The polycomb group protein EZH2 inhibits lung cancer cell growth by repressing the transcription factor Nrf2

Ziming Li a,1, Ling Xu b,1,2, Naiwang Tang a, Yunhua Xu a, Xiangyun Ye a, Shengping Shen a, Xiaomin Niu a, Shun Lu a,⇑, Zhiwei Chen a,⇑

⇑Corresponding authors. Fax: +86 21 62801109.
E-mail addresses: shunlushjtu@163.com (S. Lu), zhiwei_chenchen@163.com (Z. Chen).
1 These authors contributed equally to this work.
2 Fax: +86 21 6439 8310.

a Shanghai Lung Tumor Clinical Medical Center, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai 200030, People’s Republic of China
b Department of Oncology, Longhua Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 200032, People’s Republic of China

1. Introduction

Post-translational modification of histones by methylation is an important and widespread type of chromatin modification that is known to influence biological processes in the context of development and cellular responses [1]. Histone lysine methylation is a critical player in the regulation of gene expression, cell cycle, genome stability, and nuclear architecture [2]. Lysines can be monomethylated (me1), dimethylated (me2) or trimethylated (me3) on their ε-amine groups [1]. The most extensively studied histone methylation sites include histone H3 lysine 4 (H3K4), H3K9, H3K27, H3K36, H3K79 and H4K20 [1]. Trimethylation of histone H3 lysine 27 (H3K27me3), catalyzed by the PcG enhancer of zester homolog 2 (EZH2), is associated with transcriptional repression; whereas H3K4 methylation, catalyzed by the trithorax homolog myeloid-lymphoid leukemia (MLL), is associated with transcriptional activation [3]. Importantly, many genes involved in development, stem cell maintenance, and differentiation are targets of H3K27 and H3K4 methylation. Alteration of histone methyl modifiers and methyl-binding proteins correlates with increased incidence of various cancers [4,5]. One typical example is the H3K27 methyltransferase EZH2. EZH2 is up-regulated in a number of cancers, including prostate cancer [6], breast cancer [7], and lymphomas [8].

Lung cancer is one of the leading causes of cancer-related death in developed countries. Despite decades of intensive efforts, the prognosis of lung cancer remains unfavorable and is especially poor in advanced non-small cell lung cancer (NSCLC). One of the main causes of the poor outcome in NSCLC treatment is the innate resistance to anticancer drugs. The transcription factor NF-E2-related factor 2 (Nrf2) is a basic-leucine-zipper transcription factor originally identified as a pivotal factor for protecting cells from oxidative and electrophilic insults [9]. Nrf2 effector genes include a majority of anti-oxidative, phase II detoxifying enzymes, and ATP-dependent drug efflux pumps [10,11]. Activation of Nrf2 in cancer cells provides advantages for cell proliferation and survival from exposure to anticancer drugs [12,13]. Several studies have implicated the role of Nrf2 in tumor growth and drug-resistance in NSCLC [11,14–17]. Our recent work showed that hMOF acetylates Nrf2 and promotes its nucleus retention in human NSCLC [18]. However, how Nrf2 is controlled at the transcription level remains unknown.

Here in this study, we report that EZH2 represses NSCLC progress by epigenetically inhibiting Nrf2 expression.
2. Materials and methods

2.1. Patients

54 patients with NSCLC were included in this study. The patients were recruited at Shanghai Lung Tumor Clinical Medical Center, Shanghai Chest Hospital, Shanghai Jiao Tong University. The 11 adjacent normal lung tissues were obtained from NSCLC patients undergoing surgery. The clinical characteristics of the patients is listed in Table 1. The diagnosis of lung cancer was established using World Health Organization (WHO) morphological criteria. Both the adjacent normal tissues and the lung cancer tissues were confirmed by a pathologist. Clinicopathological parameters including age, gender, smoking history, histologic grade, tumor status, lymph node metastasis and histologic type were analyzed. A written form of informed consent was obtained from all patients, and the study was approved by the Clinical Research Ethics Committee of Shanghai Jiao Tong University.

2.2. Cells and cell culture

Human lung cancer cell line A549 was obtained from Riken Bio-Resource. The cells were cultured in high glucose-containing DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin.

