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*Original Article*

# Long non-coding RNA ANRIL overexpression predicts poor prognosis and promotes metastasis and proliferation in perihilar cholangiocarcinoma

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**Abstract:** Perihilar cholangiocarcinoma (PHCC) is still a great challenge for doctors because of its aggressive feature and poor survival rate. The molecular mechanisms of PHCC initiation and progression remain obscure at present. Accumulating evidence discovered that long non-coding RNAs (lncRNAs) are crucial regulators in cancer progression. However, the functional role of lncRNAs in PHCC has been rarely reported. The present study found lncRNA ANRIL was markedly overexpressed in PHCC cell lines and clinical specimens compared with normal human cholangiocyte cell line and tumor adjacent tissues, respectively. Statistical analysis of PHCC clinicopathological characteristics with ANRIL expression data found that high ANRIL expression was correlated with advanced TNM stage and vascular invasion ($P < 0.05$). Besides, ANRIL up-regulation was investigated to be associated with poor overall survival and progression-free survival of PHCCs. In addition, both univariate and multivariate COX regression analyses revealed that high ANRIL expression was an independent risk factor of PHCC poor prognosis. ANRIL up-regulation promoted migration and invasion of PHCC cells on Transwell assay and Matrigel assay, while ANRIL deficiency inhibited PHCC cells metastasis. Moreover, PHCC cells with ANRIL overexpression exhibited obviously increased proliferation ability on Colony formation assay and MTT assay. However, ANRIL interference suppressed proliferation significantly. In conclusion, ANRIL could promote PHCC clinical progression, proliferation and metastasis. LncRNA ANRIL may serve as a promising prognostic biomarker and therapeutic target of PHCC.

**Keywords:** Long non-coding RNA ANRIL, metastasis, proliferation, prognosis, perihilar cholangiocarcinoma

## Introduction

Perihilar cholangiocarcinoma (PHCC), originating from the epithelial cells of the bile duct, accounts for about 50% of cholangiocarcinoma (CC) [1]. Despite numerous advances in the diagnosis and treatment strategies of PHCC in recent years, the prognosis of PHCC patients remains poor at present. Rates of 5-year survival following surgical resection with negative margins range from 11% to 41% [2]. PHCCs are characterized by genetic and epigenetic alterations, chromosome aberration, aggressive nature and desmoplastic stroma [3], which make PHCC resistant to most existing adjuvant therapies. So the carcinogenesis and developmental mechanisms of PHCC should be further elucidated to establish more effective therapeutic strategies.

Long non-coding RNAs (LncRNAs), defined as non-protein-coding RNA molecules longer than 200 nucleotides, have attracted great attention in the past few years [4]. Accumulating evidence revealed that lncRNAs play widespread roles in gene regulation and other cellular processes, including but not limited to imprinting, histone-code regulation, gene activation, gene repression, lineage determination, cell proliferation, cell apoptosis and stem cell characteristics [5-7]. Intriguingly, recent literatures discovered that lncRNAs are essential regulators in cancer biology [8-11]. Aberrant lincRNAs are strongly associated with cancer progression [12]. Thus, lincRNAs may be utilized as cancer diagnostic and prognostic biomarkers and potential therapeutic targets [8]. LncRNA antisense non-coding RNA in the INK4 locus (ANRIL) was first reported to be deleted in the melano-

ma-neural system tumor syndrome family [13]. Further investigations found ANRIL was up-regulated in multiple cancers and could facilitate tumor proliferation and metastasis [14-23]. Moreover, lncRNA ANRIL was presented as a prognostic marker in gastric cancer [24], nasopharyngeal carcinoma [21], cervical cancer [25], ovarian cancer [26] and breast cancer [27]. However, to the best of our knowledge, the functional role of lncRNA ANRIL in PHCC has not been illuminated yet.

This study investigated the expression and clinical significance of lncRNA ANRIL in PHCC cell lines and clinical tissues. Besides, the role of ANRIL in predicting PHCC prognosis was also revealed. In addition, multivariate COX regression analysis identified high ANRIL expression as an independent risk factor of overall survival (OS) and progression-free survival (PFS). Finally, ANRIL was demonstrated to promote metastasis and proliferation of PHCC cells *in vitro* assays. These results suggested that ANRIL could promote the development of PHCC, and may serve as an essential prognostic marker and therapeutic target in PHCC.

