderson Cancer Center. One hundred forty-nine specimens were selected randomly: 56 were obtained from patients given adjuvant platinum-based chemotherapy, and 93 were obtained from patients who did not receive this therapy. Detailed clinical and pathologic information on the patients is presented in Supplementary Table S1. The study protocol was approved by the MD Anderson Institutional Review Board.

mRNA and microRNA analyses
Total RNA was extracted from cell lines and frozen tumor specimens using TRI Reagent (Life Technologies). Spectrophotometric analysis using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientiﬁc) was performed to determine the RNA quantity in cell lines and tumor specimens, and the quality of RNA was assessed using Agilent BioAnalyzer RNA Nanochips (Agilent Technologies). RNA extracted from lung adenocarcinoma cell lines was subjected to qRT-PCR analysis using a High Capacity RNA-to-cDNA Kit and TaqMan Gene Expression PCR assays (Applied Biosystems) to detect their EZH2 message levels using GAPDH as an endogenous control. In addition, TaqMan microRNA assay (Applied Biosystems) was used to detect the levels of miR-101 expression using U6 as an endogenous control. An ABI PRISM 7300 Sequence Detection System (Applied Biosystems) under standard PCR assay cycling conditions with triplicate specimens was used to determine relative levels of expression miR-101 in cell lines with the 2−ΔΔCT method and ABI 7300 SDS software program (version 1.4; Applied Biosystems). Total RNA extracted from tumor specimens was used to determine EZH2 and miR-101 expression levels in the specimens using Illumina WG-6 v.3 mRNA and Agilent Technologies V3 human microRNA arrays, respectively. Illumina v.3 datasets of 275 primary lung adenocarcinomas and squamous cell carcinomas (SPORE dataset; GSE41271) have been deposited in the Gene Expression Omnibus (GEO) repository (15).

VEGF stimulation
Cells lines were serum-starved for 24 hours and stimulated in fresh medium with 50 ng/mL VEGF-A (Cell Signaling Technology). Cells were then incubated under normoxic conditions, and protein lysates were collected.
18 hours later. Western blot analysis was carried out using specific antibodies against EZH2 (AC22), H3K27me3 (C36B11), HIF1α (HIF1α antibody), VEGFR-2 (55B11; Cell Signaling Technology), and E2F3 (ab54945; Abcam).

**Transfection of lung adenocarcinoma cells with siRNAs and human KDR cDNA and cell migration/proliferation assays**

Lung adenocarcinoma cell lines were transfected with three gene-specific siRNA (siRNA-3) duplexes for the EZH2, KDR, and HIF1α genes, respectively, and a control siRNA (OriGene Technologies) at a final concentration of 10 nmol/L using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. To verify the knockdown efficiency of each gene, mRNA and proteins were collected from transfected cells for qRT-PCR and Western blot analysis. VEGFR-2 (KDR) full-length human cDNA and control plasmid constructs were purchased from OriGene Technologies, and the lung adenocarcinoma cell line A549 was transfected with KDR plasmid cDNA (pKDR) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. A cell migration assay was carried out using Boyden chambers as described previously (13). Also, cell proliferation assays were carried out using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay according to the assay manufacturer’s protocols.

**Treatment with 3-deazaneplanocin A**

The day after placement of NSCLC cell lines in tissue-culture dishes, the media in the dishes were replaced with fresh complete media or complete media containing 3-deazaneplanocin A (DZNep; Cayman Chemical) at increasing concentrations. To determine the effect of treatment with DZNep on EZH2 expression and assess apoptosis, cell lines were treated with DZNep at different concentrations (0, 2.5, and 5.0 μmol/L) for 72 hours. Also, protein lysates were collected from subconfluent cultures after 72 hours of growth in media with 5% FBS and in the presence or absence of DZNep and subjected to Western blot analysis with specific antibodies against EZH2 (AC22), trimethylated H3K27me3 (C36B11), and cleaved PARP (PARP-C; Asp214; Cell Signaling Technology).

**MTS assay and treatment of lung adenocarcinoma cells with platinum agents and a VEGFR-2 inhibitor**

Cisplatin, carboplatin, and the VEGFR-2 inhibitor AZD2171 (cediranib) were purchased from Selleck Chemicals. To determine the median half-maximal inhibitory concentrations (IC₅₀) of these drugs, lung adenocarcinoma cells were seeded in octuplicate at a density of 2,000 cells per well in 96-well plates. The following day, cells were treated with the drugs at increasing concentrations, and an endpoint viability assay was performed after 72 hours of treatment using MTS assays (Promega). The dual-drug studies (cisplatin + DZNep, carboplatin + DZNep, and AZD2171 + DZNep) were carried out in a similar manner.

