Original Paper

Repression of PDK1- and LncRNA HOTAIR-Mediated EZH2 Gene Expression Contributes to the Enhancement of Atractylenolide 1 and Erlotinib in the Inhibition of Human Lung Cancer Cells

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Key Words

Atractylenolide 1 • Erlotinib • PDK1 • HOTAIR • EZH2 • Human lung cancer cells

Abstract

Background/Aims: We previously showed that the major bioactive compound of *Atractyloides macrocephala* Koidz atractylenolide 1 (ATL-1) inhibited human lung cancer cell growth by suppressing the gene expression of 3-Phosphoinositide dependent protein kinase-1 (PDK1 or PDPK1). However, the potentially associated molecules and downstream effectors of PDK1 underlying this inhibition, particularly the mechanism for enhancing the anti-tumor effects of epidermal growth factor receptor-tyrosine-kinase inhibitors (EGFR-TKIs), remain unknown.

Methods: Cell viability and cell cycle distribution were measured using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and flow cytometry assays, respectively. Western blot analyses were performed to examine the protein expressions of PDK1 and of zeste homolog 2 (EZH2). The levels of long non-coding RNA (lncRNA) and HOX transcript antisense RNA (HOTAIR) were examined via qRT-PCR. RNA-binding protein immunoprecipitation assays were used to analyze HOTAIR interaction with EZH2. The promoter activity of the EZH2 gene was determined using Secrete-Pair Dual Luminescence Assay Kit. Exogenous expressions of PDK1, HOTAIR, and EZH2 were conducted via transient transfection assays. A xenografted tumor model was used to further evaluate the effect of ATL-1 in the presence or absence of erlotinib *in vivo*. **Results:** We showed that the combination of ATL-1 and EGFR-TKI erlotinib

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further inhibited growth and induced cell arrest of the human lung cancer cells, determined by both MTT and flow cytometry assays. ATL-1 inhibited the protein expression and the promoter activity of EZH2, which was reversed in cells with PDK1 overexpression. Interestingly, ATL-1 inhibited the expression levels of HOTAIR. While silencing HOTAIR inhibited the expressions of PDK1 and EZH2, overexpression of HOTAIR reduced the ATL-1-reduced PDK1 and EZH2 protein expressions and EZH2 promoter activity. In addition, ATL-1 reduced the HOTAIR binding to the EZH2 protein. Moreover, we found that exogenously expressed EZH2 antagonized the effect of ATL-1 on cell growth inhibition. Consistent with the *in vitro* results, ATL-1 inhibited tumor growth and the expression levels of HOTAIR, protein expressions of EZH2 and PDK1 *in vivo*. Importantly, there was synergy of the combination of ATL-1 and erlotinib in this process. **Conclusion:** Here, we provide the first evidence that ATL-1 inhibits lung cancer cell growth through inhibiting not only the PDK1 but also the lncRNA HOTAIR, which results in the reduction of one downstream effector EZH2 expression. The novel interplay between the HOTAIR and EZH2, as well as repressions of the PDK1 and HOTAIR coordinate the overall effects of ATL-1. Importantly, the combination of ATL-1 and EGFR-TKI erlotinib exhibits synergy. Thus, targeting the PDK1- and HOTAIR-mediated downstream molecule EZH2 by the combination of ATL-1 and erlotinib potentially facilitates the development of an additional novel strategy to combat lung cancer.

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Introduction

Lung cancer is one of the most aggressive malignancies and is a leading cause for cancer deaths worldwide with the highest incidence and mortality rate [1]. Lung carcinoma is commonly classified as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and the latter accounts for approximately 80-85% of all cases. The current standard of treatment for lung cancer is the surgical resection followed by adjuvant radiation therapy and chemotherapy, as well as new targeted therapies. However, the 5-year survival rate is still low despite advances in diagnostics and therapeutics due to early detection deficiency, resulting in recurrence and metastasis [1, 2]. The small-molecule inhibitors of epidermal growth factor receptor-tyrosine-kinase inhibitors (EGFR-TKIs), such as erlotinib or gefitinib, have been well established as a therapeutic option for a well-defined subset of lung cancer patients [3, 4]. However, almost all patients eventually acquire resistance to these TKIs, which is a major obstacle of this otherwise promising therapeutic approach [4, 5]. Thus, a detailed understanding of the mechanisms underlying this development and progression of NSCLC is essential to improving the diagnosis, prevention, and therapy. In addition, finding alternative novel therapeutic modalities, especially for enhancing the treatment efficacy of EGFR-TKIs for lung cancer patients is also greatly desirable.

Natural products have been considered as potential agents for the prevention and treatment of cancer. Atractylenolide I (ATL-I) is a natural sesquiterpene lactone and one of the main naturally occurring compounds isolated from *Atractylodes macrocephala* Koidz. It shows a wide spectrum of pharmacological functions such as anti-inflammatory, anti-oxidant, and anti-tumor effects on various cell types including cancer cells [6-10]. One study showed that ATL-I potently inhibited proliferation and induced apoptosis through inactivating the notch pathway in gastric cancer cells [11]. Another report found that ATL-I significantly sensitized the response of ovarian carcinoma cells to paclitaxel by reducing the protein expression of IL-6, vascular endothelial growth factor (VEGF), and survivin, thus enhancing apoptosis and cell growth inhibition. This suggests that the combination of ATL-I and paclitaxel may be considered as a promising strategy for the treatment of ovarian carcinoma [10]. We previously demonstrated that ATL-1 inhibited the growth of lung cancer cells through the extracellular signal-regulated kinase 1/2 (ERK1/2)-mediated suppression of transcription factor signal transducer and activator of transcription 3 (Stat3) and SP1 protein, resulting in inhibition of PDK1 gene expression [12]. However, the detailed molecular mechanism underlying the anti-lung cancer effects still remains unknowns.

