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Research article

Jaceosidin inhibits the progression and metastasis of NSCLC by regulating miR-34c-3p/Integrin α2β1 axis

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ABSTRACT

Non-coding RNAs are crucial for cancer progression, among which miR-34c-3p has been demonstrated to be a tumor suppressor in non-small cell lung cancer (NSCLC). In this study, we attempt to identify flavonoids that can up-regulate miR-34c-3p expression, evaluate the anti-cancer activity of the flavonoids and explore its underlying mechanism in NSCLC cells. Six flavonoids were screened by RT-qPCR and we found that jaceosidin significantly increased miR-34c-3p expression in A549 cells. We found that jaceosidin inhibited the proliferation, migration and invasion of A549 and H1975 cells in a dose-relevant manner, indicated by cell counting kit (CCK-8) assay, wound healing assay, transwell assay and EdU assay, we observed that jaceosidin inhibited the proliferation, migration and invasion of A549 and H1975 cells in a dose-relevant manner. Further research suggested that miR-34c-3p bound to the transcriptome of integrin α2β1 and then inhibited its expression, leading to the inhibitory effect on the migration and invasion of NSCLC. Our study sheds some light on anti-tumor of jaceosidin and provides a potential lead compound for NSCLC therapy.

1. Introduction

Cancer is the most serious hazard to human health, among which pulmonary neoplasm is the major cause of tumor related

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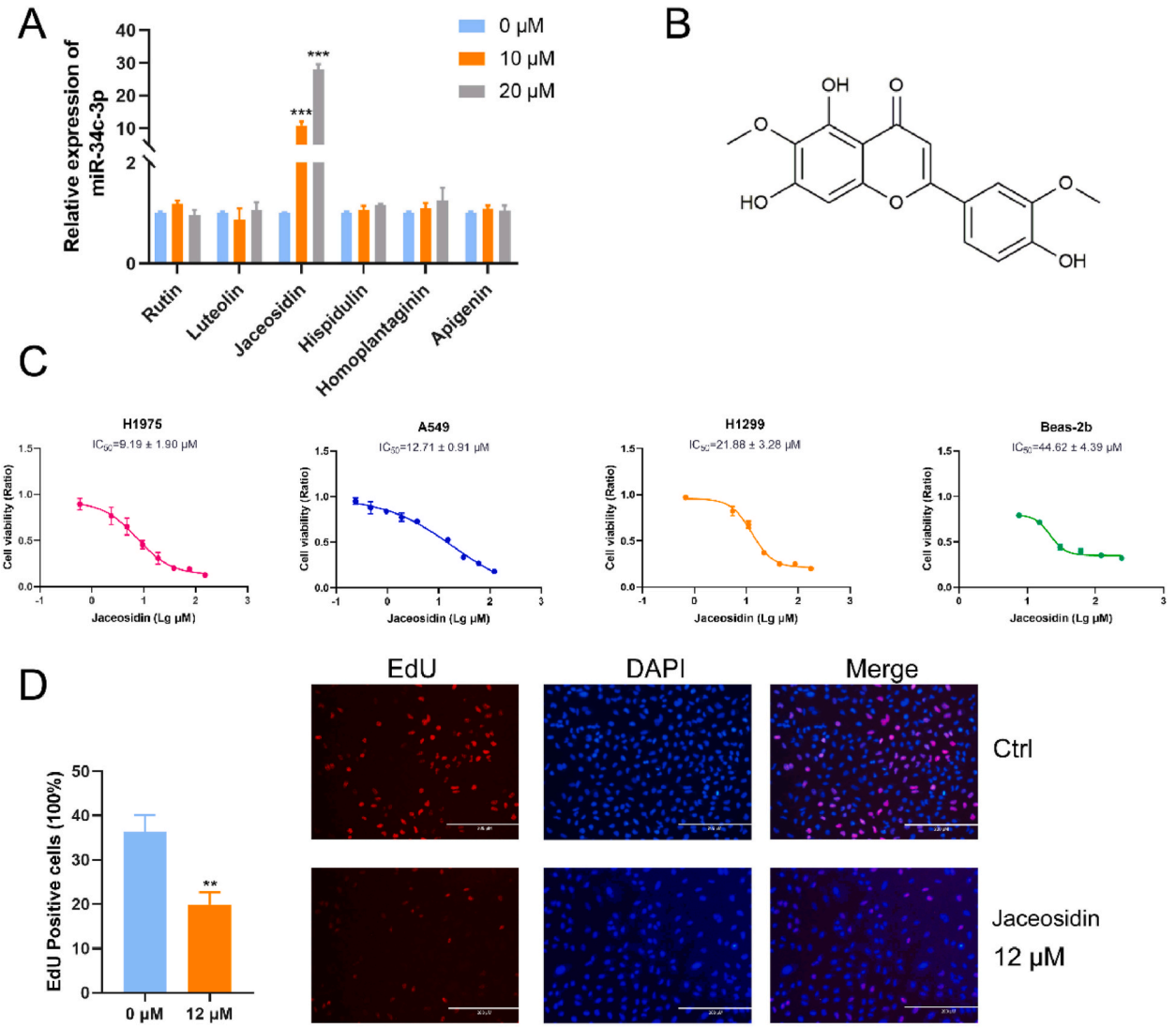

Fig. 1. Jaceosidin increased the miR-34c-3p level and decreases the proliferation in NSCLC cells. A. RT-qPCR assay detected the effect of six components for miR-34c-3p expression. B. The chemical structural formula of Jaceosidin. C. The IC_{50} values of jaceosidin against H1975, A549, H1299, and Beas-2b cells detected by CCK-8 assay. D. Jaceosidin suppressed on the proliferation of A549 cells indicated by EdU assay, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, n ≥ 3.

mortality globally. Non-small cell lung cancer (NSCLC) is a frequent histological subtype of lung cancer that account for approximately 85% of patients [1]. The therapy of NSCLC has made significant breakthroughs in the last few decades. However, obstacles such as drug resistance still seriously affect the efficacy of NSCLC treatment [2,3]. Moreover, high morbidity and mortality of lung cancer have forced a sustained effort to explore more effective treatment. Identifying promising lead compounds from natural products for NSCLC therapy remains an important and effective approach.

