FEBS Letters 589 (2015) 805–811

FEBS Letters

journal homepage: www.FEBSLetters.org

MicroRNA-575 targets BLID to promote growth and invasion of non-small cell lung cancer cells

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ARTICLE INFO

Article history:
Received 12 November 2014
Revised 10 February 2015
Accepted 11 February 2015
Available online 26 February 2015

Edited by Tamas Dalmay

Keywords:
Non-small cell lung cancer
MicroRNA-575
BLID
Oncogene

ABSTRACT

This study was designed to detect miR-575 expression and function in non-small cell lung cancer (NSCLC). A higher expression of miR-575 in NSCLC tissues was observed compared with adjacent non-neoplastic tissues. Furthermore, re-introduction of miR-575 significantly promoted cell proliferation, migration, and invasion in the NSCLC line. Moreover, we showed that BLID is negatively regulated by miR-575 at the posttranscriptional level, via a specific target site within the 3′UTR. Overexpression of BLID counteracted miR-575-induced proliferation and invasion in NSCLC cells. The expression of BLID is frequently downregulated in NSCLC tumors and cell lines and inversely correlates with miR-575 expression. The findings of this study contribute to the current understanding of the functions of miR-575 in NSCLC.
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1. Introduction

Lung cancer is the most frequent cause of cancer-related death in many countries [1]. Approximately 85% of all lung cancer cases are categorized as non-small lung cancer (NSCLC) [2]. Although the survival rate for lung cancer has increased because of improvement in diagnosis and treatment, the prognosis of NSCLC remains poor [3]. Lung carcinogenesis is a multistep process, involving activation of oncogenes and inactivation of tumor suppressor genes [4]. To date, the molecular network of lung carcinogenesis remains only partly clarified [5]. Therefore, a better understanding of the molecular mechanisms in NSCLC will be helpful to develop novel therapeutic targets and strategies for the treatment of human NSCLC [6].

MicroRNAs (miRNAs) are endogenous single-stranded non-coding RNA that regulate gene expression by base pairing with target mRNAs, leading to mRNA cleavage or translational repression [7,8]. Through these mechanisms, miRNAs regulate up to 30% of human genes and have a major influence on various biological processes, including cell differentiation, proliferation, apoptosis, stress resistance, fat metabolism, and development [9–11]. Many reports have proved a role of miRNAs in the etiology and pathogenesis of cancer [12]. MiRNAs are frequently deregulated in many types of cancer such as gastric cancer, breast cancer, and hepatocellular cancer [13–15]. Recently, increasing evidence has shown that miRNAs are also involved in NSCLC pathogenesis, providing new insights for NSCLC treatment [16,17].

Extensive researches have shown that miR-575 was deregulated and functioned as an oncogene in many tumors, including gastric cancer, lymphoblastic cancer, and leukemia [18–20]. However, the role of miR-575 in NSCLC cell remains unclear. In this study, we demonstrated that increased miR-575 expression was a characteristic molecular change in NSCLC. We also showed that miR-575 promoted growth and invasion of NSCLC cell, functioning as an oncogene by directly targeting BLID.

2. Materials and methods

2.1. Ethics statement

All of these patients agreed to participate in the study and gave written informed consent. Both this study and consent were approved by the ethical board of the institute of The Second

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http://dx.doi.org/10.1016/j.febslet.2015.02.013
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Affiliated Hospital of Harbin Medical University and complied with the Declaration of Helsinki.

2.2. Patients and tumor samples

Twenty primaries NSCLC and their corresponding adjacent non-tumour lung tissues samples (located > 3 cm from the tumor) from the same specimens were collected from the The Second Affiliated Hospital of Harbin Medical University from 2011 to 2013. No patients had received blood transfusion, radiotherapy, or chemotherapy before surgery. Tissue samples were cut into two parts, one fixed with 10% formalin for histopathological diagnosis, and the other snap-frozen in liquid nitrogen, and stored in liquid nitrogen until RNA extraction.

2.3. Cell culture

Four Human NSCLC cell lines, A549, SPC-A1, H1299, and H1650, and immortalized human bronchial epithelial cell line 16HBE were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM media (Invitrogen, Carlsbad, CA, USA) containing 10% FBS at 37 °C with 5% CO_2.

2.4. Plasmids and cell transfection

MiR-575 mimic/inhibitor and controls were purchased from RiboBio (Guangzhou, China). A549 cells were seeded in six-well plates at 30% confluence one day prior to transfection. Transfection with miR-575 mimic/inhibitor or the controls was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and transfected into the cells to a final oligonucleotide concentration of 10 nmol/l.