Long noncoding RNAs (lncRNAs) are defined as transcribed RNA molecules that are longer than 200 nucleotides and have no obvious protein coding capacity. Among these, HOX transcript antisense intergenic RNA (HOTAIR), a noncoding RNA transcribed from the *HOXC* locus with an approximate length of 2.2 kb, has been reported to reprogram chromatin organization and promote tumor progression. Overexpression of HOTAIR has been shown in several types of human cancers and has been linked to metastasis, cancer progression, and epithelial-to-mesenchymal transition through multiple signaling mechanisms. Thus, the importance of HOTAIR in cancer biology has sparked interest toward using HOTAIR as a biomarker and potential therapeutic target [13-15]. Silencing of HOTAIR or inhibition of miR-125a-5p promoted cancer cell apoptosis by releasing caspase 2 in several cancer cells [16]. HOTAIR levels were highly expressed in serous ovarian cancer (SOC) tissues compared to normal controls. Furthermore, the overexpression of HOTAIR correlated with an advanced stage and a high histological grade of ovarian cancer. Thus, HOTAIR is an independent prognostic factor for predicting the overall survival of SOC patients [17]. HOTAIR can be explored as a biomarker in lung cancer because its elevated expression in lung tumor tissues is correlated with metastasis, drug resistance, and poor survival in patients with lung cancer [13]. Nevertheless, the potential link and the detailed mechanism underlying the true role of this lncRNA with regard to the occurrence, growth, and progression of lung malignancy still remains undetermined.

The enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase unit of polycomb repressor complexes that has been shown to possess oncogenic and tumor promoter effects through multiple mechanisms, resulting in stimulated growth and tumor progression in various cancer types [18-20]. Increasing evidence indicated that elevated expression of EZH2 was observed in many malignant tumors and was significantly associated with poor outcome in cancer patients [21]. Thus, pharmacological inhibition of EZH2 or agents triggering EZH2 degradation are promising therapeutic strategies for the treatment of various cancer types [22-24]. In studies that assess the relationship between EZH2 expression and the clinical outcomes in patients with colorectal cancer treated with anti-EGFR therapeutics, the authors showed that EZH2 expression was associated with the survival in patients with colorectal cancer who were treated with anti-EGFR therapeutics. This suggests that the EZH2 expression is a useful additional prognostic biomarker for anti-EGFR therapy [25]. In addition, one report found that lncRNA-ANCR could influence the invasion and migration of colorectal cancer cells by specifically binding to EZH2, thus regulating EZH2 expression [26]. Several natural herbals have been shown to serve as EZH2 modulators through the inhibition of histone trimethylation, the reduction of the matrix metalloproteinase gene, the modulation of tumor-suppressive microRNAs, the interaction with other transcription factors, and tumor promoters. In addition to others, they influence tumor growth, apoptosis, and progression in several cancer types [27]. We previously showed that polyphyllin I (PPI), a bioactive phytochemical extracted from the Rhizoma of *Paris polyphylla*, inhibited the growth of lung cancer cells through stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK)-mediated inhibition of p65; this results in the reduction of DNA methyltransferase 1 (DNMT1) and EZH2 gene expression [28]. β -elemene, a compound extracted from *Curcuma wenyujin*, inhibited the cell growth of nasopharyngeal carcinoma via inactivation of Stat3, and the reduction of DNMT1 and EZH2 expressions [29]. While the connections between PDK1 and HOTAIR have not been shown, the links of HOTAIR and EZH2 have been reported in several other studies. The post-transcriptional regulation of gene expression plays an important role in cellular processes; mRNA binding proteins can regulate the translation of functionally related gene products and subsequent biological cellular functions via coordinated binding to subsets of mRNAs. HOTAIR has been reported to have the ability to bind to EZH2, resulting in stimulation of tumor growth, progression, and metastasis [30-32]. However, the underlying molecular mechanism for the herbals, especially ATL-1 in combination with other agents such as EGFR-TKI in the treatment of cancer still has yet to be elucidated.

Here, we extended these studies and found that ATL-1 alone or in combination of EGFR-TKI erlotinib inhibited lung cancer cell growth through the repression of the PDK1- and HOTAIR-mediated inhibition of EZH2 expression.

