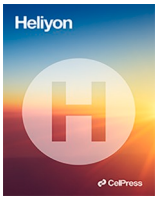
Heliyon 10 (2024) e32528

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Epigenetic silencing of miR-125a-3p promotes the progress of human cholangiocarcinoma via increasing CAC1 expression

Xiaojuan Guo [a,1], Jinxi Wang [b,1], Yunxiao Tian [a], Jianhua Yang [a], Shiqian Wu [a], Lihui Xin [a], Zhe Feng [c], Guangxu Niu [a,*]

[a] Department of Pathology, Handan Center Hospital, Handan, 056002, China
[b] The Fourth Department of General Surgery, The First Hospital of Handan, Handan, 056002, China
[c] Department of Function, Handan Center Hospital, Handan, 056002, China

ARTICLE INFO

Keywords:
Cholangiocarcinoma
microRNA
miR-125a-3p
Methylation
CAC1
Malignant progression

ABSTRACT

We aimed to investigate the dysregulation of the microRNAs(miRNAs) in cholangiocarcinoma (CCA), including its impact on the homeostasis of the transcriptome and cellular behavior. MiRNAs serve as potent epigenetic regulators of transcriptional output, targeting various signaling pathways. This study aimed to investigate the expression level, epigenetic mechanism and function of miR-125a-3 in CCA. The study data showed that the expression level of miR125a-3p was decreased in CCA tissue samples and cell lines, and it was closely related to lymph node metastasis, tissue differentiation and TNM stage. The data demonstrate a strong association between decreased miR-125a-3p expression and poorer prognosis in cholangiocarcinoma patients. miR-125a-3p acts as a tumor suppressor by inhibiting the viability, migration and invasion of CCA cells. There are CpG islands in the promoter region of miR-125a-3p gene, and the methylation of the promoter region of miR-125a-3p gene leads to the transcriptional repression of miR-125a-3p. In addition, miR125a-3p can target and regulate CAC1 mRNA and protein expression in the downstream mechanism, and the high expression of CAC1 can promote the proliferation, migration and invasion of cholangiocarcinoma cells. These data demonstrate that miR-125a-3p promoter methylation leads to silencing of its expression. Mechanically, miR-125a-3p acts as a tumor suppressor and participates in the occurrence and development of CCA through targeting CAC1 gene expression. Therefore, miR-125a-3p may serve as a new target for the diagnosis, prognostic assessment or molecular therapy of CCA.

1. Introduction

Cholangiocarcinoma, which can be anatomically divided into intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (ECC), is the most common primary biliary malignancy, accounting for approximately 10 % of primary liver tumors [1,2]. Due to its high molecular heterogeneity, patients with CCA have a poor prognosis. Most CCAs have developed to the middle and late stages at diagnosis and lost the opportunity for surgery [3]. Classic serum CCA markers, such as carbohydrate antigens CA-199 and CA-125, are less sensitive and non-specific in the identification of CCA, and the gene expression pattern of patients changes greatly

* Corresponding author. Department of Pathology, Handan Center Hospital, Zhonghua Road, Handan, 056002, China.
E-mail address: niu18303363396@126.com (G. Niu).
[1] Contributed equally.

https://doi.org/10.1016/j.heliyon.2024.e32528
Received 21 January 2024; Received in revised form 4 June 2024; Accepted 5 June 2024
Available online 15 June 2024

when CCA occurs [4,5]. Thus, search for their Sensitive biomarkers is great significance for the early diagnosis and treatment of CCA.

MicroRNA (miRNA) is an endogenous non-coding RNA of about 22 nucleotides, which can bind to the 3′UTR (untranslated region) of target mRNA in the post-transcriptional horizontal direction, which plays an important role in the occurrence and development of tumors [6,7]. The process of miRNA processing and maturation has undergone a spatial transition from the nucleus to the cytoplasm, and the whole process is coordinated by a variety of enzymes and accessory proteins to perform multi-level regulation and multi-step precise reactions [8,9]. However, the role of miRNAs in CCA has not been fully elucidated. Correa-Gallego et al. analyzed miRNA by deep sequencing technology and found that the expression of miRNA-21 and miRNA-221 in CCA patients were significantly higher than those in healthy people. There is a significant correlation between elevated miRNA expression and overall survival (OS). While downregulation of tumor suppressor miRNAs leads to cancer spread, this may be a possibility for CCA therapy [10]. miR-125a-3p was the best marker for cholangiocarcinoma and pancreatic cancer screened by Kojima et al., and was able to detect 32 out of 33 (97.0 %) in the test cohort [11]. Studies have reported that the expression of miR125a-3p is decreased in patients with non-small cell lung cancer and gastric cancer, and the silencing of miR-125a-3p expression is related to the malignant progression of tumors [12,13]. The clinical importance and tumor suppressor effect of miR-125a-3p suggest that miR-125a-3p may serve as an important diagnostic factor for gastric cancer [13]. MiR-125a-3p has resulted in the emergence of fresh diagnostic and therapeutic prospects for cancer management. However, the role and regulatory mechanism of miR-125a-3p in cholangiocarcinoma has not been reported yet.

In addition, aberrant DNA methylation is another common epigenetic modification. Studies from several groups have reported miRNA expression silencing caused by CpG island methylation or repressive histone modifications is the important mechanisms leading to tumorigenesis. Recently, it shown that miR-34 was silenced by hypermethylation in cancer, and reintroduction of miR-34a inhibits cancer cell growth and played an important role in tumorigenesis [14,15]. Interestingly, aberrant DNA methylation is induced miRNAs down-regulation, forming a field for an important mechanism for CCA development and progression.

In this study, we investigated the expression, regulation, and functional role of miR-125a-3p in cholangiocarcinoma, and we revealed whether the expression of miR-125a-3p is related to the DNA methylation status of their promoter. Mechanistically, miR-125a-3p exerted tumor suppressor function in suppressing proliferation and invasion through targeting CAC1. The study expands our understanding of the fundamental processes involved in cell growth and spread, while also presenting a novel treatment option for individuals diagnosed with CCA. This is the first study to comprehensively evaluate the expression, epigenetic regulation, and functional role of miR-125a-3p in cholangiocarcinoma.

2. Materials and methods

2.1. Research objects and main reagents

Sixty-eight cholangiocarcinoma tissues and corresponding para-cancerous normal bile duct tissues were collected, from June 2016 to December 2019. All patients underwent resection of cholangiocarcinoma at the Hepatic Surgery of Handan Center Hospital (Handan, China). Among the patients, there were 50 males and 18 females; the median age was 62 years (23–86 years old). Inclusion criteria: All patients were diagnosed with cholangiocarcinoma by two associate chief pathologists. The clinical and pathological data, including follow-up information, were complete. None of the patients had received preoperative radiotherapy, chemotherapy, or biological therapy, and they had no history of other malignancies. All patients signed informed consent forms. Peritumoral normal bile duct tissue was defined as normal bile duct tissue greater than 2 cm away from the tumor. Ethical approval for this study was obtained from the Ethics Committee (No. 2022KY099) of the Handan Central Hospital, Hebei Province and written informed consent was provided by all patients prior to the study start.

