


ORIGINAL ARTICLE

Aspirin ameliorates lung cancer by targeting the miR-98/WNT1 axis

Huizhu Gan^{1*}, Lin Lin^{2*}, Nanjun Hu¹, Yang Yang¹, Yu Gao¹, Yu Pei¹, Kang Chen¹ & Butong Sun¹ 

1 Department of Tumor Department of Hematology, China–Japan Union Hospital of Jilin University, Changchun, China

2 Department of Medical Oncology, National Cancer Center, National Clinical Research Center for Cancer, Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

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Correspondence

Butong Sun, Department of Tumor Department of Hematology, China-Japan Union Hospital of Jilin University, 126 Xiantai Street, Changchun 130033, China.
Tel: +86 431 8499 5870
Fax: +86 431 8767 7666
Email: butongsun@163.com

*Huizhu Gan and Lin Lin contributed equally to this work.

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Abstract

Background: Aspirin, an anti-inflammatory drug, has been widely investigated in the treatment of many cancer types, including colorectal, ovarian, breast, and prostate cancers. MicroRNAs (miRNAs) are the most well studied noncoding RNAs in cancers. In the current study, we were interested in defining the function of aspirin in lung cancer treatment and the related noncoding RNAs involved in this process.

Methods: The function of aspirin in lung cancer growth was evaluated by cell viability and colony formation assays. Screening of miRNAs affected by aspirin was performed through quantitative real-time PCR. Prediction of miR-98 targeting *WNT1* was performed using online bioinformatics software and was further confirmed by luciferase reporter gene analysis. The levels of miR-98 and *WNT1* were tested through immunoblotting and quantitative real-time PCR analysis in lung cancer cells under aspirin treatment.

Results: Cell viability was sharply suppressed in lung cancer cells with an increasing dose of aspirin. Aspirin markedly weakened the malignant colony formation ability of lung cancer cells. One out of six tumor suppressor miRNAs could be obviously regulated by aspirin in lung cancer cells. The inhibition of miR-98 on the luciferase activities of wild-type 3' untranslated region vectors of *WNT1* was clearly revealed in lung cancer cells. Meanwhile, the inhibitor of miR-98 increased the luciferase activities of wild-type 3' untranslated region vectors of *WNT1*. After treatment with aspirin the expression of miR-98 was induced and then its target gene, *WNT1*, was depressed in the cells.

Conclusion: Aspirin targets the miR-98/*WNT1* axis to ameliorate lung cancer development.

Introduction

Lung cancer is one of the most common causes of cancer-related death worldwide. Mono-application or combined-application chemotherapy are the traditional treatment strategies. However, the efficacy of such strategies for cancer treatment is unsatisfying, yielding poor overall survival. Aspirin, an anti-inflammatory drug, is widely applied in clinical therapy. Studies have revealed that aspirin can regulate cell proliferation, apoptosis, migration, or other processes by affecting transcription factors.^{1–3} In recent years, the anti-cancer effect of aspirin has been widely explored

and applied in the clinical treatment of colorectal cancer.^{4–6} Aspirin treatment is associated with reduced risk, overall mortality, or metastasis in colorectal cancer.⁷ It has also been investigated in ovarian, breast, prostate, and liver cancers.^{8–11} Considering the important role of inflammation in lung cancer development, we are interested in examining the role of the anti-inflammatory drug, aspirin, in the development of lung cancer.