Materials and Methods

Reagents and cell culture

The antibody against EZH2 was purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). The PDK1 antibody was obtained from Abcam (Cambridge, MA, USA). The MTT agent was purchased from Sigma Aldrich (St. Louis, MO, USA). The reagent Lipofectamine 3000 was obtained from Life Technologies (AB & Invitrogen, Carlsbad, CA, USA). ATL-1 was purchased from Chengdu Must Bio-technology Company (Chengdu, Sichuan, China). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA) unless otherwise indicated. NSCLC cells (A549 and H1299) were obtained from the Chinese Academy of Sciences Cell Bank of Type Culture Collection (Shanghai, China) and were cultured at 37°C in a humidified atmosphere containing 5% CO₂. The culture medium consisted of RPMI 1640 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Thermo Fisher Scientific Inc, Waltham, MA, USA), 100 µg/ml streptomycin, and 100 U/mL penicillin. In addition, Geneticin (G-418) Sulfate (Life Technologies, Carlsbad, CA, USA) was included at a concentration of 200 µg/mL for culturing A549-Luc cells (obtained from the Guangzhou Land Technology Co., Guangzhou, China). Once a 60-70% confluence was reached, the cells were digested with 0.25% trypsin for all other experiments.

Cell viability assay

Cell viability was examined using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously reported [33]. NSCLC cells (2×10⁴ cells/well) seeded into 96-well plates were treated with indicated agents for up to 48 h. Afterwards, MTT solution (10 µL, 5 g/L) was added and incubated at 37°C for an additional 3 h. After removing the supernatant, the solvent dimethyl sulfoxide (DMSO, 150 µL) was added and oscillated for 5-10 min. Finally, the optimal density was recorded at 490 nm absorbance using a microplate reader (Perkin Elmer, Victor X5, Waltham, MA, USA). The percentage of viable cells for the treatment group was calculated by adjusting the untreated control group. Combination-induced synergy was analyzed and expressed as a combination index (CI), using CompuSyn (ComboSyn, Inc., Paramus, NJ, USA), following the method developed by Chou and Talalay [34].

Quantitative real-time PCR (qRT-PCR)

The mRNA expression of HOTAIR was routinely quantified via quantitative real-time-PCR (qRT-PCR). The primers used in this study were designed as follows: HOTAIR forward 5'-GGTAGAAAAAGCAACCACGAAGC-3'; reverse 5'-ACATAAACCTCTGTCTGTGAGTGCC-3'; GAPDH forward 5'-AAGCCTGCCGGTGACTAAC-3'; reverse 5'-GCGCCCAATACGACCAATC-3'. QRT-PCR was performed in a 20 µL mixture containing 2 µL of the cDNA preparation, 10 µL 2×SYBR Green Premix ExTaq, and 10 µM primers on an ABI 7500 Real-Time PCR System (Applied Biosystems Inc, Grand Island, NY, USA). The qRT-PCR protocol included an initial step of 95°C for 30 sec, followed by 40 cycles of 95°C for 3 sec, and annealing at 60°C for 30 sec. Each sample was tested in triplicate. Threshold values were determined for each sample/primer pair and the 2^{-ΔΔCt} method was used to calculate the relative expression levels of HOTAIR.

HOTAIR siRNA transfection

The HOTAIR small interfering RNA (HOTAIR siRNA) and scrambled control siRNA (control siRNA) were purchased from GenePharma (GenePharma, Shanghai, China). The target sequences for HOTAIR siRNAs were as follows: The sequences of siRNA were:

si-HOTAIR1, 5'-GCCUUCUUUAUAAGCUCGU-3', 5'-ACGAGCUUAUAAGGAAGGC-3';

si-HOTAIR2, 5'-CAUAUAUCUGUUGGGCGU-3', 5'-ACGCCAACAGAUUAUUG-3';

si-HOTAIR3, 5'-GGAAGCUCUUGAAGGUUCA-3', 5'-UGAACCUUCAAGAGCUUCC-3'.

A549 and H1299 cells (2 × 10⁵/well) were transfected with a final concentration of 50 nM siRNAs, targeting the HOTAIR and control for 24 h using the Lipofectamine 3000 Transfection Reagent (Invitrogen,

CA, USA) as per the manufacturer's directions. Following the transfections, the cells were harvested for all other experiments.

Western blot analysis

The Western blot procedure was conducted as previously described [33]. In brief, equal amounts of proteins from cell lysates were applied on SDS polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) Membranes (Millipore, Billerica, MA, USA), then incubated with antibodies, such as PDK1 and EZH2 (1:1000) at 4°C overnight. Subsequently, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (1:3000, Cell Signaling, Beverly, MA, USA). Finally, the membranes were transferred to a freshly prepared ECL solution (Immobilon Western; Millipore, Billerica, MA, USA) and immunoreactive bands were visualized using chemiluminescent reagent and quantitated by Image Lab software (Bio-Rad, Hercules, CA, USA).

RNA binding protein immunoprecipitation (RIP)

The RNA binding protein immunoprecipitation assay was performed with a Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore, Temecula, CA, USA) according to the instructions provided by the manufacturer. Briefly, A549 and H1299 cells at 80–90% confluence were lysed in RIP lysis buffer on ice after washing in PBS and were then stored at –80°C until further use. Magnetic beads were prepared by initial PBS washes followed by incubation with primary antibody raised against EZH2 (5 µg of total antibody used per immunoprecipitation) at room temperature for 30 min. Extensive washes were performed prior to incubation of adsorbed magnetic beads with previously collected cell lysates. Incubation of conjugated beads with the lysate was conducted overnight at 4°C with rotation. The beads were then thoroughly washed and digested with proteinase K (30 min at 55°C) to disengage EZH2 containing ribonucleoprotein (RNP) complexes. RNA from the immunopurified RNPs were harvested *via* canonical phenol chloroform isoamyl extraction and further precipitated *via* ethanol. The immunoprecipitated RNA from the EZH2-RNPs was then subjected to cDNA synthesis and qPCR analysis.