## Materials and methods

### *Patients and tissue samples*

A total of 82 PHCC patients who underwent surgical resection at the First Affiliated Hospital of Jinan University were enrolled in this study, and written informed consents were obtained from each patient. Tumor adjacent non-tumorous tissues were isolated from at least 1 cm away from the tumor border. The collected paired tissues were frozen with liquid nitrogen following resection and stored at -80°C until being used. The exclusion criterions were receiving anticancer therapies before surgery or diagnosed with two or more malignances. This study was approved by the ethics committee of the First Affiliated Hospital of Jinan University.

### *Cell lines and cell culture*

Four PHCC cell lines, including EGI-1, TFK-1, QBC939 and FRH0201 and one normal human cholangiocyte cell line, H69, were used in this study. EGI-1 and TFK-1 were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), QBC939, FRH0201 and H69 were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). EGI-1, TFK-1, QBC939 and FRH0201 were all cultured in RPMI-1640 medium (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) and 1% Penicillin/Streptomycin (Invitrogen, USA)in 5% $CO_2$ humified atmosphere at 37°C. H69 were cultured as previously described [28].

### *RNA extraction, reverse transcription and real-time PCR*

Total RNA of PHCC clinical specimens and cell lines was extracted with TRIzol reagent (Invitrogen, USA). Reverse transcription (RT) was conducted with using the Prime-Script one step RT-PCR kit (Takara, Dalian, China) according to the manufacturer's instructions. Then, synthesized cDNA was used for the real-time PCR on the Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems, USA) with the SYBR PrimeScript RT-PCR Kit (Takara, Dalian, China). Relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. GAPDH was utilized to normalize the relative abundance of mRNAs. The following sequences of the primers were used: ANRIL forward, 5'-TGCTCTATCCGCCAATCAGG-3'; ANRIL reverse, 5'-GGGCCTCAGTGGCACATACC-3'; GAPDH forward, 5'-GCCGCATCTTCTTTTGCGTCGC-3'; GAPDH reverse, 5'-TCCCGTTCTCAGCCTTGACGGT-3'.

### *Transwell assay and matrigel assay*

For Transwell assay, transwell chamber (8.0 μm pore size; BD Biosciences, CA, USA) was used. QBC939 or EGI-1 transfected cells ($5 \times 10^4$) were suspended in serum-free medium and then plated in the top chamber with non-coated membrane. For the Matrigel assay, the transwell membrane was pre-coated with matrigel (BD Biosciences, CA, USA). While medium containing 10% FBS was added to the lower chamber. The established models were subjected to incubation for 24 hours. Subsequently, cells migrated to the lower chamber were fixed with methanol for 30 min, stained with giemsa for 30 min, followed by visualization with a phase-contrast inverted microscope.

### *Colony formation assay*

To evaluate the proliferation ability of PHCC cells, colony formation assay was conducted. Cells (1000 cells per dish) were seeded in 6-wellplates and maintained in RPMI-1640 medium containing 10% FBS. Ten days later,