Over the past decade, non-coding RNAs (ncRNAs) have mainly been implicated in the regulation of gene expression, the cell signaling pathway, and the development and

therapy of disease, including cancer.^{12,13} As members of ncRNAs, microRNAs (miRNAs) often degrade messenger RNA (mRNA) or inhibit gene translation via interaction with the 3' untranslated region (UTR) of mRNAs in the cells.¹⁴ An increasing number of studies have indicated the important role of miRNAs in some aspirin-involved cancer treatments.^{15,16} MiR-145 can function as a tumor-suppressive gene in several cancers, including lung, breast, gastric, ovarian, and prostate cancers.^{17–24} MiR-5195 inhibits the progression of lung cancer by directly targeting *MYO6*.²⁵ MiR-98 can bind to *TGFBR1* to depress cell proliferation and metastasis in lung cancer.²⁶ In miR-4317-restrained lung cancer, *FGF9* and *CCND2* serve as the key target genes.²⁷ MiR-886 can play a suppressive role in the development of lung cancer.²⁸ In a mouse model, tumor suppressor let-7 can destroy the growth of lung cancer.²⁹ In this study we were interested in searching for aspirin-targeted ncRNAs in lung cancer development.

We aimed to decipher the role of aspirin in lung cancer treatment and the associated underlying mechanism. We reveal that aspirin can effectively inhibit lung cancer growth in vitro. We explored the novel mechanism of aspirin treatment, by which aspirin can induce tumor suppressor miR-98 and then restrain its target gene *WNT1* to suppress lung cancer cell proliferation. Our findings suggest another effective therapeutic strategy for lung cancer.

Methods

Cell lines

A549 and H1299 cell lines were acquired from American Type Culture Collection (ATCC, Rockville, MD, USA). All cell lines were cultivated in 10% fetal bovine serum (Gibco, Rockville, MD, USA) and supplemented with Dulbecco's modified Eagle medium (Gibco) containing penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37°C with 5% CO₂.

Cell viability analysis

The cell proliferation ability was determined by methylthiazolyl-tetrazolium (MTT) assay. Cells were seeded on 96-well plates with at least three replicates at a density of 3000 cells/well. After 10 hours of incubation to form confluent monolayers, the media were replaced with medium containing aspirin for another 24 hours and 10 µL MTT (5 mg/mL in phosphate buffered saline [PBS]) was then added to each well. Four hours later, the medium was removed and MTT was dissolved in 150 µL dimethyl sulfoxide per well. The absorbance values were measured at optical density 490nm using an absorbance reader.

Colony formation

Cells were seeded in 12-well plates at a density of 500 cells/well. Twenty-four hours later, different treatments were administered. The cells were subsequently incubated for another 15–20 days.

RNA extraction and PCR

Total RNA was extracted using TRIzol reagent. For each sample, 1 µg RNA was reverse transcribed into complementary DNA. The mRNA levels were measured by reverse transcription-PCR and real-time PCR using SYBR PCR Master Mix (Takara, Dalian, China). The relative quantification of the mRNAs was performed according to the comparative method ($2^{-\Delta\Delta Ct}$, Applied Biosystems User Bulletin no. 2P/N 4303859); the ΔCt value for each sample is the average of triplicates.

Luciferase reporter gene assay

A549 cells were plated into 24-well plates at a density of 4×10^4 cells/well. The cells were cotransfected with reporter gene plasmids (pGL3-*WNT1*-wild type [wt] or pGL3-*WNT1*-mutant [mut]) at a dose of 100 ng/well and pRL-TK plasmid (40 ng/well) (Promega, Madison, WI, USA) and corresponding miRNA and miRNA inhibitors. The cells were collected after 48 hours and the luciferase activity was evaluated according to the manufacturer's instructions. Each experiment was repeated at least three times.

Immunoblotting analysis

Immunoblotting assay was performed as previously reported. Total proteins were extracted after corresponding treatments. The same amount of protein from each sample was analyzed. The primary antibodies, anti-WNT1 and anti-β-actin, were purchased from GeneTex, Inc. (Irvine, CA, USA) and Proteintech (Chicago, IL, USA) and then diluted as recommended by the instructions. Secondary antibodies, such as goat anti-rabbit or anti-mouse antibodies (Sigma-Aldrich, St Louis, MO, USA) were then incubated with the blots and visualized using the Bio-Rad GelDoc system (Hercules, CA, USA). The band quantification was performed using BandScan software (Glyko Software, Novato, CA, USA).

