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Circular RNA SMARCA5 inhibits cholangiocarcinoma via microRNA-95-3p/tumor necrosis factor receptor associated factor 3 axis

Guangxin Wang[a,*], Xia Gao[b,*], Zhijun Sun[a], Tianyou He[a], Chaogang Huang[a], Shouwei Li[a] and Haocheng Long[a]

Enhancing research indicatedthat circular RNA (circRNA) acted a critical part in cholangiocarcinoma (CHOL) development. This research aims to discover the role of circRNA SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 5 (SMARCA5) in CHOL bio-progression, which has been proved to be downregulated in CHOL tissues. In this study, quantitative reverse transcription polymerase chain reaction was used to reveal the level and linkage of circRNA SMARCA5, miRNA-95-3p and TNF receptor-associated factor 3 gene (TRAF3) in CHOL tissues and cancer cells. The target sites of circRNA SMARCA5 and miRNA-95-3p were forecast by Starbase, and Targetscan was conducted to forecast the potential linkage points of TRAF3 and miRNA-95-3p, and which is affirmed by double luciferase reporter assay. CCK-8 and flow cytometry assay was carried to indicate cell viability. And apoptosis-related protein was counted by caspase3 activity and Western blot assay. CircRNA SMARCA5 was downregulated in CHOL cell lines and cancer samples. Besides, over-expression of SMARCA5 inhibited cell growth and promoted apoptotic rate. Dual-luciferase reporter assays presented that miRNA-95-3p could link with circRNA SMARCA5. Moreover, miRNA-95-3p was discovered highly expressed in CHOL. Interference of miRNA-95-3p repressed cell proliferation and raised the apoptosis. Importantly, TRAF3 was validated to be a downstream of miRNA-95-3p. Strengthen of miRNA-95-3p reversed the inhibitory impact of circRNA SMARCA5-plasmid transfection, and the results of miRNA-95-3p inhibitor were reversed by si-TRAF3. CircRNA SMARCA5 is involved in CHOL development by interosculating miRNA-95-3p/TRAF3 axis and may become a novel approach for treating CHOL. *Anti-Cancer Drugs* 34: 1002–1009 Copyright © 2023 The Author(s). Published by Wolters Kluwer Health, Inc.

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Keywords: cholangiocarcinoma, miRNA-95-3p, SMARCA5, TRAF3

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Introduction

Cholangiocarcinoma (CHOL) is one of the most common cancers in humans. Recently, its morbidity and mortality rates remain high. According to the 2018 American Cancer Society survey, the number of CHOL cases in the USA was 140 250, of which 75 610 are men and 64 640 are women, ranking third in the number of new cases of male and female tumors. In terms of estimated mortality, CHOL also ranks third among all types of tumors [1]. Although the prevention, diagnosis and treatment of CHOL have a big improvement, its morbidity and mortality are still in the top several tumors [2–4]. At present, surgery is still the first choice for radical treatment of CHOL, combined with radiotherapy, chemotherapy, targeted therapy and other treatment methods, the 5-year survival rate after surgery is about 50%, showing that CHOL is still a threat to human health problems.

CircRNAs (Circular RNAs) are a type of noncoding RNA and form a circular conformation by covalent bonds. CircRNAs are formed by back-splicing by noncanonical splicing [5]. CircRNAs are a kind of RNA without translation ability and have been affirmed to control tumor bio-functions, such as chemotherapeutic resistance, epithelial-mesenchymal transition, proliferation, and so on [6–8]. Some reports have indicated some kinds of circRNAs that are related to the pathogenesis of cancer, for instance, in cervical cancer, circRNA 0000285 was upregulation [9]. CircRNA WHSC1 was participated in endometrial cancer via microRNA (miRNA)-646 and nucleophosmin 1 [10]. CircRNA 001783 was indicated, which controlled the progression of breast cancer [11]. CircRNA SMARCA5 is a brand-new circRNA that has been verified to repressed cell metastasis and proliferation in myeloma [12]. And circRNA SMARCA5 has also

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been reported that inhibited non-small cell lung cancer through homeobox A9 [13]. Although the part of circRNA SMARCA5 in kinds of human cancer has been published, the underlying mechanism of its regulation of CHOL is unclear, so we want to discover its mechanism.

miRNA is a conserved small RNAs with a length of about 18–20 bp [14]. Studies have found that many miRNAs are involved in the development of many diseases by regulating posttranslation [15,16]. Recently, some findings for miRNA in CHOL report the critical role of miRNA during the development of CHOL, such as miRNA-885-5p [17], miRNA-876 [18] and miRNA-92b [19]. And miRNA-95-3p was involved in osteosarcoma [20]. Nevertheless, the function of miRNA-95-3p and related mechanisms in CHOL have not been clarified.

TRAF3, was translated by tumor necrosis factor receptor-associated factor 3 gene, was a family of multifunctional adaptor proteins that bind to surface receptors and recruit other proteins to form multiprotein signaling complexes that facilitate cellular responses [21]. Based on previous research, FN1 is associated with the biological functions of several types of human diseases, for instance, diabetes [22], severe acute respiratory syndrome coronavirus [23] and renal disease [24]. However, the correlation between TRAF3 and CHOL has not been detected.