2.2. Culture of cholangiocarcinoma cell lines and cell transfection

Human cholangiocarcinoma cells (RBE, HuccT-1, QBC939) were purchased from Genechem. Subculture routinely in RPMI 1640 (Gibco, USA) with 10 % fetal bovine serum (FBS, Gibco, USA) in an incubator at 37 °C, saturated humidity and 5 % CO_2. After the cell passage three times, adjust the cell density to 2×10^5 and inoculate it into a 6-well plate. The cell line was treated with 5-Aza-dC (final concentration 10 μmol/L [16], Sigma, USA), and the liquid was changed every 24 h for 2 days. On the 3rd day, the cells were harvested and DNA and RNA were extracted after culturing for 24 h in medium containing 10 % FBS.

The cells were seeded into six-well plates and cultured overnight until 80 % confluence. MiRNA mimics/inhibitor (Genepharma, China) or pcDNA3.1-CAC1(GenScript, China) were transiently transfected using Lipofectamine 2000™ (Invitrogen, USA) according to the manufacturer's instruction. The cells were collected after 24 h of transfection for subsequent experiments.

2.3. qRT-PCR detection of miRNA-125a-3p expression in cholangiocarcinoma tissues and cell lines

Extract the total RNA in tissues and cells according to the instructions of TRIzol reagent, prepare a reverse transcription system according to the instructions of the TIANGEN (TIANGEN Biochemical Technology Co., Ltd.) reverse transcription kit, and synthesize the first-strand complementary DNA, that is, cDNA. The primers of miR125a-3p were designed according to the instructions of the matching fluorescence quantitative detection kit, and qRT-PCR amplification was carried out. The primer sequences for miR-125a-3p are (F)5′-GGCGACAGGTGAGGTTCTT-3′ and (R)5′-GCAGGGTCCGAGGTATTC-3'. The primer sequences for U6 are (F) 5′-GCTTCGGCAGCACATATACTAAAAT-3' (R)5′-CGCTTCACGAATTTGCGTGTCAT-3'. Using U6 as the internal reference, the relative expression of miR-125a-3p was analyzed, and the amplification reaction conditions were as follows: 40 cycles of 95 °C for 2 min,

denaturation at 95 °C for 20 s, annealing at 60 °C for 34 s, and 72 °C for 30 s. The relative expression level was expressed as $2^{-\Delta\Delta Ct}$.

2.4. miRNA-125a-3p promoter search and primer design for methylation-specific PCR (MSP)

UCSC (University of California Santa Cruz) gene sequence database was used to search the miR-125a-3p promoter region sequence to obtain the 2kd region upstream of the transcription start point. MethPrimer CpG island prediction software (http://www.urogene.org/methprimer/) analyzed the methylation of miR-125a-3p promoter region, and found that miR125a-3p has a 400bp CpG island in the proximal promoter, and the CG dinucleotide sequence density is greater than 50 %. In this experiment, primers for MSP detection were designed within the scope of this CpG island in combination with primer design software, and the primers were located in the promoter region (−272bp ~ −107bp), and then design primers in this area.

2.5. Genomic DNA extraction from CCA cell lines and methylation-specific PCR (MSP)

Using conventional phenol/chloroform extraction method, DNA was extracted from CCA cell lines before and after 5-Aza-dC treatment, tumor tissues and paracancerous normal tissues. For quantification by UV spectrophotometry, 2 μg of DNA was taken from each sample, and the samples were transformed and purified according to the instructions of the EpiTect Fast DNA purification kit (Qiagen, Germany).

The MSP primer (M) sequence is: Upstream primer 5′- AGGTTAGCGAGGGATAGGAC-3′, downstream primer 5′- CGAAC-CAAAAAAACCTAACG-3’; Unmethylated primer (U) sequence: upstream primer 5′-GGAGGTTAGTGAGGGATAGGAT-3′, downstream primer 5′-CAAACCAAAAAAACCTAACACA-3’. Reaction conditions: pre-denaturation at 95 °C for 10 min, denaturation at 95 °C for 45 s, annealing for 45 s, extension at 72 °C for 1 min, and after 35 cycles, extension at 72 °C for 7 min. The MSP amplification products were subjected to 2 % agarose gel electrophoresis, and the images were analyzed by UV gel electrophoresis imaging and image analysis system. It was determined to have methylation if a visible band was observed in the methylation reaction. The primers used in this study were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

2.6. Cell proliferation

The ability of cellular proliferation was determined by MTS and colony formation assays. For MTS assay, RBE and HuccT1cells transfected with miR-125a-3p mimics and inhibitor respectively were seeded in 96-well plates. The assays were performed by adding 20 μL of the CellTiter 96®AQ_{ueous} One Solution Reagent (Promega) directly to the culture wells at 0, 24, 48, 72, and 96 h of incubation, followed by incubation at 37 °C for 2 h. The absorbance was measured at 490 nm on a Microplate reader (Thermo Fisher Scientific).

For colony formation assay, HuccT1 and RBE cells were incubated in 6-well plates at a concentration of 3000 cells/well. Then medium was replaced and cells were allowed to culture for 7 days. The surviving colonies were fixed in 4 % paraformaldehyde and stained with 0.4 % crystal violet solution. The images were acquired using a microscope.

2.7. Migration and invasion assays

The cell migration ability was measured by a Transwell assay. HuccT1 and RBE cells in 200 μl serum-free medium (1×10^5 cells) were plated on non-Matrigel-coated in upper chamber (Corning Company), and 600 μL of RPMI-1640 containing 20 % serum medium was filled in lower chamber. To perform the invasion assay, cells in 200 μl serum-free medium were reseeded on 50 μL 1 × Matrigel® Basement Membrane Matrix (BD Biosciences)-coated chambers. After incubating for 24 h, the outer membrane was fixed with 0.1 % crystal violet and enumerated underneath a light microscope (Olympus BX53, Japan).

2.8. Western blot analysis

According to the manufacturer’s instructions, total proteins extracts were separated from using RIPA buffer (Solarbio Biotechnology, China). Protein lysates were separated by 10 % SDS-PAGE and transferred to PVDF membranes (Millipore). Then, the membranes were blocked with 5 % skimmed milk for 1h. Then membranes were probed overnight with anti-CAC1 Polyclonal antibody (1:1000, GTX118514, Gene Tex, USA) or β-Actin Antibody (ACTB) antibody (1:3000, P30002, Abmart, Shanghai) at 4 °C. The membranes were washed three times and incubated with secondary antibodies at room temperature for 45 min. Immunoreactive bands were visualized using ECL Western blot kit (Solarbio PE0010, Peking, China). ACTB was used as an internal control.

2.9. Luciferase reporter assay

To explore the targeting of CAC1 by miR125a-3p, the putative miR125a-3p binding sites in the 3′ UTR of CAC1 or its mutant sequence was cloned into the pGLO-basic vector (Promega). Cells were transfected with the plasmid pGLO-CAC1-WT or pGLO-CAC1-MUT were co-transfected into HuCCT1 with miR125a-3p mimics or a mimics NC. MiRNA mimics (40 nM) or mimics NC vector together with plasmid pTK-renilla were co-transfected. According to the manufacturer instructions, Luciferase activity was assessed using a Dual-Luciferase Reporter Assay System (Promega) after 48 h pGLO-CAC1-WT and pGLO-CAC1-MUT was constructed by Genepharma Co., Ltd.

