



Licochalcone A Inhibits Proliferation and Metastasis of Colon Cancer by Regulating miR-1270/ADAM9/Akt/NF- κ B axis

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Abstract

Background: We aimed to explore the therapeutic effect and molecular mechanism of licochalcone A (LCA) on colon cancer.

Methods: This study was carried out in 2020-2021 in Nanjing Tongren Hospital, China. Colon cancer HCT116 cells were treated with different concentrations of LCA. Cell counting kit-8, colony formation and flow cytometry assays were used to analyze cell viability, proliferation and apoptosis. Wound healing and transwell experiments were used to measure cell migration and invasion ability. The expression of ADAM9 and apoptosis-related proteins in different LCA treatment groups was detected by western blot. HCT116 cells were transfected with ADAM9 small interfering RNAs (siRNAs) or overexpression vectors. The database screened the upstream miRNA targeting ADAM9 and predicted the targeted binding site between miR-1270 and ADAM9, which was verified by a dual-luciferase reporter assay. Rescue experiments were performed to confirm the effects of the miR-1270/ADAM9 axis on cell proliferation and metastasis.

Results: LCA decreased cell growth ($P<0.05$), migration ($P<0.05$), and invasion ($P<0.05$) of colon cancer cells and inhibited ADAM9 expression in a dose-dependent manner. LCA affected the functions of colon cancer cells by negatively regulating the expression of ADAM9. MiR-1270, increased by LCA, targeted and suppressed ADAM9 expression significantly ($P<0.001$). ADAM9 overexpression restrained miR-1270 mimic and LCA-induced changes in cell proliferation, migration, and invasion, and promoted apoptosis in HCT116 cells significantly ($P<0.01$). LCA and miR-1270 mimic inactivated the Akt/NF- κ B pathway, while ADAM9 overexpression rescued it.

Conclusion: LCA exhibited antitumor efficacy in HCT116 cells by inhibiting the Akt/NF- κ B signaling pathway by regulating the miR-1270/ADAM9 axis.

Keywords: Licochalcone A; Colon cancer; miR-1270; ADAM9; Akt/NF- κ B pathway

Introduction

Colon cancer is a common cancer in the world, with the second highest mortality. The 5-year survival rate in patients with advanced or metastatic disease remains low owing to the early metastasis and high recurrence rate of colon cancer

(1). Therefore, it is urgent to develop more effective treatments for colon cancer.

Licochalcone A (LCA), a novel flavonoid isolated from the roots of *Glycyrrhiza inflata* (licorice), is a characteristic chalcone of licorice (2). The highest



content of LCA in licorice can be up to 10 mg/g, which is much higher than that of other components (3). As an oriental folk medicine, LCA is commonly used to treat gastric ulcers, bronchitis, and sore throat (4). There is substantial evidence showing that LCA exhibits the properties of anti-inflammatory (5), antioxidant (6), antiparasitic (7), and antitumor (8,9). Development in nanobiomedicine shows that nanoparticles and micelles with LCA improve its solubility, bioavailability, and pharmacological activities (10). The above evidence suggests that LCA can be developed into a potential anticancer drug with the advances in pharmaceuticals and biotech medicines.

Interestingly, the molecular mechanism of LCA is different when it is used to treat different types of malignant tumors. LCA exhibits antitumor activity by regulating several signaling pathways (8,9). LCA also suppresses the invasion in glioma cells via the ERK and a disintegrin and metalloproteinase 9 (ADAM9) signaling pathways (9). LCA eliminated programmed cell death ligand-1 (PD-L1) expression by blocking the interaction between p65 and Ras and enhancing the tumor cell-killing activity of cytotoxic T lymphocytes (8). The pathogenesis of colon cancer is extremely complex, which may be the result of the accumulation of polygenic mutations. The ADAM protein family belongs to the zinc protease superfamily, which is a kind of metalloproteinase (11). As transmembrane proteins, ADAMs have cell adhesion and protease activity because they contain both disintegrin and metalloprotease domains (12). ADAM proteins play a regulatory role in tumor progression (9,13). ADAM9, an 84 kDa membrane cell surface protein, mediates cellular adhesion and causes a notable induction of fibroblast cell motility (11). ADAM9 acts as an oncogene in several types of human cancers (14-16). Silencing the ADAM9 gene protects mice from alcohol-induced acute injury in the liver (17). Also, its inhibition suppresses breast cancer cell proliferation by inactivating the AKT/ NF- κ B signaling (14). Recent advances in interactions between non-coding RNAs and ADAM9 reveal that the antitumor efficacy of ADAM9 is regulated by many microRNAs, and lncRNAs (18). Sig-

nificantly, the role of ADAM9 in promoting invasion and 5-fluorouracil resistance in colon cancer has also been reported (19). However, the interaction between LCA-mediated antitumor efficacy and ADAM9 signaling has not been reported in colon cancer.