Non-coding RNAs assume a significant part in cell development and metabolism, as well as the progression of several disease, such as cancer. MicroRNA (miRNA) is a kind of small, single-stranded and high-conserved non-coding RNA. The miR-34 family is a kind of tumor suppressor miRNA that has been shown to be disordered in various human malignancies [4]. As early as 2007, miR-34 genes, miR-34a as well as miR-34b/c, has been demonstrated to be directly regulated by the tumor suppressor p53 [5–8]. Due to a lack of 5′-phosphate, mature miR-34 has been found to be inactive in cancer cells. However, when cells are stimulated (e.g., DNA damage), these inactive miR-34 are quickly activated by 5′-end phosphorylation [9]. And suppressing miR-34a has been showed to facilitate Epithelial-mesenchymal transition (EMT), invasion and metastasis of colorectal cancer (CRC) [10]. In addition, miR-34c-3p over-expression significantly reduced cell proliferation and induced apoptosis in KRAS mutated lung cancer cells [11].

Our previous study showed that some exosomes derived from NSCLC cell lines with low miR-34c-3p level obviously reduced miR-34c-3p expression in recipient cells and increased the integrin α2 (ITGA2) and integrin β1 (ITGB1) expression, thereby promoting migration and invasion of recipient cells [12]. Based on this, we speculate that the miR-34c-3p overexpression level in NSCLC can

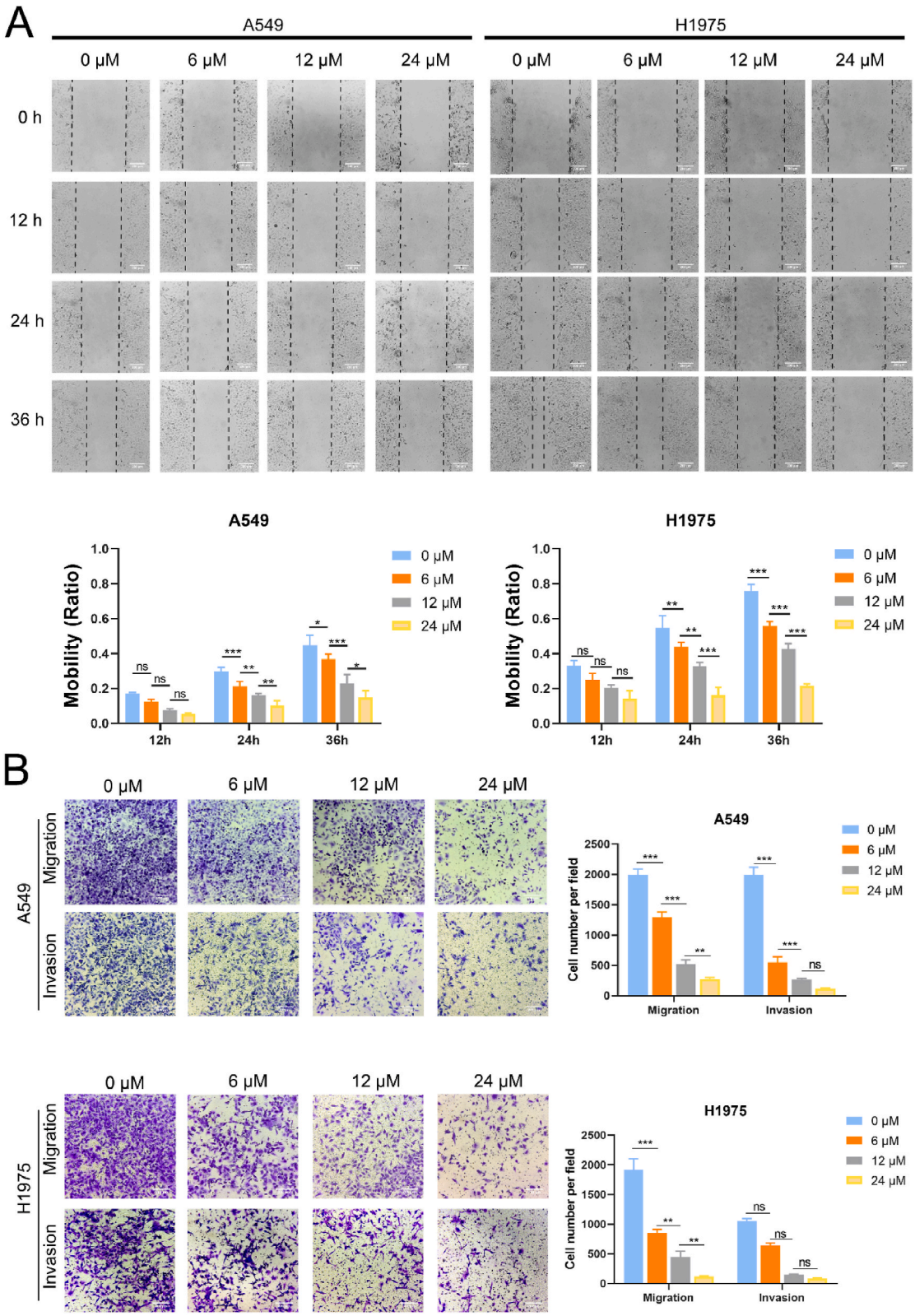

Fig. 2. Jaceosidin inhibited the metastatic ability of NSCLC *in vitro*. A. Jaceosidin inhibited the migration and invasion of A549 and H1975 cells indicated by wound healing assay. B. The inhibitory effect of jaceosidin on A549 and H1975 cells migration and invasion detected by transwell migration and invasion assay. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, n ≥ 3.