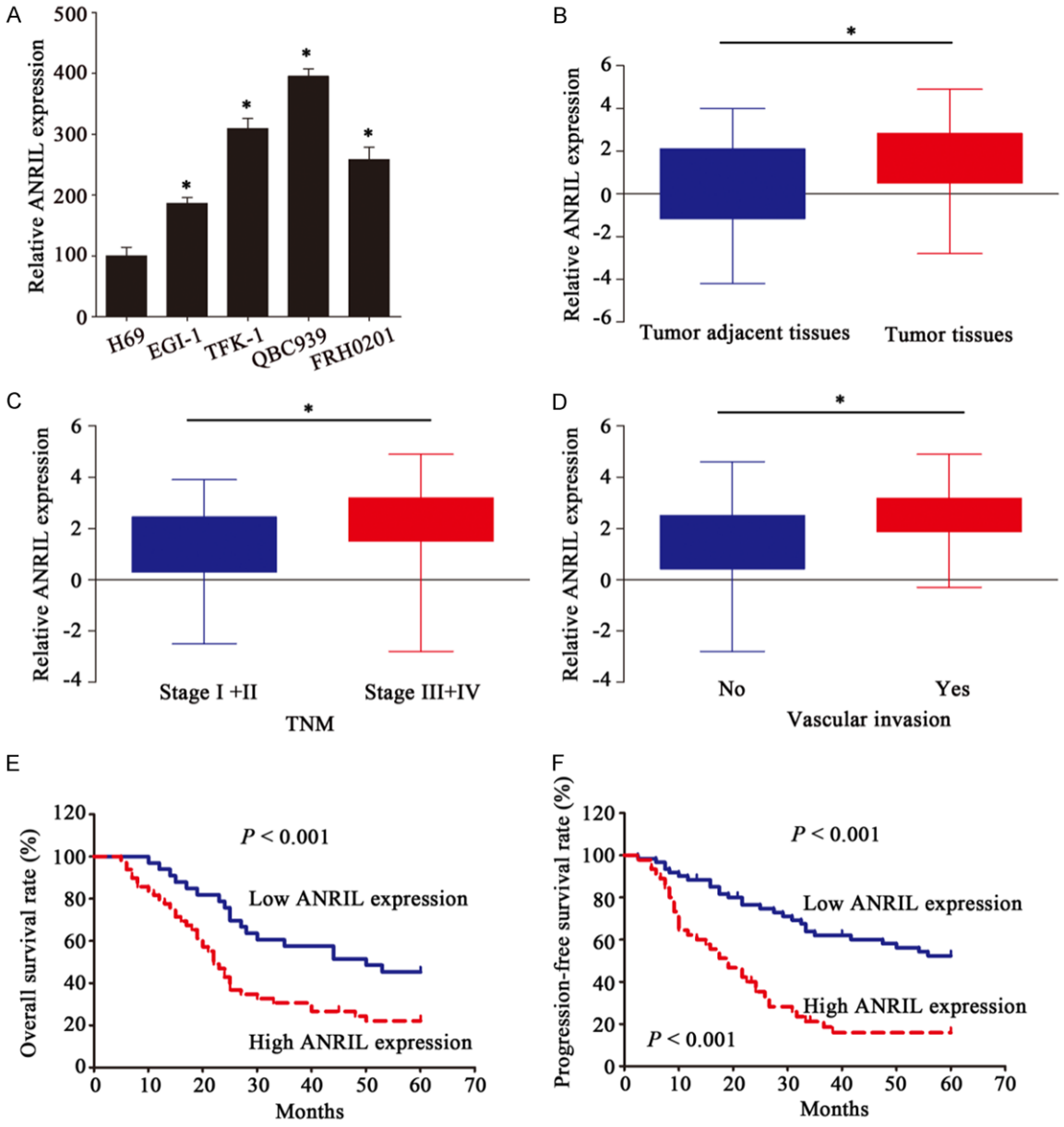


**Figure 1.** Evaluation of lncRNA ANRIL expression level and clinical significance in PHCC. (A) LncRNA ANRIL expression levels were determined by qRT-PCR in human PHCC cell lines (TFK-1, EGI-1, QBC939 and FRH0201) and the normal human cholangiocyte cell line (H69). (B) Relative expression of ANRIL was examined by qRT-PCR in PHCC tissues and paired tumor adjacent tissues. (C) Relative expression of ANRIL was examined by qRT-PCR in PHCC tissues with TNM stage (I+II) and stage (III+IV). (D) Relative expression of ANRIL was examined by qRT-PCR in PHCC tissues with/without vascular invasion. (E) The overall survival of PHCC patients with low ANRIL expression was compared with patients with high ANRIL expression. (F) The progression-free survival of PHCC patients with low ANRIL expression was compared with patients with high ANRIL expression. $P < 0.05$ in (A-F) was calculated with Student's *t*-test. $P < 0.001$ in (E and F) were analyzed by log-rank test.

the colonies was fixed, stained and then manually counted.