We aimed to investigate the effect of LCA treatment on ADAM9 expression and the proliferation and metastasis of colon cancer cells, with the involvement of miRNAs regulating ADAM9.

Methods

Cell culture and transfection

HCT116 and SW480 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA) in a humidified incubator at 37°C with 5% CO₂.

The miR-1270 mimics, miR-1270 inhibitor (antisense oligonucleotides of miR-1270), si-ADAM9 (small interfering RNAs of ADAM9), pcDNA3.1-ADAM9 overexpression vector, and all negative controls were designed by GenePharma Co., Ltd. (Shanghai, China). The transfections into the HCT116 cells were carried out by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The specific sequences are as follows: si-ADAM9:

5'-CCITCCAGAGTATTGTGACGGCAA-3', si-NC: 5'-TGCGCTAGGCCTCGGTTGC-3', miR-1270 mimic: 5'-CUGGAGAUUAUGGAAGAGCUGUGU-3', miR-1270 inhibitor: 5'-ACACAGCUCUCCAUAUCUCCAG-3', scrambled sequences of mimics: 5'-UUUGUACUACACAAAAGUACUG-3', and scrambled sequences of inhibitor: 5'-CAGUCCUUUUGUGUAGUACAA-3'.

Clinical samples

Three pairs of colon cancer tissues and adjacent normal tissues were collected from March 2020 to June 2020 in Nanjing Tongren Hospital, China. Three patients have provided written informed consent. Each tissue sample was diag-

nosed as colon cancer by two different professional pathologists. Clinical assays in this study were approved by the Ethics Committee of the Affiliated Hospital of Nanjing University of Traditional Chinese Medicine (2018NL-171-02, Nanjing, China)

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from HCT116 cells or colon cancer tissues using the TRIzol reagent (Omega, USA) and subsequently transcribed to cDNA with the PrimeScript™ RT Reagent Kit (TaKaRa, China). The qRT-PCR analysis of mRNA was carried out by using the SYBR Green qPCR kit (Qiagen, China). The specific primer sequences are as follows: miR-1270 forward: 5'-CTGGAGATATGGAAGAGCT-3', reverse: 5'-CAGTGCGTGTTCGTGGAGT-3'; ADAM9 forward: 5'-GTGTCCGGTGGTTGCTGT-3', reverse: 5'-AATAGGGCCTAGGGGCTTCTC-3'; GAPDH forward: 5'-CTCTGCTCCTCCTGTTTCGAC-3', reverse: 5'-GCGCCCAATACGACCAAATC-3'; and U6 forward: 5'-AACCTTATATCGGGCGGGA-3', reverse: 5'-TTACGGCGATGCATAAT-3'. The relative expression levels of mRNA or miRNA were calculated using the $2^{-\Delta\Delta C_t}$ methods

Cell Counting Kit-8 (CCK-8) Assay

The CCK-8 analysis was carried out to confirm the effect of LCA treatment on the proliferative activity of colon cancer cells. In brief, HCT116 and SW480 cells were seeded in 96-well plates and cultured in a humidified cell culture incubator at 37°C for seven days. The CCK-8 reagent (10 μ l, Keygen, China) was added to each well and cultured for 2 h. The absorbance at 450 nm was detected with a microplate reader.

Flow Cytometry

The level of apoptosis was detected by flow cytometry. HCT116 and SW480 cells treated with transfections and/or LCA for 48 h were digested with trypsin and then collected. Then, the Annexin V-FITC Apoptosis Detection Kit (E. BioSocion, USA) was used to quantify the apoptotic

cells according to the manufacturer's instructions. Subsequently, the cells were stained with propidium iodide and then analyzed with flow cytometry to detect the percentage of apoptotic cells.

Colony Formation Assay

To analyze the effects of LCA on the clonogenic activity of HCT116 cells, we performed a colony formation assay. The cells were seeded on 6-well plates (5×10^3 cells/well) and cultured at 37 °C for 14 days, with replaced fresh medium every other day. The cells were then stained with 0.1% crystal violet dye (Beyotime, China). The number of colonies was counted with an inverted microscope.