2.3. Quantitative real-time PCR (q-PCR)

Total RNA samples were extracted from tissues or cells with TRIzol regent (TaKaRa). cDNA was synthesized from 2 μg of RNA with One Step RT-PCR Kit (TaKaRa). q-PCR was performed with the SYBR Green (TaKaRa) detection method on an ABI-7500 RT-PCR system. The EZH2 primers (NM_004456), Nrf2 primers (NM_006164), NQO-1 primers (NM_000903), and HO-1 primers (NM_002133) were purchased from OriGene Technology.

2.4. Western blot

Tissues and lung cancer cells were lysed with cell lysis buffer (Beyotime) supplemented with protease inhibitor cocktail. 40 μg total proteins were applied to 12% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to PVDF membranes, followed by blocking in the buffer containing 5% fat-free dry milk. The membranes were then probed with indicated antibodies overnight, and then washed and incubated with HRP-conjugated secondary antibodies (Zhongshanjinqiao) for 2 h and finally visualized using Chemiluminescent ECL reagent (Vigorous Biotechnology). The following antibodies were used: Anti-GAPDH (Cell Signaling Technology, #2118), anti-EZH2 (Cell Signaling Technology, #4905), anti-Nrf2 (Abcam, #ab31163), anti-H3K27me3 (Abcam, #ab6002), anti-Histone H3 (Abcam, #ab1791).

2.5. Adenovirus/retrovirus packaging and retroviral transduction

To prepare EZH2-expressing adenovirus, the human EZH2 cDNA was inserted into D-TOPO vector (Invitrogen). The D-TOPO-EZH2 plasmid was cloned into the pAd/CMV/VS-DEST vector (Invitrogen) using LR Clonase (Invitrogen). The plasmid was linearized with PacI and was transfected into 293A cells for production of adenovirus. For Ad-Ctrl, the GFP cDNA was inserted into D-TOPO vector instead of EZH2 cDNA. Control shRNA and specific shRNAs targeting EZH2 or Nrf2 were purchased from Invitrogen, and the corresponding sequences were cloned into the pSIREN-RetroQ plasmid (Addgene) for retrovirus production with 293T cells. The targeting sequences were listed in Supplementary Table 1.

For transduction, 293T cells were incubated with virus-containing supernatant in the presence of 8 mg/ml polybrene. After 48 h, infected cells were selected for with puromycin (2 mg/ml). Then the clones were picked and cultured for further experiment.

2.6. Chromatin immunoprecipitation (ChIP)

ChIP was carried out using the ChIP-IT Express Enzymatic kit (Active Motif) according to the manufacturer's instructions. In brief, chromatin from cells was cross-linked with 1% formaldehyde (10 min at 22 °C), sheared to an average size of ~500 bp and then immunoprecipitated with anti-EZH2, anti-lgc or anti-H3K27me3 antibodies. q-PCR was performed to the test the binding ability of EZH2 to Nrf2 promoter regions. The detailed information on EZH2 binding regions and the primers were given in Supplementary Fig. 1 and Table 2.

2.7. Cell proliferation assay

Cell proliferation was monitored by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation/Viability Assay kit (R&D SYSTEMS) in according to the guidelines.

2.8. Soft sugar colony formation assay

Soft sugar colony formation assay was performed as described previously [19]. Cell colonies were stained with 0.005% crystal violet and analyzed using a microscope. The colony number in each well was calculated.

2.9. Luciferase assay

We designed the luciferase assay in according to the results of ChIP assay, and the region where EZH2 binds most frequently was chose for luciferase assay. In detail, the human Nrf2 (−750 to −D bp) promoter was cloned into the pGL4 reporter vector (Promega) to generate an Nrf2-luc reporter vector. 293T cells were

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cultured in triplicate in 24-well plates until reaching 80% confluence. The cells were then co-transfected with Nrf2-Luc/p-RL-luc and EZH2 expressing or control vectors as indicated in the figure legend. Dual luciferase assays (Promega) were performed as described by the manufacturer.

2.10. Xenograft mice experiment

Xenograft mice experiments were performed as described previously [20]. In brief, equal numbers of A549 cells expressing either control or EZH2 knockdown/overexpression vectors were injected subcutaneously, within 30 min of harvesting, over the right and left flanks in male nu/nu mice between 4 and 6 weeks of age. Tumor growth was monitored using calipers. The mice were killed and the tumors were weighted 3 weeks after inoculation. N = 10 in each group.

