

# LncRNA ANCR Suppresses the Progression of Hepatocellular Carcinoma Through the Inhibition of Wnt/ $\beta$ -Catenin Signaling Pathway

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**Objective:** Our study aimed to investigate the effect of anti-differentiation noncoding RNA (*ANCR*) on hepatocellular carcinoma (HCC) and its potential molecular mechanisms.

**Methods:** The expression of *ANCR* was detected by qRT-PCR in both HCC tissues and HCC cells. Moreover, the relationship between *ANCR* expression and clinical parameters in HCC patients was investigated. The proliferation, cell clones, migration, invasion and apoptosis of MHCC97H and HCCLM3 cells were measured by MTT assay, colony formation assay, transwell assay and flow cytometry, respectively. The expressions of N-cadherin, vimentin, E-cadherin, cleaved caspase-3, Bax, Bcl-2, Wnt1,  $\beta$ -catenin and GSK-3 $\beta$  in MHCC97H and HCCLM3 cells were measured by Western blot.

**Results:** Our results showed that *ANCR* was lowly expressed in both HCC tissues and HCC cells. *ANCR* expression was closely associated with tumor size, tumor-node-metastasis (TNM) stages and vascular invasion in HCC. *ANCR* could dramatically inhibit cell proliferation, migration and invasion, as well as promote apoptosis in MHCC97H and HCCLM3 cells. *ANCR* could significantly increase the expression of cleaved caspase-3, Bax, E-cadherin and GSK-3 $\beta$  but reduce the expression of Bcl-2, N-cadherin, vimentin, Wnt1 and  $\beta$ -catenin in MHCC97H and HCCLM3 cells. In addition, Wnt/ $\beta$ -catenin pathway inhibitor (IWP-2) partially reversed the effects of silencing *ANCR* on the proliferation, migration, invasion and apoptosis of HCCLM3 cells.

**Conclusion:** Our study demonstrated that *ANCR* can suppress cell proliferation, migration and invasion, as well as promote apoptosis of HCC cells via modulation of the Wnt/ $\beta$ -catenin signaling pathway.

**Keywords:** hepatocellular carcinoma, ANCR, proliferation, apoptosis, metastasis, Wnt/ $\beta$ -catenin pathway

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers and remains the second leading cause of cancer-related death all over the world.<sup>1</sup> Recently, despite the advances in therapeutic approaches, the 5-year survival of HCC patients still remains low due to the high incidence of metastases and recurrence.<sup>2,3</sup> Therefore, it is urgent to find new therapeutic targets and diagnostic biomarkers for the treatment of HCC.

Long noncoding RNAs (lncRNAs) are a group of RNA molecules of more than 200 nucleotides in length and lack the ability of coding protein.<sup>4</sup> More and more studies have indicated that lncRNAs can play an important role in various human

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cancers.<sup>5,6</sup> In addition, lncRNAs can reportedly modulate malignant behaviors of tumor cells, such as cell proliferation, cell cycle, apoptosis, migration and invasion.<sup>7,8</sup> For example, lncRNA *MCM3AP-AS1* as an oncogene could accelerate cell proliferation and cell cycle progression, and suppress cell apoptosis in HCC.<sup>9</sup> Yang et al<sup>10</sup> have indicated that lncRNA SNHG7 promotes the proliferation, migration and invasion of HCC cells through the regulation of *miR-122-5p*. In the recent years, anti-differentiation noncoding RNA (*ANCR*), a newly identified long noncoding RNA, is proved to be a tumor suppressor gene in multiple human cancers, such as breast cancer,<sup>11</sup> non-small cell lung cancer<sup>12</sup> and osteosarcoma.<sup>13</sup> However, the potential effect of *ANCR* on HCC has not been clearly elucidated.