Wound healing assay

A wound-healing assay was carried out to evaluate the migration ability of HCT116 cells. The cells were inoculated into 96-well plates and cells were washed when the fusion degree was approximately 80%. The artificial wound was formed on the cell monolayer with a pipette tip (10 μ l). The plates were observed by a microscope after incubating for 48 h, and the wound healing rate was quantified by the ImageJ software (NIH, USA).

Transwell Assay

The invasive ability of the HCT116 cells was examined using Transwell chambers (8 μ m pore size). Briefly, the treated cells (1×10^4) were resuspended and planted into the upper chamber in serum-free DMEM, and 500 μ l of DMEM containing 10% FBS was added to the lower chamber. The cells in each group were cultured at 37°C for 48 h. Subsequently, the invaded cells through the pores were fixed and stained with crystal violet (Beyotime). Finally, the invaded cells were counted by a microscope.

Western blot analysis

The HCT116 cells in each group were lysed with cell lysates containing proteinase inhibitors (Gibco). The protein concentration was measured with a BCA protein analysis kit (Beyotime), loaded into SDS-PAGE gel, and transferred to PVDF membranes (MILI Bay, Massachusetts). The

membrane was sealed using 5% skimmed milk (Gibco) and the recovered proteins were analyzed by immunoblotting with primary antibodies and HRP-bound secondary antibodies. The main antibodies are as follows: ADAM9 (1:1000, ab186833, Abcam, UK); survivin (1:5000, ab76424, Abcam); Bcl-2 (1:1000, ab32124, Abcam); Bax (1:5000, ab32503, Abcam); p-AKT (1:1000, ab38449, Abcam); AKT (1:2000, ab188099, Abcam); p-P65 (1:1000, ab76302, Abcam); P65 (1:2000, ab32536, Abcam); and the internal control GAPDH (1:3000, ab8245, Abcam).

Dual-luciferase reporter assay

The binding relationship of miR-1270 to ADAM9 was analyzed by the Dual-Luciferase Assay Kit (Promega, Madison). To synthesize luciferase reporter plasmids, the sequences of ADAM9 and its mutant sequences were inserted into the psiCHECK2 vector, namely ADAM9-WT and ADAM9-MUT, respectively. HCT116 cells were co-transfected with miR-1270 mimics, miR-1270 inhibitor, scrambled sequences, and the above luciferase reporter (ADAM9-WT and ADAM9-MUT) using Lipofectamine® 2000. After 48 h of incubation, the luciferase activity was measured by a Dual-Luciferase Reporter Assay System (Promega, Madison).

Statistical analysis

All graphs were statistically analyzed using GraphPad Prism 8.0 (GraphPad Software, USA). All data are presented as the mean \pm standard deviation (SD) and compared by the Student's *t* test (between two groups). $P < 0.05$ was considered statistically significant.

Results

LCA decreases the proliferation and metastasis of colon cancer cells

The chemical structure of LCA is shown in Fig. 1A. The CCK-8 assay demonstrated that LCA could reduce the viability of HCT116 and SW480 cells in a concentration-dependent manner, and

the cell viability was the lowest at the highest concentration of LCA (40 μ M) (Fig. 1B).

LCA treatment notably increased the level of apoptosis of the HCT116 cells in a concentration-dependent manner, as shown by the flow cytometry results that the 40 μ M LCA induced the highest percentage of apoptotic cells compared with the other groups ($P < 0.001$, Fig. 1C). LCA treatment greatly repressed the expression of Survivin and Bcl-2 with increased concentration, but apparently promoted Bax expression, which was highest in the cells treated with 40 μ M LCA (Fig. 1D).

LCA treatment markedly weakened the proliferation, migration, and invasion ability of HCT116 cells in a concentration-dependent manner (Fig. 1E-G). Cells treated with the highest LCA concentration showed the lowest ability of colony formation ($P < 0.001$, Fig. E), wound healing ($P < 0.001$, Fig. F), and invasion ($P < 0.001$, Fig. G) compared with the control cells.