Statistical analysis

The results were expressed as means ± standard error of the mean. Statistical differences between the two groups were analyzed by Student's *t*-test. Non-significant

differences were marked as “ns.” The criteria for statistically significant differences were: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Results

Aspirin stifles the proliferation of lung cancer A549 and H1299 cells

Aspirin is reported to exert an anti-proliferative effect in multiple cancers, including colorectal and breast cancers, and hepatocellular carcinoma,^{6,30–32} however whether aspirin can disturb lung cancer growth has not yet been clarified. To answer this question, we performed cell viability and colony formation experiments with lung cancer A549 and H1299 cell lines and treated the cells with saline or different doses of aspirin. Administration of an increasing concentration of aspirin (2.5 mM and 5.0 mM) significantly reduced cell viability (Fig 1a,b). Furthermore, colony formation assay showed that aspirin treatment obviously

destroyed the ability of A549 cells to develop malignancy (Fig 1c,d). It is worth noting that aspirin actually has the ability to suppress lung cancer cell proliferation.

Screening of microRNAs involved in aspirin-depressed lung cancer

NcRNAs, including long non-coding RNAs (lncRNAs) and miRNAs have been thoroughly investigated in many types of cancers. In particular, they are frequently considered as tumor genes or tumor suppressive genes for miRNAs. Reports have revealed that some miRNAs, including miR-145, miR-5195, miR-98, miR-4317, miR-886, and let-7, can inhibit cancer development.^{18,25–29}

We were interested in whether these miRNAs are involved in aspirin-depressed lung cancer. We used 5.0 mM of aspirin for the treatment of lung cancer A549 and H1299 cells and then analyzed the level of these candidate miRNAs through quantitative real-time PCR assay. We found that compared to saline-treated cells, all of these