**Xenograft studies**

Female athymic nude mice, 6- to 8-weeks old, were injected subcutaneously in the flank with 1 × 10⁶ of HCC4006 cell lines. Tumors were allowed to grow for 2 weeks; once tumors were palpable, mice were randomized into treatment groups of 8 mice per group for the tumor growth experiments. The following treatments were administered in cohorts of 8 mice for each treatment: vehicle alone, DZNep PBS 1 mg/kg (50 μL, intraperitoneal injection), AZD2171 (Cediranib) was suspended in 1% (w/v) aqueous polysorbate 80, 1.5 mg/kg (50 μL, oral gavage), or the combination at the indicated doses (DZNep 1 mg/kg + AZD2171 1.5 mg/kg). DZNep was administered thrice per week treatment for 3 weeks and AZD2171 was administered daily. Tumors were measured twice a week with calipers. Tumor volumes were calculated according to the formula (L x W²)/2. All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at The University of Texas MD Anderson Cancer Center.

**Statistical analysis**

Data obtained from cell culture assays and qRT-PCR and Western blot analyses were summarized using descriptive and inferential statistics accompanied by graphs of relative expression with the Prism software program (version 5.0; GraphPad Software). Western blot analyses were carried out multiples times and normalized to β-actin protein by densitometric scanning and quantified using NIH ImageJ software. The patients’ demographic and clinical information was compared using the χ², Fisher exact, Wilcoxon rank-sum, and Kruskal–Wallis tests. Overall survival (OS) and recurrence-free survival (RFS) distributions were estimated using the Kaplan–Meier method, and the distributions in the groups of patients were compared using the log-rank test. Cox proportional hazard models were used for regression analysis of survival data and conducted for OS duration, defined as the time from surgery to the patient’s death or last contact and RFS duration, defined as the time from surgery to tumor recurrence or the patient’s last contact. The follow-up duration was censored at 5 years.

**Results**

**EZH2 and miR-101 expression in lung adenocarcinoma cell lines**

To explore the mechanisms of regulation and role of EZH2 in lung cancer pathogenesis *in vitro*, we first characterized EZH2, miR-101, and VEGFR-2 expression in lung adenocarcinoma cell lines. We screened a panel of eight lung adenocarcinoma cell lines for expression of EZH2 mRNA and protein using qRT-PCR and Western blotting, respectively. In addition, we examined the global levels of H3K27me3 and VEGFR-2 expression in these cells using Western blotting. We found that all eight lung adenocarcinoma cell lines had detectable expression of EZH2 mRNA and protein and of H3K27me3 and high, low, and lack of VEGFR-2 protein expression (Fig. 1A and B). Because miR-101 is known to posttranscriptionally downregulate the
expression of EZH2, we examined miR-101 expression in the same set of cell lines using qRT-PCR. Consistent with the expression of EZH2, we observed low levels of miR-101 expression in all eight cell lines (Fig. 1C).

Activation of the VEGF/VEGFR-2 pathway upregulates tumor expression of EZH2 in a VEGFR-dependent manner

On the basis of previous reports describing an association between increased activity of the VEGF/VEGFR-2 pathway and EZH2 in tumor endothelial cells (8, 9), we sought to determine whether VEGF/VEGFR-2 pathway can regulate the expression of EZH2 in a larger panel of lung adenocarcinoma cells. We found that upon VEGF stimulation, serum-starved cell lines had increased expression of EZH2 protein and mRNA (Fig. 1D). We also observed increased H3K27me3 levels in cells treated with VEGF (Fig. 1D). Interestingly, these changes in EZH2 and H3K27 were more common in lung adenocarcinoma cell lines with high expression of VEGFR-2 (HCC4006, HCC461, HCC1171, H2085, and CALU-1) than in cell lines with low expression of it (HCC515, HCC193, and H1993), and the changes did not occur at all in a cell line lacking VEGFR-2 expression (A549, H2073, HCC827, and H23; Fig. 1D and Supplementary Fig. S1A and S1B). To determine whether VEGFR-2 expression is required for induction of EZH2 expression in the presence of VEGF, we knocked down VEGFR-2 expression using treatment with a VEGFR-2–specific siRNA (siKDR) in HCC461 and HCC4006 cells, which have high levels of VEGFR-2 protein expression. In both cell lines, treatment with siKDR resulted in decreased VEGFR-2 expression than that in nontransfected cells or cells transfected with scrambled siRNA (control; Fig. 2A and B). Moreover, EZH2 mRNA and protein and H3K27me3 expression levels in HCC461 cells remained unchanged in response to exposure to VEGF, whereas the expression levels...
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Figure 2. Knockdown of VEGFR-2 expression by treatment with siRNA decreased the expression of EZH2 and H3K27me3 in lung adenocarcinoma cell lines stimulated by VEGF. A and B, Western blots of VEGFR-2 (siKDR), EZH2, H3K27me3, and H3 in the lung adenocarcinoma cell lines HCC461 and HCC4006 upon knockdown of VEGFR-2 expression by treatment with siRNA-3 following VEGF stimulation. A and B, bottom, EZH2 mRNA expression in lung adenocarcinoma cells as determined using qRT-PCR. *P < 0.05; **P < 0.001; ***P < 0.003. C, Western blot of VEGFR-2, EZH2, H3K27me3, and H3 expression in A549 cells transfected with VEGFR-2 (pKDR). C, bottom, EZH2 mRNA expression in lung adenocarcinoma cells as determined using qRT-PCR. *P < 0.05.