LCA restrains the proliferation and metastasis of colon cancer cells by repressing ADAM9

The expression of ADAM9 decreased gradually with the increase of LCA concentration (Fig. 2A). Both ADAM9 knockdown (si-ADAM9) and LCA treatment greatly inhibited the expression of ADAM9 in HCT116 cells ($P < 0.001$), while ADAM9 overexpression plasmids significantly upregulated ADAM9 expression in LCA-treated cells ($P < 0.001$, Fig. 2B). Furthermore, the decreased cell viability and proliferation levels, as well as the increased apoptotic cell percentage in si-ADAM9-treated cells, were comparable with those in the cells treated with LCA (Fig. 2C-F). Reversely, the overexpression of ADAM9 obviously rescued the proliferation ($P < 0.001$, Fig. 2C), migration ($P < 0.001$, Fig. 2E), and invasion ($P < 0.001$, Fig. 2F) in the HCT116 cells treated with LCA. Also, ADAM9 overexpression suppressed LCA-induced apoptosis of HCT116 cells ($P < 0.001$, Fig. 2D). Moreover, as expected, overexpression of ADAM9 reversed the effects of LCA treatment on HCT116 cell invasion and migration (Fig. 2E-F).

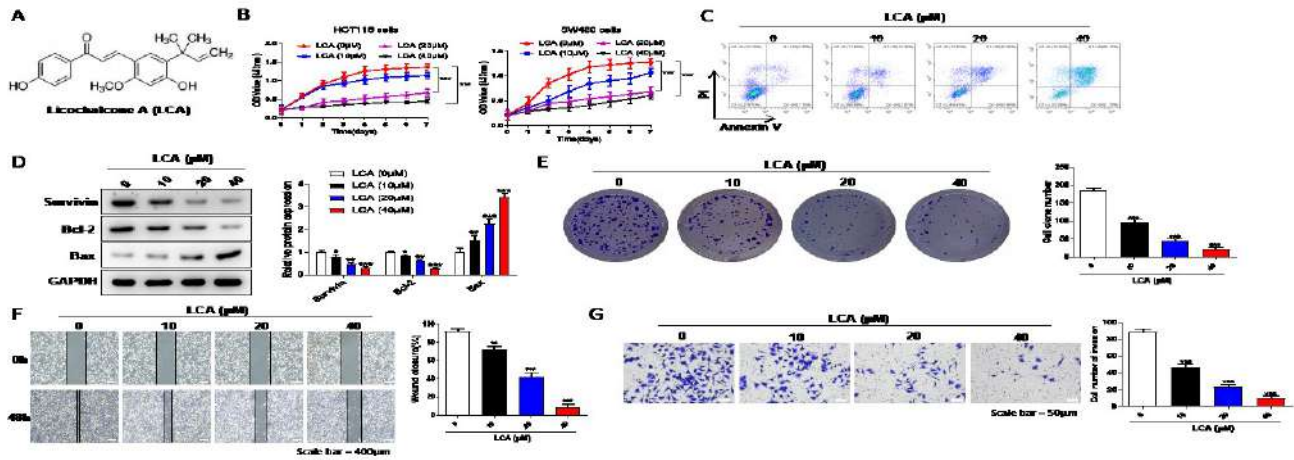


Fig. 1: Licochalcone A (LCA) decreases the proliferation and metastasis of colon cancer cells. (A) The chemical structure of LCA. (B) HCT116 and SW480 cells were treated with LCA at different concentrations of 0, 10, 20, and 40 μ M. Then, cell viability was detected by CCK-8 assay. (C) Apoptosis was detected using flow cytometry. (D) The expression of apoptosis-related proteins was analyzed by western blot. (E) Colony forming experiments, (F) wound healing assays, and (G) Transwell experiments were carried out to measure the cell proliferation, migration and invasion ability, respectively. ** and *** note $P < 0.01$, and 0.001 compared with control, respectively