Transient transfection assays

The procedure was conducted as described in a previous study [35]. Briefly, cells were seeded at a density of 2.5×10^5 cells/well in 6-well dishes and grown to 60% confluence. For each well, 2 µg of the control or wild type pEZX-PG04-EZH2 promoter constructs (purchased from GeneCopoeia, Inc., Rockville, MD, USA) with or without 0.2 µg of internal control secreted alkaline phosphatase (SEAP) were co-transfected into cells with the Lipofectamine 3000 reagent. The preparation of cell extracts and measurement of luciferase activities were determined using the Secrete-Pair Dual Luminescence Assay Kit (GeneCopoeia, Inc., Rockville, MD, USA) and normalized with SEAP activity within each sample. In the separated experiment, 2 µg of control (pCMV6) and the expression constructs containing Myc/FLAG-tagged ORFs of human PDK1 and EZH2, and control (pcDNA3.1) or pcDNA 3.1-HOTAIR obtained from OriGene Technologies, Inc. (Rockville, MD, USA) and GeneCopoeia, Inc., respectively, at a final concentration of 2 µg/mL, were transfected into the cells with the Lipofectamine 3000 reagent. The cells were then incubated for 24 h at 37°C and treated with ATL-1 for the indicated time for all other experiments.

In vivo xenograft model of human lung tumor growth

Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Guangdong Provincial Hospital of Chinese Medicine (with Ethics Approval Number 2017036) and the National Institutes of Health Guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). A total of 40 eight-week-old female nude mice obtained from Beijing Vital River Experimental Animal Co. Ltd. (Beijing, China) were maintained at the Animal Center of Guangdong Provincial Hospital of Chinese Medicine. A549 cells, carrying the luciferase reporter gene (A549-Luc, obtained from the Guangzhou SoJuBio Technology Co., Guangzhou, China) (1×10^7 cells), were subcutaneously injected into the mice. Xenografts were allowed to grow until initial measurement (100–150 mm³) was obtained using calipers. Mice were randomly divided into control and ATL-1 (75 mg/kg), erlotinib alone (50 mg/kg), and the combination of ATL-1 and erlotinib groups, which were administered daily via intraperitoneal injection for up to 20 days (n = 10 per group) according to previous studies [36–38]. Next, mice were anesthetized via inhalation of 2% isoflurane. The substrate D-luciferin (Caliper Life

Sciences, Hopkinton, MA, USA) was injected into the peritoneal cavity at a dose of 150 mg/kg in 100 μ L. The intensity of bioluminescence imaging (BLI) signal was determined using the IVIS-200 Imaging System (Xenogen/Caliper, Alameda, CA, USA). Tumor volume measurements were calculated using the formula for an oblong sphere: volume = (width² $\times$ length) $\times$ 2. Quantification of bioluminescence was reported as photons/sec. The body weights of the mice were measured once a week. All mice were euthanized on day 20 in accordance with the Guide for the Care and Use of Laboratory Animals. The corresponding xenografted tumors were removed and weighed, and then processed for the detection of the PDK1 and EZH2 protein levels by Western blot, and HOTAIR levels by qRT-PCR.

Statistical analysis

Almost all data were expressed as mean $\pm$ SD of three independent experiments. Differences between groups were assessed by one-way ANOVA and significance of difference between particular treatment groups was analyzed by Tukey's Multiple Comparison Test for multiple comparison using GraphPad Prism software version 5.0 (GraphPad Software, Inc. La Jolla, CA, USA). Asterisks in the figures indicate significant differences of experimental groups in comparison with the corresponding control condition. A *P*-value below 0.05 was deemed significant.

Results

Combination of ATL-1 and EGFR-TKI further inhibited growth and induced cell cycle arrest of lung cancer cells

We previously showed that ATL-1 inhibited lung cancer cell growth by suppressing PDK1 gene expression [12]. In the current study, we tested whether ATL-1 could enhance the anti-tumor effect of EGFR-TKI erlotinib. We observed that the combination of ATL-1 (70 mg/mL) and erlotinib (10 μ M) further inhibited growth in A549 and H1299 cells (Fig. 1A). The CI values were 0.42805 and 0.66521 in A549 and H1299 cells, respectively, indicating moderate synergy. Subsequently, we examined the nature of the cell cycle arrest induced by ATL-1. We found that A549 and H1299 cells treated with ATL-1 in either presence or absence of erlotinib for 24 h resulted in an increase in the proportion of cells at the G0/G1 phase, while reducing that of cells at the S phase by ATL-1 or erlotinib alone; stronger effects were seen in the combination treatment group as detected via flow cytometry (Fig. 1B). These results suggest that the combination of ATL-1 and erlotinib exerted enhanced effects in the inhibition of lung cancer cell growth.