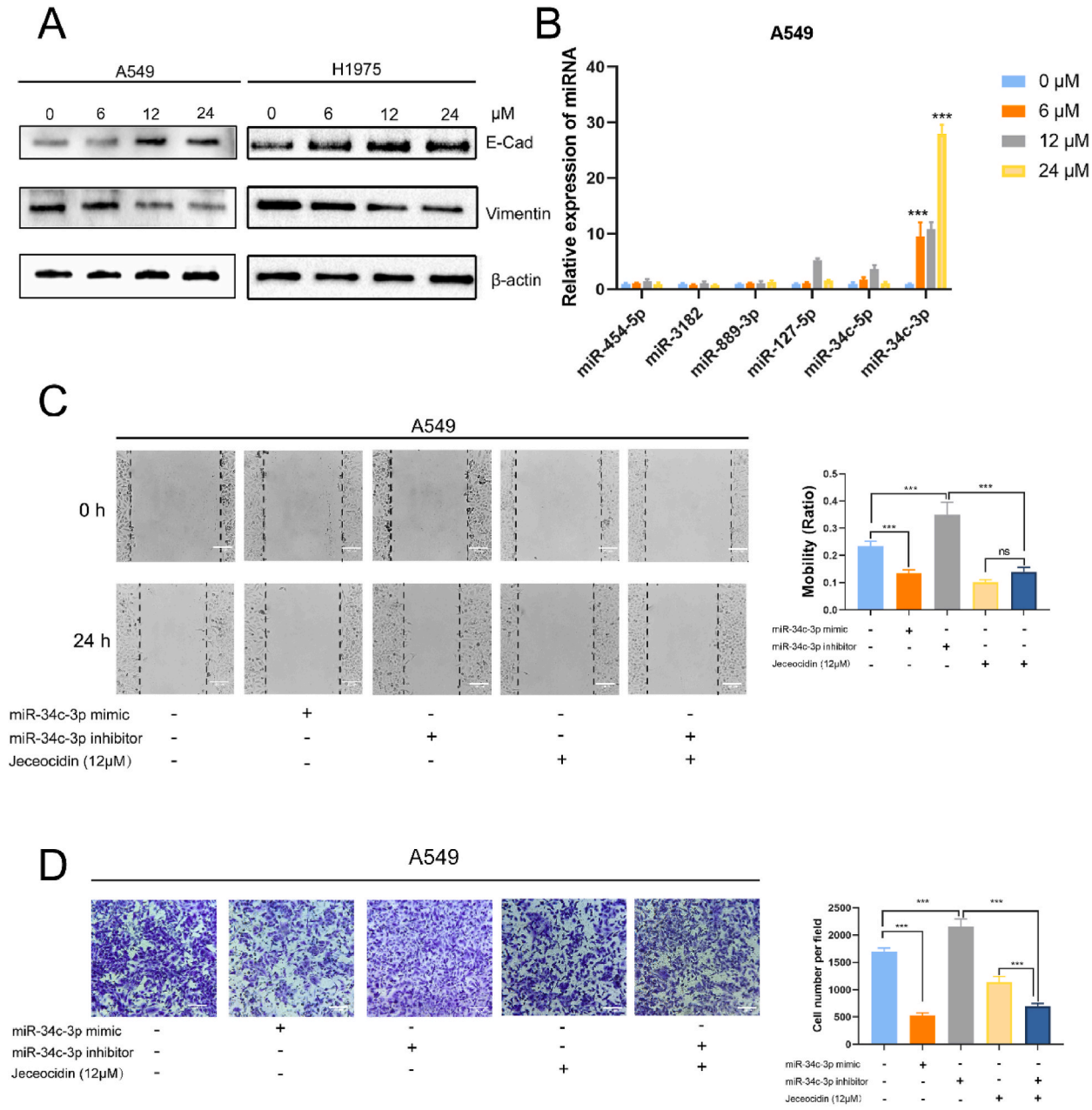

Fig. 3. Jaceosidin regulated the expression of EMT markers and miR-34c-3p *in vitro*. A. The effect of jaceosidin on the expression of EMT markers were regulated in H1975 and A549 cells B. Jaceosidin up-regulated the miR-34c-3p expression in a dose-dependent manner. C/D. Jaceosidin treatment attenuated down-regulated miR-34c-3p expression mediated effect on the migration and invasion of A549 cells, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, n $\geq$ 3. The non-adjusted images of Western blotting were shown in Supplementary Material (Fig. S3).

suppress the metastasis and infiltration of NSCLC cells. Therefore, we sought natural active compounds which can increase miR-34c-3p expression. Flavonoid, a class of natural polyphenol compounds, has been showed with excellent anti-tumor activity in many cancers, such as lung cancer and breast cancer [13]. Flavonoids are anticancer drugs which could inhibit lung cancer, while their molecular mechanism remains to be further explored [14].

Herein, we screen a flavonoid that can upregulate miR-34c-3p level from six flavonoids, including rutin, luteolin, jaceosidin, hispidulin, homoplantaginin, apigenin, and found that jaceosidin significantly increased miR-34c-3p expression in A549 cells [15,16]. CCK-8 assay showed that jaceosidin posted perfect anti-cancer effect *in vitro* and *in vivo*. Further research demonstrated that jaceosidin increased miR-34c-3p levels, and then suppressed the expression of ITGA2 and ITGB1, leading to the inhibitory effect on the migration and invasion ability of NSCLC cells and blockade of its downstream ITGA2B1/FAK/SRC/MMP2/MMP9 signaling pathway. In