2.5. RNA isolation and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using a reverse transcription kit (Tiangen). The expression of miR-575 was quantified by quantified reverse transcription polymerase chain reaction (RT-qPCR) using TaqMan microRNA assays (Applied Biosystems). U6 was used as an internal control. The expression of BLID was quantified by RT-qPCR using SYBRGreen assays (Takara, Da-liang, China) with GAPDH as an internal control. The primers are listed in Supplementary Table S1.

2.6. Cell proliferation

Cell proliferation was performed using the Cell Counting Kit-8 (CCK8) (Dojindo, Shanghai, China). Cells were cultured in 10% CCK-8 diluted medium at 37 °C until visual color conversion occurred. Proliferation rates were determined at 0, 24, 48, and 72 h after transfection. Optical density (OD) values were determined at a wavelength of 450 nm.

2.7. Cell migration and invasion assay

For the migration assays, cells (5×10^4) were added into the upper chamber of the insert (BD Bioscience, 8-μm pore size). For the invasion assays, cells (1×10^5) were added into the upper

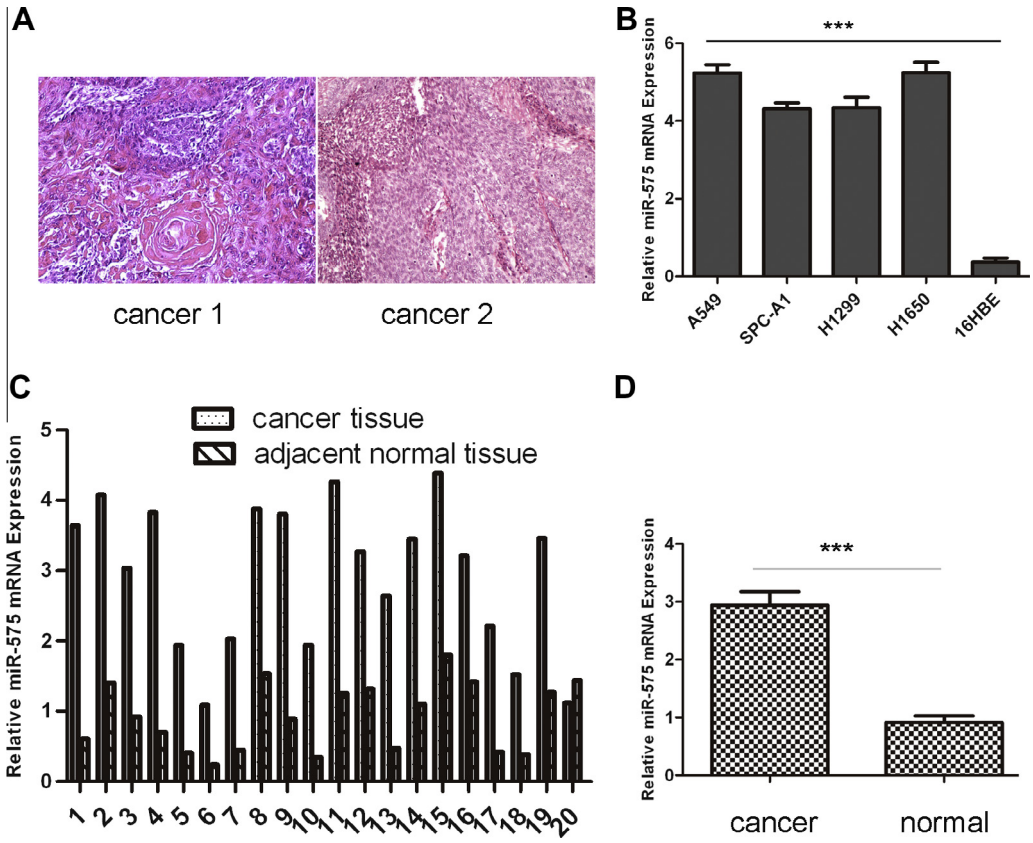

Fig. 1. The expression of miR-575 was up-regulated in NSCLC. (A) The two patients who were diagnosed as NSCLC in H&E staining (original magnification, ×100). (B) The expression relative expression levels were determined by qRT-PCR in Human NSCLC cell lines (A549, SPC-A1, H1299 and H1650) and normal bronchial epithelial cell line 16HBE. (C) qRT-PCR analysis of miR-575 expression in 20 pairs NSCLC tissues and their corresponding adjacent normal lung tissues. The expression of miR-575 was normalized to U6 snRNA. (D) Relative miR-575 expression levels in NSCLC tissues and their corresponding adjacent normal lung tissues. Student's *t* test was used to analyze the significant differences between the tumor and normal tissues. $^{***}P < 0.001$.