ATL-1 inhibited the mRNA levels of HOTAIR independent of PDK1

Although the current understanding of the transcriptional gene regulation is extensive, posttranscriptional gene regulation still remains largely unexplored. The increased expression of lncRNA has recently been shown to be involved in cancer cell growth through the regulation of PDK1 signaling [39]; to further clarify the precise functions of lncRNA signaling in this process, we first examined the effect of ATL-1 on HOTAIR, one of the lncRNA in the current study. We found that ATL-1 inhibited the mRNA levels of lncRNA HOTAIR in A549 and H1299 cells (Fig. 2A). Interestingly, we observed that the silencing of HOTAIR inhibited cell growth and also PDK1 protein expression in both A549 and H1299 cells (Fig. 2B-C). In contrast, overexpression of HOTAIR blocked the ATL-1-inhibited PDK1 protein expression (Fig. 2D), while exogenously expressed PDK1 had no effect on the ATL-1-inhibited HOTAIR mRNA levels (Fig. 2E). In summary, the results above indicate that HOTAIR acted upstream of PDK1 and that ATL-1 inhibited the expression of HOTAIR, thus reducing the PDK1 protein levels in this process.

Fig. 1. Combination of ATL-1 and EGFR-TKI further inhibited growth and induced cell cycle arrest of lung cancer cells. A, A549 and H1299 cells were treated with ATL-1 (70 mg/mL) and gefitinib (10 μ M) for 24 h, followed by measuring cell viability by MTT assays and the Chou-Talalay method expressed as CI values as described in the Materials and Methods section, respectively. B, A549 and H1299 cells were treated with ATL-1 (70 mg/mL) and erlotinib (10 μ M) for up to 24 h. Afterwards, the cells were collected and processed for analysis of the cell cycle distribution via flow cytometry. The percentages of the cell population in each phase (G0/G1, S and G2/M) were assessed by Multicycle AV DNA Analysis Software. *indicates significant difference as compared to the untreated control group ($p < 0.05$).

ATL-1 reduced EZH2 expression through inhibition of PDK1 and HOTAIR

We further assessed the potential downstream effectors of PDK1 and HOTAIR. Because the oncogenic and tumor promoter effects of EZH2 and links to HOTAIR [18-20, 40], we started to examine the EZH2 expression in this process. We showed that ATL-1 inhibited protein expression of EZH2 in A549 and H1299 cells (Fig. 3A). Interestingly, overexpressed PDK1 was found to overcome the effect of ATL-1 on the expression of EZH2 protein (Fig. 3B) and the promoter activity (Fig. 3C) in A549 and H1299 cells.

Moreover, as expected, while silencing HOTAIR reduced the EZH2 protein levels (Fig. 4A), the excessive expression of HOTAIR neutralized the ATL-1-reduced EZH2 protein and the promoter activity (Fig. 4B-C); notably, HOTAIR-resisted ATL-1-reduced EZH2 protein was not observed in cells with silenced PDK1 gene (A549 and H1299; Fig. 4D). The findings indicate that the repressions of PDK1 and HOTAIR were involved in the ATL-reduced EZH2 expression at both translational and transcriptional levels. The latter was required in the presence of PDK1 in this process. Several studies have shown HOTAIR to be physically associated with EZH2, leading to regulation of EZH2 and other gene expressions in various cancer cells [32, 41, 42]. Thus, in the current study, to further test whether HOTAIR is associated with EZH2 protein, RIP experiments were performed on cell extracts using antibodies against EZH2. We observed that ATL-1 reduced HOTAIR RNA binding to EZH2 protein (Fig. 4E), suggesting that

Fig. 2. ATL-1 inhibited the levels of HOTAIR independent of PDK1. A, A549 and H1299 cells were exposed to ATL-1 for 24 h, followed by measuring the levels of HOTAIR by qRT-PCR. B-C, A549 and H1299 cells were transfected with the control or HOTAIR siRNAs for 24 h before exposing to ATL-1 for an additional 24 h, followed by measuring the cell growth and the PDK1 protein expression by MTT and Western Blot, respectively. D, A549 and H1299 cells were transfected with the control and the HOTAIR overexpression vector for 24 h before exposing the cells to ATL-1 for an additional 24 h. Afterwards, the PDK1 protein was determined by Western Blot. E, A549 and H1299 cells were transfected with the control and PDK1 overexpression vector for 24 h before exposing the cells to ATL-1 for an additional 24 h. Afterwards, the HOTAIR levels were determined by qRT-PCR. Insert on the upper panel represented the protein levels of PDK1 as determined by Western blot. GAPDH was used as an internal control. The figures are representative cropped gels/blots that have been run under the same experimental conditions. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the ATL-1 alone ($P < 0.05$).

the physical association between EZH2 protein and HOTAIR mRNA may also be involved in the anti-lung cancer effect of ATL-1 in this process.

Effects of the combination of ATL-1 and erlotinib on the expressions of PDK1, HOTAIR, and EZH2

We further investigated whether there was an additive potential or synergy of the combination of ATL-1 and EGFR-TKI erlotinib in this process. Other studies demonstrated the more beneficial responses of the combining of either ATL-1 [10] or erlotinib with other agents in the inhibiting tumor growth [43]. As expected, we found that the combination of ATL-1 and erlotinib further inhibited the expressions of PDK1, EZH2 proteins, and HOTAIR levels in A549 and H1299 cells (Fig. 5A-C). The aforementioned results indicated additive or synergistic effects.