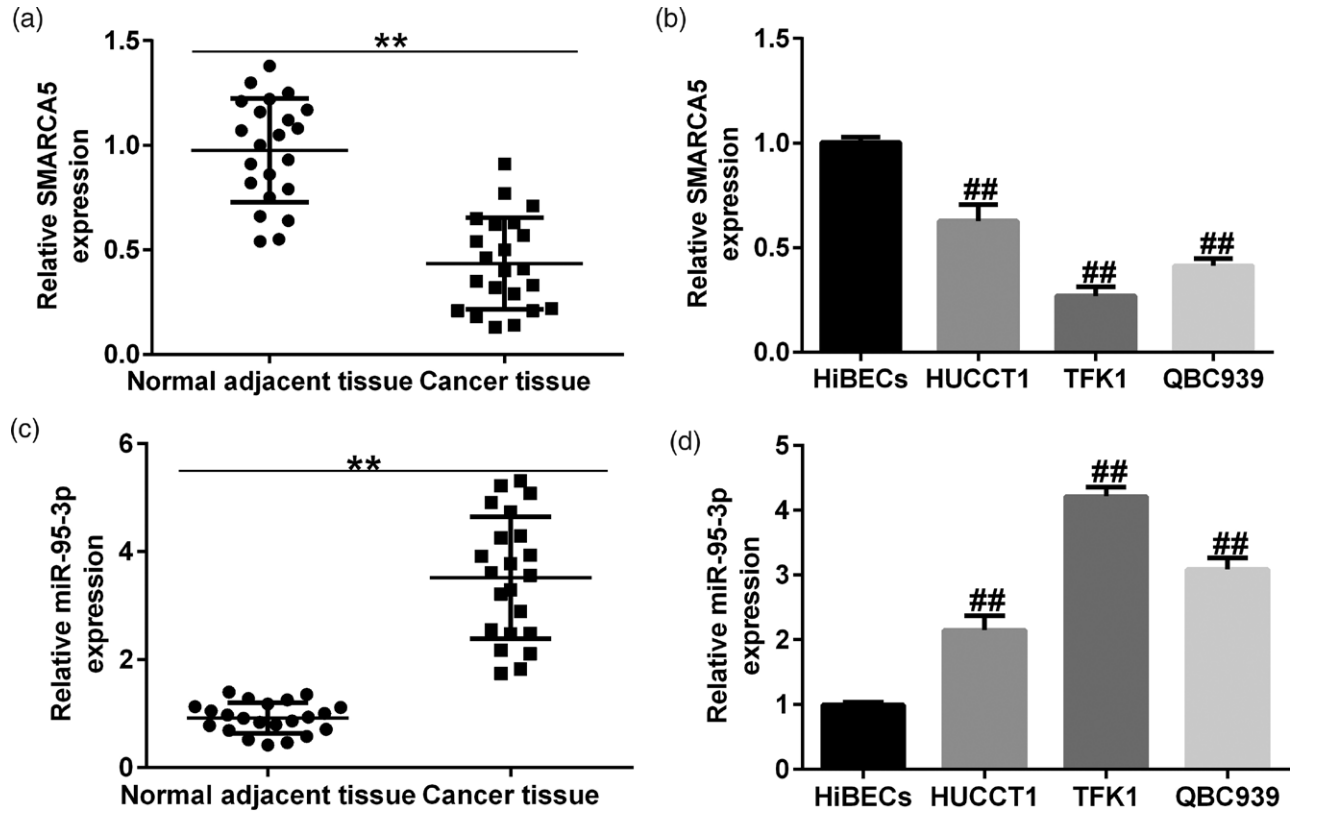
Therefore, to further explore the pathogenesis of CHOL is helpful to the demand for novel treatment strategies, which has important practical significance. In summary, this study aims at figuring out if circRNA SMARCA5 regulates CHOL process among the miRNA-95-3p/TRAF3 axis. Our study figures out novo biomarkers for the treatment of CHOL.

Protocols and material

Clinical sample collection

A total of 22 CHOL tissues and adjacent tissues were obtained from patients from Wuhan Third Hospital (Tongren Hospital of Wuhan University). Inclusion criteria: (a) none of the patients obtain anti-tumor therapy, chemotherapy or radiotherapy before surgery, and (b) final diagnosis was identified by concerned pathological determination. Exclusion criteria: (a) patients who acquired chemotherapy or radiotherapy. This study was sanctioned by Ethics Committee of Wuhan Third Hospital (Tongren Hospital of Wuhan University). All patients signed patient informed consent. Tissues were stored at –80 °C before use.

Fig. 1



Level of circRNA SMARCA5 and miRNA-95-3p in CHOL. (a and b) The level of circRNA SMARCA5 in cancer tissue and cancer cells. (c and d) The level of miRNA-95-3p in tumor samples and cancer cells. Data are exhibited as average ± SD of triple experiments. $^{**}P < 0.01$; $^{\#\#}P < 0.01$ vs. HiBECs. CHOL, cholangiocarcinoma; circRNA, circular RNA; HiBECs, human normal biliary cells.