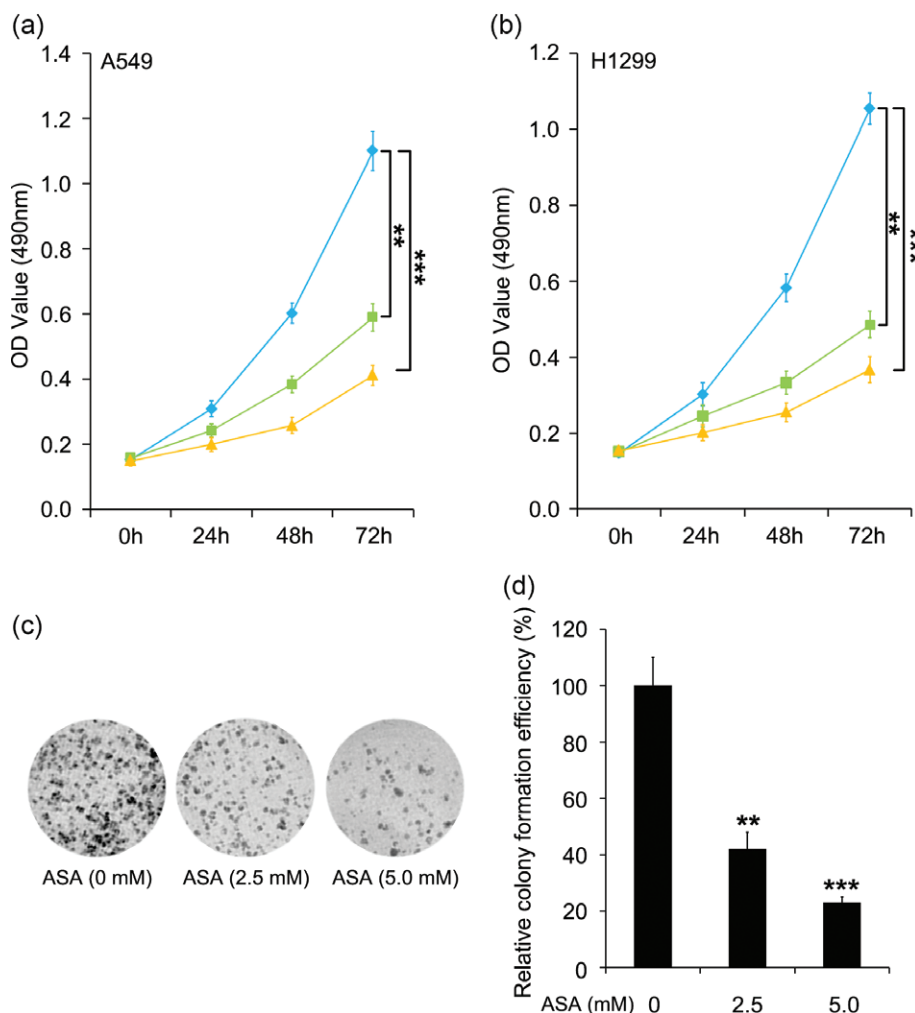


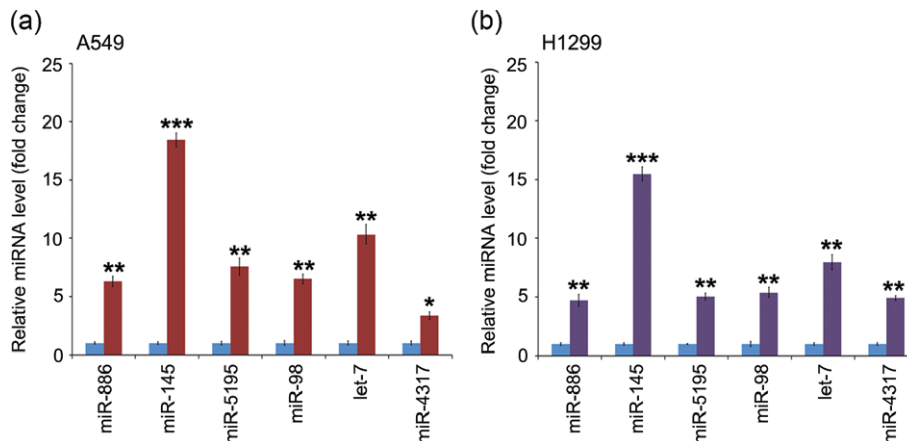
Figure 1 Aspirin (ASA) inhibits the proliferation of lung cancer A549 and H1299 cells. **(a,b)** Methyl-thiazolyl-tetrazolium assay of A549 and H1299 cell lines under different doses was used to evaluate the effect of ASA on cell proliferation. (—◆—) ASA (0 mM), (—■—) ASA (2.5 mM), and (—▲—) ASA (5.0 mM). **(c,d)** Colony formation assay and the relative colony formation efficiency of the A549 cell line after treatment with different concentrations of aspirin was used to analyze the function of ASA in the malignancy of lung cancer cells. *** $P < 0.001$; ** $P < 0.01$. OD, optical density.

tumor suppressor miRNAs were induced by aspirin in the two lung cancer cell lines (Fig 2a,b). One of these six miRNAs, miR-98, increased most sharply after the cells were treated with aspirin. Overall, our data imply that miR-98 is involved when aspirin has a treatment effect on lung cancer.

