Huaier suppresses proliferation and induces apoptosis in human pulmonary cancer cells via upregulation of miR-26b-5p

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ABSTRACT
Various studies have reported that Huaier possesses anti-tumor effects. However, the mechanisms are not completely elucidated. Here, we found 66 differentially expressed miRNAs in Huaier-treated pulmonary adenocarcinoma A549 cells, with upregulation of miR-26b-5p. Transfection of A549 cells with miR-26b-5p mimic inhibited proliferation and induced apoptosis, while transfection of Huaier-treated A549 cells with a miR-26b-5p inhibitor reversed the effects of Huaier. EZH2 was verified as the target of miR-26b-5p. Thus, our findings indicate that Huaier might suppress proliferation and induce apoptosis in lung cancer cells via a miR-26b-5p-EZH2-mediated approach, which provides a new perspective for understanding the anti-tumor effects of Huaier.

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1. Introduction
Lung cancer is the second most commonly diagnosed cancer and the leading cause of cancer-related death in both men and women in the United States [1]. There is now growing importance to develop safer, natural, non-toxic compounds as chemotherapeutic/chemopreventive agents. In this regard, traditional Chinese medicines (TCMs) have recently gained attention as possible anticancer agents with few side effects [2,3].

Trametes robiniophila murris (Huaier) is a fungus found in China and has been applied in TCM for approximately 1600 years [4]. However, the necessary clinical studies have only been conducted in recent years. Proteoglycans, which contain 41.53% polysaccharides, 12.93% amino acids, and 8.72% water, have been identified as the major effective ingredients of Huaier extracts [5,6]. Recently, accumulating evidence has indicated that Huaier has antitumor activities, such as stimulation of apoptosis in breast cancer cells [7,8], inhibition of angiogenesis [9]. Nevertheless, the underlying mechanisms of Huaier activities in lung cancer remain unclear.

MicroRNAs (miRNAs) are small, non-coding, endogenous RNAs that regulate gene expression at the post-transcriptional level. Many studies have shown that aberrantly expressed miRNAs may act as oncogenes or tumor suppressors involved in the tumorigenesis and aggression of human cancers [10,11]. Since miRNAs play crucial roles in human cancer, we hypothesized that miRNAs may be involved in mediating the anti-tumor effects of Huaier.

In the present study, to investigate involvement of miRNA in the effects of Huaier, we examined the differences in the miRNA expression profiles of Huaier-treated and untreated A549 cells by miRNA microarray analysis, and then explored the potential role of specific miRNA in anti-tumor effects of Huaier. Our data provided important insights into the mechanisms of anti-tumor effect of Huaier.

2. Materials and methods
2.1. Cells and cell culture
Human embryonic kidney (HEK) 293T and non-small cell lung cancer (NSCLC) cell lines, including A549, H1299, H1975, Hcc827,
and H460, were obtained from American Type Culture Collection (ATCC). Lung carcinoma 95-D cells were obtained from the Cell Bank of the Chinese Academy of Science. A549, 95-D, H1299, Hcc827, and H1975 cells were cultured in RPMI-1640 (Hyclone, UT, USA). HEK293T and H460 were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, CA, USA). All media were supplemented with 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, and 100 μg/mL streptomycin (Thermo, CA, USA). All cells were incubated in a humified atmosphere of 5% CO2 at 37 °C in an incubator.

2.2. Preparation of Huaier

Electuary ointment of Huaier (Gaitianli, Jiangsu, China) was dissolved in phosphate-buffered saline (PBS) to obtain a 100 mg/mL stock solution. The solution was then sterilized with a 0.22-μm filter and stored at -20 °C before use.

2.3. Cell proliferation assay

Cell proliferation was analyzed using the carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling method (Invitrogen, CA, USA). The stock solution of CFSE was 5 mM. A549 cells were suspended in PBS with 5 μM CFSE (5 × 10^6 cells/mL) for 15 min at 37 °C to stabilize CFSE labeling. Labeled A549 cells were then seeded into 12-well plates at a density of 1 × 10^5 cells/well. After 24 h, A549 cells were treated with Huaier or transfected with miRNA mimic, inhibitor, or EZH2 shRNA. CFSE fluorescence was measured by flow cytometry on a FACSAriaII flow cytometer (Becton–Dickinson, CA, USA). The cell proliferation index (P.I.) was analyzed using specialized software. P.I. was the sum of the cells in all generations divided by the calculated number of original parent cells.