chamber of the insert precoated with Matrigel (BD Bioscience). In both assays, cells were plated in medium without serum, and medium containing 10% FBS in the lower chamber as chemoattractant. After several hours of incubation, cells that did not migrate or invade through the pores were carefully wiped out with cotton wool. Then the inserts were stained with 20% methanol and 0.2% crystal violet, imaged, and counted with an IX71 inverted microscope (Olympus).

2.8. Luciferase assays

HEK293T cells (8000 cells per well) were seeded in 96-well plates the day before transfection. A mixture of 100 ng indicated BLID-3′UTR, 200 ng of BLID or BLID-miR-575 and 20 ng Renilla plasmid (containing no 3′UTR) was transfected into HEK293T cells with Lipofectamine 2000 in each well. Forty-eight hours later, Firefly and Renilla luciferase activities were measured using a Dual-Luciferase Reporter System (Promega). The Renilla luciferase activities were used as an internal control for transfection efficiency.

2.9. Rescue assays of BLID gene expression

The full-length BLID cDNA (which included the ORF and 3′-UTR) was PCR-amplified and cloned into the pcDNA3.1 vector to generate the pcDNA-BLID constructs. Cells in 6-well plates were first transfected with either the miR-575 or a scrambled dsRNA (20 nM). After 24 h in culture, these cells were then co-transfected with 2 μg of the pcDNA-BLID plasmid DNA or with 2 μg of the pcDNA empty vector plasmid. At the indicated time, cells were harvested and assayed.

2.10. Western blot

Protein was isolated from cell and tissues using the M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology, Rockfield, IL). Protein concentration was detected by the Pierce BCA assay (Thermo Fisher, Rockford, IL, USA). Equal amounts of cell lysate were resolved by SDS–PAGE and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). Membranes were blocked with the following antibodies: rabbit anti-BLID (1:2000, Cell Signaling, Danvers, MA, USA), rabbit anti-GAPDH (1:5000, Santa Cruz, Dallas, TX, USA). Bound antibodies were detected with secondary HRP-conjugated antibodies (Santa Cruz) and visualized by Pierce enhanced chemiluminescent substrate (Thermo Fisher).

3. Result

3.1. miR-575. was up-regulated in human NSCLC specimens and cell lines

The two patients who were diagnosed as NSCLC in H&E staining (Fig. 1A). miR-575 levels were frequently upregulated in NSCLC cell

