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Inhibitor of Differentiation 1 (ID1) Facilitates the Efficacy of Sorafenib in Non-Small Cell Lung Cancer Cells through Suppressing Epithelial to Mesenchymal Transition

Authors' Contribution:

Study Design A

Data Collection B

Statistical Analysis C

Data Interpretation D

Manuscript Preparation E

Literature Search F

Funds Collection G

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Background: Sorafenib, which is a multitargeted kinase inhibitor, has shown some antitumor effects in patients with non-small cell lung cancer (NSCLC). However, the potential target of sorafenib's antitumor activity is largely unknown. Moreover, definitive predictive biomarkers of benefit have rarely been reported.

Material/Methods: The alteration in inhibitor of differentiation 1 (ID1) expression in NSCLC cells with sorafenib treatment was detected by western blotting. The sensitivity of NSCLC cells to sorafenib was observed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay. Loss-of-function and gain-of-function experiments were performed to observe the role of ID1 expression in epithelial to mesenchymal transition (EMT) progression.

Results: Initially, we observed that ID1 was downregulated in NSCLC cells treated with sorafenib. The response of NSCLC cells to sorafenib was inhibited by the transfection of small interfering RNAs (siRNAs) targeting ID1. In contrast, the transfection of ID1-overexpressing plasmids improved the response of NSCLC cells to sorafenib. Further experiments indicated that ID1 is expressed at high levels in epithelial H460 cells and expressed at low levels in mesenchymal H358 cells. Loss-of-function and gain-of-function experiments suggested that ID1 negatively regulates EMT in NSCLC.

Conclusions: The expression of ID1 is dose-dependently inhibited by sorafenib, and the overexpression of ID1 contributes to the antitumor activity of sorafenib by suppressing EMT development. Our results indicate that ID1 might be a potential target for the antitumor activity of sorafenib in NSCLC and that targeting ID1 is a feasible strategy to improve the sensitivity of NSCLC cells to sorafenib.

MeSH Keywords: **Carcinoma, Non-Small-Cell Lung • Inhibitor of Differentiation Protein 1 • Physiological Effects of Drugs • Sorafenib**

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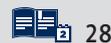
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Background

Worldwide, patients with lung cancer have a high mortality rate [1]. Lung cancer is divided into 2 broad histological classes: small-cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [2]. Patients with NSCLC are poorly sensitive to traditional chemotherapy drugs [3]. Currently, significant advances in the understanding of NSCLC treatment over the past few years have been achieved. Molecular-targeted agents have been attracting increasing attention in NSCLC therapy because they have shown promising results for improving progression-free survival and overall survival [4]. Epidermal growth factor receptor (EGFR)-targeted therapy is currently approved for lung cancer [5]. Clinical trials have proven that patients with EGFR-mutated NSCLC derive substantial benefit from EGFR inhibitors, such as erlotinib [6]. However, many patients with NSCLC do not have this kind of molecular alteration [7]; therefore, the exploration of other drugs as a substitute for EGFR inhibitors in nonresponsive patients is urgently needed.

As a multi-target tyrosine kinase inhibitor, sorafenib exerts encouraging antitumor activity in various types of cancers by inhibiting RAS/RAF/ERK pathway-regulated cell proliferation and VEGFR2-involved angiogenesis [8,9]. It has been identified as the standard of chemotherapy for advanced hepatocellular carcinoma [10]. Recently, several clinical trials have been conducted to observe the response of NSCLC patients to sorafenib, and stable disease was observed in 30 of 51 (59%) patients treated with 400 mg of sorafenib twice daily [11]. However, initial treatment with sorafenib is sensitive, and resistance eventually develops. Therefore, identifying effective biomarkers to stratify NSCLC patients who could be responsive to sorafenib and identifying the resistance mechanisms are necessary.

Inhibitor of differentiation 1 (ID1), which belongs to the family of helix-loop-helix transcription factors, exerts diverse functions in multiple steps of cancer development, including cell proliferation, invasion and migration. Moreover, it has been demonstrated to be responsible for sorafenib resistance in hepatocellular carcinoma [12]. In NSCLC, ID1 was proven to be upregulated in tumor tissues. Patients with high levels of ID1 expression have a poor prognosis [13]. The expression of ID1 was downregulated by the treatment of paclitaxel and cisplatin through the protein ubiquitination/proteasome degradation system [14]. In this study, we discovered that ID1 expression is inhibited in NSCLC cells with sorafenib treatment, and further studies suggested that epithelial to mesenchymal transition (EMT) is responsible for ID1-regulated sorafenib efficacy in NSCLC.