Overexpression of VEGFR-2 (pKDR) increased the expression of EZH2 and H3K27 following VEGF stimulation in A549 cells.

decreased in HCC4006 cells after knockdown of VEGFR-2 expression (Fig. 2A and B). In addition, we investigated overexpression of VEGFR-2 induced by transfection of a VEGFR-2 cDNA (pKDR) construct to determine whether it can induce expression of EZH2 in the presence of VEGF. To that end, we transfected pKDR into the A549 cell line, which lacks expression of VEGFR-2, and observed significantly increased expression of EZH2 mRNA and protein and H3K27me3 level in response to exposure to VEGF than that in control cells transfected with an empty vector (Fig. 2C).

EZH2 expression is regulated by E2F3 via the VEGF/VEGFR-2 pathway

VEGF is known to transcriptionally regulate the expression of EZH2 in tumor endothelial cells via the E2F transcription factors, mainly E2F1 and E2F3 (8, 16). To determine whether this regulatory mechanism is also present in tumor cells, we analyzed the E2F1 and E2F3 mRNA expression in lung adenocarcinoma cell lines stimulated with VEGF. We found that VEGF induced expression of E2F3 transcripts and protein but not of E2F1 in cell lines expressing VEGFR-2 (HCC515, HCC193, HCC4006, HCC461, and HCC1171; Supplementary Fig. S2A and S2B). We did not observe this effect in the A549 cell line, which lacks VEGFR-2 expression (Supplementary Fig. S2A and S2B) Additional experiments demonstrated that increased expression of E2F3 in response to VEGF stimulation was dependent on the presence of VEGFR-2, as knockdown of VEGFR-2 expression abrogated the response of HCC461 and HCC4006 cells to VEGF stimulation (Supplementary Fig. S2C). Moreover, we found increased expression of E2F3 in response to VEGF stimulation in A549 cells transfected with VEGFR-2 cDNA pKDR (Supplementary Fig. S2D). These findings suggest that the VEGF/VEGFR-2 pathway plays key role in regulation of EZH2 expression via E2F3 in lung adenocarcinoma cell lines.

HIF1α also increases EZH2 via the VEGF/VEGFR-2 pathway in a hypoxia-independent manner

In addition to regulation of EZH2 expression via VEGF/VEGFR-2/E2F3, a recent report on breast tumors stated that a hypoxic tumor microenvironment increases EZH2 expression via induction of HIF1α expression (11). In addition, we recently observed that VEGF regulates the expression of HIF1α independently of hypoxia in NSCLC cell lines that overexpress VEGFR-2 (13). We confirmed these previous results in the present study and observed responses of HIF1α and EZH2 expression to VEGF stimulation. Specifically, we detected a pronounced increase in coexpression of EZH2 and HIF1α in response to VEGF stimulation in lung adenocarcinoma cell lines with high expression of VEGFR-2 (HCC4006, HCC461, and HCC1171; Fig. 3A). The increase in coexpression was less pronounced in cell lines with low VEGFR-2 expression (HCC515 and HCC193) and absent from a cell line lacking VEGFR-2 expression (A549; Fig. 3A).

The increased expression of HIF1α in response to VEGF stimulation was reduced by knocking down expression of VEGFR-2 in HCC461 and HCC4006 cells (Supplementary Fig. S2C). Moreover, we found increased expression of HIF1α in response to VEGF stimulation in A549 cells transfected with VEGFR-2 cDNA (Supplementary Fig. S2D). To
determine whether HIF1α expression is required for induction of EZH2 expression in the presence of VEGF, we knocked down HIF1α expression using an HIF1α-specific siRNA (siHIF1α) in HCC461 and HCC4006 cells, which exhibited strong responses to stimulation with VEGF. siHIF1α produced lower HIF1α expression in both cell lines than in control cells transfected with scrambled siRNA and in nontransfected cells (Fig. 3B and C). We also found that increased expression of EZH2 and H3K27me3 in response to VEGF stimulation was reduced by knocking down expression of HIF1α, which was more evident in HCC4006 than HCC461 cells (Fig. 3B and C). These results suggested that increased expression of HIF1α mediated by stimulation with VEGF can upregulate expression of EZH2 in lung adenocarcinoma cell lines expressing VEGFR-2. In addition, we evaluate whether a hypoxic microenvironment induces the expression of EZH2 in lung cancer. HCC4006 and HCC461 cells were plated and incubated in normoxia (20% oxygen) or hypoxia (1% oxygen). Hypoxia increased the coexpression of EZH2 and HIF1α in lung adenocarcinoma cell lines (Supplementary Fig. S3A and S3B).