*MTT assay*

For the MTT assay, PHCC cells (2000 per well) were plated in 96-well plates. At different time points (0, 1, 2, 3 and 4 d after initial incubation), MTT (10 μl, 10 mg/ml) was added to the medium. Then, an additional 4 h incubation was followed. Subsequently, the formed formazan blue was dissolved with DMSO (100 μl). Finally, the absorbance was measured using a microplate spectrophotometer (Dynex technol-

**Table 1.** Correlation between lncRNA ANRIL expression and PHCC clinicopathological characteristics

| Parameters | NO. of patients | ANRIL (Low/High) | *P* value |
|---|---|---|---|
| Age | | | 0.822 |
| < 60 years | 41 | 17/24 | |
| ≥ 60 years | 41 | 16/25 | |
| Gender | | | 0.185 |
| Male | 47 | 16/31 | |
| Female | 35 | 17/18 | |
| Vascular invasion | | | 0.001 |
| No | 56 | 29/27 | |
| Yes | 26 | 4/25 | |
| Differentiation grade | | | 0.132 |
| Well + Moderate | 58 | 25/23 | |
| Poor + Undifferentiation | 24 | 8/16 | |
| Tumor size | | | 0.159 |
| < 3 cm | 37 | 18/19 | |
| ≥ 3 cm | 45 | 15/30 | |
| T stage | | | 0.546 |
| T1+T2 | 67 | 28/39 | |
| T3+T4 | 15 | 5/10 | |
| N stage | | | 0.107 |
| N0 | 51 | 24/27 | |
| N1 | 31 | 9/22 | |
| M stage | | | 0.245 |
| M0 | 79 | 33/36 | |
| M1 | 3 | 0/3 | |
| TNM stage | | | 0.003 |
| I+II | 41 | 23/18 | |
| III+IV | 41 | 10/31 | |

ogies, Sullyfield, VA) at a wavelength of 490 nm.

*Statistical analysis*

All continuous data was presented as mean values ± standard deviations (M ± SD). The significance of differences between groups was compared by $\chi^2$ test, Fisher's exact test or Student's *t*-test as indicated. Survival curves were calculated with Kaplan-Meier method, and differences between different groups were assessed with the log-rank test. The effects of variables on survival were assessed using univariate and multivariate Cox regression methods. All statistical analysis was conducted with the SPSS 19.0 software (IBM, USA). The significance level was set at $P < 0.05$ indicating statistically different.

## Results

*ANRIL is up-regulated and facilitates tumor progression in PHCC*

The expression of lncRNA ANRIL was first detected in PHCC cell lines by qRT-PCR assay, which showed that ANRIL was up-regulated in PHCC cells compared with normal human cholangiocyte cell line, H69. (**Figure 1A**) Besides, ANRIL expression was further examined in PHCC tissues and paired tumor adjacent tissues by qRT-PCR. Interestingly, PHCC tissues exhibited much higher level of ANRIL relative to paired tumor adjacent tissues (0.63 ± 2.11 vs. 1.69 ± 1.75, $P < 0.001$). (**Figure 1B**) Moreover, patients with advanced TNM stage (stage III and IV) exhibited much higher level of ANRIL expression than those with stage I and II (1.18 ± 1.61 vs. 2.20 ± 1.75, $P = 0.004$). (**Figure 1C**) In addition, ANRIL expression was also increased significantly in patients with vascular invasion compared with those without vascular invasion (1.29 ± 1.78 vs. 2.55 ± 1.34, $P < 0.001$). (**Figure 1D**) These results suggested that ANRIL may promote carcinogenesis and cancer development in PHCC.

To confirm our observations, all the patients were divided into 2 groups with the mean T/A value (determined by comparing ANRIL expression in PHCC tissue with that in paired tumor adjacent tissue) serving as the cutoff score ($M_{T/A} = 1.03$). And correlation analysis between ANRIL expression and PHCC clinicopathological characteristics was performed. As expected, ANRIL overexpression was revealed to be associated with advanced TNM stage and vascular invasion ($P < 0.05$), but not correlated with other clinicopathological features, such as age, gender, differentiation grade, tumor size, T, N and M stage ($P > 0.05$). (**Table 1**) Taken together, ANRIL is up-regulated in PHCC and could facilitate PHCC progression.