Material and Methods

Cell cultures, small interfering RNA (siRNA) and reagents

Human lung adenocarcinoma cell line A549, large cell lung cancer cell line NCI-H460, and NSCLC cell line H358 were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Chemically synthesized small interfering RNAs (siRNAs) targeting ID1 were purchased from Santa Cruz Biotechnology. ID1 over-expression plasmid pcDNA3-ID1 was purchased from Addgene. Cells were transfected with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instruction. siRNAs were used at the final amount of 300 pmol in 60 mm dish. We purchased 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) from Sigma-Aldrich. Sorafenib was purchased from LC Laboratories.

Western blot

Cells were lysed in lysis buffer. Proteins (40 µg) were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel, then transferred electrophoretically onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocked with 5% non-fat milk in TBST (tris-buffered saline plus Tween 20) at room temperature for 1 hour, the membranes were incubated with primary antibody overnight at 4°C. Then the membranes were washed with TBST and incubated with secondary antibody at room temperature for 45 minutes. The bands were visualized using the enhanced chemiluminescence detection system (Pierce).

Real-time quantitative polymerase chain reaction (PCR)

Total RNA was extracted using TRIzol (Beijing Solar Biotechnology Co., Ltd.) reagent according to the manufacturer's instructions. The primers used for amplification of human genes were as follow:

ID1-F GATTCTCAGCAGTCATCG, ID1-R ATGATCTAGTGGTCGG ATCTGG; platelet-derived growth factor receptors (PDGFR)-F CCCCCTTTCTGGCCTGATG, PDGFR-R CCACCTTTCTTGGCGGGGTA; EGFR-F AGCAGAGACCCACACTACCA, EGFR-R GTAGTCAGGG TTGTCCAGGC. The PCR array was performed as previously described [15]. Briefly, the reverse transcription products were loaded on a real-time PCR System (Eppendorf International). Relative messenger RNA (mRNA) levels of ID1, PDGFR, and EGFR were calculated based on the Ct values and normalized using GAPDH expression. All experiments were performed in triplicate.

MTT assay

Changes of cell viability were analyzed using the MTT assay. Briefly, cells were seeded in 96-well plates at a density of 2.5×10^3 cells per well. After attached overnight, cells were