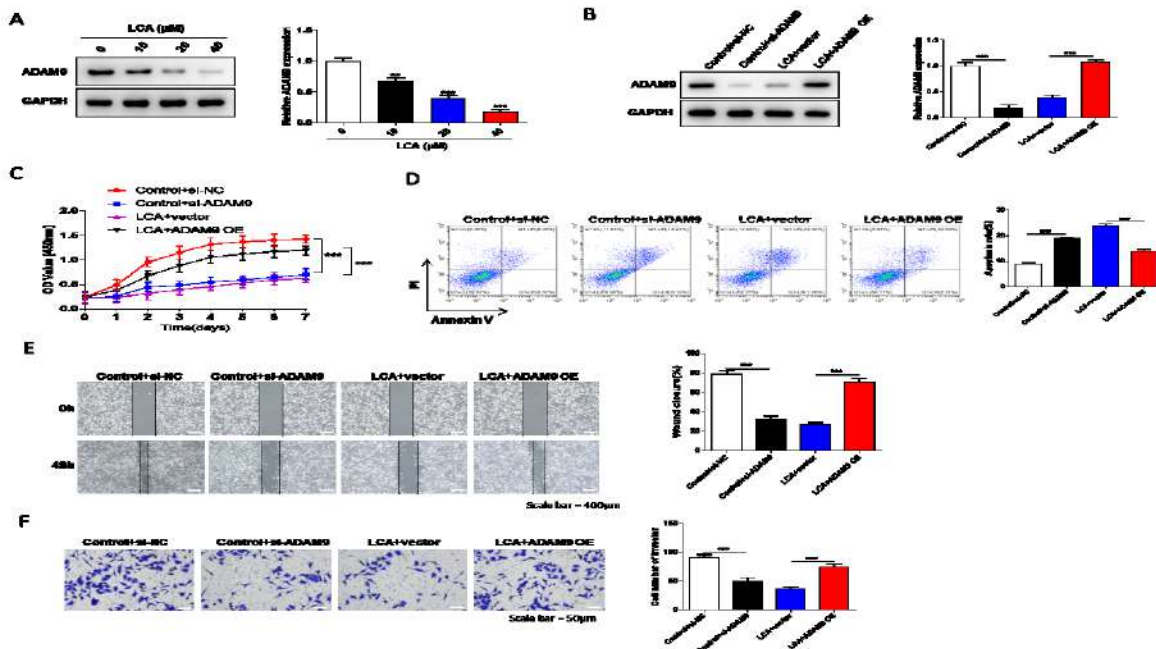


Fig. 2: Licochalcone A (LCA) inhibits the proliferation and metastasis of colon cancer cells by repressing ADAM9 (A) After being treated with LCA at concentrations of 0, 10, 20, and 40 μ M, the expression of ADAM9 in HCT116 cells was detected by western blot. (B-F) HCT116 cells were transfected as follows: Control+si-NC, Control+si-ADAM9, LCA+vector, and LCA+ADAM9 OE (overexpression), and the treatment concentration of LCA was 40 μ M. (B) ADAM9 expression was detected by western blot. (C) The viability and (D) apoptosis of HCT116 cells were analyzed by CCK-8 and flow cytometry, respectively. (E) The wound healing assay was performed to assess the migration ability. (F) Transwell experiment was performed to assess the invasion ability of HCT116 cells in each transfected group. ** and *** note $P < 0.01$ and 0.001 compared with controls, respectively

MiR-1270 targets ADAM9 in colon cancer cells

Five miRNAs were common to the two databases (Fig. 3A and B). The levels of four miRNAs were significantly decreased in the tumor tissues compared with controls ($P<0.001$) and the downregulation of miR-1270 was the most significant (Fig. 3B).

The binding activity of miR-1270 to ADAM9 was predicted through the ENCORI website (Fig. 3C) and was verified using the dual-luciferase reporter gene assay (Fig. 3D). We found that the luciferase activity in the ADAM9 WT group was greatly decreased by the miR-1270 mimic ($P<0.001$) and notably increased with the miR-1270 inhibitor ($P<0.001$), but no obvious changes were observed in the luciferase activity between cells with ADAM9 MUT (Fig. 3D). Western blotting analysis showed that the expression of ADAM9 was prominently restrained by miR-1270 mimic ($P<0.001$), but was obviously enhanced in the miR-1270 inhibitor group ($P<0.001$, Fig. 3E).

Next, we determined that the viability of HCT116 cells in the miR-1270 mimic group was reduced prominently but increased significantly in the ADAM9 overexpression group, and it was notably recovered in the miR-1270 mimic+ADAM9 OE group ($P<0.001$, Fig. 3F). The flow cytometry results showed that the trend of

cell apoptosis was opposite to that of cell viability (Fig. 3G). The migration and invasion abilities of HCT116 cells were markedly weakened in the miR-1270 overexpression (mimic) group ($P<0.001$) and were significantly enhanced in the ADAM9 overexpression group. Simultaneous overexpression of miR-1270 and ADAM9 substantially restored the metastatic ability of HCT116 cells ($P<0.001$ vs oe-ADAM9, Fig. 3H-I).

LCA regulates the miR-1270/ADAM9/Akt/NF- κ B pathway in colon cancer cells

miR-1270 was upregulated in HCT116 cells with increasing LCA concentrations (Fig. 4A). In addition, we found that the phosphorylation levels of the Akt and NF- κ B proteins decreased significantly with LCA concentrations (Fig. 4B). The expression of p-Akt and p-p65 proteins were the lowest in the HCT116 cells treated with 40 μ M LCA.