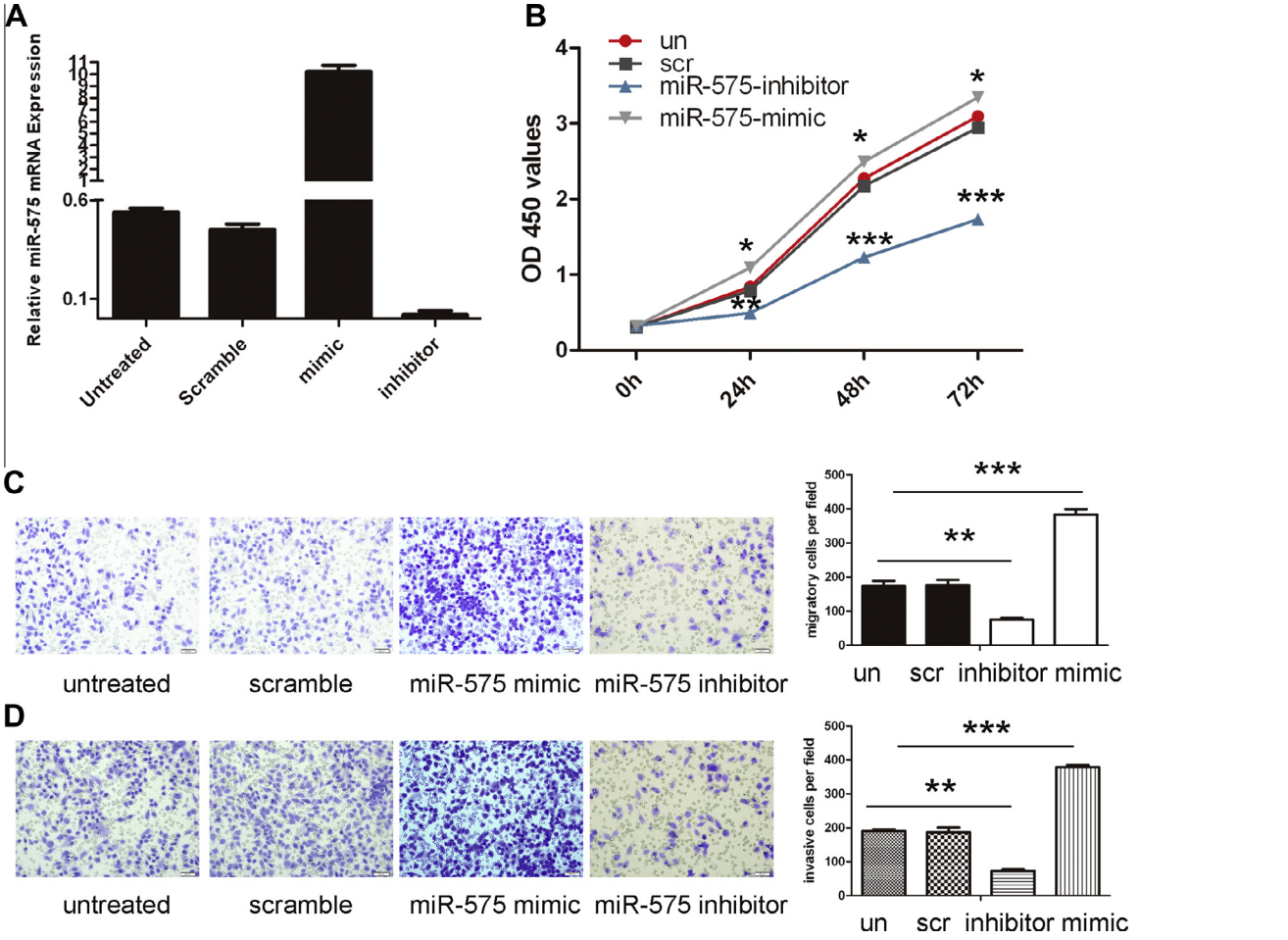

Fig. 2. Overexpression of miR-575 promoted proliferation, migration and invasion of NSCLC cells. (A) qRT-PCR analysis of miR-575 expression after the transfection of miR-575 mimics, inhibitors or scramble or no transfection. (B) The CCK8 assay used to evaluate the proliferation of A549 cells after transfection with the miR-575 mimics, inhibitors or scramble or no transfection. (C) Migration assays of A549 cells after treatment with miRNA mimics, inhibitors or scramble or no transfection; the relative ratio of migratory cells per field is shown. (D) Transwell analysis of A549 cells after treatment with miRNA mimics, inhibitors or scramble or no transfection; the relative ratio of invasive cells per field is shown below, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

lines compared with normal bronchial epithelial cell line (Fig. 1B). In addition, we tested miR-575 expression in NSCLCs obtained from 20 patients. As shown in Fig. 1C, 95% (19 of 20) of carcinoma tissues showed increased miR-575 expression compared with normal counterparts, and the average expression level in carcinoma tissues (combination) was significantly higher compared with normal lung tissues ($P < 0.001$, Fig. 1D).

3.2. miR-575. promoted NSCLC cell proliferation, migration and invasion

We next evaluated the effects of miR-575 on the proliferation, migration and migration of A549 cells using the CCK8, the migration assays and Transwell assay. NSCLC cells were transfected with scrambled control oligo or miR-575 mimics and inhibitors, which exhibited high transfection efficiency (Fig. 2A). A549 cells transfected with miR-575 mimic showed increased proliferation (Fig. 2B); in contrast, knockdown of miR-575 had the opposite effect on cell proliferation (Fig. 2B). Moreover, the percentage of migrated cells was higher in cells transfected with miR-575 mimic and lower in cells transfected with miR-575 inhibitor (Fig. 2C). Furthermore, the percentage of invasive cells was significantly higher in cells transfected with miR-575 mimic and lower in cells transfected with miR-575 inhibitor (Fig. 2D). Taken together, these results demonstrated that miR-575 promoted cell proliferation, migration and invasion in NSCLC cell.