*ANRIL overexpression predicts poor prognosis in PHCC*

To further investigate the clinical significance of ANRIL in PHCC, the correlation between ANRIL expression and PHCC prognosis was analyzed. Kaplan-Meier test and log-rank test verified that patients with high ANRIL expression had much poorer OS rates and shorter PFS periods than those with low ANRIL expression. ($P < 0.001$) (**Figure 1E**, **1F**)

**Table 2.** Univariate and multivariate analysis of clinicopathologic features for overall survival and progression-free survival of PHCC patients

| Parameters | Overall survival | | | Progression-free survival | | |
|---|---|---|---|---|---|---|
| | HR | 95% CI | *P* value | HR | 95% CI | *P* value |
| Univariate analysis | | | | | | |
| Age ≥ 60 years vs. < 60 years | 0.983 | 0.573-1.685 | 0.950 | 1.006 | 0.595-1.699 | 0.983 |
| Gender Female vs. Male | 0.709 | 0.406-1.236 | 0.225 | 0.758 | 0.443-1.297 | 0.312 |
| Vascular invasion Yes vs. No | 1.721 | 0.980-3.022 | 0.059 | 1.716 | 0.992-2.968 | 0.054 |
| Differentiation Poor + Undifferentiated vs. Well + Moderate | 1.405 | 0.788-2.505 | 0.249 | 1.594 | 0.915-2.777 | 0.100 |
| Tumor size ≥ 3 cm vs. < 3 cm | 0.973 | 0.567-1.670 | 0.922 | 1.007 | 0.595-1.703 | 0.980 |
| T stage (T3+T4) vs. (T1+T2) | 1.406 | 0.723-2.733 | 0.316 | 1.439 | 0.760-2.726 | 0.264 |
| N stage N1 vs. N0 | 2.666 | 1.545-4.602 | < 0.001 | 2.421 | 1.422-4.121 | 0.001 |
| M stage M1 vs. M0 | 14.387 | 3.814-54.270 | < 0.001 | 11.678 | 3.236-42.147 | < 0.001 |
| TNM stage (III+IV) vs. (I+II) | 3.353 | 1.895-5.934 | < 0.001 | 3.318 | 1.905-5.779 | < 0.001 |
| ANRIL High vs. Low | 1.544 | 1.263-1.887 | < 0.001 | 1.492 | 1.234-1.805 | < 0.001 |
| Multivariate analysis | | | | | | |
| N stage N1 vs. N0 | 0.750 | 0.331-1.697 | 0.489 | 0.681 | 0.322-1.441 | 0.315 |
| M stage M1 vs. M0 | 5.287 | 1.321-21.165 | 0.019 | 4.583 | 1.192-17.621 | 0.027 |
| TNM stage (III+IV) vs. (I+II) | 3.323 | 1.415-7.805 | 0.006 | 3.484 | 1.594-7.618 | 0.002 |
| ANRIL High vs. Low | 1.403 | 1.146-1.717 | 0.001 | 1.346 | 1.113-1.627 | 0.002 |

Univariate COX analysis discovered that high ANRIL expression was a risk factor of poor OS (HR = 1.544, 95% CI = 1.263-1.887, $P < 0.001$) and PFS (HR = 1.492, 95% CI = 1.234-1.805, $P < 0.001$) (**Table 2**). Further analysis with multivariate COX regression analysis found that high ANRIL expression was an independent risk factor of PHCC poor OS (HR = 1.403, 95% CI = 1.146-1.717, $P < 0.001$) and PFS (HR = 1.346, 95% CI = 1.113-1.627, $P = 0.002$) (**Table 2**). Together, these findings indicate that ANRIL may be highlighted as an essential prognostic marker of PHCC patients.