As a mediator in aspirin-reduced lung cancer, miR-98 directly binds to the 3' untranslated region of *WNT1* and depresses its expression

The most extensive function of miRNAs is degrading mRNA or inhibiting gene translation by binding to the 3'UTR of mRNAs.¹⁴ We predicted the target genes with a 3'UTR that might be bound by miR-98 using TargetScan (<http://www.targetscan.org/>). *WNT1* was one of the candidate target genes. Increasing evidence reveals that *WNT1* can drive lung cancer progression.^{33,34} We hypothesized whether *WNT1* is a target gene in aspirin-induced miR-98 in lung cancer. Our data showed that there was a putative binding site of miR-98 within the 3'UTR of *WNT1* mRNAs (Fig 3a). We cloned the 3'UTR region of *WNT1* (wt or mut) into pGL3-control plasmid to detect whether miR-98 targets *WNT1* (Fig 3b). As shown in Figure 3c, the luciferase activities of the wt reporter gene gradually decreased along with the elevated dose of miR-98, while the administration of miR-98 did not change the luciferase activities of the mut reporter gene. In parallel, the application of anti-miR-98 obviously elevated the luciferase activity of pGL3-*WNT1*-wt but not pGL3-*WNT1*-mut (Fig 3d). Notably, the addition of anti-miR-98 counteracted the inhibition of pGL3-*WNT1*-wt activity caused by aspirin (Fig 3e). These results indicate that miR-98 can impede *WNT1* expression by targeting the 3'UTR in lung cancer that has been impacted by aspirin treatment.

Figure 2 Screening of the microRNAs (miRNAs) involved in aspirin-depressed lung cancer. (a) Analysis of miRNAs of A549 cell lines after aspirin (ASA) treatment was performed through quantitative real time PCR. (■) ASA (0 mM) and (■) ASA (5 mM). (b) Evaluation of miRNA changes in H1299 cell lines after ASA was added to the cells. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.



Aspirin regulates the miR-98/ *WNT1* axis to suppress lung cancer

Based on the observation that miR-98 targets *WNT1* and aspirin can induce miR-98 in lung cancer, we clarified and confirmed the mechanism by which aspirin inhibits the proliferation of lung cancer cells. After the administration of aspirin (2.5 mM and 5.0 mM), the miR-98 level was increased 7–16-fold in A549 cells and at least 5–12-fold in H1299 cells (Figs 4a, b). In addition, immunoblotting analysis showed that the miR-98 target gene was dose-dependently downregulated in both A549 and H1299 cells (Figs 4a, b). Thus, we conclude that aspirin is capable of ameliorating lung cancer by modulating the miR-98/ *WNT1* axis.

Discussion

Lung cancer remains the leading cause of cancer-related death worldwide. The development of lung cancer is associated with inflammation and fibrosis. However, the efficacy of current strategies of treatment remains unsatisfactory, with poor overall survival. As a classic anti-inflammatory drug, aspirin is widely applied in clinical therapy. It has also been well studied in numerous cancers, including ovarian, breast, prostate, and liver cancers.^{8–11} In this investigation, we were interested in determining the function of aspirin in lung cancer progression.

We found that increasing concentrations of aspirin inhibited cell viability. We also observed that aspirin administration could destroy the ability of lung cancer cells to develop malignancy. Thus aspirin suppresses lung cancer growth.

In recent years, novel targets, including miRNAs and lncRNAs, have emerged as therapy for cancer.¹⁴ Some miRNAs can serve as a tumor suppressor of lung cancer progression. MiR-145 can function as a tumor-suppressive gene in several types of cancers, including lung, breast, gastric, ovarian, and prostate cancers.^{17–24} MiR-5195 can