3.3. miR-575. directly targeted BLID 3′UTR and negatively regulates its expression

To determine the target gene of miR-575 on NSCLC cells, miRDB, micrrna.org and PicTar were used to predict the potential targets of miR-575. As shown in Fig. 3A, there was complementarity between has-miR-575 and the BLID 3′UTR. We further used luciferase reporter assay to confirm the direct regulation of BLID by miR-575 in NSCLC cell lines. Overexpression of miR-575 in A549 cells resulted in decreased luciferase expression (Fig. 3B). In addition, miR-575 mimics decreased BLID expression in NSCLC cell line (. and miR-575 inhibitor can increased the expression of BLD in NSCLC cell line Fig. 3C and Fig. 3D and E). These results suggested that miR-575 regulated endogenous BLID expression at least partially by degrading its mRNA in NSCLC cells.

3.4. Overexpression of BLID impaired the miR-575-induced promotion of proliferation and invasion

We performed rescue experiments to further validate that BLID was involved in the function of miR-575 in NSCLC cells. The BLID expression vector pcDNA3.1-BLID was used to restore BLID expression. The expression of BLID was reduced when cells were transfected with pcDNA-BLID and miR-575 mimic after 24 h (Fig. 4A). Ectopic expression of BLID decreased cell proliferation and migration (Fig. 4B and C; see group pcDNA-BLID + Scramble and

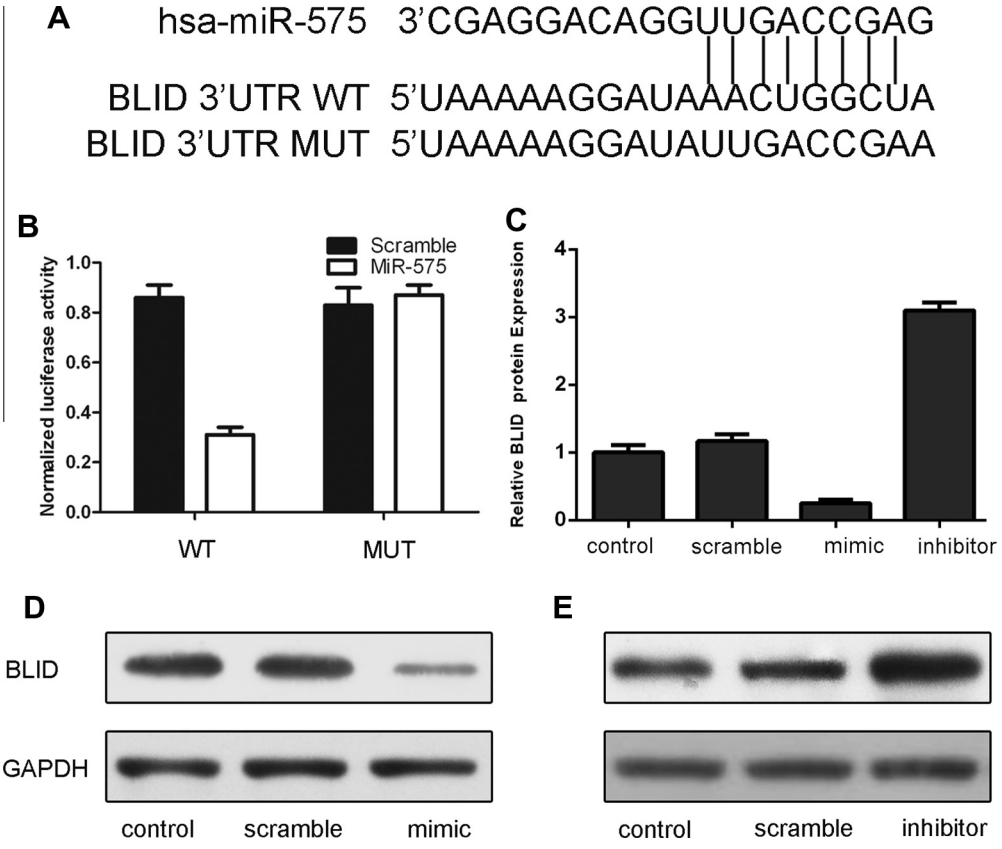

Fig. 3. miR-575 targeted BLID in NSCLC cells. (A) The sequences of miR-575 binding sites within the human BLID 3′UTRs and schematic reporter constructs, in this panel, BLID-WT represent the reporter constructs containing the entire 3′UTR sequences of BLID. BLID-MUT represent the reporter constructs containing mutated nucleotides. (B) The analysis of the relative luciferase activities of BLID-WT, BLID-MUT. The error bars are derived from triplicate experiments. (C) qRT-PCR analysis of BLID mRNA expression in A549 cells after treatment with miRNA mimics or scramble or no transfection. The expression of BLID was normalized to GAPDH. (D) Western blot analysis of BLID expression in A549 cells transfected with miR-575 mimics or scramble or no transfection. GAPDH was also detected as a loading control. (E) Western blot analysis of BLID expression in A549 cells transfected with miR-575 inhibitor or control or scramble. GAPDH was also detected as a loading control.