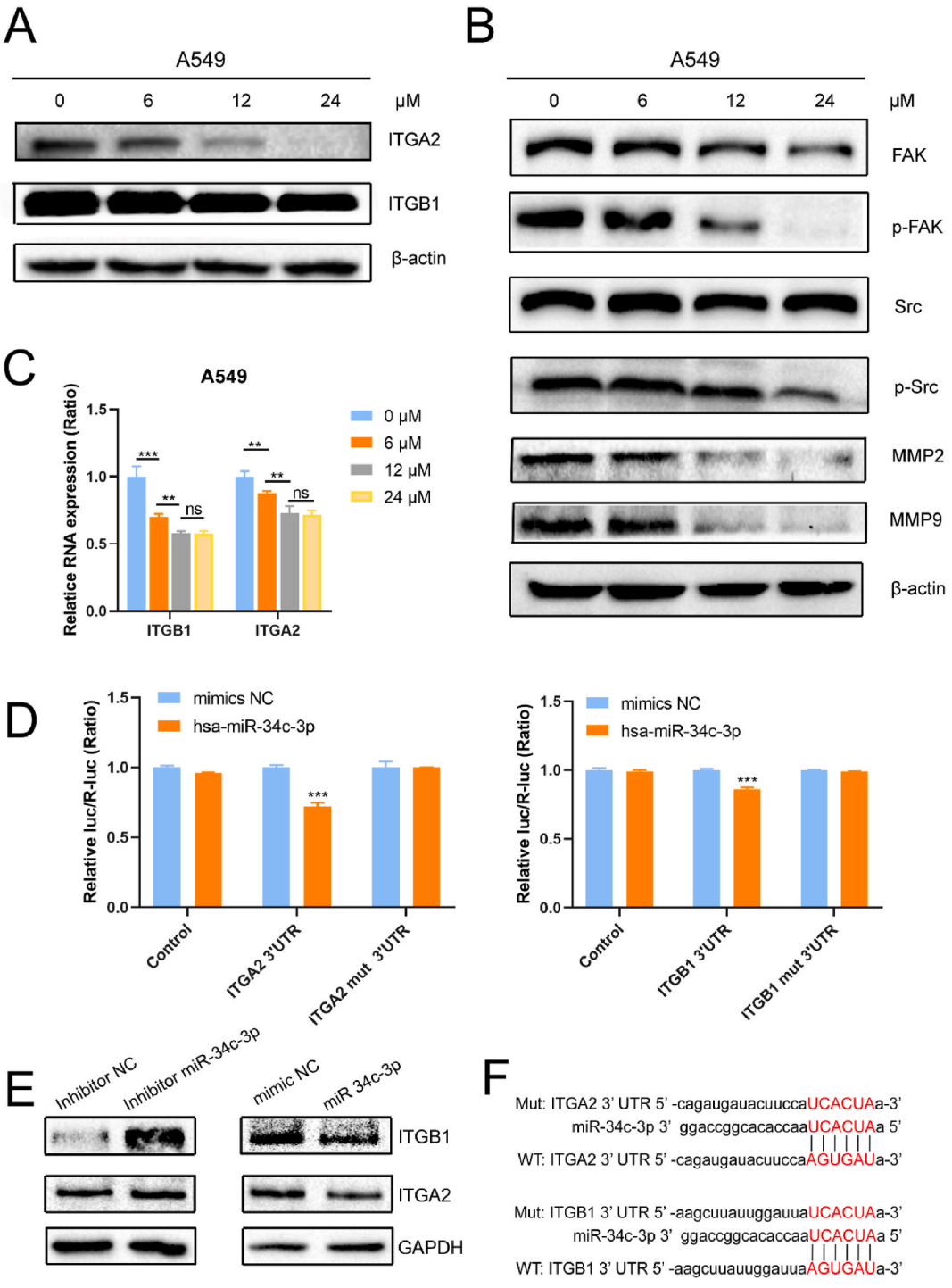

Fig. 4. Jaceosidin suppressed ITGA2 and ITGB1 expression in NSCLC. A. Western blotting assay showed jaceosidin suppressed ITGA2 and ITGB1 expression in A549 cells. B. Jaceosidin down-regulated the downstream pathway-related proteins of ITGA2B1. C. Jaceosidin inhibited mRNA level of ITGA2 and ITGB1 in A549. D. The binding relationship between miR-34c-3p and mRNA of ITGA2 and ITGB1 were verified with dual-luciferase reporter assay. E. MiR-34c-3p negatively regulated the expression of ITGA2 and ITGB1. F. ITGA2B1-WT and ITGA2B1-MUT luciferase reporter plasmids containing miR-34c-3p binding sites were constructed. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, n $\geq$ 3. The non-adjusted images of Western blotting were shown in Supplementary Material (Figs. S4–S6).

conclusion, our study suggests that jaceosidin is a potential lead compound for NSCLC treatment, provides some conducive clues for the development of NSCLC therapy strategies.

2. Results

2.1. Jaceosidin increased miR-34c-3p expression and inhibits the proliferation of NSCLC cells

We selected six potential flavonoids by reviewing the literature, including rutin, luteolin, jaceosidin, hispidulin, homoplantaginin and apigenin to evaluate their effect on miR-34c-3p [15,16]. RT-qPCR assay showed that only jaceosidin could significantly upregulate the miR-34c-3p in A549 cells (Fig. 1A). Jaceosidin (Fig. 1B) is a bioactive flavone abundant in the traditional medicinal plants of genus *Artemisia* [17]. Jaceosidin has been demonstrated perfect anti-cancer effect in several cancer, such as breast cancer and renal cancer [18,19]. However, whether it can suppress NSCLC is still largely unclear. Therefore, we conducted CCK-8 assay to estimate its effect on the proliferation of three NSCLC cell lines and BEAS-2B cells. The result showed that jaceosidin inhibited the proliferation of H1975, A549, H1299 and Beas-2b with an IC_{50} value of 9.19 ± 1.90 μM, 12.71 ± 0.91 μM, 21.88 ± 3.28 μM and 44.62 ± 4.39 μM, respectively (Fig. 1C). This result indicated that jaceosidin had a potent inhibition toward NSCLC cells and its effect on normal cells was inconspicuous. And EdU assay demonstrated that jaceosidin markedly inhibited proliferation of A549 cells (Fig. 1D).

2.2. Jaceosidin inhibited the metastasis of NSCLC in vitro

We further evaluated the effect of jaceosidin in NSCLC cells migration and invasion using wound healing assay and transwell migration and invasion assay. The wound healing assay showed that jaceosidin treatment significantly inhibited the migration of A549 cells and H1975 cells in a dose-dependent manner. The mobility of A549 cells in 0, 6, 12 and 24 μM group were 17.18 ± 0.55%, 12.31 ± 1.39%, 7.54 ± 0.86% and 5.42 ± 0.63% in 12 h; 29.8 ± 2.34%, 21.3 ± 2.77%, 16.08 ± 1.06% and 10.12 ± 2.83% in 24 h; 44.87 ± 5.67%, 36.65 ± 3.03%, 22.66 ± 5.32% and 14.91 ± 3.70% in 36 h, respectively. For H1975 cells, the mobility with increasing administration concentration were 32.94 ± 3.07%, 24.97 ± 3.68%, 20.43 ± 1.59%, 14.14 ± 4.50% in 12 h; 54.50 ± 7.23%, 43.81 ± 2.58%, 32.59 ± 2.34%, 16.08 ± 4.63% in 24 h; 75.78 ± 3.87%, 55.79 ± 2.56%, 42.90 ± 2.78%, 21.64 ± 0.97% in 36 h, respectively (Fig. 2A); Similar results were observed in transwell migration and invasion assay, the migration and invasion cells were obviously decreased after jaceosidin treatment (Fig. 2B). These results suggested that jaceosidin significantly inhibited the migration and invasion of A549 and H1975 cells in a dose-relevant manner.