2.4. Apoptosis assay

After treatment with varying concentrations of Huaier for different times or transfection with miRNA mimic or Huaier and inhibitor for 48 h, A549 cells were harvested. Apoptosis was measured by propidium iodide and Annexin V-FITC double-staining (KeyGEN BioTECH, Nanjing, China). Flow cytometry analysis was performed as described above. Experiments were conducted three times.

2.5. miRNA microarray

A549 cells were treated with 4 mg/mL Huaier for 48 h and the experiments were repeated three times. Total RNA was extracted and purified using mirVana PARIS (Ambion, TX, USA). Before miRNA microarray analysis, we pooled the RNA generated from three respective times. The miRNA microarray was carried out at Shanghai Biotechnology Corporation (Shanghai, China). Data analysis was performed using GeneSpringGX software 11.0 (Agilent Technologies, Santa Clara, USA).

2.6. RNA extraction and real-time PCR analysis for miRNA

Total RNA from all lung cancer cell lines was extracted using TRIzol (Invitrogen, CA, USA). Total RNA from pulmonary alveolar epithelial cells was purchased from PriCells (Wuhan, China). Quantitative real-time reverse transcription (qRT)-PCR was conducted using TaqMan miRNA Reverse Transcription kit, TaqMan Small RNA Assays kit, and TaqMan Universal PCR Master Mix (Applied Biosystems, CA, USA). U6 served as an internal control. Values are shown as 2^{−ΔΔCt}=(2^{ΔCt(untreated)})(2^{ΔCt(treated)})/(2^{ΔCt(control)}).

2.7. Transient transfection of miRNA mimic and inhibitor

miR-26b-5p mimic, inhibitor, and controls were purchased from Ambion. The transfection was carried out with Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer’s instructions. The miR-26b-5p mimic was used at a final concentration of 100 nM, while the miRNA inhibitor was used at a final concentration of 200 nM. Forty-eight hours after transfection, apoptosis was analyzed, and Western blotting was performed.

2.8. miRNA target predictions

The target genes of miR-26b-5p were predicted by the miRBase (Version 19) [12], which included MiRDB, TargetScan, PicTar, MiRanda, and other computational prediction methods. The predicted targets that were related to proliferation and apoptosis were screened first.

2.9. Plasmid constructs and luciferase reporter assay

The full-length EZH2 3′-UTR containing the putative miR-26b-5p recognition element was amplified from the genome of human
A549 cells by PCR (sense: 5'-TATCTAGACATCTGCTACCTCCTCCC-3', antisense: 5'-ATGCGGCCGCGATTCAACAAGGCAA-3'). The mutated 3'-UTR of EZH2 was also amplified (sense: 5'-TATCTAGACATCTGCTACCTCCTCCC-3', antisense: 5'-ATGCGGCCGCGATTCAACAAGGCAA-3'). The mutated 3'UTR Wild-type and mutated PCR products were cloned into the region downstream of the reporter gene between the XbaI and NotI sites of the pRL-TK vector (Promega, WI, USA). Two constructs were confirmed by sequencing.

Luciferase reporter assays were carried out in HEK293T cells. HEK293T cells were seeded into 96-well plates and transfected with either 100 ng reporter constructs or corresponding mutants together with 10 ng of pGL3 control (Promega, WI, USA) and 10 pmol miR-26b-5p mimic or 20 pmol inhibitor using Lipofectamine LTX and Plus Reagent (Invitrogen). Luciferase activity was measured by the Dual-Glo Luciferase Reporter Assay system (Promega). All assays were performed in triplicate.

2.10. EZH2 knockdown

Two shRNAs targeting human EZH2 and the shRNA negative control sequence were as follows: shRNA#1, GAGGTTCAGACGAGCTGAT; shRNA#2, AGACTCTGAATGCAGTTGC [13]; shRNA control (OriGene, MD, USA), GGATTTCAGTCGATGTAC was a sequence that targets firefly luciferase and does not match the specific regions of human mRNA, as determined by BLAST analysis. The shRNA constructs were separately cloned into the RNAi-ready pSiREN-RetroQ vector (Clontech, CA, USA) and were confirmed by sequencing. Recombinant plasmids were then transfected into A549 cells with Lipofectamine LTX and Plus Reagent (Invitrogen).