Cell cultured

Human normal biliary cell and cancer cell (HUCCT1, TFK1 and QBC939) and 293T cells for dual-luciferase assay were obtained from the ATCC. The cells were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (Vivacell, Shanghai, China) supplemented with 1% penicillin and streptomycin (Vivacell) and 20% fetal bovine serum (Vivacell) in an atmosphere of 5% CO_2, at 37 °C.

Bioinformatics information collected

The binding points of miRNA-95-3p on circRNA SMARCA5 were predicted by Starbase, and TRAF3 fragments containing miRNA-95-3p binding points were forecasted by TargetScan 7.2 (https://www.targetscan.org/vert_80/).

Dual-luciferase reporter gene assay

The wild-type (WT) of SMARCA5 or the mutative (MUT) SMARCA5 sequence, and TRAF3 was synthesized into a pGL3-U6-puromycin vector (Tsingke, Beijing, China) in a luciferase activity. Subsequently, the 293T cells were cotransfection by SMARCA5-WT (or TRAF3-WT) or SMARCA5-MUT (or TRAF3-MUT) with mimic control and mimic of miRNA-95-3p by using jetPRIME, according to supplier's step (Polyplus, France). The activity was counted by using a reporter system 24 h after infection(Promega, Madison, Wisconsin, USA). The results were related to Renilla luciferase.

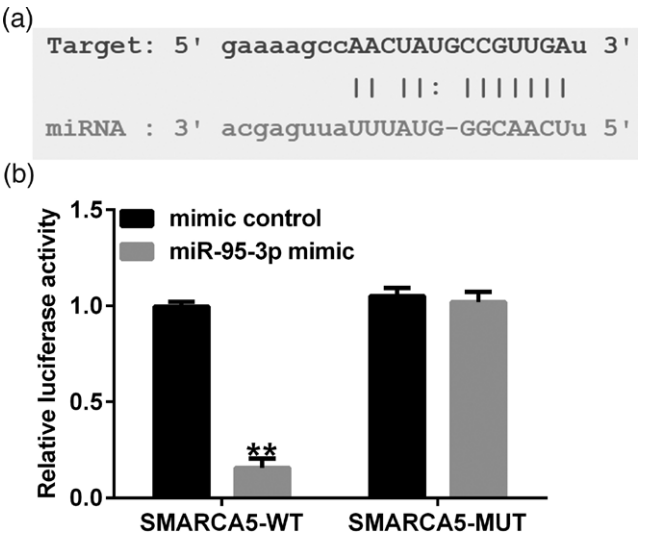
Cell transfection

For control of expression of miRNA-95-3p, inhibitor of miRNA-95-3p and inhibitor control (miRNA-95-3p inhibitor: 5′-AAAUGUCUGUUGAAUUGAAAU-3′ and inhibitor control: 5′-AUACACCUUGCUUUACUU-3′), mimic of miRNA-145-5p (5′-UUCAACGGGUAUUUAUUGAGCA-3′) and mimic control (5′-GCUUAUUCGGUUGGCUUAGC-3′) were purchased from Genscript (Nanjing, China). To knock down the expression of TRAF3, siRNA for TRAF3 (si-TRAF3: 5′-GGAAGAUUCGCGACUACAAGC-3′) was utilized and nonspecific control (control-siRNA: 5′-CACGATAAGACAATGTATTT-3′) were also purchased from Thermo Fisher (Fermantas, Lithuania). SMARCA5 whole sequence was ligated into pCMV4 vector (Genscript, Nanjing, China). All these sequences were transfected onto cells that grown to 65% confluence with jetPRIME (Polyplus). After 48 h cultured at 37 °C, 5% CO_2, cells were collected after transfection.

qRT-PCR assay

Following the supplier's step, total RNA was recovered from cells by using ISOLATION TRIzol buffer (Aladdin, China), and cDNA was got from reverse transcribed RNA with the Titan One Tube RT-PCR Kit (Merck, USA). The level of miRNA was detected by TransScript Probe SuperMix (Transgene, Nanjing, China) and the quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used PerfectStart Sybr qPCR Mix (Transgene). The expression levels were calculated by $2^{-\Delta\Delta Ct}$ assay. Primers of SMARCA5, miRNA-95-3p, U6, TRAF3 and β-actin were stated below: U6: 5′-CGCCCTCTTCAGCAGTTATACTA-3′ (F) and 5′-CTTCACGCCTTTGCGGCTCAT-3′ (R); β-actin: 5′-CATGTACGTTGCTATCCAG-3′ (F) and 5′-GCGAGAGGAGCACAGATACCACCAA-3′ (R); SMARCA5: 5′-ATACTCAACTCAGCAGGCAAGAT-3′ (F) and 5′-TTACTACATCAGCAGTCGCAAGA-3′ (R); miRNA-95-3p: 5′-CTGGTGGAGGGATGGATGAA-3′ (F) and 5′-GGCCCGATCACAAACTCATC-3′ (R); TRAF3: 5′-CAGACTAACCCGCCGCTAAAG-3′ (F) and 5′-GATGCTCTCTTGACACGCTGT-3′ (R).