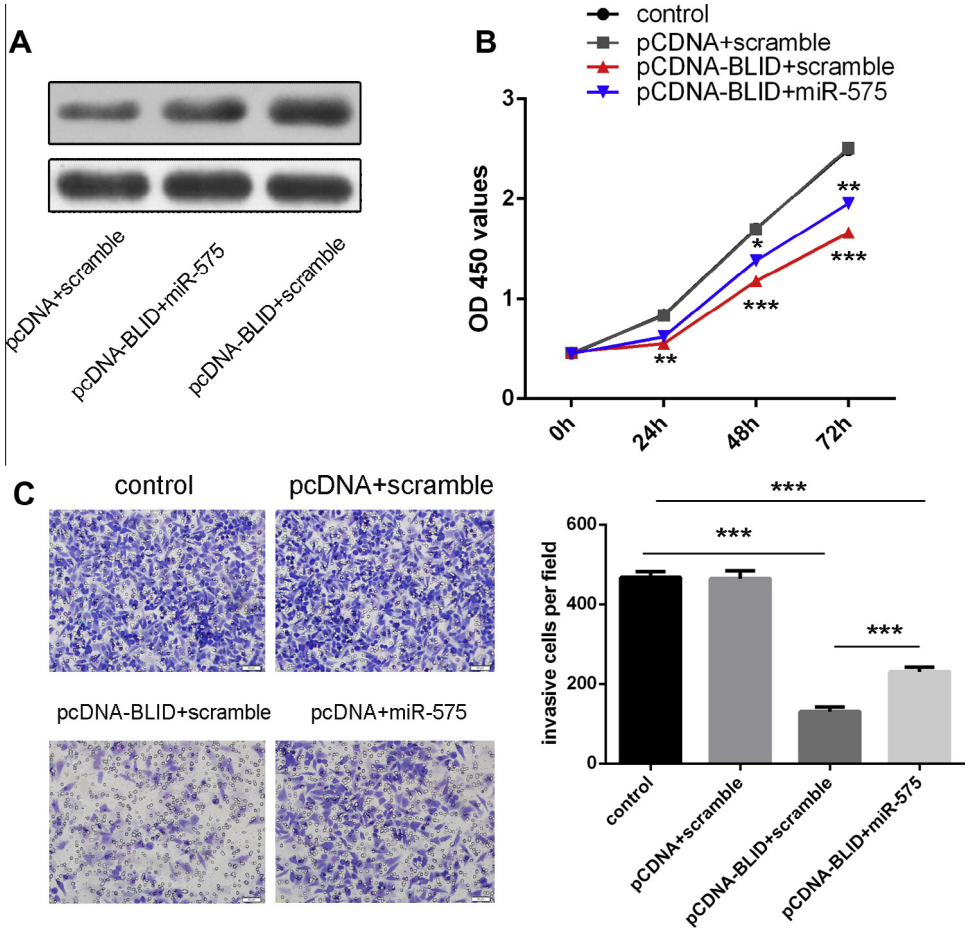

Fig. 4. Overexpression of BLID impaired miR-575-induced proliferation and invasion in NSCLC cell. (A) Western blot analysis of BLID expression in A549 cells co-transfected with either miR-575 mimics or scramble and pCDNA-BLID or pCDNA empty vector; GAPDH was also detected as a loading control. (B) The CCK8 assay used to evaluate the proliferation of A549 cells transfected with different combinations. (C) Invasion analysis of A549 cells treated with different combinations. The relative ratio of invasive cells per field is shown right. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

group pcDNA + Scramble). In addition, decreased cell proliferation (Fig. 4B) was accompanied by decreased cell invasion (Fig. 4C). Thus, we concluded that the promotion of cell growth and invasion by miR-575 was a consequence of decreased BLID expression in the NSCLC cells.