Fig. 2. miR-26b-5p was raised after Huaier treatment and was downregulated in lung cancer cells. (A) A549 cells were treated with Huaier (4 mg/mL) or PBS for 48 h. Unsupervised hierarchical clustering based on miRNA expression profiles in Huaier-treated versus untreated A549 cells at fold change > 2. (B) The relative expression of miR-26b-5p was confirmed by qRT-PCR in A549 cells exposed to Huaier (4 mg/mL) for 48 h. Data are shown as relative $2^{-\Delta \Delta Ct}$ values. (C) The relative expression levels of miR-26b-5p in normal pulmonary alveolar epithelial cells and six lung cancer cell lines were analyzed by qRT-PCR. Means from triplicate experiments in all cases, bars, SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 

The primers for detection of EZH2 mRNA levels were as follows: sense primer, 5'-TTGTGGGGAAGCCTGAATATGC-3'; antisense primer, 5'-TTGTGGGGAAGCCTGAATATGC-3’ [14]. EZH2 mRNA and protein levels were evaluated at 24 h and 48 h after transfection, and cell proliferation and apoptosis rates were analyzed by flow cytometry.

### 2.11. Western blotting

Cells were lysed in RIPA buffer and stored at –80°C. Cell lysates were separated by electrophoresis on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and then transferred onto PVDF membranes. Membranes were probed with antibodies against EZH2.

Fig. 3. Effects of miR-26b-5p on cell proliferation and apoptosis in A549 cells. (A, C and E) A549 cells were transiently transfected with miR-26b-5p mimic or control (100 nM) for 48 h. Cell proliferation was measured by CFSE assay. Cell apoptosis was assessed by flow cytometry. Results are the means ± SDs of three independent experiments. (B, D and F) A549 cells were treated with 4 mg/mL Huaier and 200 nM miR-26b-5p inhibitor (Huaier + inhibitor) or Huaier and inhibitor control (Huaier + control) for 48 h. Cell proliferation and apoptosis assay were performed as mentioned above. Representative histograms depicting cell proliferation and apoptosis are shown. *P < 0.05, **P < 0.01.
(1:1000, Cell Signaling Technology, MA, USA), β-catenin (1:1000, SantaCruz, CA, USA), bcl-2 (1:1000, SantaCruz), and β-actin (1:5000, Sigma, USA) in 5% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST). A goat anti-mouse secondary antibody (1:10,000, Sigma) was used. Labeled bands were detected using an ECL chemiluminescent kit (Thermo, USA).

2.12. Statistical analysis

Statistical analysis was performed with Student’s t-tests. Differences with P values of less than 0.05 were considered statistically significant. The experiments were performed in triplicates and repeated three times.

3. Results

3.1. Huaier suppressed proliferation and induced apoptosis of A549 cells

Huaier has been reported to exert antitumor effects in a number of cancers [9,14–16]. Consistently, we observed that Huaier treatment (2–8 mg/mL for 24–72 h) suppressed the proliferation of A549 cells (Fig. 1A) and induced A549 cell apoptosis (Fig. 1B and C). According to these results, we chose treatment with 4 mg/mL Huaier for 48 h as the optimal treatment time/concentration for further experiments.

3.2. Huaier upregulated miR-26b-5p expression in A549 cells

Next, we investigated the effects of Huaier on miRNA expression profiles in A549 cells. Huaier treatment caused obvious changes in miRNA expression, as shown by miRNA microarray analysis (Fig. 2A). Of 1886 human miRNAs, 66 exhibited changes of more than 2-fold in expression after Huaier treatment, with 33 upregulated miRNAs, including miR-150, miR-192, miR-296, miR-26b and so on, and 33 downregulated miRNAs, including miR-503, miR-575, miR-188, miR-494 and so on. Combining data from microRNA chip results and literature searches, we analyzed the functions of the differentially expressed miRNAs and identified miR-26b as an miRNA known to be significantly downregulated in various cancers [17,18] and inhibits proliferation and induces apoptosis in cancers [19,20]. Hence, we selected miR-26b-5p for validation by the Taqman MicroRNA Assays. The upregulation in miR-26b-5p expression level in Huaier-treated A549 cells as revealed by real-time RT-PCR was consistent with the results from miRNA array analysis (Fig. 2B). Importantly, we found that miR-26b-5p expression was much lower in six NSCLC cell lines than in normal epithelial cells (Fig. 2C). Together, these results provided

![Graphs and images related to the validation of EZH2 as a target of miR-26b-5p](image-url)
strong evidence that the low expression of miR-26b-5p in NSCLC cells was altered after Huai er treatment.