2.3. Jaceosidin regulated the expression of EMT markers and miR-34c-3p in vitro

Western blotting detected whether jaceosidin affect the expression of epithelial-mesenchymal transition markers. The Western blotting assay showed that jaceosidin promoted E-cadherin expression and inhibited Vimentin expression in H1975 and A549 cells in a dose-dependent manner (Fig. 3A). RT-qPCR assay indicated that jaceosidin up-regulated miR-34c-3p expression in a dose-relevant manner (Fig. 3B). Subsequently, we found that miR-34c-3p overexpression inhibited A549 cell migration and invasion, while suppressing miR-34c-3p expression had opposite effect. Moreover, we also found that jaceosidin treatment obviously attenuated low-expressed miR-34c-3p mediated stimulative effect on the migration and invasion ability (Fig. 3C & D). These results indicated that jaceosidin inhibited the EMT and migration and invasion of NSCLC cells, and this effect might be associated with its effect on miR-34c-3p.

2.4. ITGA2 and ITGB1 were direct targets of miR-34c-3p

Our previous research found that ITGA2 and ITGB1 were direct targets of miR-34c-3p in exosomes, we further evaluated whether jaceosidin affect ITGA2 and ITGB1 expression using Western blotting assay and RT-qPCR assay. The result of Western blotting assay showed that jaceosidin obviously inhibited the ITGA2 and ITGB1 expression in A549 cells (Fig. 4A). Moreover, jaceosidin also affected the expression of downstream pathway of FAK, SRC, MMP2 and MMP9 (Fig. 4B). Besides, the mRNA expression level of ITGA2 and ITGB1 were also inhibited by jaceosidin (Fig. 4C). Next, we performed dual-luciferase reporter assay to confirm whether ITGA2 and ITGB1 are the direct targets of miR-34c-3p in NSCLC. ITGA2B1-WT and ITGA2B1-MUT luciferase reporter plasmids containing miR-34c-3p binding sites were constructed (Fig. 4F). Interestingly, miR-34c-3p consistently reduced the luciferase activity for the 3′ UTRs of ITGA2 and ITGB1 (Fig. 4D), which indicated that ITGA2 and ITGB1 were direct targets of miR-34c-3p. Furthermore, Western blotting assay showed that miR-34c-3p overexpression could down-regulate the ITGA2 and ITGB1 level, while miR-34c-3p knockdown resulted in opposite effect (Fig. 4E). These results validated the interaction of miR-34c-3p with ITGA2 and ITGB1.

2.5. Jaceosidin inhibited tumor growth on the xenograft model of A549 cells

We established the xenografts model by subcutaneously injecting A549 cells in Balb/c-nude m The result showed that jaceosidice to further evaluate the anti-tumor effect of jaceosidin. Nude mice were randomly allocated to including control group, jaceosidin (25 mg/kg) group and jaceosidin (50 mg/kg) group. The mice were intraperitoneally injected with 200 μL 0.5% CMC-Na +1% DMSO, 25 mg/kg and 50 mg/kg jaceosidin for seven consecutive days after the tumor volume reached 100 mm^3. After jaceosidin administration, representative images and weight changes of nude mice in different concentration groups were recorded (Fig. 5A & B and Fig. 6). The

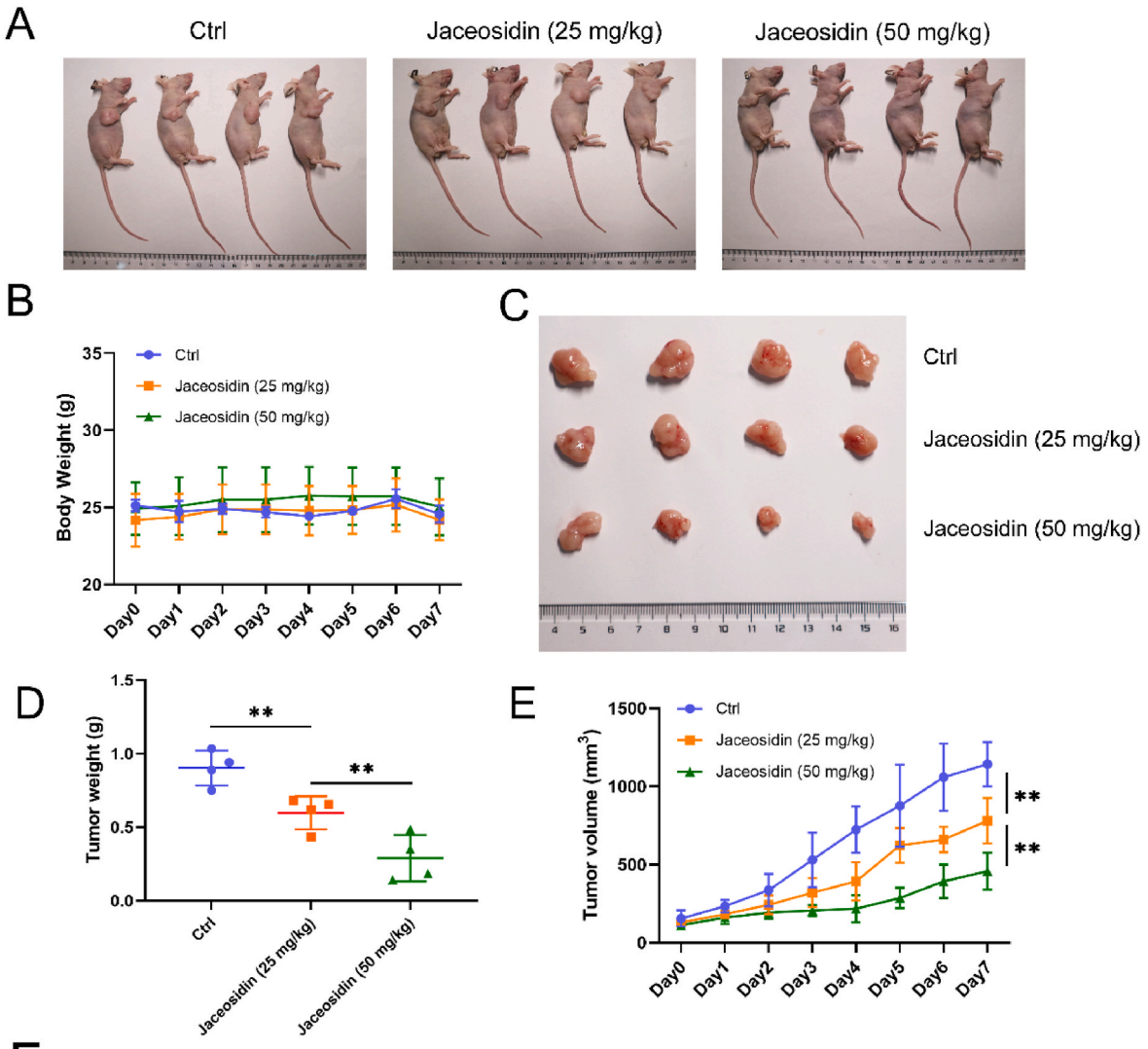

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Fig. 5. Jaceosidin inhibited tumor growth on the xenograft model of A549 cells. A. After jaceosidin administration, representative images of nude mice in different concentration groups were displayed. B. The body weight curve of nude mice was shown. C. The image of tumor was displayed. D. Tumor weight statistics was presented. E. The tumor volume curves of were shown. F. The images of HE staining and immunohistochemical assay (The scale for large figures are 200 μm and the scale for small figures are 500 μm). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$, n ≥ 3.