*ANRIL promotes metastasis and proliferation of PHCC cells*

To extend our understanding of the functional role of ANRIL in PHCC, ANRIL was knocked down with siRNA or overexpressed in QBC939 cells and EGI-1 cells, respectively. (**Figure 2A**, **2B**) As expected, ANRIL deficiency obviously inhibited migration and invasion of QBC939 cells on Transwell assay and Matrigel assay. (**Figure 2C**) Consistently, ANRIL ectopic expression markedly promoted the motility of EGI-1 cells. (**Figure 2D**) Furthermore, both colony formation assay and MTT assay confirmed that ANRIL interference reduced the proliferation ability of QBC939 cells. (**Figure 3A**, **3B**) Accordingly, ANRIL up-regulation significantly facilitated proliferation of EGI-1 cells. (**Figure 3C**, **3D**) Above all, ANRIL could promote metastasis and proliferation of PHCC cells.

## Discussion

LncRNA ANRIL is encoded in the chromosome 9p21 region, a hotspot for disease-associated polymorphisms, and there is an altered cellular proliferation component in almost all the diseases associated with the locus [29]. The effect of ANRIL on cellular proliferation has been well elucidated. Naemura *et al.* [15] reported that ANRIL could regulate the proliferation of colorectal cancer cells in both two- and three-dimensional culture. In nasopharyngeal carcinoma, ANRIL was examined to promote cell proliferation by reprograming cellular glucose metabolism, which may provide ATP provision for cell proliferation [21]. Besides, ANRIL was also demonstrated to regulate the proliferation of bladder cancer cells and epithelial ovarian cancer cells through mediating cell apoptosis or senescence [16, 18]. In addition, recent studies confirmed that ANRIL could also facilitate metastasis and correlated with chemotherapy resistance in various cancers [19, 20, 23, 26, 30]. Moreover, lncRNA ANRIL was considered as a prognostic marker in gastric cancer [24], nasopharyngeal carcinoma [21], cervical cancer [25], ovarian cancer [26] and breast cancer [27], which shed new light on utilizing ANRIL as an effective diagnostic marker and