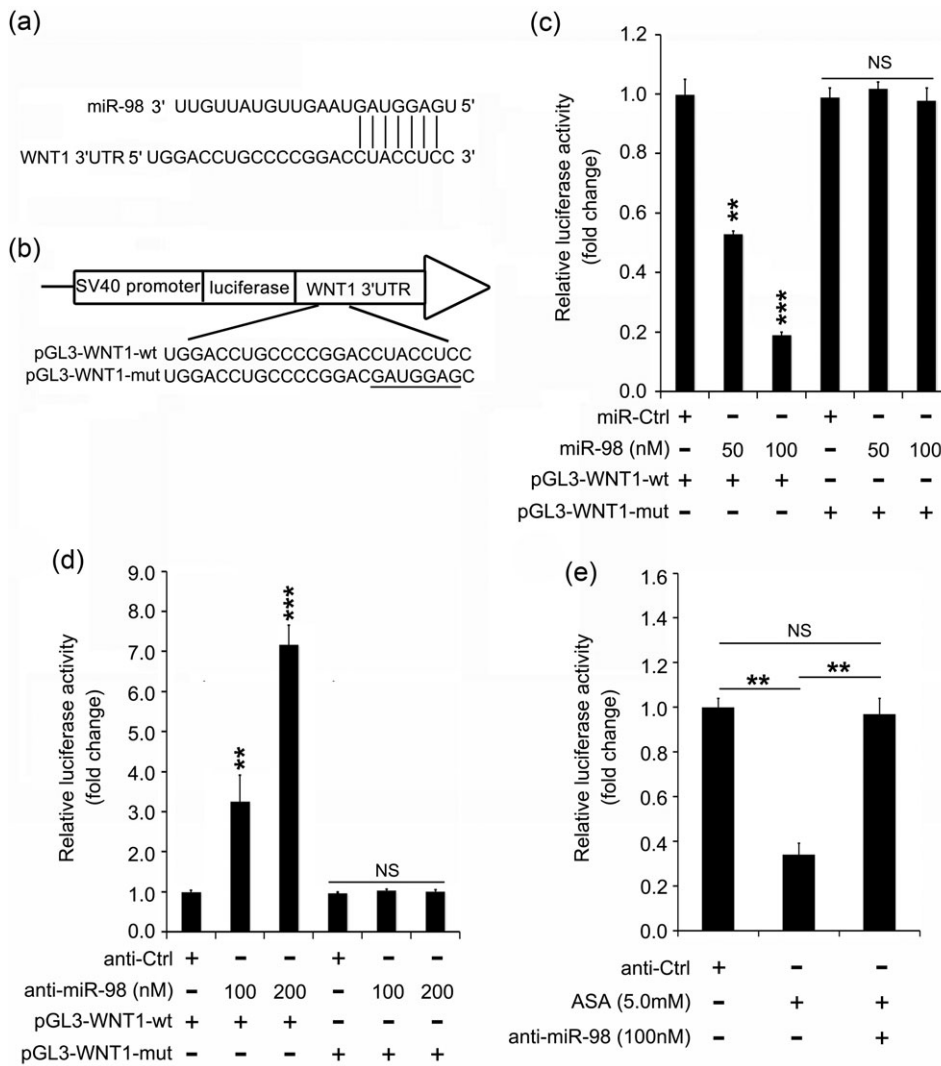


Figure 3 miR-98 restrains the level of *WNT1* by directly targeting the 3' untranslated region (3'UTR) of messenger RNAs (mRNAs). (a) Prediction of the targeting of miR-98 on *WNT1* by Targetscan online software. (b) Construction of wild-type (wt) or mutant (mut) vectors containing binding sites of miR-98 with 3'UTR of *WNT1* mRNA. (c,d) Analysis of miR-98 (or its inhibitor)-regulated *WNT1* in an A549 cell line by luciferase reporter gene assay. (e) Effect of anti-miR-98 and/or aspirin (ASA) on *WNT1* 3'UTR vector in A549 cell line by luciferase reporter gene assay. *** $P < 0.001$; ** $P < 0.01$; ns, not significant.