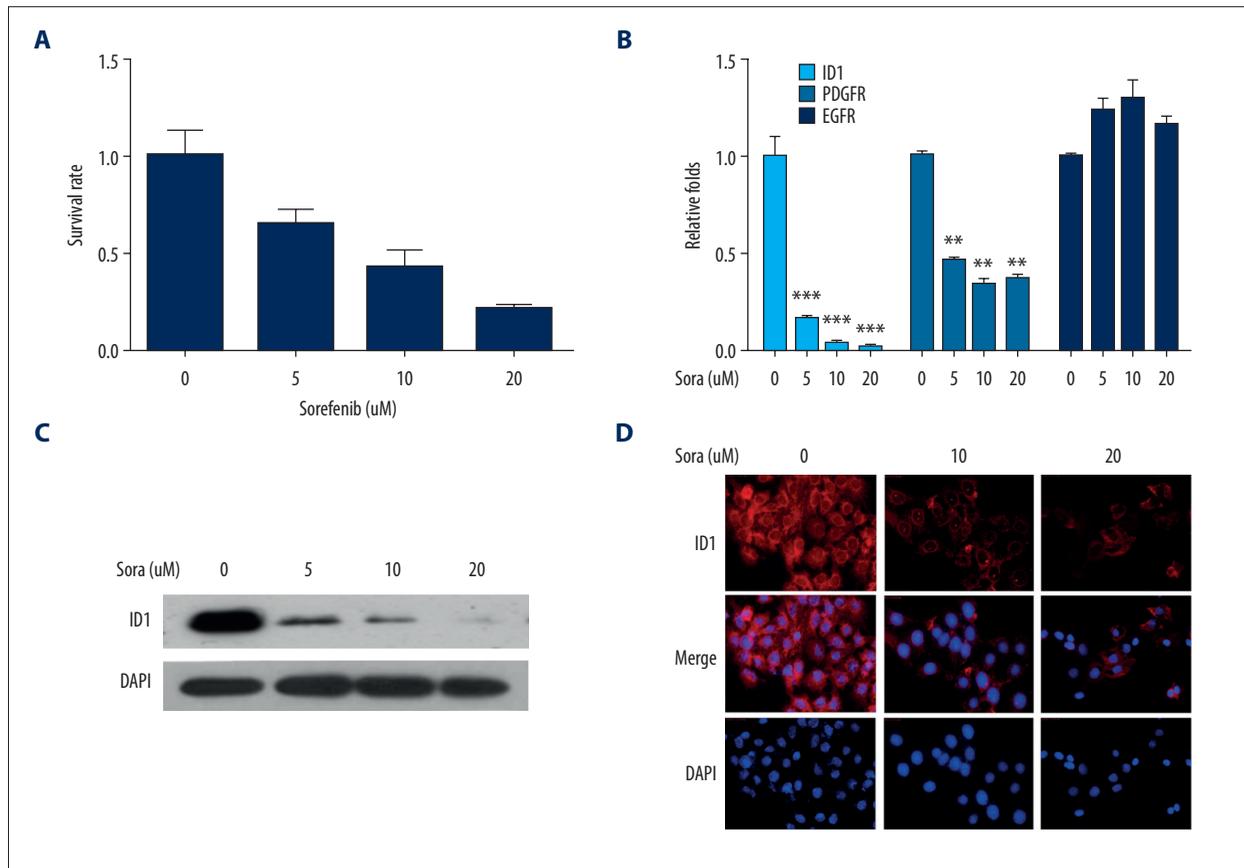


Figure 1. Sorafenib inhibits ID1 expression. **(A)** Response of cells to sorafenib detected by MTT assay. The average of 3 independent experiments and SD are shown. **(B)** H460 cells were treated with different concentrations of sorafenib. Real-time quantitative PCR was performed to determine the alterations in mRNA expression of ID1, PDGFR, and EGFR. ** $P < 0.01$; *** $P < 0.001$. **(C)** H460 cells were treated with different concentrations of sorafenib. Western blot experiments were carried out to determine the alteration in ID1 protein expression. **(D)** Immunofluorescence staining for ID1 (red) was conducted in sorafenib-treated H460 cells, and merged images were obtained. DAPI fluorescence is shown in blue. ID1 – inhibitor of differentiation 1; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; SD – standard deviations; PCR – polymerase chain reaction; mRNA – messenger RNA; PDGFR – platelet-derived growth factor receptors; EGFR – epidermal growth factor receptor; DAPI – 4',6-diamidino-2-phenylindole.

incubated with 0.5% dimethyl sulfoxide (DMSO) (vehicle control) or different concentrations of sorafenib for 4 hours or 48 hours. Then, 5 uL of 5 mg/mL of MTT in PBS was added to each well for 3 to 4 hours at 37°C. At last, 100 uL of DMSO was added to each well to dissolve formazan crystals. The absorbance was measured using a microplate reader at a wavelength of 570 nm.

Immunofluorescence staining

Cells were cultivated on appropriate 6-well coverslips and fixed with 4% paraformaldehyde (PFA) for 15 to 20 minutes. Primary antibodies including monoclonal antibody anti-E-cadherin (1: 100, Cell Signaling Technologies, Danvers, MA, USA), anti-vimentin (1: 100, Abcam, Cambridge, MA, USA) and anti-ID1 (1: 100, Abcam, Cambridge, MA, USA) were used. Cells were co-stained with DAPI (4',6-diamidino-2-phenylindole)

to detect nuclei. The results were analyzed using fluorescence microscopy.

Wound healing assay

Cells were transfected with pcDNA3-ID1 expressing vector or siRNA for ID1 using Lipofectamine 2000. Empty vector pcDNA3 or negative control siRNA served as a control. After 24 hours of transfection, a micropipette's tip was used to make a scratch, producing a clean wound area. Cells were maintained in incubator and photographed at indicated time points under the microscope.

Statistical analyses

Statistical analyses were done using GraphPadPrism version 6.05. Data are shown as mean ± standard deviation (SD).