Fig. 2



CircRNA SMARCA5 targets miRNA-95-3p. (a) The conjunction points of miRNA-95-3p on SMARCA5 WT and MUT. (b) The luciferase reporter activity of miRNA-95-3p co-transfection with SMARCA5 WT and MUT. Data are exhibited as average ± SD of triple experiments. $^{**}P < 0.01$ vs. mimic control. CircRNA, circular RNA; MUT, mutative; WT, wild-type.

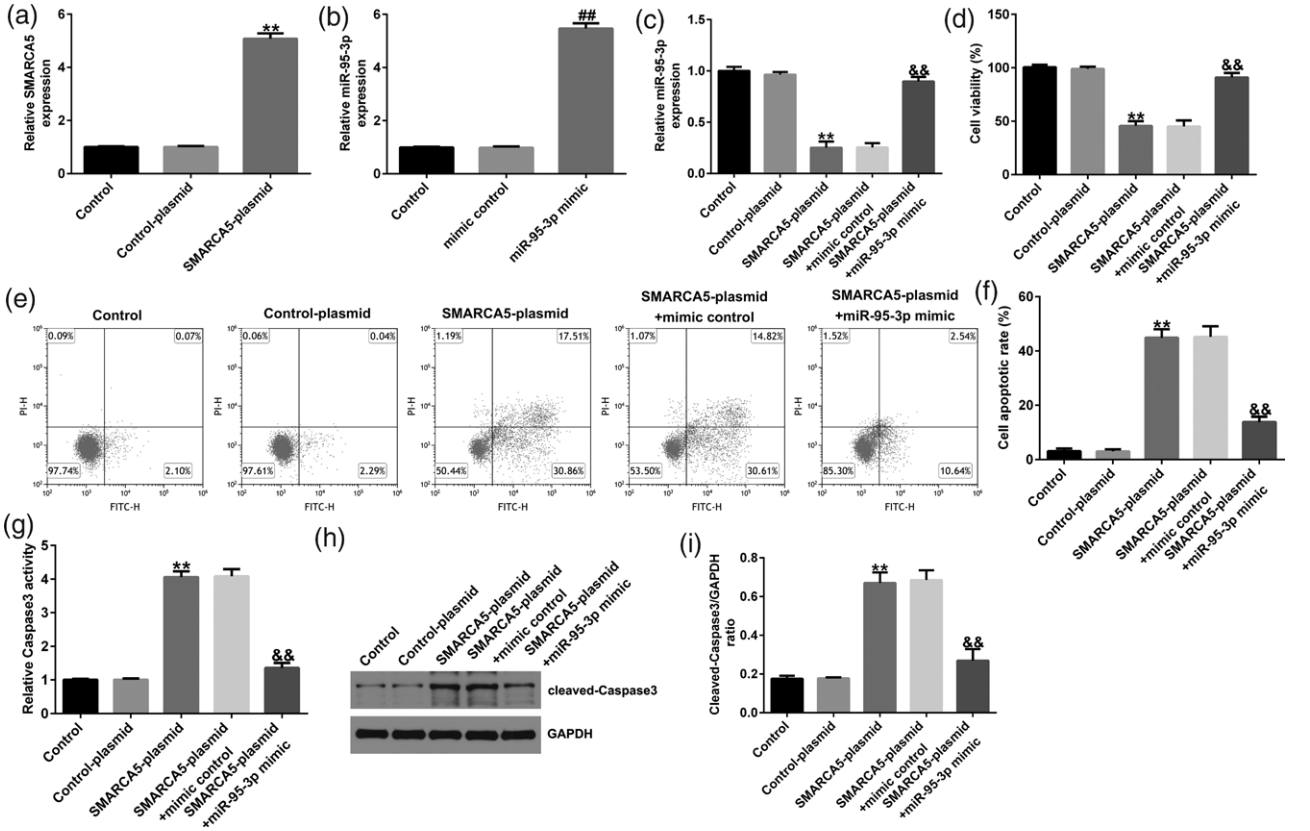
Cell viability detection

The capability of cell growth was tested by Cell Counting Kit-8 (CCK-8) reagent (Solarbio, Beijing, China). After transfection, cancer cells were resuspended and split to 96-well plates with 5×10^3 cells and incubation with 10-μl CCK-8 reagent for 1–2 h at 37 °C, 5% CO_2 in the dark. Optical density numerical values were detected at 490 nm using spectrophotometer (Bio-rad, Hercules, California, USA).

Cell apoptosis assay

A total of 2×10^5 transfection cancer cells were gathered and cultured with 5-μl annexin-V FITC and 5-μl propidium iodide (Beyotime, Shanghai, China) at room temperature in the dark for 30 min. Then, cell apoptotic rate was analyzed by flow cytometry (Beckman Coulter, Miami, Florida, USA).

Fig. 3



Impact of circRNA SMARCA5 over-expression in CHOL cells by regulating miRNA-95-3p. (a) The efficiency of circRNA SMARCA5-plasmid transfection. (b) The efficiency of miRNA-95-3p mimic transfection. (c) The level of miRNA-95-3p with circRNA SMARCA5-plasmid and miRNA-95-3p mimic cotransfection calculated by RT-qPCR. (d) Cell viability was counted by CCK-8. (e and f) Apoptosis ratio of TFK1 cell was detected by Annexin-C/PI assay. (g) The activity of caspase3. (h and i) The level of cleaved caspase3 was detected by Western blot assay. Data are exhibited as average ± SD of triple experiments. $^{**}P < 0.01$ vs. Control-plasmid; $\#\#P < 0.01$ vs. mimic control; $\&\&P < 0.01$ vs. SMARCA5-plasmid+mimic control. CCK-8, Cell Counting Kit-8; CHOL, cholangiocarcinoma; circRNA, circular RNA.