Overexpression of miR-1270 notably decreased the phosphorylation levels of Akt and NF- κ B ($P<0.01$), while overexpression of ADAM9 enhanced their phosphorylation levels (Fig. 4C). Moreover, simultaneous treatment of miR-1270 mimic and ADAM9 overexpression restored their phosphorylation levels increased by ADAM9 overexpression single ($P<0.01$, Fig. 4C).

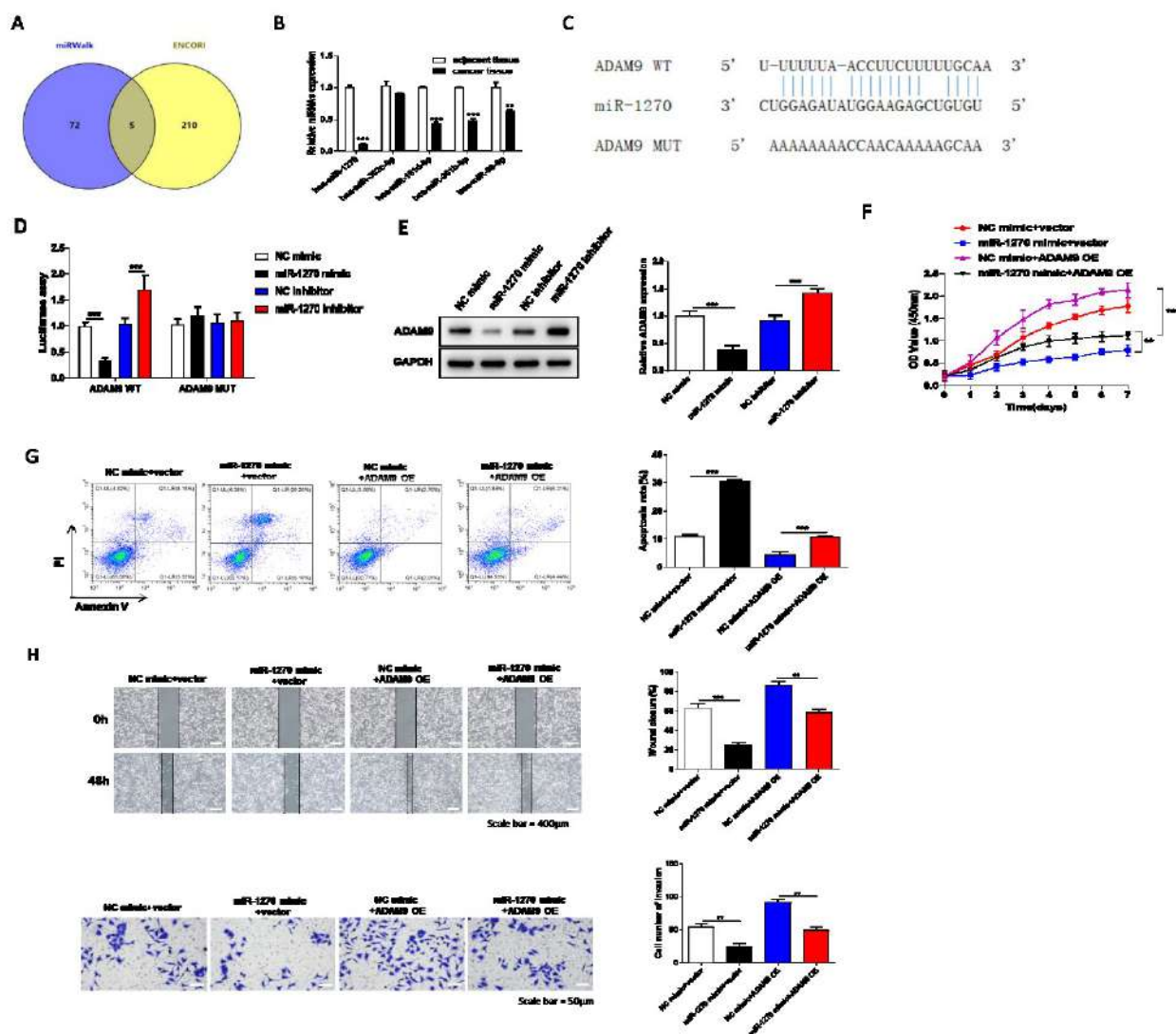


Fig. 3: MiR-1270 targets ADAM9 to regulate HCT116 cell proliferation, migration, apoptosis, and invasion. (A) The upstream miRNAs targeting ADAM9 were screened through the miRWalk and ENCORI websites. (B) The common miRNAs in clinical samples of colon cancer were verified with qRT-PCR. (C) The binding site of miR-1270 and ADAM9 was predicted through the ENCORI website. (D) A dual-luciferase reporter gene assay was used to verify the combination of miR-1270 and ADAM9. (E) ADAM9 expression regulated by miR-1270 was analyzed by western blot. (G-I) HCT116 cells were transfected with miR-1270 mimic, ADAM9 overexpression (OE) vector, and their negative controls, respectively. (F) CCK-8 