2.11. Statistical analysis

All values are expressed as the means ± S.E.M. At least three replicates were applied for each experiments if no additional information was indicated in this study. Statistical differences among groups were determined using either Student’s t test or one-way ANOVA. The correlation of EZH2 mRNA level with patients’ clinicopathological variables was analyzed by the χ² test or Fisher’s exact test. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. EZH2 is decreased in NSCLC and low EZH2 level predicts poor survival

To investigate EZH2 function in human NSCLC, we examined changes in EZH2 mRNA and protein expression in human NSCLC and adjacent normal tissues. The results showed that EZH2 expression was significantly down-regulated in NSCLC tissues at mRNA and protein levels (Fig. 1A and B), and EZH2 level decreased with the development of cancer (Fig. 1B). Those data implicates that EZH2 may participate in the development and prognosis of NSCLC. To test this hypothesis, we analyzed the correlation between mRNA level of EZH2 and overall and disease-free survival in patients with NSCLC. Markedly, low expression of EZH2 was correlated with poor overall and disease-free survival (Fig. 1C and D). In addition, low EZH2 level was correlated with large tumor size, high metastasis, and high disease stage (Table 1). Those results indicate that EZH2 expression is down-regulated in human NSCLC tissues and that low level of EZH2 predicts poor survival.

3.2. EZH2 regulates lung cancer cell growth in vitro and in vivo

To explore the role of EZH2 in lung cancer cell growth, we performed cellular proliferation and colony formation experiments in vitro and xenograft mice experiments in vivo. We knocked down EZH2 expression in A549 lung cancer cells and found that EZH2 knockdown promoted A549 cell proliferation (Fig. 2A, Supplementary Fig. 1A and B). In contrast, when EZH2 was overexpressed, the proliferation rate of A549 cells decreased significantly (Fig. 2B). Next, we evaluated the colony formation capacity. The results showed that EZH2 knockdown up-regulated colony formation activity of A549 cells (Fig. 2C, Supplementary Fig. 1C, D and 2A), while EZH2 overexpression reduced colony formation activity of A549 cells (Fig. 2D, Supplementary Fig. 2B). Those results indicate that EZH2 inhibits lung cancer growth in vitro, which prompted us to confirm the role of EZH2 in lung cancer in vivo. Therefore, we performed xenograft mice experiments. Our results showed that EZH2 knockdown increased the average tumor weight (Fig. 2E, Supplementary Fig. 3A), whereas EZH2 overexpression decreased the average tumor weight of lung cancer (Fig. 2F, Supplementary Fig. 3B). Taken together, our findings demonstrate that EZH2 inhibits lung cancer growth in vitro and in vivo.

![Fig. 1. EZH2 expression decreases in NSCLC and low EZH2 level predicts poor survival.](image-url)

(A) EZH2 mRNA level is decreased in human NSCLC tissues. EZH2 mRNA levels were determined with q-PCR in adjacent normal lung tissues versus lung cancer tissues. The boxes represent the interquartile range; bars represent the median. N = 11 in normal group; n = 54 in NSCLC group. (B) EZH2 protein level is decreased in human NSCLC tissues. Protein was extracted from adjacent normal lung tissues and lung cancer tissues from different stages, and the protein was subjected to Western blot analysis with indicated antibodies. N: Normal lung tissues; I: Stage I; II: Stage II; III: Stage III. (C, D) Kaplan–Meier curve comparing time to survival between NSCLCs with the low (<25th percentile) versus high (>25th percentile) EZH2 expression was determined using the q-PCR results. (C) Overall survival. (D) Disease-free survival.
3.3. EZH2 is correlated with Nrf2 expression in NSCLC tissues