3.5. BLID was inversely expressed with miR-575 in NSCLC and normal tissues

The mRNA and protein expression of BLID was frequently down-regulated in NSCLC cell lines (A549, SPC-A1, H1299, and H1650) compared with immortalized human bronchial epithelial cell line (16HBE) (Fig. 5A and B). BLID mRNA was expressed at lower levels (100%) in tumor tissues compared with normal tissues (Fig. 5C). Furthermore, the protein levels of BLID in NSCLC were also lower compared with adjacent normal lung tissues by western blot (Fig. 5C). As shown in Fig. 5E, when the BLID levels were plotted against miR-575 expression, a significant inverse correlation was obtained (two-tailed Pearson's correlation analysis, $r = -0.83$; $P < 0.01$).

4. Discussion

Recent studies have revealed that miRNAs regulate the expression of many genes involved in lung disease, including cellular stress, cell differentiation, proliferation and invasion [21–23]. NSCLC is a multistep process with complex mechanisms. A better understanding of the detailed mechanisms might be useful to find new therapeutic targets and strategies for the treatment of NSCLC. Our research indicated that the expression of miR-575 was higher in NSCLC tissues than in matched normal lung tissues. In addition, overexpression miR-575 increased cell proliferation, migration and invasion in NSCLC cells. Moreover, we also identified BILD as a direct target of miR-575. Our findings, together with those other groups, suggest that miR-575 has a fundamental role in NSCLC tumorigenesis by promoting cell proliferation and invasion.

Although miR-575 was reported to associate to EBV-transformed lymphoblastoid, and gastric cancer [18–20,24,25], its expression in NCSLC remains to be determined. As shown in this report, the expression of miR-575 was higher in NCSLC than the corresponding normal adjacent tissues. To assess the role of miR-575 in NSCLC development, we investigated the function effects of miR-575 on various aspects of NSCLC biology, including cell proliferation, migration and invasion. Inhibition expression of miR-575 inhibited NSCLC cell proliferation, migration and invasion. These results suggest that miR-575 might act as a new oncogene whose up-regulation contributes to the progression and metastasis of NCSLC.

In this study, we used four miRNA target prediction algorithms to identify the potential targets of miR-575. Complementary sequence of miR-575 is identified in the 3′UTR of BLID mRNA. BLID 3′UTR reporter experiment showed a significant decrease in luciferase activity when contransfected with miR-575. And BLID protein and mRNA was downregulated in miR-575 overexpression