Fig. 3. ATL-1 reduced EZH2 expressions through inhibition of PDK1. A, A549 and H1299 cells were treated with indicated doses of ATL-1 for 24 h. Afterwards, the cell lysate was harvested and the protein expression of EZH2 was measured by Western blot. GAPDH was used as loading control. B, A549 and H1299 cells were transfected with the control and the PDK1 overexpression vectors for 24 h before exposing the cells to ATL-1 for an additional 24 h. Afterwards, the cell lysate was harvested and the protein expression of EZH2 was measured by Western blot. C, A549 and H1299 cells were transfected with the control and the PDK1 overexpression vectors, and with a wild type human EZH2 promoter reporter construct ligated to luciferase reporter gene and an internal control secreted alkaline phosphatase for 24 h, followed by treating with ATL-1 for an additional 24 h. Afterwards, the promoter activities were determined using the Secrete-Pair Dual Luminescence Assay Kit as described in the Materials and Methods section. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the ATL-1 alone ($P < 0.05$).

Overexpressed EZH2 had no effect on PDK1 and HOTAIR

Given the observations of the potential links of PDK1, HOTAIR, and EZH2 in this process, we examined the potential regulatory loops among these next. We showed that either excessive expressed EZH2 had no effect on the ATL-1-reduced PDK1 protein or HOTAIR mRNA levels in A549 and H1299 cells (Fig. 6A-B). However, exogenously expressed EZH2 overcame ATL-1-inhibited cell growth (Fig. 6C). These findings indicated that inhibition of EZH2, which is one of the downstream effectors of PDK1 and HOTAIR, was required to mediate the inhibitory effect of ATL-1 on lung cancer cell growth.

ATL-1 inhibited tumor growth, HOTAIR expression levels, and protein expressions of PDK1 and EZH2 *in vivo*

To further investigate the antitumor effects of ATL-1 in combination of EGFR-TKI *in vivo*, we labeled A549 cells with a luciferase reporter system. The activity of ATL-1 against the growth of lung cancer cells *in vivo* was examined in A549-Luc cancer xenografts in nude mice. The tumor progression rate was assessed via *in vivo* luciferase bioluminescent imaging. We showed that in the treatment group, the signals in the tumor are significantly lower than that

Fig. 4. ATL-1 reduced EZH2 expressions through inhibition of HOTAIR. **A**, A549 and H1299 cells were transfected with the control or HOTAIR siRNAs for 24 h before exposing to ATL-1 for an additional 24 h, followed by measuring the EZH2 protein expression by Western Blot. **B**, A549 and H1299 cells were transfected with the control and the HOTAIR overexpression vectors for 24 h before exposing the cells to ATL-1 for an additional 24 h. Afterwards, the EZH2 protein expression was determined by Western blot. **C**, A549 cells were transfected with the control and the HOTAIR overexpression vectors, and a wild type human EZH2 promoter reporter construct ligated to luciferase reporter gene and an internal control secreted alkaline phosphatase for 24 h, followed by treating with ATL-1 for an additional 24 h. Afterwards, the promoter activities of EZH2 were determined using the Secrete-Pair Dual Luminescence Assay Kit as described in the Materials and Methods section. The figures are representative cropped gels/blots that have been run under the same experimental conditions. **D**, A549 and H1299 cells were transfected with the control or the PDK1 siRNAs, followed by exposing the cells to the control and the HOTAIR overexpression vectors for 24 h before exposing the cells to ATL-1 for an additional 24 h. afterwards, the PDK1 and EZH2 protein levels were measured by Western Blot. **E**, Cells lysates from A549 cells treated with ATL-1 for 24 h, Afterwards, Immunoprecipitations were performed using the anti-EZH2 monoclonal antibody and the IgG1 isotype control. The levels of HOTAIR in immunoprecipitates were examined by qRT-PCR. The relative enrichment of the HOTAIR was determined after normalized to the input. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the ATL-1 alone ($P < 0.05$).

in the vehicle-treated control one (Fig. 7A). Administration of ATL-1, erlotinib alone, and a combination of both with a dosage of 75 mg/kg/day (ATL-I) and erlotinib (50 mg/kg) caused a significant suppression of growth of the established A549 xenografted tumors compared to that of the vehicle-treated control animals as shown via tumor weight and sizes (Fig. 7B-D). Moreover, consistent with the results of the *in vitro* data, the reduced expressions of PDK1, EZH2, and HOTAIR from fresh tumors that were harvested from the afore-mentioned experiment were observed in the ATL-1, or erlotinib-treated groups compared to that in the control group (Fig. 7E-F). It is worth noting that the combination treatment showed even greater inhibitory effects (Fig. 7E-F).

Fig. 5. The effects of the combination of ATL-1 and erlotinib on the expressions of PDK1, HOTAIR and EZH2. A-C, A549 and H1299 cells were treated with ATL-1 (70 mg/mL) and erlotinib (10 μ M) for 24 h, followed by measuring the PDK1, EZH2 protein expressions, and HOTAIR mRNA levels by Western blot and qRT-PCR, respectively. The figures are representative cropped gels/blots that have been run under the same experimental conditions. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the ATL-1 alone ($P < 0.05$).