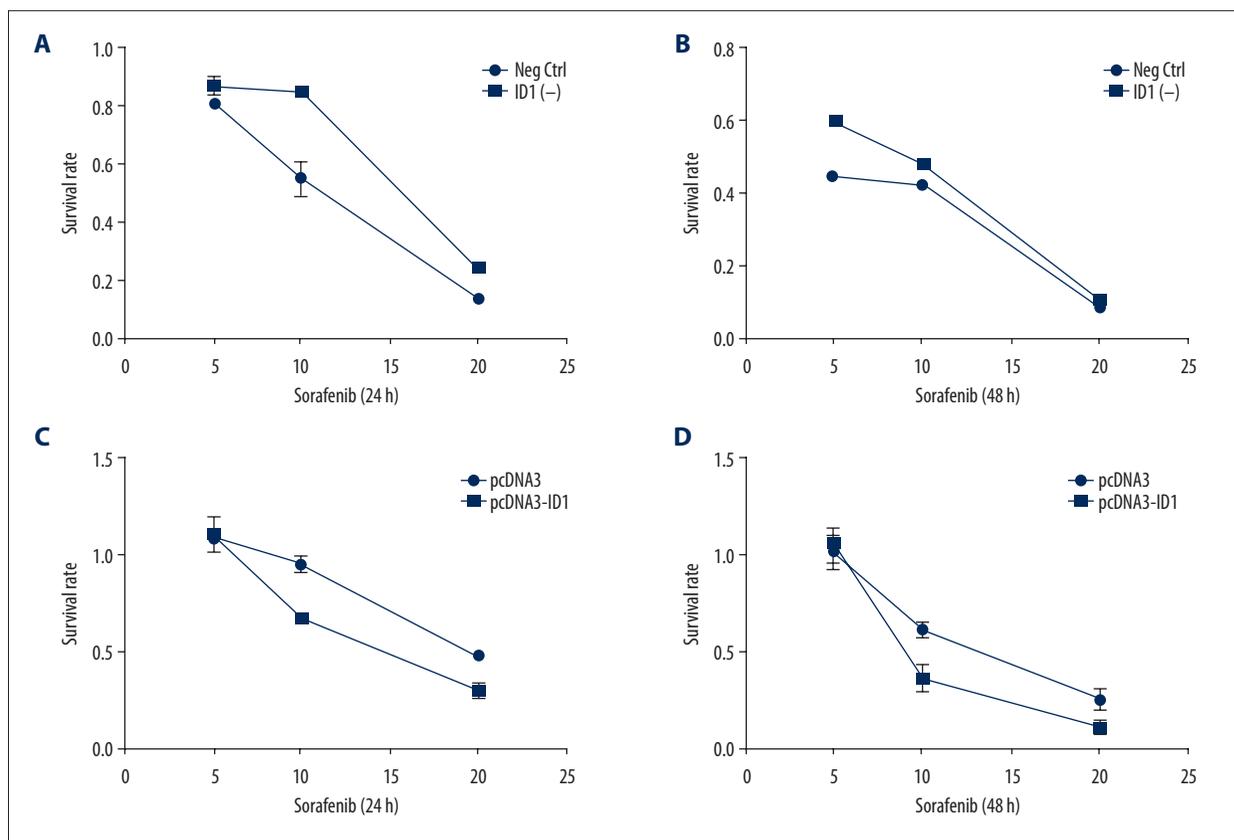


Figure 2. ID1 downregulation enhances sorafenib efficacy. (A, B) H460 cells were transfected with siRNAs targeting ID1. Following treatment with various concentrations of sorafenib for 24 hours (A) and 48 hours (B), the cell survival rate was determined by MTT assay. (C, D) H358 cells were transfected with ID1 overexpression plasmids. Following treatment with various concentrations of sorafenib for 24 hours (C) and 48 hours (D), the cell survival rate was determined by MTT assay. ID1 – inhibitor of differentiation 1; siRNAs – small interfering RNAs; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

Differences between groups were considered significant when the *P*-values were ≤ 0.05 .

Results

Sorafenib inhibited ID1 expression at both the mRNA and protein levels

Initially, we treated H460 cells with different concentrations of sorafenib and observed that cell survival was reduced by sorafenib in a dose-dependent manner (Figure 1A). Next, we observed the effect of sorafenib on ID1 expression. In contrast to the modest downregulation of PDGFR and EGFR, ID1 mRNA expression was markedly inhibited (Figure 1B). This result implies that ID1 might be a target for sorafenib in NSCLC. Western blot analysis confirmed that the expression of ID1 protein was suppressed by sorafenib in a dose-dependent manner (Figure 1C). Moreover, immunofluorescence staining of ID1 protein was weak when cells were incubated with sorafenib (Figure 1D).

ID1 overexpression enhanced the efficacy of sorafenib in NSCLC

The aforementioned data demonstrated that ID1 expression was prevented by sorafenib, and we speculated that ID1 expression has an effect on sorafenib efficacy in NSCLC. To verify this hypothesis, we introduced siRNAs targeting ID1 to downregulate ID1 and pcDNA3-ID1 plasmids to upregulate ID1. Cells in the experimental group and control group were treated with the same concentrations of sorafenib. MTT assays were conducted to observe the response of the cells in the different groups to sorafenib. According to the results shown in Figure 2A and 2B, the survival rate was higher in cells with ID1 knockdown than in the negative control group; in contrast, cells transfected with pcDNA3-ID1 overexpression plasmids were more sensitive to sorafenib (Figure 2C, 2D). The results indicated that ID1 knockdown inhibited sorafenib efficacy, while the overexpression of ID1 enhanced the efficacy of sorafenib in NSCLC.