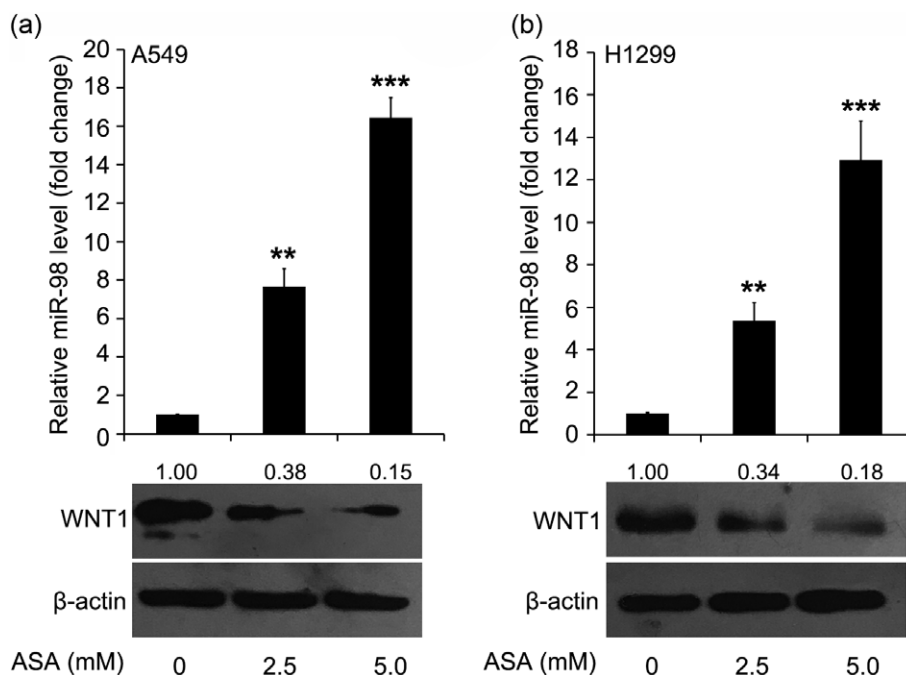
inhibit the progression of lung cancer by directly targeting *MYO6*.²⁵ MiR-98 can bind to *TGFBR1* to depress cell proliferation and metastasis in lung cancer.²⁶ In miR-4317-restrained lung cancer, *FGF9* and *CCND2* serve as the key target genes.²⁷ MiR-886 is capable of playing a suppressive role in the development of lung cancer.²⁸ Tumor suppressor let-7 can destroy the growth of lung cancer in a mouse model.²⁹

In this study we treated lung cancer cells with increasing concentrations of aspirin and evaluated changes in the candidate miRNAs to determine whether miRNAs are involved in aspirin-inhibited lung cancer. Our findings revealed that one tumor suppressor miRNA, miR-98, was obviously upregulated by aspirin. We attempted to further decipher the underlying mechanism of the anti-proliferation effect of aspirin. Sequence analysis and website prediction indicated that miR-98 might bind to the

3'UTR of *WNT1*. We therefore conducted mutation of the miR-98 binding site on 3'UTR of *WNT1*, and the results from luciferase reporter gene assay indicated that the activity of mutated 3'UTR of *WNT1* could not be downregulated by miR-98 mimics or stimulated by its inhibitor. We then showed that aspirin could induce the level of miR-98 to decrease the level of *WNT1* in lung cancer.

Our findings indicate that aspirin is a novel mechanism to ameliorate lung cancer. Aspirin plays an inhibitory role in the growth of lung cancer. As a tumor suppressor miRNA, miR-98 can be induced by aspirin treatment in lung cancer. *WNT1* serves as a mediator in aspirin/miR-98-depressed lung cancer; therefore, aspirin can ameliorate the development of lung cancer by sabotaging the miR-98/*WNT1* axis. Our findings highlight the important role of this newfound mechanism in lung cancer progression as a potential medication for lung cancer.

Figure 4 Aspirin (ASA) regulates the miR-98/WNT1 axis to suppress lung cancer. The level change of miR-98 and its target gene, *WNT1*, in (a) A549 or (b) H1299 cells treated with different doses of ASA by quantitative real-time PCR and immunoblotting assay. BandScan was used to quantify immunoblotting bands. *** $P < 0.001$; ** $P < 0.01$.



Acknowledgments

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Disclosure

No authors report any conflict of interest.

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