2.10. Statistical analysis

SPSS 22.0 software was used for statistical analysis of the results of this study. Measurement data were expressed as mean ± standard deviation. The comparison between CCA tissue and its corresponding paracancerous tissue was performed by paired *t*-test, and the relationship between methylation test results and patients' clinicopathological data was performed by χ^2 test. Survival analysis was performed by Kaplan-Meier method, and univariate and multivariate prognostic analysis was performed by Cox method. $P < 0.05$ indicated that the difference was statistically significant.

3. Results

3.1. miR-125a-3p is upregulated in CCA and associated with the prognostic of patients

The expression of miR-125a-3p in CCA and corresponding para-cancerous tissues were detected by qRT-PCR. The results showed that the expression of miR-125a-3p in CCA tissues were significantly lower than those in corresponding para-cancerous tissues (Fig. 1A). Analysis of the clinicopathological data of 68 CCA patients found that the expression of miR-125a-3p was associated with the pathological grade, TNM stage, lymph node metastasis, but not related to the patient's gender and age (Table 1). In addition, Kaplan-Meier survival analysis showed that patients with low miR-125a-3p expression (fold change < median, n = 39) had significantly lower survival overall (Fig. 1B). Univariate and multivariate Cox regression analysis indicated that miR-125a-3p downregulation was an independent prognostic marker in CCA patients (Table 2).

3.2. miR-125a-3p represses CCA cell proliferation, migration and invasion in vitro

The expression of miR-125a-3p was significantly decreased in cholangiocarcinoma (Fig. 2A). To investigate the biological function of miR-125a-3p in CCA cell lines, RBE and HuCCT1 were transfected with miR-125a-3p mimics and inhibitor, respectively, the transfection efficacy of miR-125a-3p mimics and miR-125a-3p inhibitor was measured by qRT-PCR. As shown in Fig. 2B, compared with the control group, the expression of miR-125a-3p in the mimics group was significantly increased, and the expression of miR-125a-3p in the inhibitor group was significantly decreased. MTS and colony formation assays showed that high expression of miR-125a-3p significantly reduced cell viability and colony formation compared with the control group, miR-125a-3p downregulation promoted cells proliferation (Fig. 2C, D and 2E). In addition, Transwell analysis showed that miR-125a-3p mimic-transfected cells migrated and invaded less than control cells, miR-125a-3p inhibitor-transfected cells migrated and invaded more than control cells (Fig. 2F, G, 2H and 2I). MiR-125a-3p was found to suppress key oncogenic phenotypes like proliferation, migration and invasion in cholangiocarcinoma cell lines.

3.3. Methylation of the promoter region of miR-125a-3p gene in CCA leads to silencing of its expression

It is known that many tumor-suppressor miRNAs are silenced epigenetically by DNA methylation and/or histone modification, though these alterations also can activate the expression of onco-genes with leading to tumor progression [17,18]. The online meth-primer analysis showed that there were dense CpG islands in the upstream 5′ region of the miR-125a-3p gene (Fig. 3A). After 5-Aza-dC treatment, the expression of miR-125a-3p was increased in all three CCA cell lines (RBE, HuccT-1, QBC939) (Fig. 3B). In addition, the methylation degree of miR-125a-3p gene in RBE, HuccT-1, and QBC939 cell lines was decreased, and the unmethylation degree of HuccT-1 and QBC939 cell lines was increased (Fig. 3C). The results of MSP analysis showed that the methylation rate of miR-125a-3p in CCA tissues was significantly higher than that in para-cancerous normal tissues (Fig. 3D). Analysis of the clinico-pathological data of 68 CCA patients found that the methylation status of this gene was associated with the pathological grade and TNM stage (Table 1). In 68 CCA tissues, the relative expression level of miR-125a-3p in tissues with miR-125a-3p gene methylation was

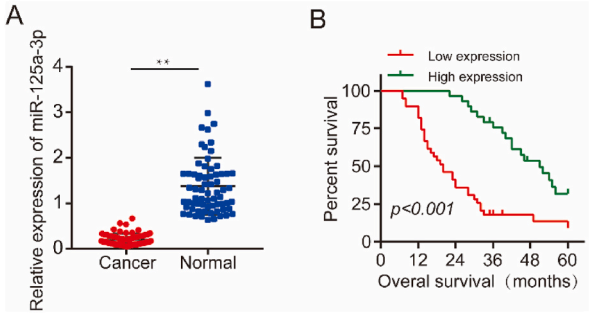


Fig. 1. miR-125a-3p is upregulated in CCA and associated with the prognostic of patients. a. miR-125a-3p expression levels in paired cholangiocarcinoma tissues and corresponding para-cancerous normal bile duct tissues detected by qRT-PCR (n = 68). b. Kaplan-Meier curves show that CCA patients with very high miR-125a-3p expression levels show significantly prolonged overall survival (green Kaplan-Meier curve) compared to patients with lower miR-125a-3p expression levels (red Kaplan-Meier curve). $^{**}P < 0.01$.

Table 1
Expression and methylation status of miR125a-3p in human ESCC tissues and their relationship with clinicopathologic features of ESCC tissues.

Characteristics	*N*	Expression of miR125a-3p			Methylation of promoter		
		$\overline{x} \pm s$	*t*	*P*	*N(%)*	χ^2	*P*
Total	68	0.203 ± 0.129	−15.578	<0.001[a]	41(60.29 %)	7.536	0.006[a]
Gender							
Male	50	0.207 ± 0.121	0.383	0.705	33(66.00 %)	2.569	0.109
Female	18	0.192 ± 0.153			8(44.44 %)		
Age (years)							
≥62	38	0.194 ± 0.131	−0.644	0.522	22(57.89 %)	0.207	0.649
< 62	30	0.214 ± 0.127			19(63.33 %)		
Lymph node metastasis							
Negative(N0)	20	0.256 ± 0.119	2.320	0.028[a]	5(25.00 %)	14.743	<0.001[a]
Positive(N1/2/3)	48	0.181 ± 0.128			36(75.00 %)		
Pathological differentiation							
Well/moderate	40	0.234 ± 0.144	2.654	0.010[a]	15(37.505)	21.083	<0.001[a]
Poor	28	0.159 ± 0.089			26(92.86 %)		
TNM stage							
I+II	32	0.259 ± 0.131	3.682	<0.001[a]	14(43.75 %)	6.911	0.009[a]
III+IV	36	0.153 ± 0.106			27(75.00 %)		

[a] a < 0.05

Table 2
Univariate and multivariate Cox regression analysis for clinicopathological features associated with prognosis of 68 CCA patients.