**miR-101 regulates EZH2 via the VEGF/VEGFR-2 pathway**

Recently, investigators showed that EZH2 expression is regulated at the posttranscriptional level by miR-101 (10, 12). Of note, in endothelial cells, VEGF downregulates miR-101 expression, resulting in upregulation of EZH2 expression (9). To determine the effects of VEGF stimulation on miR-101 expression and whether decreased miR-101 expression correlates with EZH2 expression, we stimulated lung adenocarcinoma cell lines with VEGF and analyzed miR-101 expression in the cell lines using qRT-PCR. We found that VEGF stimulation led to downregulation of
miR-101 expression and increased EZH2 expression. This phenomenon was more pronounced in cell lines with high expression of VEGFR-2 (HCC4006, HCC461, and HCC1171), less pronounced in those with low expression of VEGFR-2 (HCC515 and HCC193), and absent from a cell line lacking expression of VEGFR-2 (A549; Fig. 3D). Down-regulation of miR-101 expression in response to VEGF stimulation was reduced by knocking down miR-101 in HCC461 and HCC4006 cells (Supplementary Fig. S2C, bottom). This finding was more evident in HCC4006 cells than in HCC461 cells. Furthermore, we observed downregulation of miR-101 expression together with an increase in EZH2 expression in response to VEGF stimulation in A549 cells transfected with VEGFR-2 cDNA (Supplementary Fig. S2D, bottom). These results suggested that the VEGF/VEGFR-2 pathway regulates EZH2 expression via miR-101 in lung adenocarcinoma cell lines. In addition, we evaluated the expression of miR-101 in hypoxia condition in lung adenocarcinoma cell lines. Hypoxia condition--decreased miR-101 expression correlates with increase of EZH2 expression in lung adenocarcinoma cell lines (Supplementary Fig. S3C).

Moreover, to determine whether increased EZH2 is the result of decreased miR-101 by stimulation with VEGF, we overexpressed miR-101 by transfection of an miR-101-3p mimic in HCC461 cells and stimulated in the presence or absence of VEGF. Transfection of miR-101 inhibits the expression of EZH2 mRNA and protein and remained unchanged in response to exposure to VEGF in HCC461 cells (Supplementary Fig. S4A–S4C). These results confirm that miR-101 regulates EZH2 expression in lung adenocarcinoma cell lines.

Effect of inhibition of EZH2 activity and expression by treatment with a small-molecule inhibitor EZH2 activity and siRNA

To determine the effects of abrogation of EZH2 activity in lung adenocarcinoma cell lines, we selected a subset of cell lines based on their EZH2 protein expression profiles (HCC4006, low expression; A549, intermediate expression; and H2073, high expression). In these three cell lines, EZH2 activity was inhibited pharmacologically by treatment with DZNep, and EZH2 gene expression was knocked down by treatment with siRNA. Researchers recently identified DZNep, a small molecule that efficiently inhibits EZH2 activity, by depleting EZH2 expression levels and inhibiting trimethylation of H3K27 in cancer cells via treatment with this molecule in a dose-dependent manner (17, 18). To determine the effect of DZNep on cell viability, we treated the cell lines with it at increasing doses (range, 0–10 μmol/L) and observed that DZNep only slightly decreased cell viability by 15% in H2073 and A549 cells, and 30% in HCC4006 cells (Supplementary Fig. S5). This response plateaued at higher concentrations, suggesting that DZNep had a cytostatic effect on these cell lines. In addition, we determined whether DZNep decreased the expression of EZH2 and induced apoptosis in these cell lines by treating them with the molecule at different doses (0, 2.5, and 5.0 μmol/L) for 72 hours. We found DZNep treatment decreased the expression of EZH2 in a dose-dependent manner (Fig. 4A, left), and reduced levels of H3K27 and increased the expression of PARP-C in all of the cell lines (Fig. 4A, left). DZNep is also known to mediate the depletion of other components of PRC2, such as embryonic ectoderm development (17). We validated the results of treatment with DZNep by knocking down EZH2 expression in these cell lines using treatment with siRNA. Similar to our results with DZNep, Western blot analysis of protein lysates of cell lines treated with EZH2-specific siRNA demonstrated lower EZH2 expression (Fig. 4A, right) and H3K27me3 expression concomitant with higher PARP-C level (Fig. 4A, right) than that in nontransfected cells and cells transfected with scrambled siRNA (control). Thus, we found targeting EZH2 pharmacologically or genetically resulted in decreased EZH2 levels, reduced H3K27 methylation, and increased markers of apoptosis.