It has been reported that the degradation of ID1 protein is modulated by the ubiquitin-proteasome system, in which

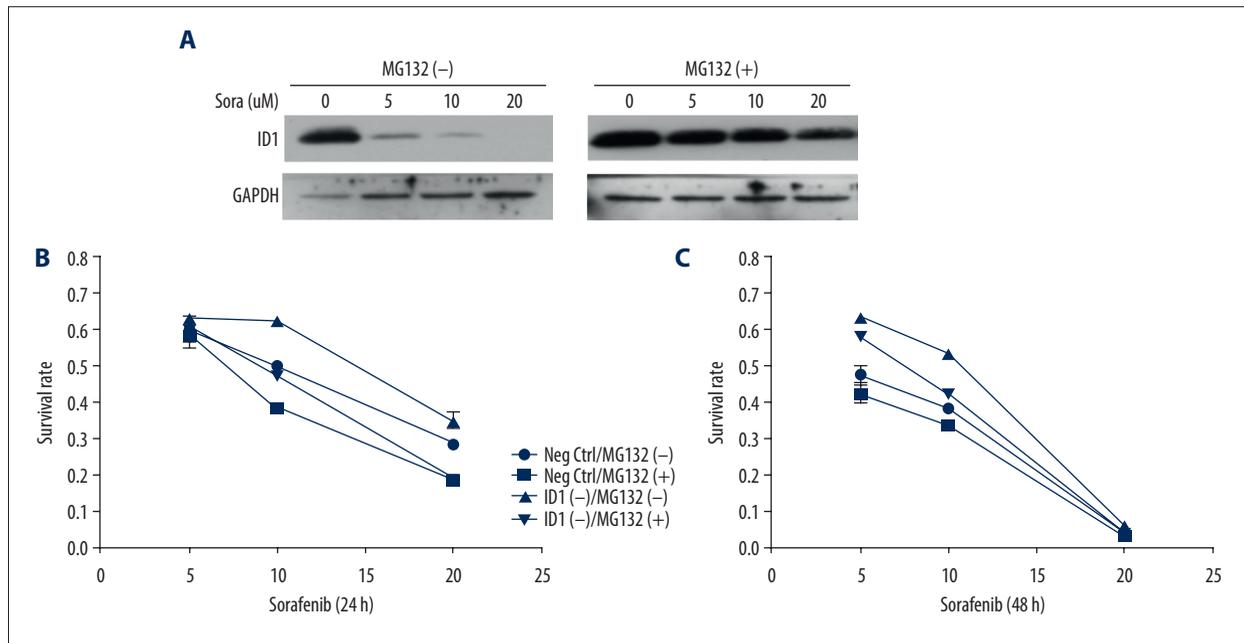


Figure 3. MG132 inhibits the effect of sorafenib on ID1. (A) H460 cells were incubated with various concentrations of sorafenib for 24 hours, with or without pretreatment with MG132. Western blotting was performed to determine the alterations in ID1 expression. (B, C) H460 cells transfected with siRNAs targeting ID1 were incubated with various concentrations of sorafenib, with or without pretreatment with MG132. The MTT assay was used to determine the cell survival rate. ID1 – inhibitor of differentiation; siRNAs – small interfering RNAs; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

ID1 protein is tagged by certain types of polyubiquitin chains and then selectively recognized and removed by the proteasome [16,17]. We observed that the reduced efficacy of sorafenib with downregulated ID1 protein expression was relieved by MG132, which is the most commonly used agent to inhibit proteasome activity (Figure 3A). Then, we observed that the application of MG132 abolished the inhibitory effect of ID1 knockdown on sorafenib efficacy, which was evidenced as the cell survival rate was decreased when cells were pretreated with MG132 (Figure 3B, 3C), demonstrating that the upregulation of ID1 contributed to sorafenib efficacy.