Variables	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	*P*	HR (95 % CI)	*P*
Age (<62 vs. ≥62)	1.040(-0.686–0.410)	0.590	1.2250 (0. 659-2.37q)	0.494
Gender (male vs. female)	1.285 (−0.724–0.617)	0.933	0.778(0.379–1.597)	0.494
Lymph node metastasis (negative vs. positive)	2.114(1.123–3.981)	0.020[b]	1.500 (0.776–2.897)	0.228
Pathological differentiation (well/moderate vs. poor)	3.168(1.805–5.559)	<0.001[a]	4.618(2.179–2.179)	<0.001
TNM stage (I+II vs. III+IV)	2.081 (1.194–3.629	0.010[b]	1.998(1.026–3.890)	0.885
miR-125 (high vs. low)	0.280 (0.155–0.506)	<0.001[a]	0.195 (0.097–0.392)	<0.001

***$P < 0.001$.
[a] $P < 0.01$.
[b] $P < 0.05$.

significantly lower than that in unmethylated tissues (Fig. 3E).

3.4. miR-125a-3p regulates CAC1 expression

Targetscan and Miwalk databases were selected to predict miR-125a-3p target genes, and it was found that the miR-125a-3p seed region was bound to the CAC1 3′-UTR region. The score was high, so it was speculated that CAC1 might serve as its candidate target gene (Fig. 4A). The expression of CAC1 mRNA and protein was significantly decreased in HuCCT1 cells after transfected with miR-125a-3p mimics, whereas the expression of CAC1 was upregulated in RBE cells after transfected with miR-125a-3p inhibitor (Fig. 4B and C). Furthermore, CAC1 expression was negatively correlated with miR-125a-3p in cholangiocarcinoma tissues (Fig. 4D). Luciferase reporter assay was performed to validate whether CAC1 was a direct target of miR-125a-3p. The results showed that compared with the control group, miR-125a-3p mimics significantly inhibited the luciferase activity of pmirGLO-CAC1, while the inhibitory effect in pmirGLO-CAC1 (mut) group was not obvious (Fig. 4E). It was seen that CAC1 may serve as a downstream target gene of miR-125a-3p.

3.5. miR-125a-3p suppresses the proliferation, migration and invasion of cholangiocarcinoma cells by targeting CAC1 in vitro

To find out whether miR-125a-3p suppresses proliferation and migration by regulating CAC1. We first detected the biological function of CAC1 in cholangiocarcinoma. RT-qPCR was performed to test the efficiency of CAC1 overexpression, and the results showed that CAC1 was higher expression in pcDNA3.1 CAC1-transfected group than that in control group (Fig. 5A). The results of Western blot showed that the protein level of CAC1 was significantly increased after transfection with the pcDNA3.1-CAC1 plasmid (Fig. 5B). We performed cell function tests in HuCCT1 cells. Transfecting pcDNA3.1-CAC1 could promote the proliferation, migration and invasion of cholangiocarcinoma cells (Fig. 5C, D and 5E). In addition, transfecting the miR-125a-3p mimic greatly reduced cell proliferation, while co-transfecting the miR-125a-3p mimic and CAC1 was similar to control cells (Fig. 5F). Similarly, overexpressing CAC1 reversed the effect of the miR-125a-3p mimic on cell colony formation in HuCCT1 cells (Fig. 5G). Meanwhile, co-transfecting miR-125a-3p mimic and CAC1 reversed the inhibition of the miR-125a-3p mimic on migration cells in HuCCT1 cells (Fig. 5H).

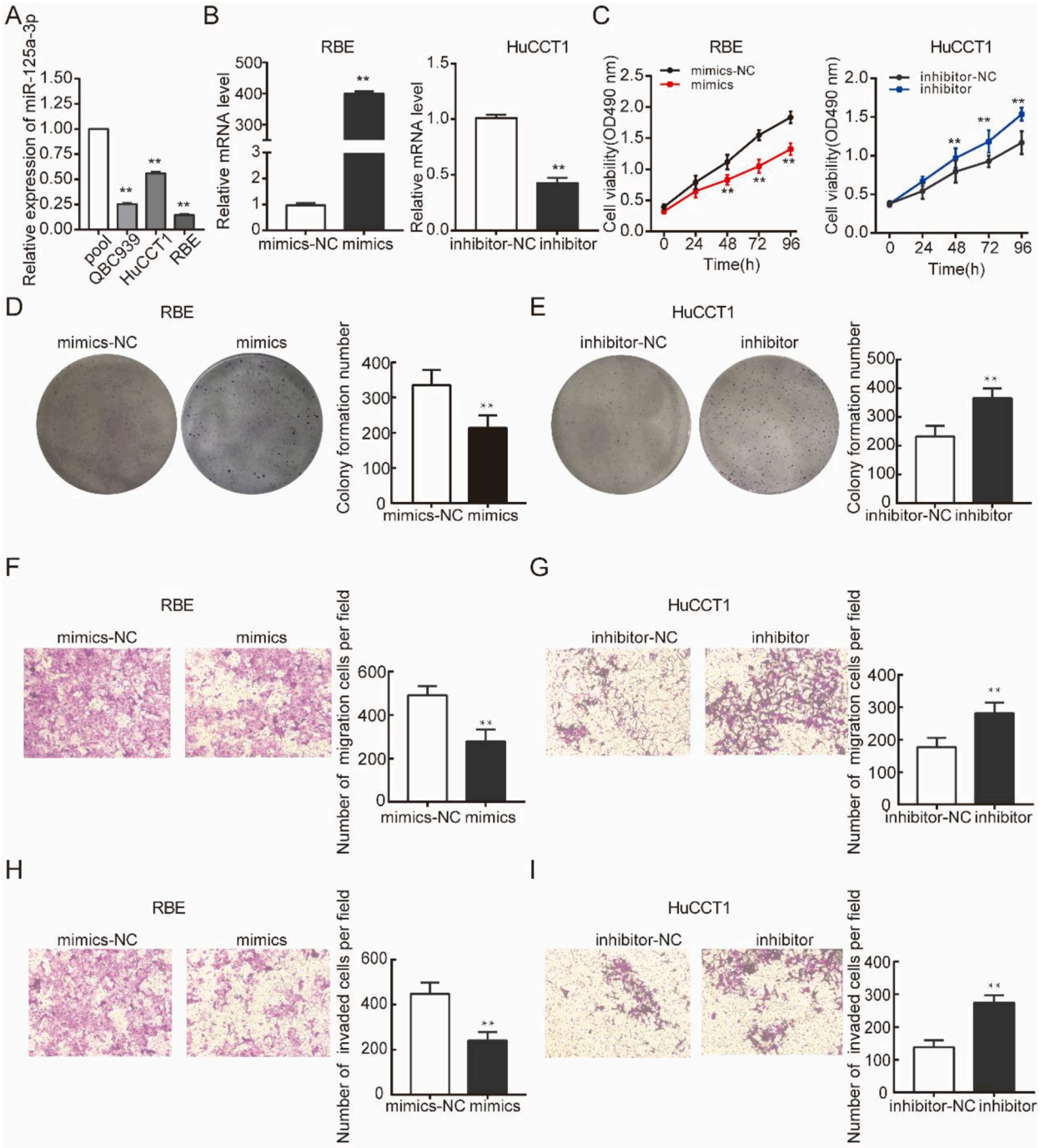


Fig. 2. miR-125a-3p represses CCA cell proliferation, migration and invasion in vitro. a. Expression levels in cholangiocarcinoma cell lines relative to normal bile duct cells analyzed by qRT-PCR. The data are shown as the mean ± S.D. **p < 0.01. b. Using miR-125a-3p mimics or miR-125a-3p inhibitor were transfected into REB and HuCCT1 cells, to overexpress or downregulate the expression of miR-125a-3p. c, d and e. MTS and colony formation assays were performed to evaluate RBE and HuCCT1 cells proliferation after miR-125a-3p overexpression or knockdown. f-i. Cell migration and invasion ability were analyzed by Transwell assays (Magnification: × 100).