Wnt/ $\beta$ -catenin pathway is a canonical Wnt signaling pathway which may modulate a series of biological processes, including cell proliferation, apoptosis, cell cycle, migration and invasion.<sup>14</sup> Moreover, accumulating evidence has suggested that Wnt/ $\beta$ -catenin pathway plays a vital role in the development of multiple cancers, including HCC.<sup>15</sup> Moreover, lncRNAs can exert anti-cancer or pro-cancer effect through modulating Wnt/ $\beta$ -catenin pathway in cancers, including HCC.<sup>16</sup> For instance, lncRNA *FAM83H-AS1* could facilitate cell proliferation, migration and invasion via regulating Wnt/ $\beta$ -catenin pathway in HCC.<sup>17</sup> However, whether *ANCR* affects HCC through regulating Wnt/ $\beta$ -catenin pathway is unknown.

In this study, we explored the effect of *ANCR* on HCC and its potential molecular mechanisms. Our cumulative data demonstrated that *ANCR* could suppress cell proliferation, migration and invasion, as well as promote apoptosis of HCC through inhibiting Wnt/ $\beta$ -catenin signaling pathway. Findings of our study may provide new theoretical foundation for deeply exploring the treatment of HCC.

## Materials and Methods

### Clinical Samples

A total of 75 HCC tissues and corresponding adjacent non-tumor tissues were collected from HCC patients underwent hepatectomy at Weihai Municipal Hospital during January 2017 to June 2019. The patients did not receive chemotherapy, radiotherapy, immunotherapy or targeted therapy prior to surgical resection. After surgery, the tissues were immediately kept in liquid nitrogen for subsequent use. Our study was approved by the Ethics

Committee of Weihai Municipal Hospital (No. 2017030), and performed in accordance with the Declaration of Helsinki. Before the study, all patients provided their written informed consent.

### Cell Cultures

The immortalized normal liver epithelial cell line (THLE-3) and human HCC cell lines (MHCC97H, HCCLM3, Huh7 and Hep-3B) were purchased from the China Center for Type Culture Collection (Wuhan, China). All cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) complemented with 10% fetal bovine serum (FBS, Gibco, USA), with 100 U/mL penicillin (Invitrogen, USA) and 100  $\mu$ g/mL streptomycin (Invitrogen, USA) in a 5% CO<sub>2</sub> incubator at 37°C.

### Transfection of Plasmid and siRNA

MHCC97H and HCCLM3 cells were planted into the 6-well plates with  $2 \times 10^5$  cells/well. Transfection was performed by Lipofectamine 3000 (Invitrogen, USA) following the manufacturer's protocol. The siRNAs targeting *ANCR* (si1-*ANCR*, si2-*ANCR*), control siRNA (si-NC), pcDNA3.1-*ANCR* (*ANCR*) and pcDNA3.1-Control (Vector) were designed and synthesized by GenePharma (Shanghai, China). The transfection efficiency was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). In addition, HCCLM3 cells in si1-*ANCR* group were cultured in DMEM containing 10  $\mu$ M IWP-2 (Wnt/ $\beta$ -catenin pathway inhibitor, Sigma, USA) for 48 h and named si1-*ANCR* + IWP-2 group.

### MTT Assay

The cell viability was detected using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) assay. The transfected MHCC97H and HCCLM3 cells were added to 96-well plates at a density of  $3 \times 10^3$  cells per well. At each time point, 20  $\mu$ L of MTT solution (5 mg/mL) was added into each well and then the 96-well plates were kept in dark for 4 h. Following removing MTT solution, DMSO (200  $\mu$ L) was added to each well. Finally, the absorbance at 570 nm was detected by using a microplate reader.

### Colony Formation Assay

After 48 h of transfection, MHCC97H and HCCLM3 cells ( $3 \times 10^3$  cells/well) were seeded into a 6-well plate and cultured for 14 days. Subsequently, the cells were fixed with paraformaldehyde and then stained with

crystal violet at room temperature for 20 min. Finally, the number of colonies were counted under an inverted light microscope.