3.3. miR-26b-5p inhibited proliferation and induced apoptosis in A549 cells and inhibition of miR-26b-5p reversed the effects of Huai er

Given that miR-26b-5p was upregulated in Huai er-treated A549 cells, we transiently transfected miR-26b-5p mimic into A549 cells or miR-26b-5p inhibitor together with 4 mg/ml Huai er to investigate the effects of miR-26b-5p. There was a significant reduction in the proliferation index (P.I.) following transfection with the miR-26b-5p mimic (P.I. = 14.26 ± 0.38) compared with the control group (P.I. = 18.54 ± 1.68; Fig. 3A and C). A549 cells treated with 4 mg/ml Huai er and miR-26b-5p inhibitor resulted in an increase in the P.I. (P.I. = 15.33 ± 1.63) in comparison to the group treated with Huai er and inhibitor control (P.I. = 7.67 ± 1.53; Fig. 3B and D). Moreover, flow cytometry analysis showed that the rate of apoptosis increased more than 2.5-fold in A549 cells.

Fig. 5. EZH2 knockdown in A549 cells and effects of Huai er and miR-26b-5p on the expression of EZH2, β-catenin, and bcl-2 proteins. (A) EZH2 mRNA was analyzed by qRT-PCR at 24 h post-transfection with EZH2 shRNA or the negative control. Means are from triplicate experiments in all cases, and error bars indicate SDs. **P < 0.01. (B) Western blotting for EZH2 protein level after induction of EZH2 shRNA expression for 48 h. (C and D) The proliferation index and apoptosis rates were evaluated in empty vector- and shRNA-transfected A549 cells after 48 h by CFSE assay. Means ± SDs are depicted. **P < 0.01. (E) Western blotting for EZH2, β-catenin, and bcl-2 at 48 h post-treatment with 4 mg/ml Huai er in A549 and H1299 cells. (F) Western blotting for EZH2, β-catenin and bcl-2 in A549 and H1299 cells after transfection with miR-26b-5p mimic, mimic control, both 4 mg/ml Huai er and miR-26b-5p inhibitor (Huai er + inhibitor) or both Huai er and inhibitor control (Huai er + control) for 48 h. β-Actin used as an internal control.
transfected with the miR-26b-5p mimic as compared to the negative control (Fig. 3E). In addition, cells treated with 4 mg/mL Huaier and miR-26b-5p inhibitor exhibited a 50% drop in the rate of apoptosis as compared with the group treated with Huaier and inhibitor control (Fig. 3F). These results strongly suggested that miR-26b-5p played a critical functional role in the anti-tumor effects of Huaier.

3.4. EZH2 was a direct target of miR-26b-5p

To further elucidate the mechanism through which miR-26b-5p participated in the anti-tumor effects of Huaier, we next attempted to identify the functional targets of miR-26b-5p. Among the target genes predicted from miRBase, EZH2, which is upregulated in multiple cancers [21–23] and is involved in proliferation and apoptosis of cancer cells [24,25] became our research interest.

We firstly measured changes of EZH2 mRNA expression in NSCLC cell lines by qRT-PCR. Interestingly, we found a negative correlation between miR-26b-5p and EZH2; the expression of EZH2 was upregulated in NSCLC cell lines (Fig. 4A), while miR-26b-5p was downregulated (Fig. 2C). Additionally, luciferase reporter assays demonstrated that the luciferase activity of the reporter containing the wild-type EZH2-3’-UTR, but not the seed-region deletion mutant, was decreased after transfection with the miR-26b-5p mimic (Fig. 4B and C). Conversely, when pRL-TK-EZH2-3’-UTR was co-transfected with miR-26b-5p inhibitor, there was an obvious increase in luciferase activity compared with the miR-26b-5p negative control; this effect was not observed in the mutant group (Fig. 4D). Therefore, our data supported that the EZH2 was a direct target of miR-26b-5p.