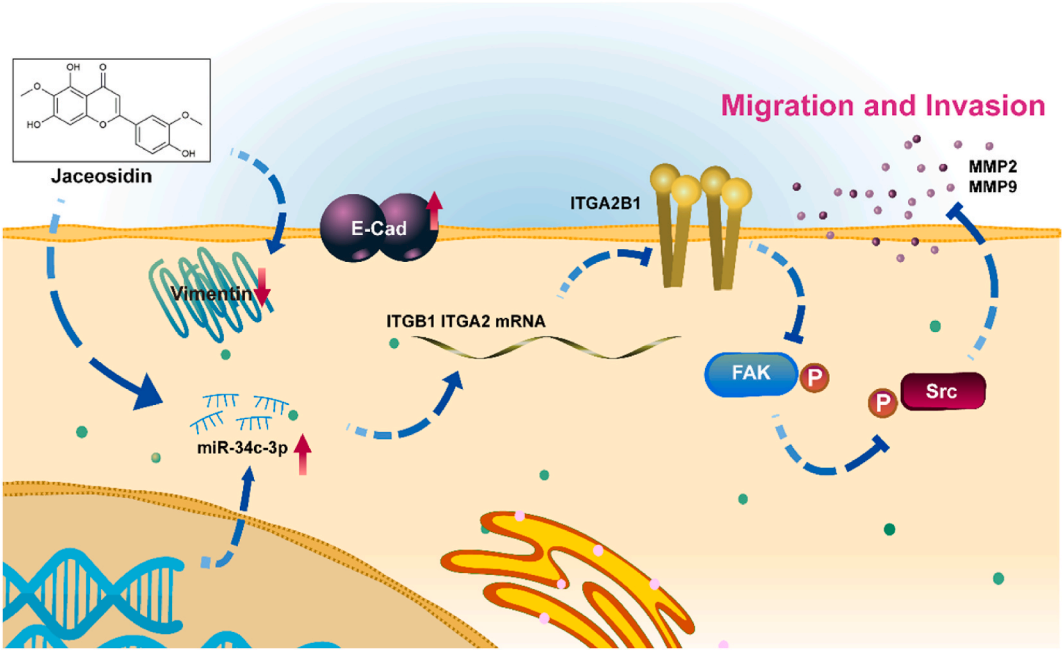

Fig. 6. The graphic abstract of this study.

result showed that jaceosidin administration obviously inhibited the tumor growth, indicated that the tumor volume in jaceosidin group was obviously decreased when compared with that in control group (Fig. 5C, D & 5E). And HE staining and immunohistochemistry showed that jaceosidin up-regulated E-cadherin expression and down-regulated the expression of Vimentin and ITGA2B1 in tumor tissues (Fig. 5F).

3. Discussion

Despite great strides have been made in the treatment of cancer, malignant tumor remains the main source of death all over the world. Among them, lung cancer has become the major type of cancer that threats human health. Albeit extraordinary headway has been made in the therapy of lung cancer, chemotherapy resistance is still a significant obstruction. Consequently, it is urgent to develop new therapeutic pharmaceuticals. With the discovery of a variety of active ingredients and their pharmacological effects, natural products have been widely used in clinical practice. Flavonoids, derived from many natural plants, have been found to regulate various diseases including cancer. For instance, acacetin has been found that it could induce mitochondrial superoxide generation, DNA damage and reactive oxygen species (ROS) generation in breast cancer, and induce necroptosis [20]. Li's research points out that quercetin inhibits the proliferation, vitality and EMT of nasopharyngeal carcinoma (NPC) and enhances the sensitivity of NPC to cisplatin, and this effect may be caused by its inhibition effect on Yes-associated protein expression [21]. Wei's review discussed flavonoids monomer compounds with anti-lung cancer effects, including isoorientin, luteolin, etc [22]. Flavonoids have shown amazing effects in anti-tumor effect in lung cancer, whereas their molecular mechanisms need to be further explored.

miRNA is a single-stranded non-coding RNA molecule involved in the regulation of post-transcriptional gene expression. miRNAs can play an important role in tumor progression by binding to 3′ UTR of mRNA to suppress expression [23]. The miR-34c-3p discussed in this study has been found to involve in variety diseases. For example, Jiang' research pointed out that, the exosomes derived from lung that with low miR-34c-3p level obviously promoted the proliferation and migration of mesenchymal stem cells (MSCs), thus alleviating phosgene-induced lung injury [24]. In silica-induced pulmonary fibrosis-related EMT progression, miR-34c-3p overexpression inhibited EMT process and eliminated pulmonary fibrosis [25]. Besides, miR-34c-3p can be used as markers of tumor development and regulate tumor progression. In oral squamous cell carcinomas, miR-34c-3p inhibits the proliferation of cancer cells by targeting SCL7A11, and then induce erastin-induced ferroptosis [26]. MiR-34c-3p is low expressed in osteosarcoma (OS) tissues, and exerts as a tumor inhibitor by regulating the MARCKS expression [27]. However, the detailed mechanism of miR-34c-3p in progression and metastasis of NSCLC is still largely unclear. Our previous study found that low miR-34c-3p level in exosomes increased

the expression of ITGA2 and ITGB1 in NSCLC, thereby promoting NSCLC progression [12]. Based on this conclusion, we attempted to find natural products that can up-regulate miR-34c-3p level and explore its underlying mechanism. Consequently, we screened the flavonoid compounds including rutin, luteolin, jaceosidin (Fig. 1B), hispidulin, homoplantaginin, apigenin. Among these six compounds, only jaceosidin showed significantly effect on up-regulating the miR-34c-3p level in A549 cells (Fig. 1A). Subsequently, we used CCK-8 assay to detect the cytotoxicity of jaceosidin, the result displayed that jaceosidin could inhibit the proliferation ability with an IC_{50} value of 9.19 ± 1.90 μM, 12.71 ± 0.91 μM, 21.88 ± 3.28 μM and 44.62 ± 4.39 μM for H1975, A549, H1299 and Beas-2b cells (Fig. 1C). Likewise, EdU assay verified the anti-proliferation activity of jaceosidin with 12 μM for A549 cells (Fig. 1D). Our results showed that jaceosidin had obviously inhibitory effect on the proliferation of NSCLC cells and its effect on normal cells was weak. Moreover, we also confirmed the inhibitory effect of jaceosidin on the migration and invasion of A549 and H1975 cells (Fig. 2A & B).