Caspase3 activity assay

Caspase3 activity of transfected cancer cells was performed by caspase3 activity kit (Novus, Littleton, Colorado, USA). In brief, culture medium and cells digested with trypsin (Thermo Fisher, Waltham, Massachusetts, USA), sample harvested and centrifuged at 600 rpm for 5 min. Caspase3 activity buffer (Novus) was cocultured with supernatant for 15 min. Supernatant was collected by centrifugation at 15 000 rpm for 10 min. Values were measured by microplate reader (Wix, China).

Western blot assay

Cancer cell lysis was prepared with RIPA cleavage buffer (Beyotime, Nanjing, China). Fifteen percent SDS-PAGE gel separated protein and then was shifted to the polyvinylidene fluoride (PVDF) membrane (Merck Millipore, Billerica, MA, USA). Tris-buffered saline Tween-20 and 5% skimmed milk powder were used to seal the PVDF membrane. Then, the PVDF membrane was incubated overnight with specific antibodies, including anticleaved-caspase3 (Affbiotech, Shanghai, China) and anti-TRAF3 (CST, Danvers, Massachusetts) at 4 °C overnight. GAPDH was used to be endogenous control. On the second day, after incubation with secondary antibody, the bands were detected by imaging system (Clinx, Shanghai, China), and the grayscale value of proteins was analyzed by Image J (version 1.46; National Institutes of Health).

Statistical analysis

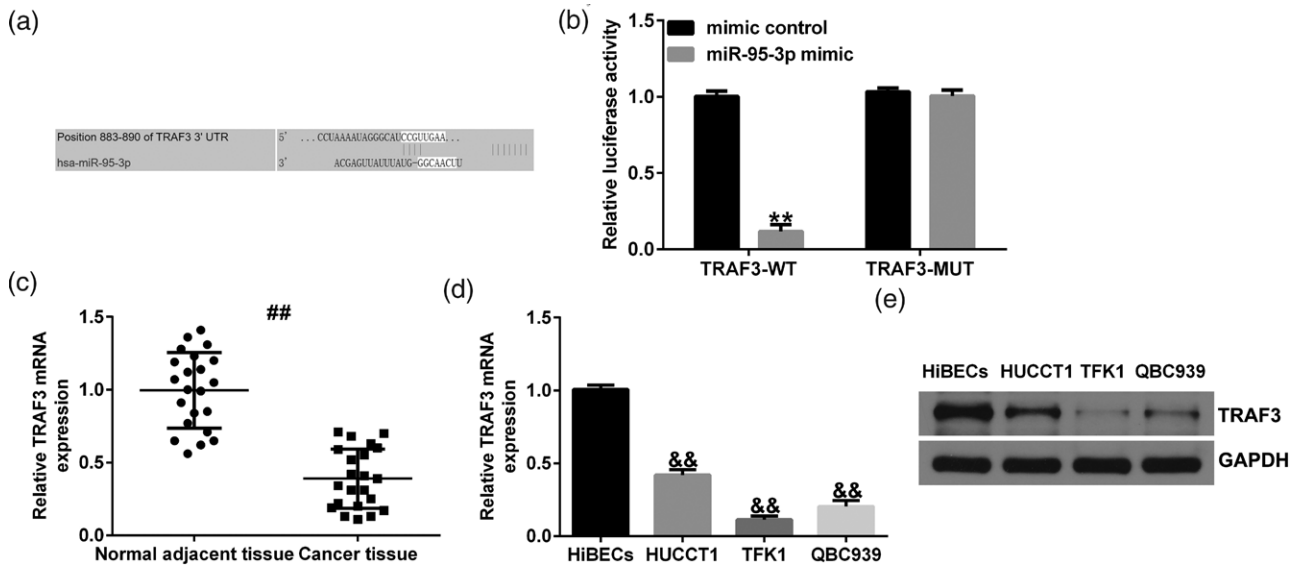
The average ± SD represents data from triple experiments. Student's *t*-test or one-way analysis of variance analysis followed by Tukey's test was applied for statistical analysis. $P < 0.05$ means statistically significant.