ID1 expression was negatively correlated with EMT biomarkers

Next, we explored the mechanisms involved in the ID1-induced enhancement of sorafenib efficacy in NSCLC. Accumulating evidence has proven that sorafenib resistance can be affected by EMT development [9]. In this study, we detected the expression levels of ID1 in different NSCLC cells with different morphologies. As shown in Figure 4A, epithelial morphology in H460 cells, mesenchymal morphology in H358 cells, and both epithelial and mesenchymal morphology in A549 cells were observed. The data were in accordance with the western blotting results showing that the expression of the mesenchymal biomarker vimentin was increased, while the expression of the epithelial biomarker E-cadherin was decreased from the

level in H460 cells to that in H358 cells. Importantly, we noticed that the ID1 protein had high expression in H460 cells, had moderate expression in A549 cells, and was negative for expression in H358 cells (Figure 4B), implying that the ID1 expression level was negatively correlated with EMT biomarkers. Immunofluorescence confirmed that the extent of ID1 staining was strong in epithelial H460 cells and low in mesenchymal H358 cells (Figure 4C).

The ID1-EMT pathway was responsible for sorafenib efficacy in NSCLC

The aforementioned data suggested that EMT played a crucial role in ID1-involved sorafenib efficacy. Western blot experiments showed that the knockdown of ID1 induced an increase in vimentin and a decrease in E-cadherin, and the opposite result was observed in cells with ID1 overexpression, as vimentin was downregulated and E-cadherin was upregulated (Figure 5A). To further confirm the role of ID1 in the EMT process, a wound healing assay was conducted to evaluate the effect of ID1 knockdown or overexpression on cell migration. As expected, ID1 knockdown led to a significant improvement in migration ability, while ID1 overexpression led to a significant reduction in migration ability, proving that ID1 knockdown promoted cell migration and that ID1 overexpression suppressed cell migration (Figure 5B).