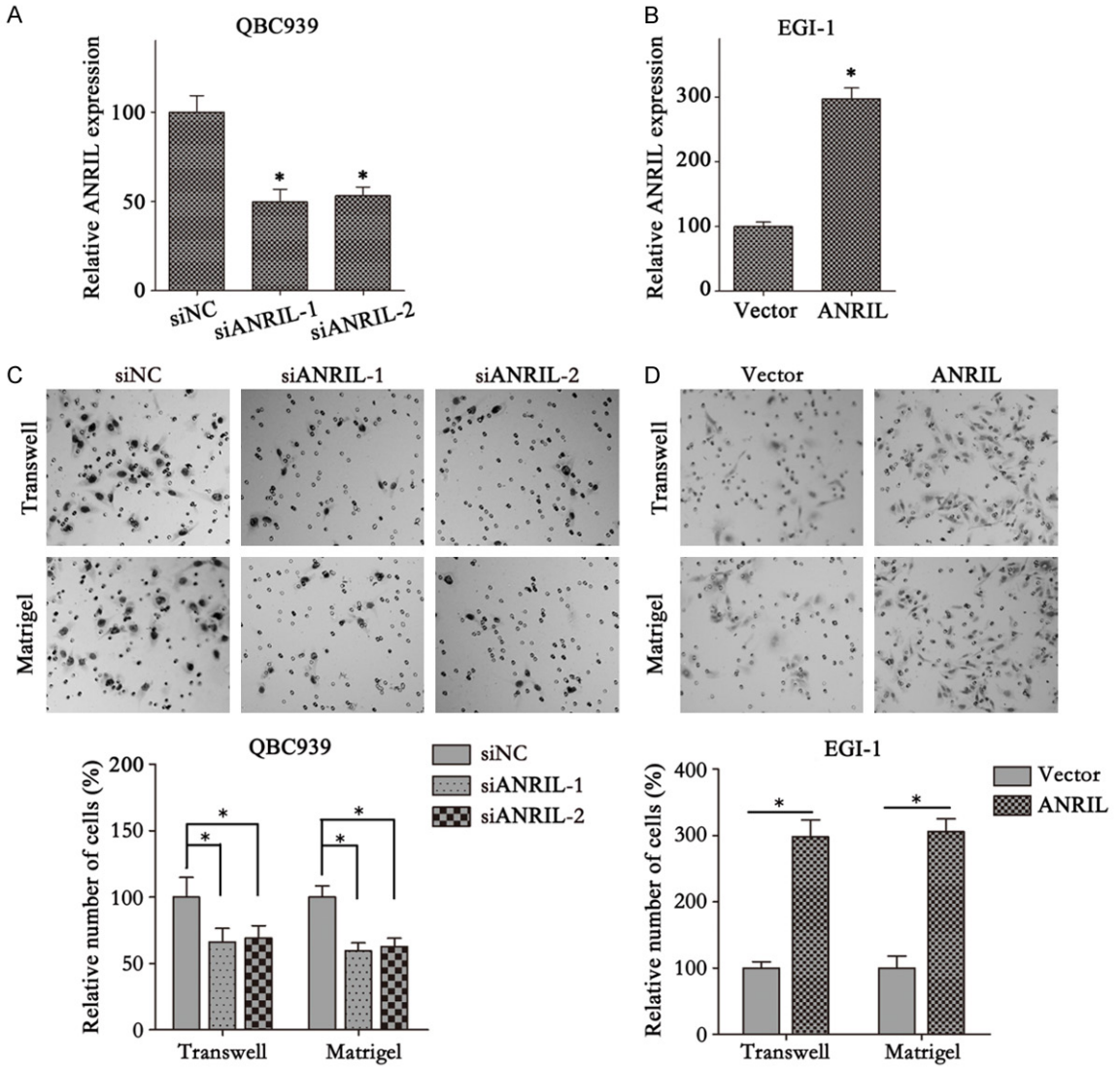


**Figure 2.** ANRIL up-regulation facilitates metastasis of PHCC cells. (A and B) Relative expression of ANRIL was quantified in PHCC cells with ANRIL interference (A) or overexpression (B) in comparison with corresponding control cells. (C) Transwell assay and Matrigel assay were adopted to verify the migration ability and invasion ability of QBC939 cells with ANRIL deficiency. (D) Transwell assay and Matrigel assay were adopted to detect the migration ability and invasion ability of EGI-1 cells with ANRIL ectopic overexpression. $P < 0.05$ was calculated with Student's *t*-test.

therapeutic target in cancer diagnosis and treatment.

Our study first revealed ANRIL was up-regulated in PHCC and associated with PHCC progression. As presented in other cancer types, ANRIL overexpression predicted poor OS and PFS in PHCC. In addition, multivariate COX regression analysis identified high ANRIL expression as an independent risk factor of OS (HR = 1.403, 95% CI = 1.146-1.717, $P < 0.001$) and PFS (HR = 1.346, 95% CI = 1.113-1.627, $P = 0.002$). It was verified that ANRIL could promote metastasis on Transwell and Matrigel assays, and increase cell proliferation abilities on colony formation and MTT assays. These results suggested that ANRIL could promote the development of PHCC, and may serve as an essential prognostic marker and therapeutic target of PHCC patients.

Numerous literatures have focused on the mechanisms of ANRIL promoting cancer development. ANRIL has been shown to epigenetically regulate its neighbor genes, CDKN2A/B, which are known tumor suppressors and have well-established roles in cell proliferation, apoptosis, senescence and aging [31, 32], and