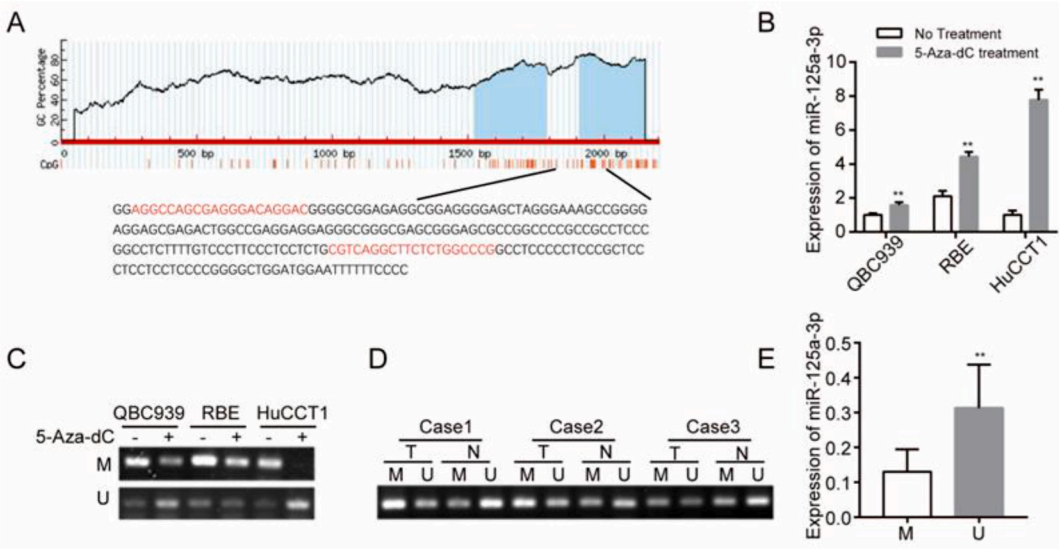


Fig. 3. Methylation of the promoter region of miR-125a-3p gene in CCA leads to silencing of its expression. a. Distribution of CpG islands in the promoter region of miR-125a-3p genome. The two MSP regions analyzed are indicated. b. Relative miR-125a-3p expression in CCA cells treated with 10 mmol/L 5-Aza-deoxycytidine (5-Aza-dC) or vehicle for 72 h, as determined by quantitative RT-PCR (RT-qPCR) analysis. c. Methylation-specific PCR (MSP) of genomic DNA from RBE, HuccT-1 and QBC939 cells treated with or without 10 mmol/L 5-Aza-dC treatment. (The original image is provided in the Supplementary file). d. MSP is performed to analyze in CCA tissues. (The original image is provided in the Supplementary file). Case 1–3: CCA case representative. e. RT-qPCR was used to detect the relative expression of miR-125a-3p in tissues with miR-125a-3p gene methylation in CCA tissues. T: Tumor, N: Normal, M: Methylation, U: Un-methylation. $^{**}P < 0.01$.

Therefore, miR-125a-3p suppresses the proliferation, migration and invasion of cholangiocarcinoma cells by promoting CAC1 expression.

4. Discussion

miR-125a-3p is one of the miRNA molecular markers screened in the peripheral blood of patients with pancreatic cancer and cholangiocarcinoma [19–21]. Compared with healthy controls, the expression of miR-125a-3p was significantly reduced in patients with cholangiocarcinoma [22,23]. However, further studies on miR-125a-3p in cholangiocarcinoma have not been reported. In this study, we first detected the expression of miR-125a-3p in the tissues of patients with cholangiocarcinoma. The results showed that compared with the para-cancerous normal control group, the expression of miR-125a-3p in patients with cholangiocarcinoma was significantly decreased, and this was related to the lymph node metastasis and clinical stage of the patients, which indicated that miR125a-3p may be related to the occurrence and progression of CCA.

The miRNA promoters underlying DNA methylation can directly lead to change for their expression levels and participate in their post-transcriptional regulation [24,25]. In order to further study the mechanism of miR-125a-3p expression silencing, we first predicted CpG island distribution on the miR-125a-3p promoter. There was a 264bp CpG island in miR-125a-3p promoter region. Therefore, we speculated that the low expression of miR-125a-3p may be related to the gene promoter methylation. Then combined with the primer design software, the primers for MSP detection were designed in the range of this CpG island. This study firstly detected the expression of miR-125a-3p in CCA cells. The study found that after treatment with methylation inhibitor 5-Aza-dC, the expression of miR-125a-3p was significantly increased in three CCA cell lines (RBE, HuccT-1, QBC939), suggesting that genomic DNA methylation may be one of the mechanisms leading to the down-regulation of miR-125a-3p expression in CCA. 5-Aza-dC can restore the normal demethylation state of genes and restore the expression of inactive genes. This study also found that the methylation rate of miR-125a-3p in CCA tissues was significantly higher than that in para-cancerous normal tissues, further suggesting that the down-regulation of miR-125a-3p expression may be closely related to the abnormal hypermethylation of CpG islands in its promoter region. Moreover, the hypermethylation of miR-125a-3p gene is related to the pathological grade of CCA tissue and the TNM stage of patients, suggesting that gene silencing caused by methylation may be related to the malignant phenotype of CCA, which may have certain reference significance for prognosis evaluation. This paper identified promoter hypermethylation as a novel mechanism leading to miR-125a-3p downregulation in this cancer type. This provides a basis for future studies to further elucidate the underlying molecular mechanisms.