EMT is a specific procedure that epithelial cells acquire a mesenchymal phenotype and transform into mesenchymal phenotypic cells, becoming motile and exerting a crucial role in tumor metastasis [28]. After jaceosidin administration, E-cadherin expression was up-regulated while Vimentin was down-regulated (Fig. 3A). Besides, we found that jaceosidin could significantly increase miR-34c-3p level of A549 cells but has no significant effect on other miRNAs (Fig. 3B). Interestingly, the migration and invasion ability caused by the miR-34c-3p down-regulation could be reversed by jaceosidin (Fig. 3C & D). In addition, the tumor growth could be impeded by jaceosidin (Fig. 5C, D & 5E). Representative images of nude mice and body weight changes were recorded after jaceosidin administration (Fig. 5A & B). HE staining and immunohistochemical analysis showed that jaceosidin obviously up-regulated E-cadherin expression while down-regulated Vimentin expression in tumor tissues (Fig. 5F). Our results indicated that jaceosidin inhibited the progression and metastasis of NSCLC by regulating the expression of EMT markers and miR-34c-3p.

To better explore the mechanism of jaceosidin in NSCLC, we performed the following experiments. First, we used Western blotting assay to uncover the molecular mechanism of jaceosidin. We found that jaceosidin significantly down-regulated ITGA2 and ITGB1 expression, and FAK, SRC, MMP2 and MMP9 expression were also reduced after jaceosidin treatment (Fig. 4A & B). Accordingly, the mRNA level of ITGA2 and ITGB1 were also interfered by jaceosidin (Fig. 4C). Second, dual-luciferase reporter assay showed that ITGA2 and ITGB1 are direct targets of miR-34c-3p, and we have also verified the interaction between miR-34c-3p and ITGA2B1 (Fig. 4D & E). As a transmembrane protein that connects cancer cells and microenvironment in series, integrin can mediate tumor metastasis and drug resistance [29]. It indicated that miR-34c-3p exerted its anti-tumor activity by inhibiting ITGA2 and ITGB1. Taken together, we speculate that jaceosidin inhibits the progression of NSCLC by regulating miR-34c-3p/Integrin α2β1 axis.

Taken together, our study showed that jaceosidin inhibited the progression and metastasis of NSCLC by regulating miR-34c-3p/Integrin α2β1 axis. Our study provided evidence for jaceosidin to be developed into potential anti-tumor agent for NSCLC therapy.

4. Materials and methods

4.1. Compound standard

Flavonoid samples used in this study were all purchased from companies. Jaceosidin, hispidulin and homoplantaginin were purchased from Sichuan Weikeqi-biotech company, China. Luteolin and Apigenin were purchased from Chengdu Must Bio-technology company, China. Rutin were purchased from Tianjin Shilan technology company, China. All these compounds were detected by HPLC (SIL-20A, Shimadzu, Japan), chromatographic column: Dikma Diamonsil C_{18} column (250 mm × 4.6 mm i.d., 5 μM). The methods of HPLC for jaceosidin, hispidulin, homoplantaginin, luteolin and apigenin are as follow: 30% acetonitrile, 70% water (0.1% phosphoric acid), 0 min; 60% acetonitrile, 40% water (0.1% phosphoric acid), 30 min. The method for rutin is as follow: 35% acetonitrile, 65% water (0.1% phosphoric acid), 30 min. All samples were dissolved in methanol respectively. The HPLC chromatogram of the flavonoid samples were provided in supplementary material (Fig. S1). The purity of all the compound samples was more than 98%.

4.2. Cell culture and reagents

The human lung cancer cell lines A549, H1975, H1299 and the human bronchial epithelial cell line BEAS-2B (iCell Bioscience Inc, China) were grown in a dampish gaseous environment with 5% CO_2 at 37 °C. The A549, H1299 and H1975 cells were cultured with RPMI 1640 medium (Gibco, Invitrogen, USA), and the BEAS-2B cells were cultured with DMEM medium (Gibco, Invitrogen, USA). The RPMI 1640 and DMEM medium were complemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, USA) and 1% penicillin-streptomycin solutions (TIANHANG Biotech Company, China) [30].

4.3. CCK-8 assay and 5-Ethyny-2′-deoxyuridine (EdU) assay

In terms of the manufacturer's protocol, we conducted CCK-8 kits (Dojindo, Japan) to detect the cell viability in terms of the manufacturer's protocol. In brief, cells were plated in 96-well plates (4×10^3 cells/well) and treated with different concentration jaceosidin. After treating jaceosidin for 72 h, cells were co-cultured at 37 °C for 1–2 h with CCK-8 solution. The absorbance was determined with wavelength at 450 nm using Bio-Rad Laboratories Model 550 Microplate reader [31]. According to the concentration-response curve, the IC_{50} value was obtained.

For EdU assay, cells were plated in 24-well plates and treated with jaceosidin (12 μM). After treating jaceosidin for 24 h, EdU (Sangon Biotech, China) was conducted basing on the manufacturer's instructions and photographs were taken using fluorescence microscope [32]. The fluorescence intensity was observed, and the positive cell rate was counted.

4.4. Wound healing assay

The A549 and H1975 cells were seeded in 6-well plate and cultured until cells density was about 100%. The monolayer cells were scratched evenly using a pipette tip to create three liner wounds. And then the cells were treated with or without different concentration of jaceosidin (0, 6, 12, 24 μM). At each specific point in time (0, 12, 24, 36 h), microscope was used to capture images and observe the wound healing. The percentage of wound healing area represented the motility of cells [33]. The calculation formula of cell motility: Cell motility rate (%) = wound healing area/total surface area × 100%.