and (G) flow cytometry experiments were performed to assess the viability and apoptosis of HCT116 cells in different transfected groups, respectively. (H) Wound healing and (I) Transwell assays were performed to detect the migration and invasion of HCT116 cells, respectively. ** and *** note $P < 0.01$ and 0.001 compared with controls, respectively

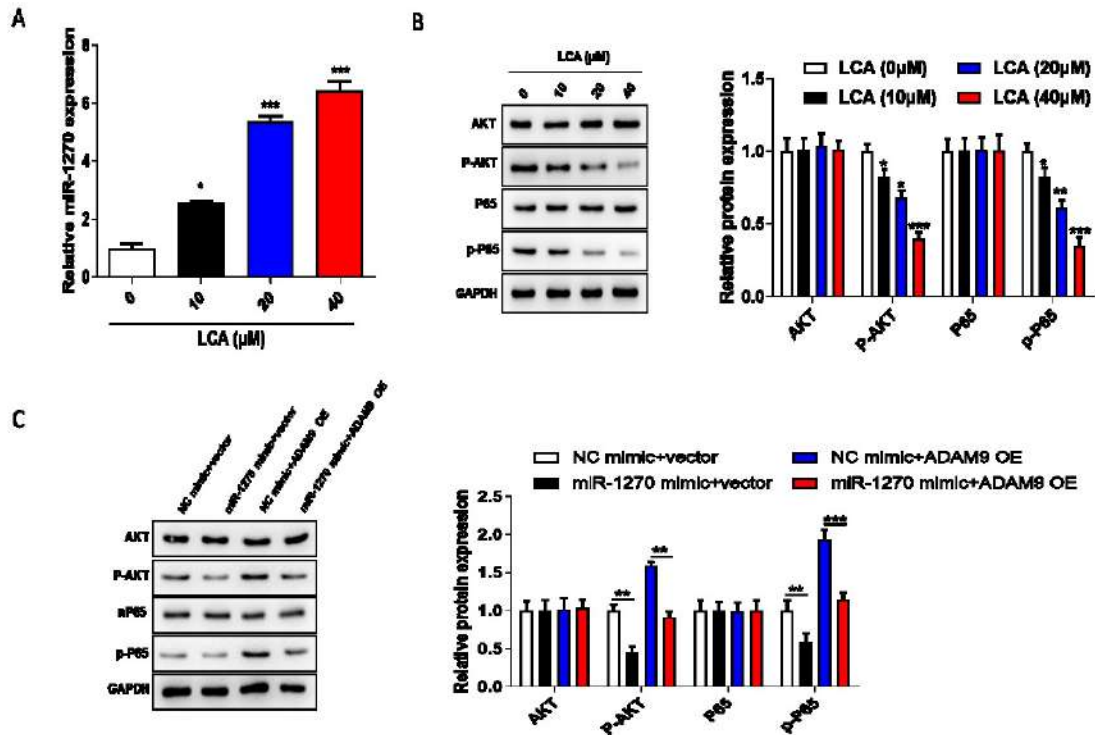


Fig. 4: Licochalcone A (LCA) regulates the miR-1270/ADAM9/Akt/NF-κB pathway in colon cancer cells (A-B) HCT116 cells were treated with LCA at concentrations of 0, 10, 20, and 40 μM. (A) The level of miR-1270 in was quantified by qRT-PCR. (B) The protein expression changes of Akt, p-Akt, NF-κB (P65) and p-NF-κB (P65) in different groups were verified by western blot. (C) HCT116 cells were transfected with miR-1270 mimic, ADAM9 overexpression (OE) vector, and their negative controls, respectively. Then, the protein expressions of Akt, p-Akt, NF-κB (P65) and p-NF-κB (P65) were verified by western blot. *, **, and *** note $P < 0.05$, 0.01 , and 0.001 compared with control, respectively