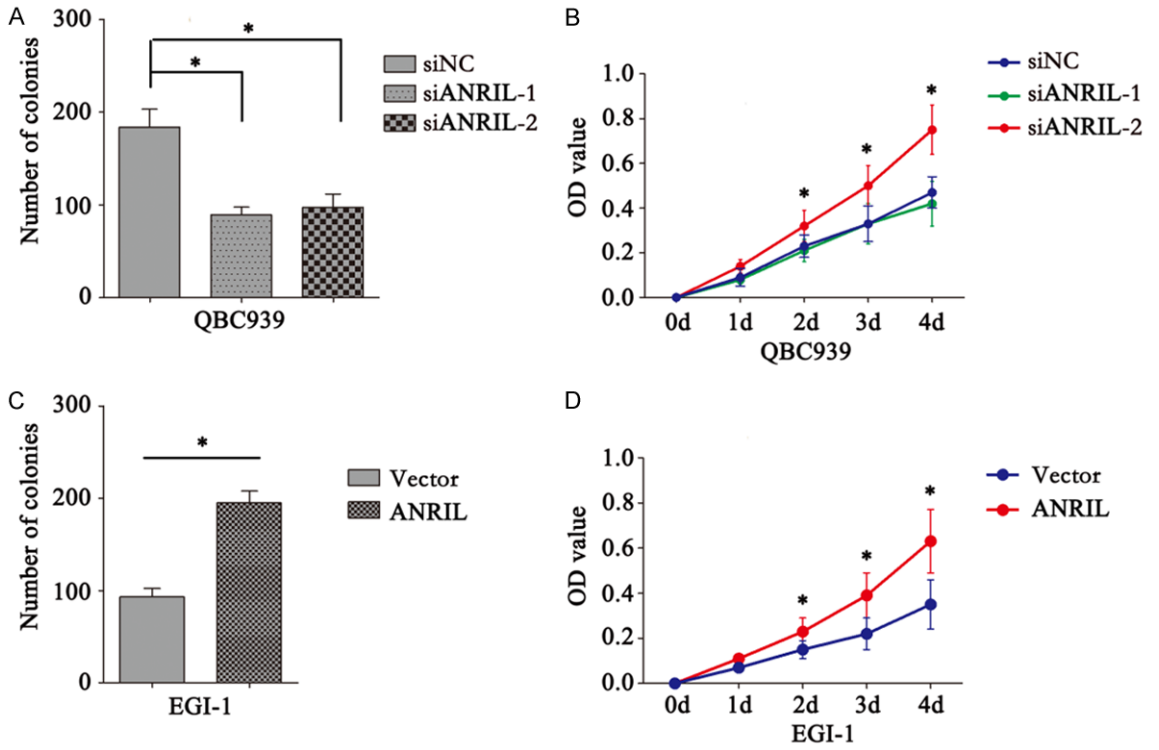


**Figure 3.** ANRIL could promote the proliferation of PHCC cells. A. Colony formation assay was conducted to evaluate the proliferation of QBC939 cells with ANRIL knock-down. B. Cell proliferation ability was assessed with MTT assay after ANRIL silencing in QBC939 cells. C. Colony formation assay was conducted to evaluate the proliferation of EGI-1 cells with ANRIL up-regulation. D. Cell proliferation ability was assessed with MTT assay after ANRIL overexpression in EGI-1 cells. $P < 0.05$ was calculated with Student's *t*-test.

thereby regulate cell proliferation and senescence [33]. Wang *et al.* [34] indicated that downregulation of lncRNA ANRIL represses tumorigenicity and enhances cisplatin-induced cytotoxicity via regulating microRNA let-7a in nasopharyngeal carcinoma. Besides, ANRIL-mediated growth promotion was also suggested to be partly caused by epigenetic repression of miR-99a/miR-449a by binding to PRC2, thus promoting gastric cancer cell proliferation [24]. Some studies declared that ANRIL could epigenetically repress Kruppel-like factor 2 (KLF2) transcription by binding with PRC2 and recruiting it to the KLF2 promoter region and thus drive carcinogenesis [14, 22, 35]. All this evidence supports the idea that ANRIL is a key regulatory molecule in mediating tumor progression through various pathways at different levels and cellular settings. The detailed mechanisms of ANRIL promote PHCC progression deserves further investigations.

In conclusion, this study confirmed ANRIL was overexpressed in PHCC and correlated with PHCC clinical progression and prognosis. ANRIL was identified as an independent risk factor of PHCC poor OS or early recurrence. In addition, ANRIL aberrant expression could regulate the proliferation and metastasis abilities of PHCC cells. These results highlighted ANRIL as a prognostic marker and therapeutic target of PHCC.

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## Disclosure of conflict of interest

None.

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