As we have showed that EZH2 controlled lung cancer growth and predicted patients' survival. We next wanted to know the underling mechanism by which EZH2 regulates lung cancer growth. Nrf2 is a transcription factor that was reported to regulate lung cancer proliferation and drug-resistance [11,15,21,22]. Accumulating data have shown the high expression of Nrf2 in...
the tissues of human NSCLC as described in the introduction. However, how Nrf2 is regulated at the transcription level remains elusive. A previous report showed that when cancer cells were treated with proteasome inhibitor MG132, EZH2 was downregulated, which was coupled with the up-regulation of Nrf1 and Nrf2 [23]. Therefore, we hypothesized that EZH2 may regulate Nrf2 expression in lung cancer cells. First, we explored the expression level of Nrf2 and its downstream genes (NQO-1 and HO-1) in lung cancer tissues and adjacent normal tissues. We found that Nrf2 was overexpressed in human NSCLC tissues (Figs. 3A and 4D). In addition, NQO-1 and HO-1, two genes transcriptionally controlled by Nrf2 and participate significantly in NSCLC growth and drug-resistance, were also up-regulated in NSCLC tissues (Fig. 3B and C). Next, we performed linear regression analysis to analyze the correlation between EZH2 mRNA level and Nrf2, NQO-1 and HO-1 mRNA levels. The results showed that EZH2 mRNA level was negatively but significantly correlated with the mRNA levels of Nrf2, NQO-1 and HO-1 (Fig. 3D–F). Interestingly, we found that EZH2 can bind to the promoter region of Nrf2 gene in lung cancer tissues and cell lines (Fig. 4A, Supplementary Fig. 4), but not to the promoter region of NQO-1 or HO-1 gene (Supplementary Fig. 5). As EZH2 is an H3K27me3 methyltransferase, we also investigated the enrichment of H3K27me3 at the promoter region of Nrf2 gene. The results showed that the H3K27 at Nrf2 promoter was trimethylated (Fig. 4B). Interestingly, EZH2 level and the methylation level of H3K27me3 at Nrf2 promoter was significantly down-regulated in NSCLC tissues compared with adjacent normal tissues (Fig. 4C). In addition, we also found the global decrease in H3K27me3 level in NSCLC tissues compared to adjacent normal tissues (Fig. 4D). Because H3K27me3 is correlated with transcription regression, we wanted to know whether low methylation of H3K27me3 enrichment at Nrf2 promoter is correlated with high Nrf2 expression. Our linear regression analysis indicated that H3K27me3 enrichment at Nrf2 promoter was negatively but significantly correlated with the expression level of Nrf2 and its downstream genes (Fig. 4E, F and data not shown).

3.4. EZH2 regulates Nrf2 expression through trimethylation of H3K27

To further demonstrate the effect of EZH2 on Nrf2 and its downstream genes, we knocked down or overexpressed EZH2 in A549 lung cancer cells. We found that EZH2 knockdown significantly up-regulated the mRNA level of Nrf2 (Fig. 5A), while EZH2 overexpression down-regulated the mRNA level of Nrf2 (Fig. 5B). In line with this finding, EZH2 knockdown decreased H3K27me3 level and up-regulated Nrf2 protein level, whereas EZH2 overexpression increased H3K27me3 level and down-regulated Nrf2 protein level (Fig. 5C). In addition, EZH2 overexpression enhanced H3K27me3 enrichment at the promoter of Nrf2 (Fig. 5D), indicating that EZH2 may inhibit Nrf2 promoter activity. Indeed, our luciferase assay showed that EZH2 overexpression inhibited the activity of Nrf2 promoter (Fig. 5E). Finally, we analyzed the mRNA level of Nrf2 downstream genes in A549 cells with EZH2 overexpression. The results showed that EZH2 overexpression significantly down-regulated the mRNA levels of NQO-1 and HO-1 (Fig. 5F). Those findings provide direct evidence that EZH2 inhibits Nrf2 expression by regulating H3K27me3. As previous report has showed that EZH2 may inhibit Nrf2 expression through regulating H3K27me3. As previous report has showed that EZH2 may inhibit Nrf2 expression through regulating H3K27me3.

3.5. Nrf2 is essential for the function of EZH2 in A549 lung cancer cell proliferation

Our data have demonstrated that EZH2 inhibited Nrf2 expression through regulating H3K27me3 methylation at Nrf2 promoter.
Another question remains unknown is whether Nrf2 is essential for the role of EZH2 in lung cancer cells. We generated A549 cells with stably Nrf2 knockdown (Fig. 6A), then we infected those cells with retrovirus carrying Ctrl shRNA, and EZH2 shRNA. We found that EZH2 knockdown was unable to affect either A549 cell proliferation (Fig. 6B) or colony formation capacity (Fig. 6C) when Nrf2 retrovirus carrying Ctrl shRNA, and EZH2 shRNA. We found that EZH2 knockdown was unable to affect either A549 cell proliferation (Fig. 6B) or colony formation capacity (Fig. 6C) when Nrf2
was knocked down, whereas modulation of EZH2 could still inhibit A549 cell proliferation and colony formation when Nrf2 shRNA was replaced by a control shRNA (Fig. 6B and C). Finally, we showed that high Nrf2 level predicted poor survival (Fig. 6D). Those findings demonstrate that Nrf2 is critically essential for the role of EZH2 in lung cancer cells.