In addition, this study further explored the downstream mechanism of miR-125a-3p regulating the malignant progression of CCA. Bioinformatics prediction software was used to predict the target genes that might be regulated downstream of miR-125a-3p, and CAC1 with a higher binding rate was selected for verification. It has been reported that the CAC1 gene, the CDK2-related cullin domain 1, is located on human chromosome 10 and is a member of the cullin family [26,27]. It is overexpressed in various cancers, such as

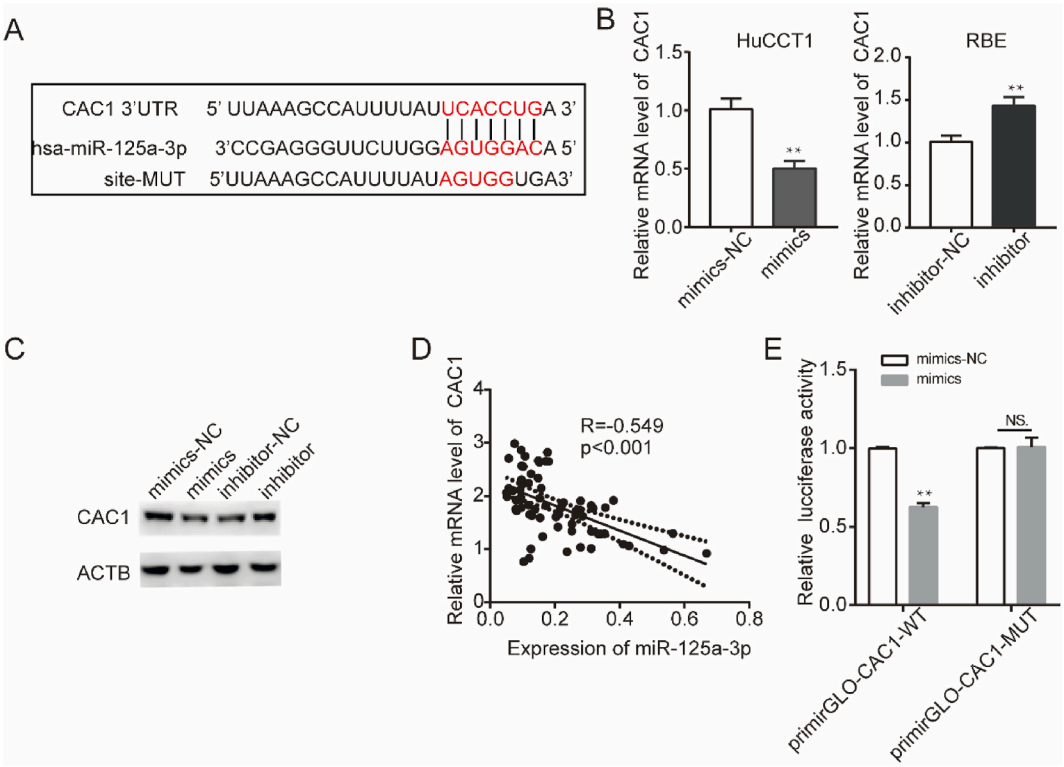


Fig. 4. miR-125a-3p regulates CAC1 expression. a. The sequences of the potential binding sites between 3′ UTR of CAC1 and miR-125a-3p. CAC1-MUT is the binding sites-mutated segments. b-c. CAC1 mRNA and protein expression levels in the indicated cholangiocarcinoma cells transfected with miR-125a-3p mimics or inhibitor. ACTB was used as an internal control for Western blot. (The original image is provided in the Supplementary file). d. Correlation between CAC1 expression and miR-125a-3p expression in cholangiocarcinoma tissues detected by RT-qPCR. e. Luciferase activities were measured 24 h after the indicated cells transfected with miR-125a-3p mimics and reporter plasmid containing wild type 3′ UTR regions of CAC1 or mutated segments. $^{**}P < 0.01$.

colorectal cancer [28], breast cancer [29], lung cancer [30], and gastric cancer [31]. In recent years, CAC1 has been shown to promote cell cycle progression and cell proliferation in some cancer cell lines [32–34]. However, the role and function of CAC1 in CCA has not been reported. In the study, it was found that miR-125a-3p has a significant targeted regulation effect on CAC1. And CAC1 has obvious promoting effect on the proliferation, migration and invasion of CCA cells.

In conclusion, the findings suggest miR-125a-3p merits further investigation as a prognostic biomarker or therapeutic target in this disease. Its promoter methylation play an important role in the occurrence and development of CCA. And miR125a-3p promoter hypermethylation may be one of the main mechanisms leading to the decreased expression of this gene in CCA tissues. At present, miR-1468-5p and miR-195 have been found to have an indicative effect on the prognosis of CCA, and their serum detection may have diagnostic significance for CCA [35–37]. Therefore, further study on the mechanism of miR125a-3p may provide a new molecular basis for the diagnosis and prognosis evaluation of CCA.

Declaration of conflicts of interest

The authors declare no potential conflicts of interest.

Funding

This study was supported by the Key Medical Research Project of Hebei Province (No. 20231957; No. 20170227)