Discussion

Our present study showed that LCA decreased the proliferation, migration, invasion, and expression of ADAM9. Also, miR-1270 expression and colon cancer cell apoptosis were upregulated upon LCA treatments, in a dose-dependent manner. miR-1270 mimic and LCA-induced similar influences on colon cancer cells, which were significantly restored by ADAM9 overexpression. Also, we found the LCA and miR-1270 inactivated Akt/NF-κB, while ADAM9 activated it. These results showed that LCA suppressed colon cancer cell proliferation and metastasis via regulating miR-1270-ADAM9 and inactivating the ADAM9/Akt/NF-κB signaling pathway.

The antitumor activity of LCA has aroused great interest recently. Reports have indicated that

LCA could inhibit many cancers, including breast cancer (20), and bladder cancer (21). It is worth noting that LCA can regulate tumor cell functions, such as inducing apoptosis, promoting tumor cell cycle arrest, and reducing angiogenesis (22). LCA might induce cell apoptosis through the inactivation of TrxR1 in colorectal cancer cells (23). Herein, we studied the effect of LCA on the viability and apoptosis of the colon cancer cell lines HCT116 and SW480. Notably, our studies confirmed that LCA markedly restrained the proliferation and metastasis of HCT116 and SW480 cells, which was consistent with previous studies about the anti-proliferative, anti-invasive, and anti-migratory effects of LCA in other tumors (24). These results suggested that LCA exhibited antitumor activity in the colon cancer cells.

Digging into the mechanism, including the interaction of signaling pathways, would help to reasonably design new treatment schemes for cancer. ADAM9 is closely associated with aggressiveness in several malignant tumors and acts as an oncogene (9,14-16,25). LCA inhibited the migration and invasive activity of glioma cells through ADAM9 without cytotoxicity (9). Fisetin suppressed the ADAM9 expression and inhibited glioma cell invasion by regulating the ERK signaling pathway (9,26). LCA also suppresses the invasion in glioma cells via the ERK signaling pathway (9). Also, LCA exhibits antitumor activity by regulating the NF- κ B and PI3K/Akt/mTOR signalings (8). Also, ADAM9 inhibition suppresses breast cancer cell proliferation by inactivating the AKT/NF- κ B signaling (14). Our data demonstrated that LCA could block the Akt/NF- κ B signaling pathway, in tune with the ADAM9 expression, in colon cancer cells. Lu et al (27) demonstrated that the inactivation of Akt and NF- κ B were in consistent with declined cell proliferation in the HCT116 cells. The antitumor efficacies of potential drugs in colon cancer cells were associated with the NF- κ B pathway (28). Our study substantiated that LCA might downregulate ADAM9 expression in colon cancer cells to reduce cell growth and block cell migration and invasion.

Subsequently, we screened the upstream regulatory miRNAs of ADAM9 to more deeply and comprehensively understand the mechanism. Abnormal upregulation of miR-1270 has been shown in human gastric cancer and papillary thyroid cancer cell lines and tumors, while the downregulation of inhibited cancer cell migration in vitro (29). Notably, upregulated ADAM9 expression was associated with a poor overall 5-year survival rate in gastric cancer patients (29). We designated miR-1270, which could be regulated by LCA treatment and targeted to inhibit ADAM9 in this study. The rescue experiments in HCT116 cells further confirmed that LCA regulated cell proliferation, apoptosis, and metastasis via regulating the miR-1270/ADAM9 axis. LCA could simultaneously regulate the expression of miR-1270/ADAM9 and decrease the AKT/NF-

κ B signaling, thus decreasing proliferation, migration or invasion, and increase apoptosis in the colon cancer cell line HCT116.

Conclusion

LCA showed an antitumor efficacy in the colon cancer cell line HCT116 through the axial regulation of miR-1270/ADAM9 and the Akt/NF- κ B signaling pathways. LCA exhibited its antitumor efficacy by suppressing cell proliferation, migration, invasion, and colony formation, and promoting cell apoptosis in HCT116 cells via suppressing the ADAM9 and Akt/NF- κ B signaling pathways, in a dose-dependent manner. These data for the first time showed that LCA might have great development potential as a drug for colon cancer treatment.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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No funding was received in this study.

Conflict of Interest

The authors declare that there is no conflict of interest.

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