Results

SMARCA5 is downregulated and miRNA-95-3p is upregulated in cholangiocarcinoma

We carried out the RT-qPCR analysis of SMARCA5 and miRNA-95-3p expression levels in CHOL. Results unveiled that SMARCA5 was lowly expressed in cancer tissue, compared with normal control group (Fig. 1a). Consistently, in Fig. 1b, SMARCA5 level was declined in cancer cells (HUCCT1, TFK1 and QBC939). Conversely,

Fig. 4



TRAF3 is a downstream mRNA of miRNA-95-3p. (a) The foreknowledge binding points between miRNA-95-3p and TRAF3 was forecasted by Targetscan 7.0. (b) The dual relative luciferase assay was used for making sure the linkage between miRNA-95-3p and TRAF3. (c) The gene level of TRAF3 in tumor sample and normalized to control. (d and e) The gene and transcription level of TRAF3 in CHOL cancer cells. Data are exhibited as average ± SD of triple experiments. $^{**}P < 0.01$ vs. Mimic control; $\#\#P < 0.01$; $\&\&P < 0.01$ vs. HiBECs. CHOL, cholangiocarcinoma; HiBECs, human normal biliary cells; miRNA, microRNA; TRAF3, TNF receptor-associated factor 3 gene.

miRNA-95-3p level was upper in cancer tissue, and also raised in cancer cells (Figs. 1c and d). Taken together, SMARCA5 and miRNA-95-3p were involved in CHOL process.

miRNA-95-3p bounds with SMARCA5

To inquire into the feasible binding points of SMARCA5 and miRNA-95-3p, bioinformatics prediction website (StarBase) was carried out, and we discovered that miRNA-95-3p contained the possibly SMARCA5 bind sites (Fig. 2a). To confirmed the linkage of SMARCA5 and miRNA-95-3p, dual-luciferase gene assay was acted in 293T cells. As Fig. 2b, the relative luciferase level of SMARCA5-WT was notably reduced, when the cells were cocultured with mimic of miRNA-95-3p. When the potential connection points were MUT, the miRNA-95-3p mimic exhibited noneffect.

Over-expression of SMARCA5 represses proliferation and raises apoptosis on cholangiocarcinoma

To confirm the effect of SMARCA5 on CHOL cells' physiological activity, we transfected SMARCA5-plasmid and miRNA-95-3p mimic into TFK1 cell. Compared with control-plasmid group, SMARCA5-plasmid significantly enhanced the level of SMARCA5 in TFK1 cells (Fig. 3a). Compared with mimic control group, miRNA-95-3p mimic notably raised the pattern of miRNA-95-3p in TFK1 cells (Fig. 3b). In TFK1 cells, SMARCA5-plasmid repressed the level of miRNA-95-3p, and this reduction was rescued by miRNA-95-3p mimic cotransfection (Fig. 3c). The viability was recorded using CCK-8 and the results indicated that cells proliferation in SMARCA5-plasmid group was suppressed, and reversed by miRNA-95-3p mimic (Fig. 3d). Besides that, flow cytometry assay revealed that SMARCA5-plasmid notably induced the apoptosis-positive cell and inhibited by miRNA-95-3p mimic (Figs. 3e and f). The caspase3 activity level was strengthened by SMARCA5-plasmid transfection and cotransfection of miRNA-95-3p mimic declined its activity (Fig. 3g). Western blot assay results showed that band pattern of cleaved-caspase3 was heavy in SMARCA5-plasmid group and disturbed with miRNA-95-3p mimic cotransfection (Figs. 3h and i).

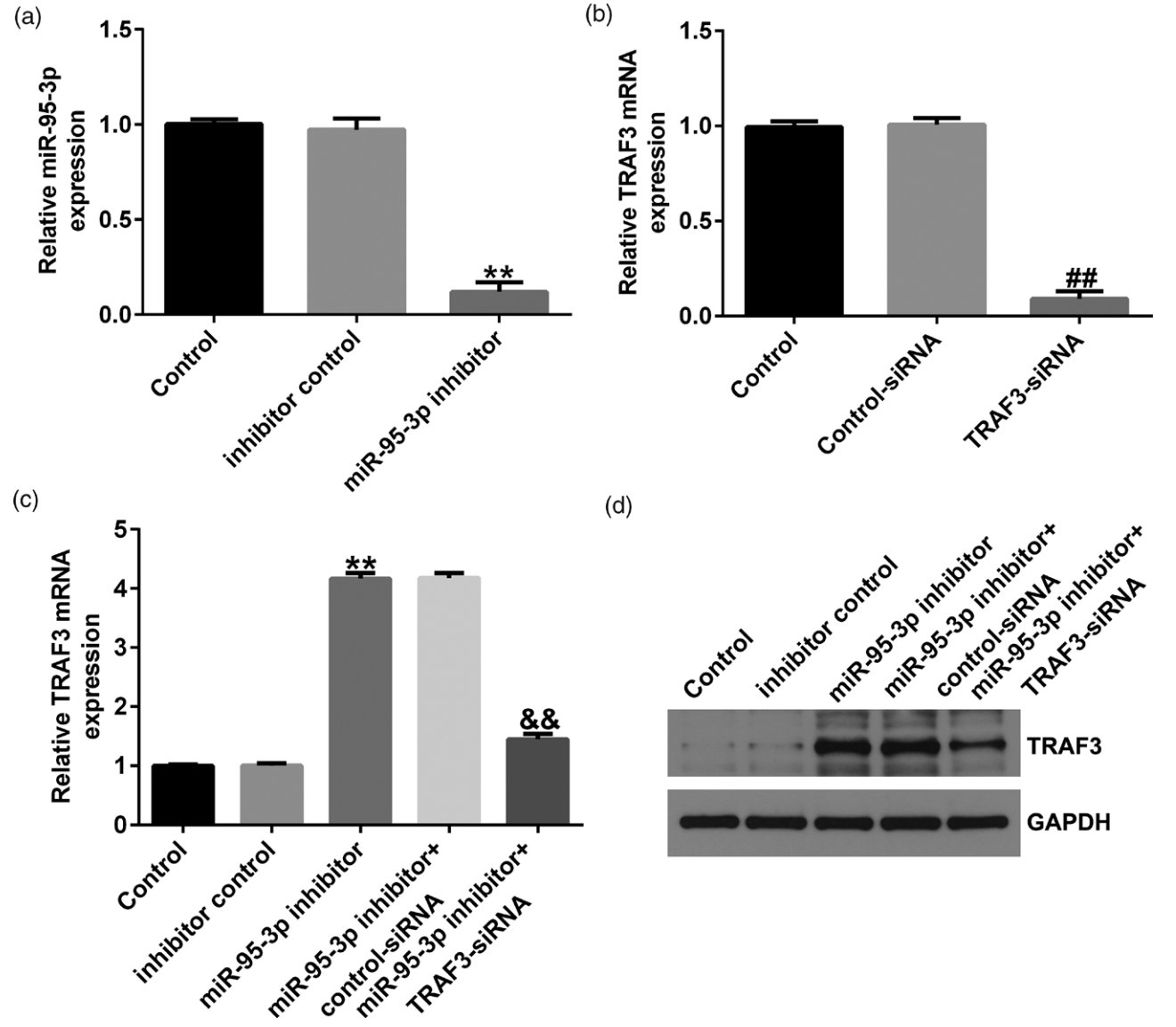
TRAF3 is a downstream target of miRNA-95-3p