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

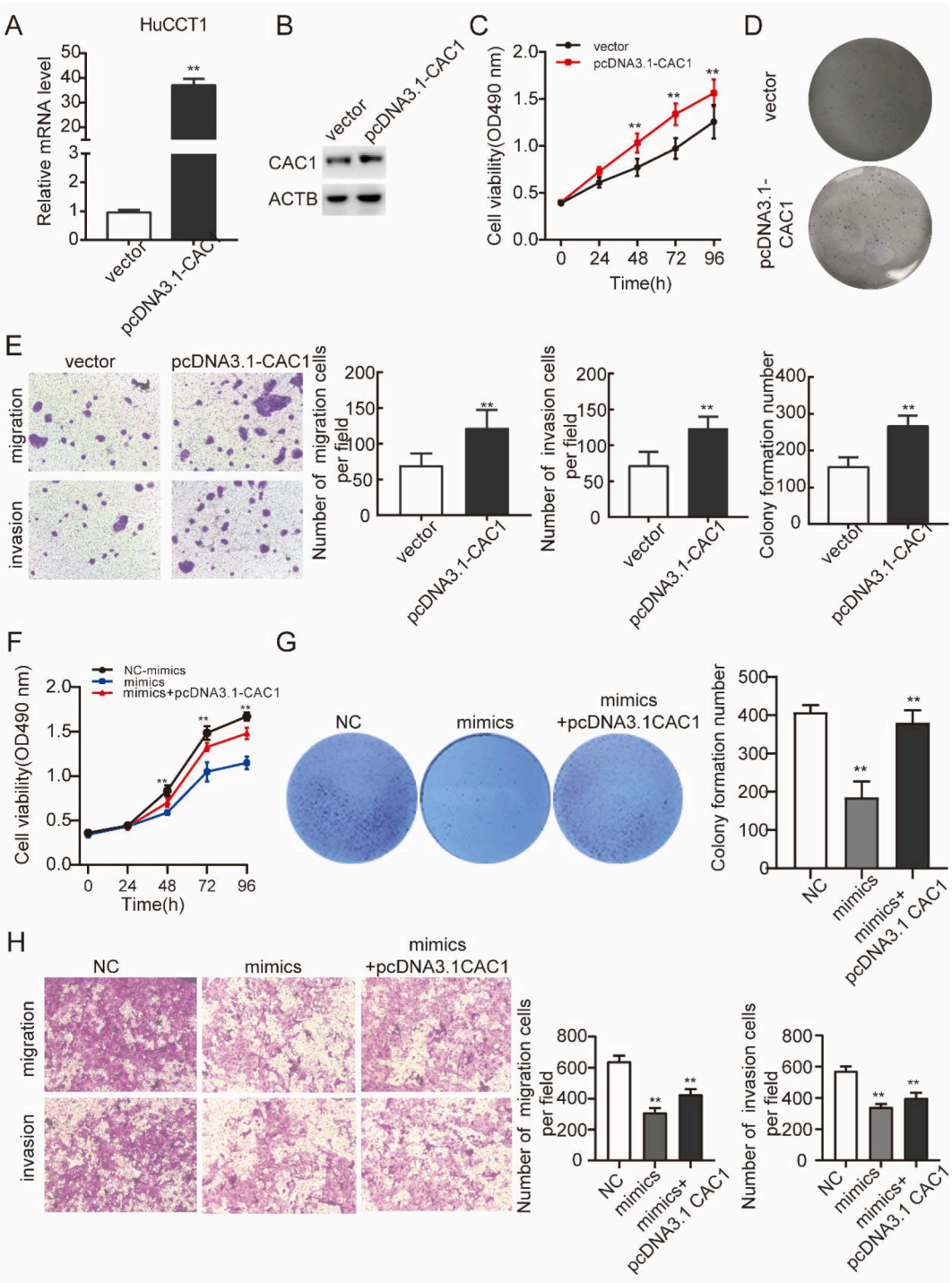


Fig. 5. miR-125a-3p suppresses the proliferation, migration and invasion of cholangiocarcinoma cells by targeting CAC1 in vitro. a. CAC1 expressions were examined using qRT-PCR after pcDNA3.1-CAC1 transfection. b. CAC1 protein expression was examined using western bolt analysis after pcDNA3.1-CAC1 transfection. (The original image is provided in the Supplementary file). c and d. MTS and colony formation assays were performed to evaluate RBE cells proliferation after CAC1 overexpression. e. Cell migration and invasion ability were analyzed by transwell assays (Magnification: × 100). f and g. MTS and colony formation assays were performed to examine the effect of miR-125a-3p/CAC1 axis on cell proliferation ability of CCA cells. h. Transwell assay was performed for evaluating the effect of miR-125a-3p/CAC1 axis on cell invasion and metastatic ability of CCA cells in each group (Magnification: × 100). $^{**}P < 0.01$.

Ethics approval and consent to participate

All experiments performed using human samples were approved by the Ethics Committee of the Handan Central Hospital, Hebei Province (Handan, China; approval no. 2022KY099), and written informed consent was provided by all patients prior to the study start.

Patient consent for publication

Not applicable.

The following are the supplementary data to this article

Multimedia component.

CRediT authorship contribution statement

Xiaojuan Guo: Writing – original draft, Methodology, Formal analysis. **Jinxi Wang:** Investigation, Formal analysis, Data curation. **Yunxiao Tian:** Methodology, Investigation. **Jianhua Yang:** Software, Resources. **Shiqian Wu:** Software, Data curation. **Lihui Xin:** Supervision, Formal analysis. **Zhe Feng:** Validation, Investigation. **Guangxu Niu:** Writing – review & editing, Project administration.

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.

Acknowledgment

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32528.

References

[1] A. Lamarca, A. Santos-Laso, K. Utpatel, A. La Casta, S. Stock, A. Forner, J. Adeva, T. Folseraas, L. Fabris, R.I.R. Macias, M. Krawczyk, M. Krawczyk, V. Cardinale, C. Braconi, D. Alvaro, M. Evert, J.M. Banales, J.W. Valle, Group: on behalf of the European Network for the Study of C, Liver metastases of intrahepatic cholangiocarcinoma: implications for an updated staging system, Hepatology 73 (2021) 2311–2325.
[2] S. Nara, M. Esaki, D. Ban, T. Takamoto, K. Shimada, T. Ioka, T. Okusaka, H. Ishii, J. Furuse, Adjuvant and neoadjuvant therapy for biliary tract cancer: a review of clinical trials, Jpn. J. Clin. Oncol. 50 (2020) 1353–1363.
[3] Y.Y. Tang, Y.N. Zhao, T. Zhang, Z.Y. Chen, X.L. Ma, Comprehensive radiomics nomogram for predicting survival of patients with combined hepatocellular carcinoma and cholangiocarcinoma, World J. Gastroenterol. 27 (2021) 7173–7189.
[4] D. Tummanatsakun, T. Proungvitaya, S. Roytrakul, T. Limpaiboon, S. Wongkham, C. Wongkham, A. Silsirivanit, O. Somintara, S. Sangkhamanon, S. Proungvitaya, Serum apurinic/apyrimidinic endodeoxyribonuclease 1 (APEX1) level as a potential biomarker of cholangiocarcinoma, Biomolecules 9 (2019).
[5] G. Xu, L. Sun, Y. Li, F. Xie, X. Zhou, H. Yang, S. Du, H. Xu, Y. Mao, The clinicopathological and prognostic value of PD-L1 expression in cholangiocarcinoma: a meta-analysis, Front. Oncol. 9 (2019) 897.
[6] P.T.B. Ho, I.M. Clark, L.T.T. Le, MicroRNA-based diagnosis and therapy, Int. J. Mol. Sci. 23 (2022).
[7] S. Tealdi, E. Ferro, C.C. Campa, C. Bosia, microRNA-Mediated encoding and decoding of time-dependent signals in tumorigenesis, Biomolecules 12 (2022).
[8] B. Kour, S. Gupta, R. Singh, Y. Sophiarani, P. Paul, Interplay between circular RNA, microRNA, and human diseases, Mol. Genet. Genom. 297 (2022) 277–286.
[9] V. Rani, R.S. Sengar, Biogenesis and mechanisms of microRNA-mediated gene regulation, Biotechnol. Bioeng. 119 (2022) 685–692.
[10] C. Correa-Gallego, D. Maddalo, A. Doussot, N. Kemeny, T.P. Kingham, P.J. Allen, M.I. D'Angelica, R.P. DeMatteo, D. Betel, D. Klimstra, W.R. Jarnagin, A. Ventura, Circulating plasma levels of MicroRNA-21 and MicroRNA-221 are potential diagnostic markers for primary intrahepatic cholangiocarcinoma, PLoS One 11 (2016) e0163699.
[11] M. Kojima, H. Sudo, J. Kawauchi, S. Takizawa, S. Kondou, H. Nobumasa, A. Ochiai, MicroRNA markers for the diagnosis of pancreatic and biliary-tract cancers, PLoS One 10 (2015) e0118220.
[12] L. Hou, P. Luo, Y. Ma, C. Jia, F. Yu, Z. Lv, C. Wu, D. Fu, MicroRNA-125a-3p downregulation correlates with tumorigenesis and poor prognosis in patients with non-small cell lung cancer, Oncol. Lett. 14 (2017) 4441–4448.
[13] L. Yan, J. Zhang, D. Guo, J. Ma, S.F. Shui, X.W. Han, IL-21R functions as an oncogenic factor and is regulated by the lncRNA MALAT1/miR-125a-3p axis in gastric cancer, Int. J. Oncol. 54 (2019) 7–16.
[14] B.D. Adams, C. Parsons, F.J. Slack, The tumor-suppressive and potential therapeutic functions of miR-34a in epithelial carcinomas, Expert Opin. Ther. Targets 20 (2016) 737–753.
[15] F. Wang, L. Kong, Y. Pu, F. Chao, C. Zang, W. Qin, F. Zhao, S. Cai, Long noncoding RNA DICER1-AS1 functions in methylation regulation on the multi-drugresistance of osteosarcoma cells via miR-34a-5p and GADD45A, Front. Oncol. 11 (2021) 685881.
[16] G. Greville, E. Llop, J. Howard, S.F. Madden, A.S. Perry, R. Peracaula, P.M. Rudd, A. McCann, R. Saldova, 5-AZA-dC induces epigenetic changes associated with modified glycosylation of secreted glycoproteins and increased EMT and migration in chemo-sensitive cancer cells, Clin. Epigenet. 13 (2021) 34.