We next investigated the effects of EZH2 inhibition on the sensitivity of the cell lines to treatment with cisplatin and carboplatin. We pretreated three lung adenocarcinoma cell lines (HCC4006, A549, and H2073) with 2.5 μmol/L DZNep for 24 hours and then treated them with cisplatin or carboplatin in the presence or absence of DZNep at a fixed concentration (2.5 μmol/L) for 72 hours. We found that the sensitivity of the three cell lines to cisplatin and carboplatin in vitro (Fig. 4B, left) was significantly higher (P < 0.05) with DZNep-based treatment than without it. This strongly suggested that DZNep-mediated inhibition of EZH2 expression and activity sensitizes lung adenocarcinoma cell lines to platinum-based drugs. We confirmed these results via knockdown of EZH2 expression by treatment with siRNA (siEZH2) and noted similarly greater sensitivity of the three cell lines to cisplatin and carboplatin (Fig. 4B, right). These findings suggest that depletion of EZH2 significantly increases the sensitivity of lung adenocarcinoma cell lines to platinum drugs. In addition, as shown above the overexpression of VEGFR-2 increased expression of EZH2. We evaluated whether this increased EZH2, produced by the overexpression of VEGFR-2 into the A549 cell line, which lacks expression of VEGFR-2, resulting in a decrease in sensitivity to cisplatin and if this can be reversed by knockdown of EZH2 or inhibition with DZNep. We observed that increased expression of VEGFR-2 induces a decrease in sensitivity to cisplatin. To evaluate whether this decrease in sensitivity to cisplatin is due to increased expression of EZH2, EZH2 activity was inhibited by knockdown using treatment with siRNA or treatment with DZNep. We observed that the decrease in sensitivities to cisplatin produced by the overexpression of VEGFR-2 was reversed by EZH2 knockdown and more strongly by inhibition with treatment with DZNep (Supplementary Fig. S6). These findings suggest a functional connection between VEGFR-2 and EZH2 to promote malignant phenotype and modulate the response to therapy. We evaluated the effect of EZH2 knockdown by siRNA on lung adenocarcinoma cell migration and proliferation. We found that knockdown of EZH2 expression resulted in significantly less migration (Fig. 4C).
Figure 4. Pharmacologic inhibition of EZH2 activity by treatment with DZNep and knockdown of EZH2 expression by treatment with siRNA decreased the expression of EZH2 and H3K27me3, induced apoptosis, increased sensitivity to treatment with cisplatin and carboplatin, and reduced migration and proliferation in lung adenocarcinoma cells. A, Western blot analysis of EZH2, H3K27me3, and PARP-C expression in the lung adenocarcinoma cell lines A549, H2073, and HCC4006 upon inhibition of EZH2 by treatment with different doses of DZNep (0, 2.5, and 5.0 μmol/L; left) and knockdown of EZH2 expression by treatment with siRNA-3 (right). Both approaches decreased the expression of EZH2 and H3K27me3 and increased the expression of PARP-C. B, pharmacologic inhibition of EZH2 by treatment with DZNep (left) and knockdown of EZH2 expression by treatment with siRNA-3 (right) decreased the viability of the lung adenocarcinoma cell lines A549, H2073, and HCC4006 exposed to cisplatin and carboplatin according to an MTS assay (data are graphed as the mean percentage increase ± percentage SD). Treatment with DZNep caused a 26-fold (P < 0.03) decrease in the cisplatin IC50 in A549 cells, a 1.7-fold (P < 0.05) decrease in it in H2073 cells, and a 2.4-fold (P < 0.05) decrease in it in HCC4006 cells. Treatment with DZNep also caused 27.7-fold (P < 0.03), 3.7-fold (P < 0.05), and 2.3-fold (P < 0.05) decreases in the carboplatin IC50 in A549, H2073, and HCC4006 cells, respectively. Knockdown of EZH2 expression caused a 3.8-fold (P < 0.05) decrease in the cisplatin IC50 in A549 cells, a 1.5-fold (P < 0.05) decrease in it in H2073 cells, and a 1.7-fold (P < 0.05) decrease in it in HCC4006 cells. In addition, knockdown of EZH2 expression caused 1.2-, 1.5-, and 1.2-fold (P < 0.05) decreases in the carboplatin IC50 in A549, H2073, and HCC4006 cells, respectively. C, inhibition of migration of A549, H2073, and HCC4006 cells by treatment with siEZH2 with and without stimulation with FBS showed decreased cell migration (*, P < 0.01). D, knockdown of EZH2 expression resulted in significantly lower rates of proliferation of HCC4006 and A549 cells (*, P < 0.05) and, to a lesser extent, lower rates of that of H2073 cells than of control siRNA-transfected and nontransfected cells.
and proliferation (Fig. 4D) of the three cell lines than of nontransfected cells or cells transfected with scrambled siRNA. Overall, the results of this *in vitro* analysis of lung adenocarcinoma cell lines supported our hypothesis that EZH2 overexpression promotes the malignant phenotype in lung adenocarcinoma cells by increasing their proliferation, migration, and resistance to platinum drugs.

**Association of EZH2 and mir-101 expression with clinicopathologic features and clinical outcome in patients with lung adenocarcinoma**

We analyzed EZH2 and mir-101 mRNA expression in lung adenocarcinoma specimens using our own Illumina WG-6 v.3 mRNA and Agilent V3 human microRNA array data sets, respectively. Specifically, we investigated the expression of EZH2 and/or mir-101 to determine whether it was associated with clinicopathologic features of the 149 surgically resected lung adenocarcinoma specimens. We found that high EZH2 expression (EZH2High) was significantly correlated with ever-smoker status (*P* = 0.001) and large tumors (*P* = 0.049; Supplementary Table S2). Also, low mir-101 expression (mir-101Low) was significantly correlated with ever-smoker status (*P* = 0.012) and male sex (*P* = 0.001) but was not correlated with tumor size.