Discussion

ATL-1 has been shown to have anti-tumor effects in various cell types including lung cells [6-10]. We recently demonstrated that ATL-1 inhibited the growth of lung cancer cells through ERK1/2-mediated suppression of transcription factors Stat3 and SP1 protein levels, resulting in inhibition of PDK1 gene expression [12]. In the current study, we demonstrated the potential synergy of the combination of ATL-1 and one EGFR-TKI erlotinib on lung cancer cell growth. We observed that the inhibition of the tumor drivers PDK1 and lncRNA HOTAIR, resulting in the reduction of EZH2 gene expression, contributed to the overall synergistic effects of the combination of ATL-1 and erlotinib.

Previously, we reported the role of PDK1 in mediating the anti-lung cancer effect of ATL-1. In the current study, we further explored the potential targets associated with the regulation of PDK1 that may also be involved in the effect of ATL-1 in the presence or absence of EGFR-TKI erlotinib. Our results indicate the role of lncRNA HOTAIR expression in mediating the anti-tumor effect of ATL-1, suggesting that the interaction and repression of the PDK1 and HOTAIR were involved in the effect of ATL-1 in this process. HOTAIR plays a critical role in the gene regulation and chromatin dynamics and appears to be dysregulated in a variety of cancers. The expression of HOTAIR is elevated in lung cancer and correlates

Fig. 6. Overexpressed EZH2 had no effects on PDK1 and HOTAIR. A-B, A549 and H1299 cells were transfected with the control and the EZH2 overexpression vector for 24 h before exposing the cells to ATL-1 for an additional 24 h. Afterwards, the protein expression of PDK1 and mRNA levels of HOTAIR were measured by Western blot and qRT-PCR, respectively. Insert on the upper panel in B represented the protein levels of EZH2 as determined by Western blot. GAPDH was used as internal control. C, A549 and H1299 cells were transfected with the control and the EZH2 overexpression vector for 24 h before exposing the cells to the ATL-1 for an additional 24 h, followed by measuring the EZH2 protein and cell viability by Western blot and MTT assays as described in the Materials and Methods sections, respectively. Insert represented the EZH2 protein expression. The figures are the representative cropped gels/blots that have been run under the same experimental conditions. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the ATL-1 alone ($P < 0.05$).

with metastasis and poor prognosis. Moreover, HOTAIR promotes proliferation, survival, invasion, metastasis, and drug resistance in lung cancer cells [13, 44]. However, so far, no connection between HOTAIR and PDK1 has been reported, and the function role of HOTAIR in mediating the anti-tumor effects of ATL-1 and EGFR-TKI remains unknown as well. For the first time, we observed the role of HOTAIR in mediating the effect of ATL-1 or EGFR-TKI in the inhibition of human lung cancer cells. Our results suggest that the repression and interaction of PDK1 and HOTAIR expression were involved in the anti-lung cancer effects of these agents. Regardless, whether epigenetically regulation of PDK1 gene promoter is activated by HOTAIR or *vice versa*, requires to be determined using Chip-qRT-PCR or RNA-pull-down assays. The expression pattern and potential function of lncRNAs in EGFR-TKI resistant NSCLC cells have also been reported. Disregulation of lncRNAs other than HOTAIR plays critical roles in EGFR-TKIs resistant NSCLC cells. The lncRNA-mediated EGFR-TKIs resistance might help us to improve clinical response rates and could be used as novel potential targets to reverse the EGFR-TKI resistance for NSCLC patients [45, 46]. Our findings implied that targeting

Fig. 7. ATL-1 inhibited tumor growth, levels of HOTAIR, and protein expressions of EZH2 and PDK1 in vivo. A, The tumor growth was monitored by injecting luciferin followed by measuring bioluminescence signals. Representative images are shown. B-C The xenografts were harvested on day 20, and the volume and weight of tumors were measured. D, The photographs of ATL-1 (75 mg/kg/day alone or in the presence or absence of and erlotinib (50 mg/kg), and vehicle-treated xenografts derived from nude mice are shown. E-F, At the end of the experiments, xenograft tumors were isolated and processed for detecting PDK1 and EZH2 proteins, and HOTAIR mRNA levels by Western blot and qRT-PCR, respectively. Values in bar graphs were given as the mean $\pm$ SD from three independent experiments. *indicates significant difference as compared to the untreated control group ($P < 0.05$). **indicates significant difference from the ATL-1 alone ($P < 0.05$). G, The diagram shows that ATL-1 inhibits lung cancer cell growth through inhibition not only of PDK1 but also of lncRNA HOTAIR, which results in reduction of the EZH2 expression. The repression of PDK1 and HOTAIR, and interplay between HOTAIR and EZH2 coordinate to the overall effects of ATL-1. Importantly, the combination of ATL-1 and EGFR-TKI erlotinib exhibits synergy.

the critical oncogenes PDK1 and HOTAIR might be involved in the additive effects or even potential synergy of the combination of ATL-1 and erlotinib in the inhibition of lung cancer cell growth.