Then, we quested the downstream mRNA of miRNA-95-3p, used the Targetscan 7.0, we found that TRAF3 contained the potential miRNA-95-3p bind points (Fig. 4a). We carried to use a dual relative luciferase method to concern this binding. We demonstrated that the over-expression of miRNA-95-3p downregulated luciferase level of TRAF3-WT reporter gene (Fig. 4b). RT-qPCR and Western blot assay outcomes hinted that TRAF3 was decreased in CHOL cancer tissues and cancer cells (Figs. 4c–f).

Inhibition of miRNA-95-3p suppresses growth and enhances apoptotic ratio in cholangiocarcinoma cells

To make sure the linkage of miRNA-95-3p and TRAF3 in CHOL, we transfected TFK1 cell with miRNA-95-3p inhibitor, TRAF3-siRNA or together. To the inhibitor control group, miRNA-95-3p inhibitor

Fig. 5



TRAF3 was negatively controlled by miRNA-95-3p. (a) The efficiency of miRNA-95-3p inhibitor transfection. (b) The efficiency of TRAF3-siRNA transfection. (c and d) The level of TRAF3 with miRNA-95-3p inhibitor and TRAF3a-siRNA cotransfection counted by RT-qPCR and Western blot assay. Data are exhibited as average ± SD of triple experiments. $^{**}P < 0.01$ vs. inhibitor control; $\#\#P < 0.01$ vs. Control-siRNA; $\&\&P < 0.01$ vs. miRNA-95-3p inhibitor + control-siRNA. miRNA, microRNA; TRAF3, TNF receptor-associated factor 3 gene.

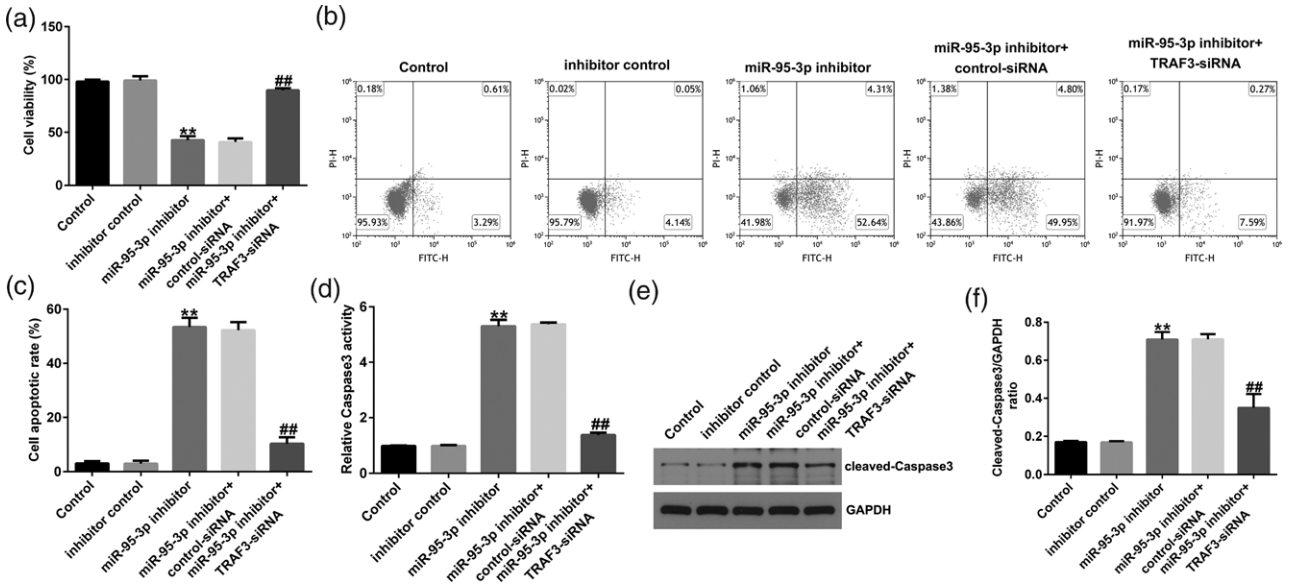
dramatically disturbed the expression of miRNA-95-3p in TFK1 cells (Fig. 5a). And transfection of TRAF3-siRNA inhibited TRAF3 levels (Fig. 5b). Western blot assay and RT-qPCR results indicated that gene and protein pattern of TRAF3 was elevated by miRNA-95-3p inhibitor, and destroyed by TRAF3-siRNA (Figs. 5c and d).