In univariate analysis, EZH2High was significantly associated with poor OS duration (HR, 1.844; 95% CI, 1.041–3.209; *P* = 0.030; Fig. 5A). These findings were confirmed in a multivariate analysis, after adjustment for age, tumor size, overall stage, and adjuvant therapy. EZH2High was significantly associated with poor OS duration (HR, 1.828; 95% CI, 1.041–3.209; *P* = 0.036, data not show). In multivariate analysis, EZH2High was marginally significantly correlated with poor OS duration (HR, 2.33; 95% CI, 0.956–5.678; *P* = 0.062) in patients with lung adenocarcinoma who received adjuvant platinum-based therapy but not in patients who did not receive this therapy (Fig. 5B and C and Supplementary Table S3). We did not find a correlation between EZH2 expression and RFS duration or between mir-101 expression and patient outcome.

**Inhibition of EZH2 activity by DZNep sensitizes lung adenocarcinoma cells to VEGFR-2–targeted therapy**

Our *in vitro* findings demonstrated that treatment with the EZH2 inhibitor DZNep increased the sensitivity of lung adenocarcinoma to treatment with cisplatin and carboplatin. We then asked whether treatment with the combination of an EZH2 inhibitor and VEGFR-2–targeting drugs provides additional therapeutic benefits in patients with NSCLC. We first sought to determine whether treatment with AZD2171, a known inhibitor of VEGFR-2 activity, decreases EZH2 expression. We treated A549, HCC461, and HCC4006 cells with different doses of AZD2171 (0, 5, and 10 nmol/L) for 48 hours. We found that this treatment decreased the expression of EZH2 in HCC4006 and HCC461 cells with different doses of AZD2171 (0, 5, and 10 nmol/L) for 48 hours. We found that this treatment decreased the expression of EZH2 in HCC4006 and HCC461 cells expressing VEGFR-2 but not in A549 cells lacking expression of VEGFR-2 in a dose-dependent manner (Fig. 6A).

![Figure 5. Association between EZH2 expression and clinical outcome in patients with lung adenocarcinoma.](https://www.aacjrournals.org)

Gene expression data on RNA extracted from frozen tumor specimens were examined using Illumina WG-6 v.3 mRNA and Agilent Technologies V3 human microRNA arrays. A, Kaplan–Meier OS curves according to EZH2 expression in all patients. B, Kaplan–Meier OS curves in patients who received adjuvant platinum-based therapy. C, Kaplan–Meier OS curves in patients who did not receive adjuvant platinum-based therapy. E, event; N, total number of cases.
In addition, we pretreated A549, HCC461, and HCC4006 cells with 2.5 μmol/L DZNep for 24 hours and subsequently exposed them to AZD2171 at various concentrations in the presence or absence of DZNep at a fixed concentration (2.5 μmol/L) for 72 hours. We found that the in vitro sensitivity to AZD2171 was significantly higher in HCC4006 and HCC461 cells expressing VEGFR-2 in a dose-dependent manner but did not do so in A549 cells lacking expression of VEGFR-2. B, pharmacologic inhibition of EZH2 activity by treatment with DZNep decreased the viability of A549, HCC4006, and HCC461 cells exposed to AZD2171 according to an MTS assay (data are graphed as the mean percentage increase ± percentage SD). DZNep increased sensitivity to AZD2171 treatment in HCC4006 (3.8-fold decrease IC50, \(P < 0.01\)) and HCC461 (1.4-fold decrease IC50, \(P < 0.05\)) and slight increase in the sensitivity of A549 cells (1.2-fold decrease in IC50) to this treatment. C, athymic nude mice were inoculated with HCC4006 cell lines and then treated with vehicle, DZNep, AZD2171, or a combination of DZNep plus AZD2171. Tumor volume was determined for each treatment. The sensitivity in vivo to AZD2171 was significantly increased in the presence of DZNep, observing an inhibition of tumor growth with the combination of DZNep plus AZD2171 versus either treatment alone (\(P < 0.05; \ldots; P < 0.003; \ldots; P < 0.001\)). D, proposed model of the tumor-cell VEGF/VEGFR-2 pathway that upregulates EZH2 expression via increased E2F3 and HIF1α and downregulated miR-101 expression, promoting the malignant phenotype in lung adenocarcinoma cells. EZH2 depletion at the genetic or protein level promotes increased apoptosis, decreased cell migration, and increased sensitivity to standard platinum-based and VEGFR-2–targeted therapy.

In addition, we pretreated A549, HCC461, and HCC4006 cells with 2.5 μmol/L DZNep for 24 hours and subsequently exposed them to AZD2171 at various concentrations in the presence or absence of DZNep at a fixed concentration (2.5 μmol/L) for 72 hours. We found that the in vitro sensitivity to AZD2171 was significantly higher in HCC4006 and HCC461 cells (\(P < 0.05\)) and slightly higher in A549 cells in the presence of DZNep than in cells treated with AZD2171 in the absence of DZNep (Fig. 6B). This response was more potent in HCC461 and HCC4006 cells, which have high VEGFR-2 expression, than in A549 cells, which lack this expression. Xenograft studies showed similar results, the sensitivity in vivo to AZD2171 in nude mice inoculated with HCC4006 cell lines was significantly increased in the presence of DZNep, observing an inhibition of tumor growth when combined EZH2 inhibition with DZNep and VEGFR-2–target therapy with AZD2171, compared with the result of mice treated with DZNep or AZD2171 alone (Fig. 6C). These results suggested that treatment with anti–VEGFR-2 drugs in combination with an EZH2 inhibitor greatly increases the sensitivity of lung adenocarcinoma to VEGFR-2–targeted therapy.