In addition, we demonstrated an important role of EZH2 in this process. Our findings indicated that the reduction of EZH2 gene expression by ATL-1 and erlotinib was involved in the enhanced inhibition of lung cancer cell growth. This strengthens the notion that EZH2 is an important target in developing novel anti-cancer strategies [22, 23]. Consistent with our results, several natural products have been reported to have potential for the prevention and treatment of several cancer types via regulation of EZH2 through multiple mechanisms [47]. In addition, the genetic, transcriptional, and posttranscriptional dysregulation of EZH2 is frequently found in several cancer types. EZH2 promoted tumorigenesis by altering the expression of numerous tumor suppressor genes. Thus, the inhibition of EZH2 expression has been shown to be involved in the control of growth and progression of several cancer types [18-20, 48], suggesting a critical role of this molecule. Previous studies indicated that

lncRNA was capable of regulating target gene expression through interactions with RNA binding proteins. More importantly, lncRNA HOTAIR is physically associated with EZH2, leading to the regulation of EZH2 and other target gene expressions in various cancer cells [32, 41, 42, 49]. Consistent with this, we observed reduced physical association between HOTAIR and EZH2 proteins treated with ATL-1, which influenced the repression of EZH2 and perhaps other genes; subsequently, this might also be involved in the effect of ATL-1 on lung cancer cell growth inhibition. Regardless, we suggest that more experiments (using methods, such as qRT-PCR-chromatin immunoprecipitation, among others) are still required to elucidate the detailed mechanism underlying the physical interaction of HOTAIR and EZH2. Expressions of EZH2 or other target genes may be involved in the ATL-1-inhibited human lung cancer growth. EZH2 is involved in multiple pathological processes related to carcinogenesis, proliferation, apoptosis, and metastasis [50, 51]. We therefore reasoned that the expression or/and activities of HOTAIR affected by ATL-1 in lung cancer cells might depend on the interaction of HOTAIR and EZH2. Links of HOTAIR and EZH2 have been reported in several other studies. HOTAIR has been shown to interact with and recruit polycomb repressive complex 2 (PRC2) to regulate the chromosome occupancy through EZH2, which then leads to histone H3 lysine 27 trimethylation (H3K27me3) of certain genes [40]. HOTAIR could also bind to EZH2; resulting in the stimulation of growth, progression, and metastasis in several cancer types [30-32, 49, 52]. Thus, blocking the ability of HOTAIR to interact with EZH2 and subsequently inhibit the HOTAIR-EZH2 pathway might unveil a novel cancer therapeutic approach [53]. We showed the regulation of EZH2 by PDK1; still, more studies are required to determine if there are true PDK1 binding sites in the EZH2 promoter or/and whether a physical association exists between the PDK1 and EZH2 proteins affected by ATL-1 in the presence or absence of erlotinib. Overall, these observations demonstrated the links and interactions among PDK1, HOTAIR, and EZH2 in the regulation of the biological functions and lung cancer cell growth treated with the ATL-1 and erlotinib, suggesting that repressions of PDK1 and HOTAIR, at both translational and transcriptional levels, have been involved in mediating the ATL-reduced EZH2 expression. This further illustrates the associated mechanism in the inhibition of PDK1 and lncRNA HOTAIR, and subsequently reduces the expression of EZH2. In addition, our results also highlight EZH2 as a rational target for therapeutic intervention in NSCLC.

Our *in vivo* results fit the observation *in vitro*, further confirming the inhibitory effect of ATL-1 and erlotinib on lung tumor growth, and regulation of PDK1, HOTAIR, and EZH2 expressions. The doses of ATL-1 used were based on another study, which showed significant inhibitory effects on tumor growth without noticeable toxicity [38]. In addition, the combination of erlotinib with other agents, showing even greater effects in inhibition of human cancer cells through multiple molecular mechanisms, have also been reported in other studies [54-56]. Our findings suggest that ATL-1 might sensitize or enhance the effect of erlotinib in the controlling human lung cancer cell growth or *vice versa* via targeting the tumor suppressor target genes PDK1 and EZH2 [57], and HOTAIR signaling axis. This implies a potential additive synergy of these agents in EGFR-TKI resistance setting. This also highlighted a critical step forward towards treating EGFR-TKI resistant human lung cancer, thus implying a potential therapeutic strategy for clinical patients. We suggest that more studies are needed to further determine the important role of EZH2 in this process, such as using cells that are stably transfected with overexpression of EZH2 gene in animal models.

Conclusion

In conclusion, our results show that ATL-1 inhibits lung cancer cell growth via inhibition not only of PDK1 but also of lncRNA HOTAIR, which results in the reduction of one downstream effectors EZH2 expression. The interplay between HOTAIR and EZH2, and the repression of PDK1 and HOTAIR coordinate the overall effects of ATL-1. Importantly, the combination of ATL-1 and EGFR-TKI erlotinib exhibits synergy (Fig. 7F). Thus, targeting the

PDK1- and HOTAIR-mediated the downstream molecule EZH2 by a combination of ATL-1 and erlotinib potentially facilitate the development of an additional novel strategy to combat lung cancer.

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Disclosure Statement

The authors declare that they have no competing interests.

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