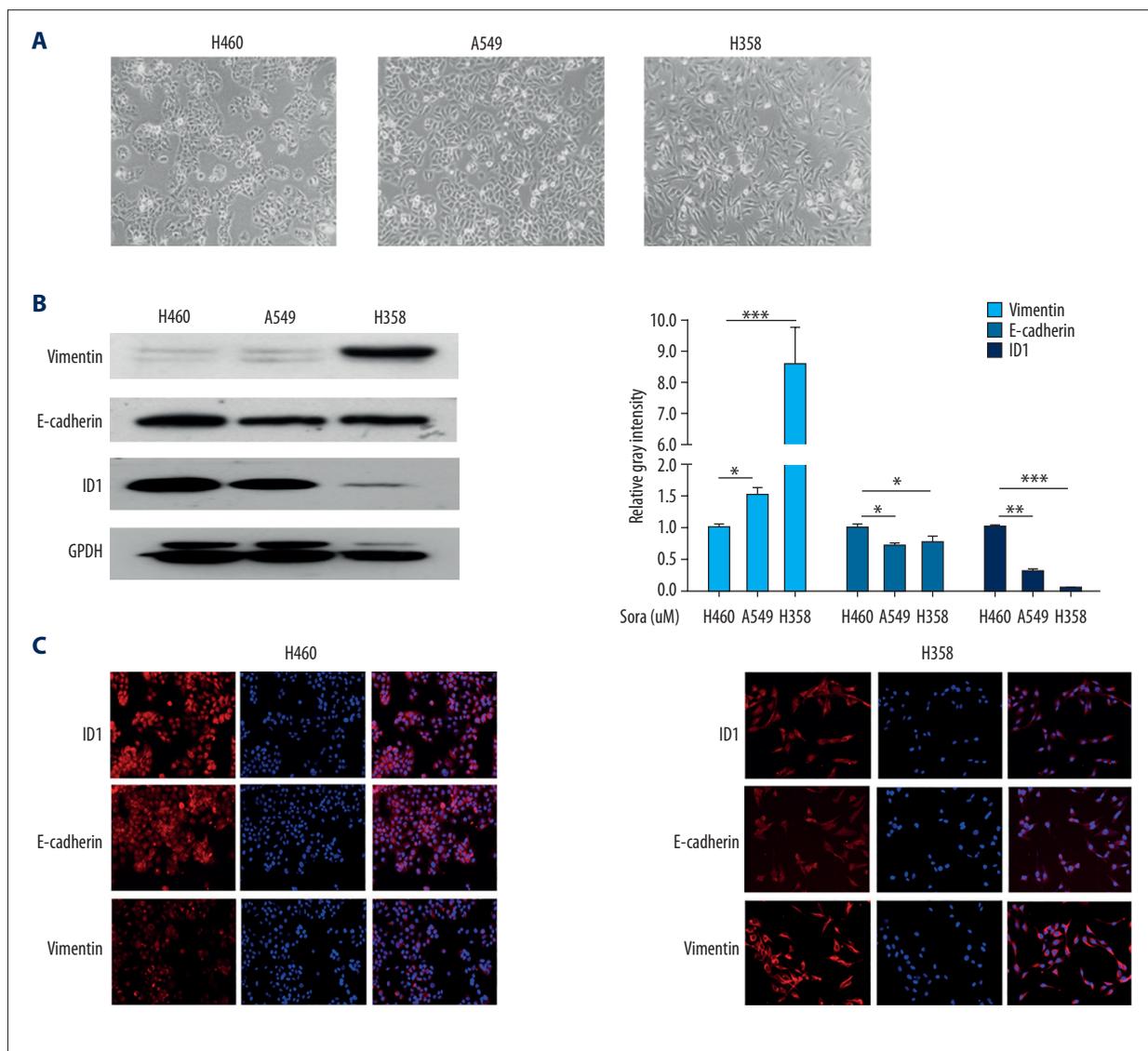


Figure 4. ID1 expression was negatively correlated with EMT biomarkers. (A) The pictographs show the morphology of H460, A549, and H358 cells. (B) Western blot analysis of EMT markers (E-cadherin and vimentin) and ID1 in H460, A549, and H358 cells. The intensity of the bands was quantified using ImageJ software and normalized to GAPDH (right panel). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (C) Immunofluorescence was used to detect the fluorescence intensity of E-cadherin, vimentin and ID1 in H460 and H358 cells. ID1 – inhibitor of differentiation 1; EMT – epithelial to mesenchymal transition.

Discussion

The majority of NSCLC patients are diagnosed in advanced stages or with metastatic disease, and most of them are unresectable. For unresectable NSCLC patients, chemotherapy is the standard treatment [18]. EGFR-tyrosine kinase inhibitors (TKIs) have been shown to exert satisfactory survival benefits in clinical trials, and therefore, EGFR-TKIs have been identified as first-line chemotherapy agents to treat NSCLC alone or in combination with cytotoxic drugs [19]. However, only a small percentage of NSCLC patients have tumors carrying an EGFR mutation, a biomarker of oncogene addiction that strongly

relates to the response to EGFR-TKIs; therefore, exploring other agents to treat NSCLC patients whose tumors do not have EGFR mutations is necessary.

Sorafenib has shown encouraging antitumor function in clinical trials as monotherapy or combined treatment in patients with advanced NSCLC [20–22]. Previous cellular and molecular studies have also proven that sorafenib alone suppresses cell proliferation in NSCLC, and when combined with other drugs, the inhibitory effect is increased [23–25]. These studies indicate that sorafenib is likely to be a promising candidate for NSCLC treatment. However, definitive predictive biomarkers