Discussion

Although EZH2 is widely overexpressed in aggressive tumors, the genetic mechanism of EZH2 upregulation in malignant epithelial cells is unknown. In the present study, we demonstrated that VEGF induced expression of EZH2 and concomitantly increased of H3K27me3 levels, in lung adenocarcinoma cells expressing VEGFR-2. This finding was more common in cells overexpressing VEGFR-2 than in cell lines with low expression or cell than lacking VEGFR-2 expression. In addition to increasing the expression of EZH2, we observed that VEGF stimulation increased the expression of E2F3 and HIF1α and downregulated miR-101 expression, at the transcriptional and posttranscriptional levels. Increased expression of EZH2 together with that of E2F3 and HIF1α and downregulation of expression of miR-101 in response to VEGF stimulation were reduced by...
knockdown of expression of VEGFR-2. This finding suggests that the VEGF/VEGFR-2 pathway plays an important role in the regulation of EZH2 expression via upregulation of E2F3 and HIF1α expression and downregulation of miR-101 expression (Fig. 6D).

VEGF and its receptor VEGFR-2 act as master regulators of angiogenesis, stimulating endothelial cell functions and enhancement of vascular permeability (19–22). VEGF overexpression is associated with tumor progression and poor prognosis (23, 24). In tumor cells as in endothelial cells, overexpression of VEGFR-2 has been associated with cell migration, proliferation, and survival (13, 24–28). In addition, stimulation with VEGF has caused overexpression of VEGFR-2 in a feed-forward loop (29). In endothelial cells, VEGF increases expression of E2F transcription factors, which bind directly to the promoter of EZH2 and thus activate its transcription (8, 9), and downregulates expression of miR-101, a negative regulator of EZH2, contributing to increased expression of EZH2 (9, 10, 12, 30). In addition, recent work by our group demonstrated that VEGF-2 overexpression product of the increasing gene copy number in NSCLC was highly associated with resistance to platinum-based chemotherapy (13). This finding suggests that activation of the VEGF/VEGFR-2 pathway in malignant cells overexpressing VEGFR-2 promotes the malignant phenotype by upregulating EZH2 expression. In malignant cells, EZH2 acts as an intermediary of the functions regulated by the VEGF/VEGFR-2 pathway promoting cell migration, proliferation, and survival.

The hypoxic microenvironment triggered by tumor growth induces expression of HIF1α, a key regulator that mediates adaptation to hypoxia responsible for the induction of expression of genes that facilitate adaptation and survival of tumor cells to hypoxia microenvironments (31, 32). In a hypoxic microenvironment, HIF1α binds to the VEGF promoter to stimulate increased VEGF production (11, 33–35). As we recently demonstrated, VEGF in turn regulates HIF1α expression levels in NSCLC cell lines independently of hypoxia (13). This suggests the existence of a paracrine or autocrine loop between VEGF and HIF1α that maintains high levels of expression of both of them and promotes angiogenesis. Of note, researchers have found that HIF1α is associated with chemoresistance of many tumor types (36–38). In addition, investigators recently showed that in breast tumors, a hypoxic state increases EZH2 expression via HIF1α (11), as the EZH2 promoter region contains consensus sequences of HIF1α response elements (11). In the present study, we showed for the first time that in malignant cells, the VEGF/VEGFR-2 pathway directly regulates the expression of EZH2 via HIF1α independently of hypoxia. This suggests that in malignant cells, the VEGF/VEGFR-2 pathway can regulate expression of EZH2 in various ways, increase the expression of E2F3 and HIF1α, and downregulate the expression of miR-101. Authors have described overexpression of both VEGF and VEGFR-2 in NSCLC cells and that it was associated with poor prognosis (13, 24). Increased activation of this pathway produced by overexpression of VEGF and VEGFR-2 may account for the increased levels of EZH2 expression that we detected in lung adenocarcinoma specimens. However, we do not rule out other regulatory mechanisms associated with EZH2 expression. In addition, recently authors have described in breast cancer that MEK–ERK activation pathway leads to EZH2 overexpression (39), possibly intrinsic status of activation of this pathway may account for the different levels of response to stimulation with VEGF/VEGFR-2 pathways.