4. Discussion

EZH2 was first reported to participate in the progression of prostate cancer ten years ago [6]. Ectopic expression of EZH2 in prostate cells induces transcriptional repression of a specific cohort of genes. Gene silencing mediated by EZH2 requires the SET domain and is attenuated by inhibiting histone deacetylase activity. Amounts of both EZH2 messenger RNA and EZH2 protein are increased in metastatic prostate cancer; in addition, clinically localized prostate cancers that express higher concentrations of EZH2 show a poorer prognosis [6]. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells [7]. Mechanistically, EZH2 mediates transcriptional silencing of the tumor suppressor gene E-cadherin by trimethylation of H3K27 [24]. EZH2 is essential for the proliferation of both transformed and non-transformed human cells [25]. Clinical evidence identified EZH2 as a novel and independent prognostic marker in endometrial cancer, and validate previous findings on prostate and breast cancer [26]. Interestingly, a recent report showed that EZH2 serves as a tumor suppressor in myelodysplastic syndromes, which was evidenced by EZH2 deletions, missense and frameshift mutations [27]. Here we showed the down-regulation of EZH2 in human NSCLC tissues. We further found that EZH2 serves as a tumor suppressor in lung cancers.

Nrf2 has long been considered to be a factor against environmental oxidative stress. However, recently evidence have showed that Nrf2 can promote cancer cell proliferation and regulate drug resistance. Accumulating data have strongly demonstrated the oncogenic role of EZH2 in human NSCLC. Loss of Keap1 or decreased Keap1 activity in cancer cells induces greater nuclear accumulation of Nrf2, causing enhanced transcriptional induction of antioxidants, xenobiotic metabolism enzymes, and drug efflux pumps [14–16]. Cancer related mutations in Nrf2 impair its recognition by Keap1-Cul3 E3 ligase and promote malignancy [28]. Nrf2 enhances cell proliferation and resistance to anticancer drugs in human lung cancer [11]. In addition, a recent report showed that gain of Nrf2 function in NSCLCs confers radio-resistance [17]. Our recent work identified acetylation affects Nrf2 function in human lung cancer. We showed that hMOF, the histone acetyltransferase required for histone H4 lysine 16 acetylation, can bind to Nrf2 and acetylates Nrf2. hMOF-dependent Nrf2 acetylation maintains its retention in the nucleus and mediates the transactivity of Nrf2, subsequently regulates lung cancer growth and drug-resistance [18]. However, the mechanism by which Nrf2 is regulated at the transcription level remains unknown. Herein we showed that Nrf2 is inhibited by EZH2. The expression of Nrf2 and its downstream genes are correlated with EZH2 expression and H3K27me3 level in human NSCLC samples. Further, EZH2 could bind to Nrf2 promoter and regulated the trimethylation of H3K27, which repressed Nrf2 transcription. In lung cancer tissues, EZH2 down-regulation or mutation leads to the reduction of H3K27me3 at the promoter of Nrf2, resulting in up-regulation of Nrf2 transcription. However, translated Nrf2 proteins might be unable to degenenate timely, as previous report has showed mutations in Keap1 in approximately 20% of patients and 50% of cell lines in NSCLC, which leads to Nrf2 phosphorylation/acetylation and nuclear translocation [14,18]. Together with our previous report, we identified the underlying mechanism by which Nrf2 is regulated at transcriptional and post-translational levels. Finally, Nrf2 was critically essential for the role of EZH2 in human lung cancer cells, and high Nrf2 level predicts poor survival.

In summary, low EZH2 expression predicts poor survival in human NSCLC. Our in vitro and in vivo evidence demonstrate that EZH2 inhibits lung cancer cell growth. Mechanistically, EZH2 binds to the promoter of Nrf2 and represses Nrf2 expression by regulating H3K27me3. Finally, we show that Nrf2 is essential for the role of EZH2 in lung cancer cells.

5. Conflict of interest

None.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.05.057.

References