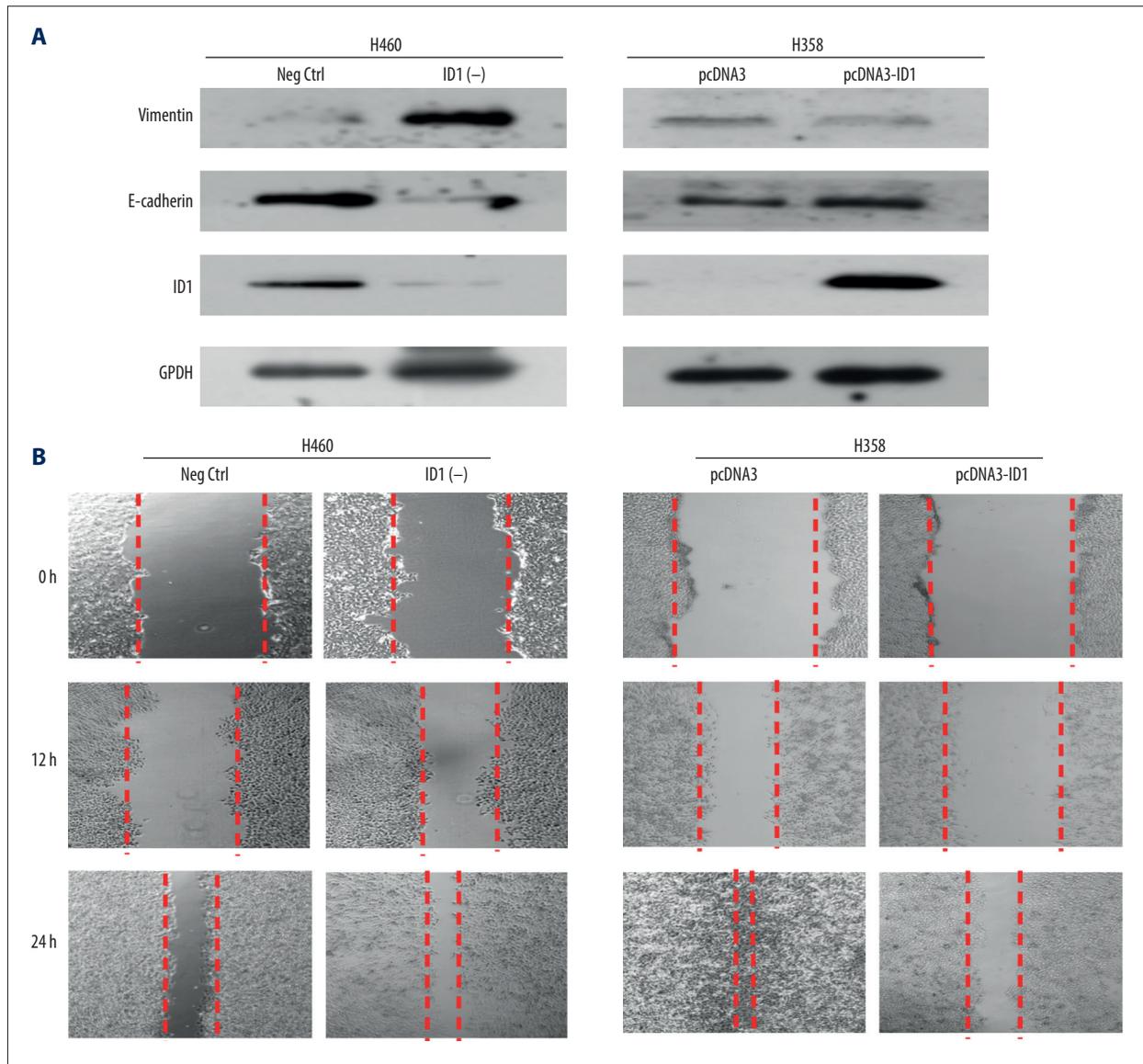


Figure 5. ID1 suppresses EMT in NSCLC. **(A)** Western blot analysis for the expression of EMT-related markers in H460 cells with a negative control and siRNAs targeting ID1 (**left panel**). Western blot analysis for the expression of EMT-related markers in H358 cells with the vector alone as the control and ID1 overexpression plasmids (**right panel**). **(B)** Representative images of wound healing in H460 cells with negative control and siRNAs targeting ID1 are shown in the **left panel**. Representative images of wound healing in H358 cells with the vector alone as the control and ID1 overexpression plasmids are shown in the **right panel**. ID1 – inhibitor of differentiation 1; EMT – epithelial to mesenchymal transition; NSCLC – non-small cell lung cancer; siRNAs – small interfering RNAs.

of benefit have rarely been reported. In this study, NSCLC cells with ID1 knockdown showed less sensitivity to sorafenib, suggesting that ID1 might be a promising biomarker for the application of sorafenib in NSCLC patients. It is well known that sorafenib exerts antitumor effects via VEGFR and PDGFR; however, the changes in these classical targets were modest, indicating that the specific targets for the antitumor activity of sorafenib in NSCLC still need to be elucidated.

As a generally negative prognostic factor in many types of cancers, ID1 was paradoxically identified as a potential molecule for enhancing drug efficacy and preventing chemoresistance. NSCLC patients with high ID1 expression showed better disease-free and overall survival after adjuvant paclitaxel and cisplatin chemotherapy [14]. A previous report also revealed that ID1 contributed to sorafenib efficacy by modulating the p16/IL6 axis in hepatocellular carcinoma [12]. In this study, our data demonstrated that ID1 expression was negatively correlated

with EMT biomarkers and that the overexpression of ID1 suppressed EMT development. The effect of EMT on chemotherapy resistance has widely been reported [26,27]. Cells incubated with sorafenib for a long time underwent EMT and became resistant to sorafenib [28]. Drug resistance in sorafenib-resistant hepatocellular carcinoma cells could be reversed by blocking EMT. In the present study, we revealed that EMT was responsible for the regulation of sorafenib efficacy by ID1.

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Conclusions

Our results indicate that ID1 is inhibited by sorafenib, and the overexpression of ID1 contributes to the antitumor activity of sorafenib by preventing EMT. Strategies that upregulate ID1 would be beneficial to NSCLC patients undergoing sorafenib treatment.

Conflict of interest

None.