Our findings suggest that EZH2 overexpression promotes a more malignant phenotype in lung adenocarcinoma cells. In addition, we demonstrated that pharmacologic and genetic depletion of EZH2 increased the sensitivity of lung adenocarcinoma cell lines to cisplatin and carboplatin, increased apoptosis, and reduced the cells’ migration and proliferation capabilities. Furthermore, our results demonstrate that high EZH2 expression in resected lung adenocarcinoma specimens was associated with poor OS durations in patients who received adjuvant platinum-based therapy but not in patients who did not receive this therapy. Interestingly, the association with clinicopathologic features revealed a significant correlation between EZH2 high expression and larger tumors. This correlation is consistent with our in vitro findings showing that the inhibition of EZH2 decreases cell proliferation. These findings suggest that EZH2 is a predictive marker of poor outcome in patients with lung adenocarcinoma treated with platinum agents and that targeting EZH2 with specific inhibitors of it improves the response of lung adenocarcinoma to platinum-based therapy and can reduce the metastatic potential and proliferation of this disease.

EZH2 has oncogenic functions and its overexpression has been associated with a malignant phenotype in tumor cells, and it is implicated to have a role in neoplastic transformation and progression in many tumors (3, 6, 11, 16). EZH2 overexpression has been linked with silencing of numerous tumor-suppressor genes that control important cellular processes, such as p15 (INK4b; ref. 40), p19 (ARF; ref. 41), cyclin A (42), E-cadherin (2), Bim (43), and RUNX3 (5). In addition, authors have extensively described EZH2 overexpression in tumor endothelial cells (3, 5, 7, 8, 44). In tumor endothelial cells, EZH2 overexpression contributes to angiogenesis by methylating and silencing vasoactive 1, an endothelial cell–specific antiangiogenic factor (8), identifying EZH2 as a key regulator of tumor angiogenesis (8, 9). The oncogenic characteristics of EZH2 and its ability to repress gene targets make it a potential therapeutic target in lung cancer cases that may regulate the epigenome to overcome therapy resistance and angiogenesis. In the present study, we demonstrated that treatment with platinum drugs in combination with depletion of EZH2 significantly improved the response of lung adenocarcinoma cells to this therapy. The mechanisms responsible for these results may be in part from induction of a change in the gene expression profile by EZH2 depletion, facilitating the reexpression of epigenetically silenced genes, favoring response to chemotherapy and inducing apoptosis in lung adenocarcinomas. Researchers recently found that EZH2 has a role in
modulation of DNA damage response (45). Specifically, EZH2 depletion results in abrogation of both G1 and G2/M cell-cycle checkpoints and promotes DNA-damaging agent-induced apoptosis in both p53+/− and p53−/− cancer cells via release of transcriptional repression of FBXO32, which binds to and directs p21 for pro tease-mediated degradation (45). Another possible explanation is that EZH2 depletion may cause structural changes in the state of compaction of chromatin that facilitates the accessibility of these genotoxic agents as cisplatin and carboplatin and their interaction with DNA, allowing DNA damage. These mechanisms support the notion that the depletion of EZH2 can overcome resistance to chemotherapy in patients receiving adjuvant platinum-based therapy and exhibit high EZH2 expression, facilitating access to DNA and inducing apoptosis by DNA-damaging agents.

In recent years, investigators have examined a number of approaches to inhibiting VEGF/VEGFR-2 signaling and blocking angiogenesis (46). However, all of the agents directed against VEGF/VEGFR-2 signaling in these studies exhibited low efficiency and high toxicity (47). This raises the need to design new, better therapeutic strategies that overcome these problems. In this context, EZH2 may be an attractive target because of its functionality in promoting angiogenesis, proliferation, migration, and survival of endothelial and tumor cells. Our present results not only demonstrate that the combination of inhibition of EZH2 and platinum-based chemotherapy improves the response of lung adenocarcinoma to this standard chemotherapy but also show for the first time that treatment with the combination of inhibition of EZH2 and targeting of VEGFR-2 with the agent AZD2171 results in a significant increase in sensitivity of tumors to these agents. In addition, these results can be translated into significant clinical benefits such as reducing the doses used, which in turn can reduce the severity of side effects of these therapies. In summary, our findings indicate that activation of the VEGF/VEGFR-2 pathway upregulates EZH2 expression via increased E2F3 and HIF1α and downregulated miR-101 expression, indicating the malignant phenotype in lung adenocarcinoma cell (Fig. 6D). EZH2 depletion decreases the malignant potential of lung adenocarcinoma, which decreases the migratory and proliferative capacity of tumor cells, while increasing apoptosis in the cells and their sensitivity to both standard and VEGFR-2–targeted therapy. In addition, our findings indicate that high EZH2 expression is associated with poor OS durations in patients with lung adenocarcinoma who received adjuvant platinum-based chemotherapy. These data presented herein identify the VEGF/VEGFR-2 pathway as a regulator of EZH2 expression in malignant cells. Furthermore, these data reveal the role of EZH2 in lung adenocarcinoma progression and identify it as a potential target for overcoming the resistance of tumors to therapy.

Disclosure of Potential Conflicts of Interest

J. V. Heymach reports receiving commercial research grants from AstraZeneca, Bayer, and Pfizer, and is a consultant/advisory board member for GSK, Novartis, Sanofi-Aventis, and Pfizer. No potential conflicts of interest were disclosed by the other authors.

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