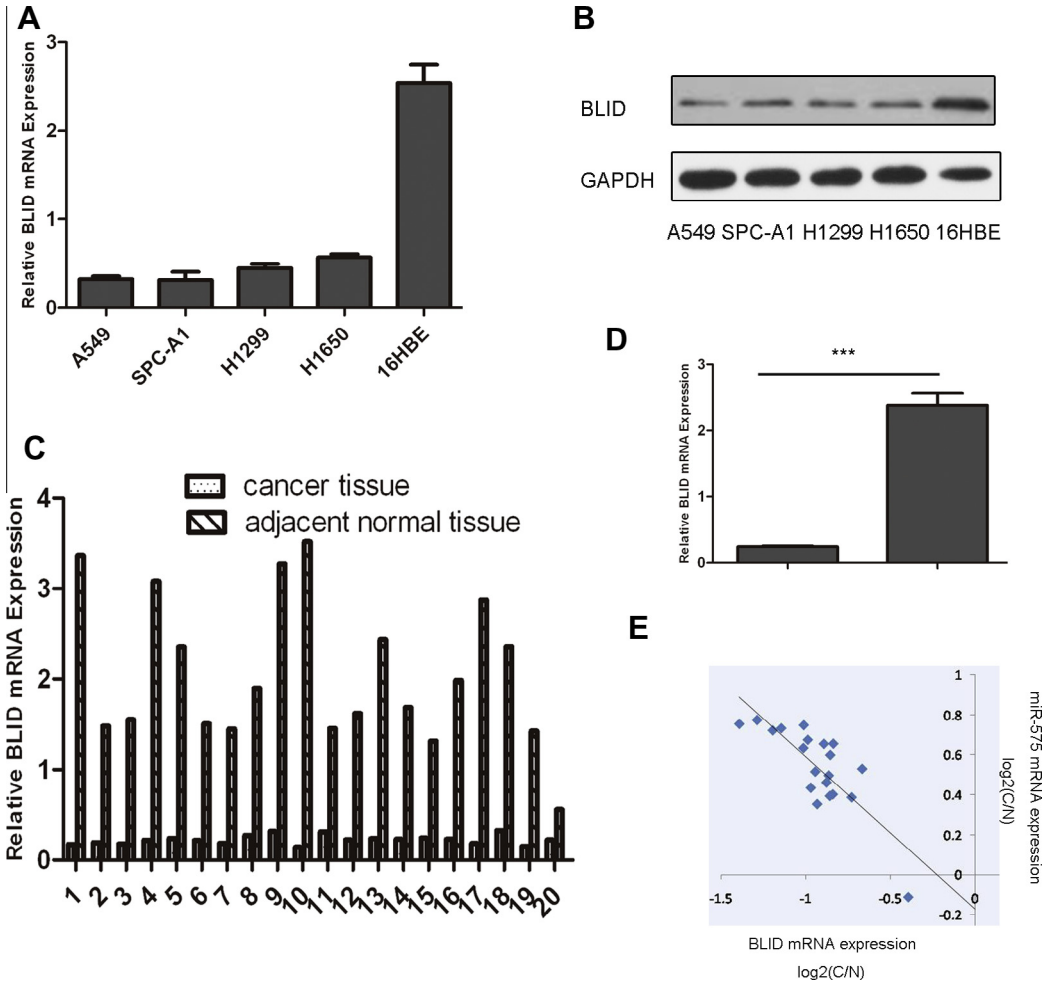

Fig. 5. BLID was inversely expressed with miR-575 in NSCLC. (A) The BLID relative mRNA expression levels were determined by qRT-PCR in Human NSCLC cell lines (A549, SPC-A1, H1299 and H1650) and normal bronchial epithelial cell line 16HBE. (B) Western blot analysis of BLID expression in Human NSCLC cell lines (A549, SPC-A1, H1299 and H1650) and normal bronchial epithelial cell line 16HBE. (C) qRT-PCR analysis of BLID expression in 20 pairs NSCLC tissues and their corresponding adjacent normal lung tissues. The expression of BLID was normalized to GAPDH. (D) Expression of BLID in NSCLC tissues was lower than that in adjacent normal lung tissues. (E) Analysis of correlation of miR-575 and BLID expression in NSCLC tissues. (Two-tailed Pearson's correlation analysis, $r = -0.83$; $P < 0.01$). Data was presented as log 10 of fold change of NSCLC tissues relative to non-tumor adjacent tissues.

group as compared with control and scrambled group. These results strongly indicated that miR-575 regulated BLID in NSCLC. BLID (BH3-like motif containing, cell death inducer) was first reported in 2004 in MDA-MB 231 human breast cancer cells [26,27]. Previous studies showed that BLID acted as a new tumor suppressor and inhibited breast cancer cell growth and metastasis via downregulating AKT pathway [28]. Also, BLID was an independent prognostic factor for overall survival and distant metastasis-free survival [29,30]. All these data strongly support a role for BLID as a new tumor suppressor gene. However, the expression and role of BLID in NSCLC remain to be determined. In this study, we indicated that the expression of BLID was lower in NSCLC tissues than in matched normal lung tissues. In addition, restored expression of BLID suppressed cell proliferation and invasion in NSCLC cells. However, the underlying mechanisms are still unclear. For NSCLC, we first demonstrated that miR-575 was a new modulatory regulator of BLID expression in NSCLC, providing one potential mechanism for role of miR-575 in NSCLC development.

5. Conclusion

In conclusion, we demonstrated that upregulated miR-575 was a common event in NSCLC and might function as an oncogene by directly targeting BLID. To the best of our knowledge, this is the first study to demonstrate that the miR-575/BLID axis regulates the proliferation, migration and invasion of NSCLC cells. These findings provide a better understanding of the pathogenesis and development of NSCLC. The miR-575/BLID axis may provide an important target for future therapy of the NSCLC.

6. Competing interests

The authors declare that they have no competing interests.

7. Authors' contributions

Conceived and designed the experiments: Hongwei Wang, Chunhua Yan, Xiaodong Shi, Jiaolin Zheng, Lili Deng, Lei Yang, Fangfei Yu, Yuxia Shao, Yuandi Yang. Performed the experiments: Hongwei Wang, Chunhua Yan. Analyzed the data: Hongwei Wang, Chunhua Yan. Contributed reagents/materials/analysis tools: Yuxia Shao. Wrote the paper: Hongwei Wang, Chunhua Yan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.02.013.

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