Contrast to inhibitor control, growth of TFK1 cells was reduced by miRNA-95-3p inhibitor (Fig. 6a). The apoptosis-positive cells were hoisted by miRNA-95-3p inhibitor transfection (Figs. 6b and c). The activity of caspase3 and expression pattern of cleaved caspase3 were raised by miRNA-95-3p inhibitor cultured (Figs. 6d–f), and the above-mentioned results were reversed by TRAF3-siRNA co-cultured.

Discussion

Currently, CHOL is the common malignant tumors in the world [25]. With the progress and development of modern medicine, targeted therapy for cancer has begun to lead a revolution. The premise of targeted therapy is to identify the mechanism of abnormal expression of genes in cancer, to improve the survival rate of cancer patients. In recent years, with the research on biomarkers of CHOL, the targeted diagnosis, treatment and prognosis of CHOL has achieved preliminary results, but more

Fig. 6



Inhibition of miRNA-95-3p suppressed cell growth and boosted cell apoptosis via TRAF3 in CHOL. (a) Cell viability was counted by CCK-8. (b and c) Apoptosis ratio of cells was detected by FCM. (d) The activity of caspase3. (e and f) The level of cleaved caspase3 was detected by Western blot assay. Data are exhibited as average ± SD of triple experiments. $^{**}P < 0.01$ vs. inhibitor control; $^{\#\#}P < 0.01$ vs. miRNA-95-3p inhibitor + control-siRNA. CCK-8, Cell Counting Kit-8; CHOL, cholangiocarcinoma; FCM, flow cytometry; miRNA, microRNA; TRAF3, TNF receptor-associated factor 3 gene.

research is needed to combat CHOL. Therefore, the key biomarkers in CHOL are an effective strategy for the treatment of CHOL.

In our research, we elaborated the molecular mechanism of circRNA SMARCA5 in CHOL. This examination confirmed that circRNA SMARCA5 was participated in CHOL process via miRNA-95-3p and TRAF3; finger out circRNA SMARCA5 served as a novo-biomarker for CHOL.

Heavily evidence uncovered that circRNAs played a critical role in CHOL. In CHOL, kinds of circRNAs are described as biomarkers. circRNA ACTN4 assisted CHOL process by YBX1 and FZD7 [26]. Yi *et al.* [27] hinted that the possible mechanism of circRNA 000585 in CHOL. Meantime, Du *et al.* [28] pointed out that circRNA NFIB repressed growth and metastasis of CHOL via MEK1. However, few researchers were done to dissect the role of circRNA SMARCA5 in CHOL. In our study, we uncovered that circRNA SMARCA5 was obviously declined in CHOL. These inventions increased the recent comprehension of role of circRNA SMARCA5 in the modulation of CHOL, which may be helpful to better rummage pathological mechanism of CHOL.

Many researchers illustrated that circRNAs regulated the process of diseases via miRNAs [29,30]. Predecessors found that many miRNAs were involved in CHOL [17–19]. Loeffler *et al.* [31] demonstrated that miRNA-451a is involved in CHOL. Salem *et al.* [32] hinted that miRNA-150 was associated with CHOL. miRNA-146b-5p was reported that it inhibited CHOL process by TRAF3 pathway [33]. Here, experimental proof identified miRNA-95-3p as the downstream of circRNA SMARCA5, as they have confirmed connection points. We expounded that the level of miRNA-95-3p was clearly upregulated and was contrastly controlled with the level of circRNA SMARCA5 in CHOL.

Previously, TRAF3 was reported as a critical role that involved in human diseases, such as inflammatory bowel disease [34], ischemia-reperfusion injury [35] and acute pancreatitis [36]. Magically, in this study, TRAF3 mRNA and protein were lowly expressed in CHOL, and it was associated with CHOL.

Conclusion

Our research presented certification that circRNA SMARCA5 modulates miRNA-95-3p/TRAF3 to repressed CHOL process. Our data demonstrate that circRNA SMARCA5/miRNA-95-3p/TRAF3 supplies a new strategy for CHOL.

Acknowledgements

G.W. and X.G. contributed to data collection, statistical analysis, data interpretation and manuscript preparation. Z.S., T.H., C.H. and S.L. contributed to data collection and statistical analysis. H.L. contributed to data collection and manuscript preparation. All authors have read and approved the final manuscript.

Availability of data and materials: the datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of interest

There are no conflicts of interest.

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