[17] J. Tao, L. Xia, Z. Cai, L. Liang, Y. Chen, J. Meng, Z. Wang, Interaction between microRNA and DNA methylation in atherosclerosis, DNA Cell Biol. 40 (2021) 101–115.
[18] P. Zhang, M. Zhang, Epigenetic alterations and advancement of treatment in peripheral T-cell lymphoma, Clin. Epigenet. 12 (2020) 169.
[19] A. Seyed Salehi, N. Parsa-Nikoo, F. Roshan-Farzad, R. Shams, M. Fathi, H. Asaszadeh Aghdaei, A. Behmanesh, MicroRNA-125a-3p, -4530, and -92a as a potential circulating MicroRNA panel for noninvasive pancreatic cancer diagnosis, Dis. Markers 2022 (2022) 8040419.
[20] S. Ling, Y. He, X. Li, M. Hu, Y. Ma, Y. Li, Z. Lu, S. Shen, B. Kong, X. Zou, K. Jiang, P. Huang, CircRHOT1 mediated cell proliferation, apoptosis and invasion of pancreatic cancer cells by sponging miR-125a-3p, J. Cell Mol. Med. 24 (2020) 9881–9889.
[21] R. Shams, S. Saberi, M. Zali, A. Sadeghi, S. Ghafouri-Fard, H.A. Aghdaei, Identification of potential microRNA panels for pancreatic cancer diagnosis using microarray datasets and bioinformatics methods, Sci. Rep. 10 (2020) 7559.
[22] K. Okamoto, K. Miyoshi, Y. Murawaki, miR-29b, miR-205 and miR-221 enhance chemosensitivity to gemcitabine in HuH28 human cholangiocarcinoma cells, PLoS One 8 (2013) e77623.
[23] C. Moriya, H. Taniguchi, K. Miyata, N. Nishiyama, K. Kataoka, K. Imai, Inhibition of PRDM14 expression in pancreatic cancer suppresses cancer stem-like properties and liver metastasis in mice, Carcinogenesis 38 (2017) 638–648.
[24] C. Lu, Y. Wei, X. Wang, Z. Zhang, J. Yin, W. Li, L. Chen, X. Lyu, Z. Shi, W. Yan, Y. You, DNA-methylation-mediated activating of lncRNA SNHG12 promotes temozolomide resistance in glioblastoma, Mol. Cancer 19 (2020) 28.
[25] B. Qiu, X. Xu, P. Yi, Y. Hao, Curcumin reinforces MSC-derived exosomes in attenuating osteoarthritis via modulating the miR-124/NF-kB and miR-143/ROCK1/TLR9 signalling pathways, J. Cell Mol. Med. 24 (2020) 10855–10865.
[26] T. La, G.Z. Liu, M. Farrelly, N. Cole, Y.C. Feng, Y.Y. Zhang, S.K. Sherwin, H. Yari, H. Tabatabaee, X.G. Yan, S.T. Guo, T. Liu, R.F. Thorne, L. Jin, X.D. Zhang, A p53-responsive miRNA network promotes cancer cell quiescence, Cancer Res. 78 (2018) 6666–6679.
[27] R. Santana, R. Zuluaga, P. Ganan, S. Arrasate, E. Onieva, M.M. Montemore, H. Gonzalez-Diaz, PTML model for selection of nanoparticles, anticancer drugs, and vitamins in the design of drug-vitamin nanoparticle release systems for cancer cotherapy, Mol. Pharm. 17 (2020) 2612–2627.
[28] N. Chen, Y. Kong, Y. Wu, Q. Gao, J. Fu, X. Sun, Q. Geng, CAC1 knockdown reverses drug resistance through the downregulation of P-gp and MRP-1 expression in colorectal cancer, PLoS One 14 (2019) e0222035.
[29] J. Kim, U.H. Park, M. Moon, S.J. Um, E.J. Kim, Negative regulation of ERalpha by a novel protein CAC1 through association with histone demethylase LSD1, FEBS Lett. 587 (2013) 17–22.
[30] T.J. Chen, F. Gao, T. Yang, A. Thakur, H. Ren, Y. Li, S. Zhang, T. Wang, M.W. Chen, CDK-associated Cullin 1 promotes cell proliferation with activation of ERK1/2 in human lung cancer A549 cells, Biochem. Biophys. Res. Commun. 437 (2013) 108–113.
[31] Q. Zheng, L.Y. Zhao, Y. Kong, K.J. Nan, Y. Yao, Z.J. Liao, CDK-associated Cullin 1 can promote cell proliferation and inhibit cisplatin-induced apoptosis in the AGS gastric cancer cell line, World J. Surg. Oncol. 11 (2013) 5.
[32] T. Fukuda, Y. Kigoshi-Tansho, T. Naganuma, A. Kazaana, T. Okajima, F. Tsuruta, T. Chiba, CACUL1/CAC1 attenuates p53 activity through PML post-translational modification, Biochem. Biophys. Res. Commun. 482 (2017) 863–869.
[33] K. Zhang, Y. Gao, J. Li, R. Burgess, J. Han, H. Liang, Z. Zhang, Y. Liu, A DNA binding winged helix domain in CAF-1 functions with PCNA to stabilize CAF-1 at replication forks, Nucleic Acids Res. 44 (2016) 5083–5094.
[34] D. Kim, D. Setiaputra, T. Jung, J. Chung, A. Leitner, J. Yoon, R. Aebersold, H. Hebert, C.K. Yip, J.J. Song, Molecular architecture of yeast chromatin assembly factor 1, Sci. Rep. 6 (2016) 26702.
[35] C. Zhou, W. Wei, J. Ma, Y. Yang, L. Liang, Y. Zhang, Z. Wang, X. Chen, L. Huang, W. Wang, S. Wu, Cancer-secreted exosomal miR-1468-5p promotes tumor immune escape via the immunosuppressive reprogramming of lymphatic vessels, Mol. Ther. 30 (2022) 976–977.
[36] V. Sanchez, F. Golyardi, D. Mayaki, R. Echavarria, S. Harel, J. Xia, S.N.A. Hussain, Negative regulation of angiogenesis by novel micro RNAs, Pharmacol. Res. 139 (2019) 173–181.
[37] L. Li, K. Piontek, M. Ishida, M. Fausther, J.A. Dranoff, R. Fu, E. Mezey, S.J. Gould, F.K. Fordjour, S.J. Meltzer, A.E. Sirica, F.M. Selaru, Extracellular vesicles carry microRNA-195 to intrahepatic cholangiocarcinoma and improve survival in a rat model, Hepatology 65 (2017) 501–514.