4.5. Transwell assay

The transwell assay was performed to determine the metastatic potential of cells. For transwell invasion assay, we used basal medium to dilute Matrigel (Corning Costar, Corning, USA) at a ratio of 1:4 and pre-coating at the upper transwell contraption chamber. For migration transwell assay, there is no need to pre-coated Matrigel. Cells were cultured in the upper transwell contraption chamber at densities of 4×10^4 cells/well. After incubating for 24 h, the cells were fixed with methanol (General-reagent, China) for 1–2 h, stained with 0.5% (w/v) crystal violet (Beyotime Biotech, China), and then the cells that on the top layer were gently wiped away [34].

4.6. RNA isolation and real-time quantitative PCR (RT-qPCR)

We used RNA extracted kit (Solarbio, China) to extract total RNA in cells and PrimeScript™ RT Master Mix (Takara, Japan) to synthesize cDNA from mRNA. Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan) was used to prime reverse transcription from miRNA into cDNA. RT-qPCR assay was subsequently executed by the TB Green® Premix Ex Taq™ II (Takara, Japan), and detected Ct values by LightCycler480 System. GAPDH and U6 were served as endogenous reference of mRNA and miRNA. The fold changes of each group were calculated by the $2^{-\Delta\Delta CT}$ formula [35]. The primers used in the study was displayed in Table S1.

4.7. Transient transfection

For RNA transfection, TransMate Reagent (Sangon Biotech, China) was used to transfect the cells with miR-34c-3p mimic, inhibitor, and negative control (NC) RNA purchased from Sangong Biotech, Inc. Experimental procedure was performed following with the manufacturer's instructions. The transfection efficiency was detected within 48 h [36].

4.8. Western blotting

To obtain total proteins, cells were hydrolyzed in lysis buffer containing RIPA, PMSF, and phosphatase inhibitors (Beyotime Biotech, China). The concentration of total protein for each sample was quantitatively measured by BCA protein assay kit (Thermo, USA). Protein samples were isolated with SDS-PAGE (Bio-Rad, USA), and later transferred to PVDF membrane (Millipore, USA). And the blots were blocked using 5% of skimmed milk for 1 h, and then incubated in specific primary antibodies for 12–16 h at 4 °C. The blots were then incubated in HRP-conjugated secondary antibodies at ambient temperature. At last, protein signals were detected using ECL solution (Vazyme, China) [37]. The antibodies used in the study were displayed in Table S2.

4.9. Dual-luciferase reporter assay

The wild type of ITGA2 or ITGB1 3′- UTR (ITGA2-WT or ITGB1-WT) and the mutant fragment of ITGA2 or ITGB1 3′-UTR (ITGA2-MUT or ITGB1-MUT) containing the predicted binding site with miR-34c-3p mimic (Tsingke Biotech, China) were subcloned into pmirGLO vector for purpose of constructing the luciferase reporter plasmids. 293T cells were co-transfected with 100 nM miR-34c-3p and 0.5 μg luciferase reporter plasmids (Tsingke Biotech, China). The luciferase activity of all groups were measured by normalization applying dual-luciferase reporter assay system (Promega, USA) 24 h after cell transfection [38].

4.10. Animal experiment

Male BALB/c nude mice (aging from 4 to 6 weeks, 18–22 g) were provided by the HFK Biotechnology company. All animal experimentation was approved by the Institutional Animal Care and Use Committee of Guangzhou Medical University. The A549 cell suspension (1×10^6 cells/200 μL/mouse) containing PBS (Gibco, Invitrogen, USA) and Matrigel was fully re-suspended and injected into the axilla of the mice. The tumor-bearing mice were arbitrarily allocated to three groups. The volume of the tumor was estimated at regular intervals. Drug administration was started when the tumor volume was about 100 mm^3. Full ultrasonic dissolution of the jaceosidin was dissolved into a uniform suspension (1% DMSO + 5% CMC-Na + normal saline) and intraperitoneal injected into mice (200 μL/mouse). The body weight and the tumor volumes were recorded every day. After 7 days of continuous administration, the mice were dissected and the tumors were excised. The tumor was fixed in 4% paraformaldehyde (Biosharp, China) for routine sections preparation. Routine sections were utilized for hematoxylin/eosin (HE) staining (Servicebio, China) and immunohistochemical (IHC) analysis according to the protocol [39]. The antibodies used in the IHC assay including E-cadherin (#3195, CST), ITGA2 (ab181548, Abcam), ITGB1 (ab179471, Abcam) and Vimentin (#5741, CST).

Table 1
Abbreviations used.

Abbreviations	Full Name
NSCLC	Non-small Cell Lung Cancer
miRNA	MircoRNA
CRC	Colorectal Cancer
EMT	Epithelial-Mesenchymal Transition
ITGA2	Integrin α2
ITGB1	Integrin β1
FAK	Focal Adhesion Kinase
MMP2	Matrix Metalloproteinase 2
MMP9	Matrix Metalloproteinase 9
FBS	Fetal Bovine Serum
NC	Negative Control
SPF	Specific Pathogen Free
HE	Hematoxylin/Eosin
IHC	Immunohistochemical

4.11. Statistical analysis

SPSS 25.0 and GraphPad Prism 8 were applied for all statistical analysis. All the data from the experiment were presented as mean ± SD of three or more repetitive except as otherwise specified. The difference between the groups was determined using *t*-test, one-way ANOVA or non-parametric test according to the result of normal distribution. Analysis of correlation was analyzed through Pearson's correlation method. P-value<0.05 was considered to be statistically significant.

Author contribution statement

Qiao-ru Guo: Conceived and designed the experiments; Performed the experiments.
Wen-min Zhou, Xue-ping Lei: Performed the experiments; Analyzed and interpreted the data.
Guo-bin Zhang: Performed the experiments; Wrote the paper.
Zhuo-fen Deng: Contributed analysis data; Analyzed and interpreted the data.
Xin-zhu Chen: Contributed analysis tools; Performed the experiments.
Fang-yun Sun: Analyzed and interpreted the data.
Yan-yan Yan: Analyzed and interpreted the data; Wrote the paper.
Jian-ye Zhang: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Abbreviations

The abbreviations used in this article are presented in Table 1.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e16158.

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