3.5. EZH2 knockdown in A549 cells and effects of Huaier and miR-26b-5p on the expression of EZH2, β-catenin, and bcl-2 proteins

We next used shRNAs to knockdown EZH2 (Fig. 5A and B) in A549 cells and investigated the effects of this intervention on proliferation and apoptosis. Knockdown of EZH2 resulted in a decrease in the P.I. (Fig. 5C) and an increase in apoptosis (Fig. 5D) compared with the shRNA control. Following treatment with 4 mg/mL Huaier or transfection with miR-26b-5p mimic for 48 h, EZH2, β-catenin, and bcl-2 protein levels were all decreased in both A549 and H1299 cells compared with untreated cells (Fig. 5E and F), while inhibition of miR-26b-5p attenuated the effects of Huaier to upregulate expression of the above protein levels in both A549 and H1299 cells (Fig. 5F). Taken together, Huaier treatment and miR-26b-5p overexpression were heavily attributed to the reduced expression of EZH2 and EZH2 related proteins. These data further confirmed that EZH2 deregulation may play an important role in anti-tumor effects of Huaier-miR-26b-5p pathways in lung cancer cells.

4. Discussion

The results of miRNA microarray analysis and Taqman MicroRNA analysis revealed that the usually low level of miR-26b-5p in lung cancer cells was increased after Huaier treatment. Transfection of miR-26b-5p mimic into A549 cells significantly reduced the proliferation and increased apoptosis in A549 cells. Importantly, inhibition of miR-26b-5p reversed the anti-tumor effects of Huaier to promote cell proliferation and suppress apoptosis. These data demonstrated that the anti-tumor mechanisms of Huaier may partly be mediated by the upregulation of miR-26b-5p.

EZH2 was then identified as a target of miR-26b-5p by luciferase reporter assay. EZH2 is the histone H3 lysine 27 methyltransferase of polycomb-repressive complex 2 and is overexpressed in multiple cancer types [26]. Cai et al. [27] reported that Let-7a inhibited proliferation and induced apoptosis by targeting EZH2 in nasopharyngeal carcinoma cells. EZH2 was also reported to be inhibited by miR-101 in renal cancer [28], glioblastoma [29], and prostate cancer [30]. Our data confirmed EZH2 as a target of miR-26b-5p by luciferase reporter assays, and shRNA-mediated EZH2 knockdown exerted the same effects as Huaier treatment and miR-26b-5p overexpression. EZH2 protein levels were downregulated after Huaier treatment and miR-26b-5p overexpression, while inhibition of miR-26b-5p attenuated the effects of Huaier to upregulate EZH2 level. These results suggested that EZH2 may be involved in the pathway of miR-26b-5p-mediated anti-tumor effects of Huaier.

To further investigate the mechanisms of EZH2 in Huaier-treated lung cancer cells, we detected several EZH2-related proteins. During normal cellular functions, cytoplasmic β-catenin is maintained at a low level through the APC/axin/GSK-3β destruction complex in the canonical Wnt signaling pathway [31]. However, EZH2 can silence Wnt pathway antagonists, resulting in activation of Wnt/β-catenin signaling, and ultimately upregulating β-catenin [32,33]. In addition, mutations in β-catenin can activate β-catenin/Tcf signaling, increase c-Myc, and elevate E2F1 expression, and enhance Bcl-2 expression in colon tumors [34]. Thus, overexpressed EZH2 may upregulate Bcl-2 by activation of β-catenin. Our data demonstrated that Huaier treatment, miR-26b-5p overexpression or inhibition affected β-catenin and Bcl-2, further linking these two proteins with EZH2. Taken together, our data suggested that EZH2, β-catenin, and Bcl-2 may contribute to subsequent cell proliferation and apoptosis after Huaier treatment. Clearly, further studies on EZH2/β-catenin/Bcl-2 pathway are warranted.

In conclusion, miR-26b-5p-mediated repression of EZH2, β-catenin, and Bcl-2 may be a pivotal regulatory mechanism through which Huaier mediates decreased cell proliferation and increased apoptosis in lung cancer cells. Our study was the first to uncover the Huaier/miR-26b-5p/EZH2 pathway in lung cancer cells, providing novel mechanisms through which to understand the antitumor effects of Huaier and further suggesting a new basis for clinical treatment.

Conflict of interest statement

The authors have no competing interests to declare.

Authors’ contributions

T.W., W.C., and S.L. carried out the molecular biology analysis, participated in the design of the study and the clinical specimen collection, and drafted the manuscript. H.L., H.W., D.K., X.H., and Q.K. carried out the clinical specimen collection, participated in the data analysis, and performed the statistical analysis. Z.L. and Y.N. conceived of and designed the study, and participated in the data analysis and coordination, and helped to draft the manuscript. All authors read and approved the final manuscript.

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