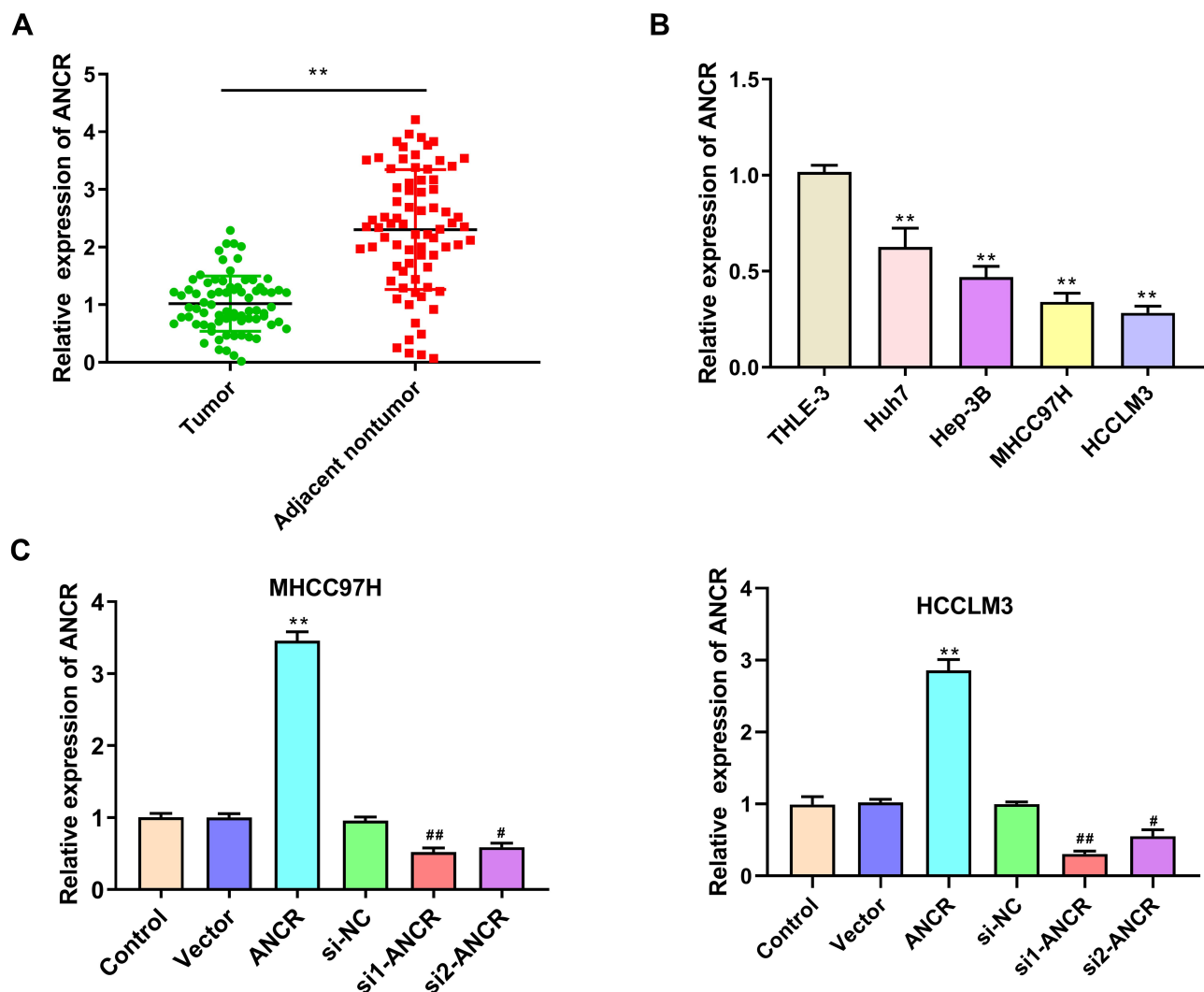
## Transwell Assay

The migration and invasion of MHCC97H and HCCLM3 cells were detected by using transwell chamber (Millipore, Billerica, USA). Briefly, the transfected MHCC97H and HCCLM3 cells were resuspended in serum-free medium and inoculated to the upper chamber (precoated with Matrigel (Millipore, Billerica, USA) for invasion) at a density  $1 \times 10^5$  cells/well. The bottom chamber was added with 500  $\mu$ L complete medium containing 10% serum. After 24 h incubation, the cells

in the upper chamber were carefully wiped with cotton swabs. The migrating or invading cells in the lower chamber were fixed with paraformaldehyde for 20 min and then stained with crystal violet (Sigma, USA) for 30 min. Finally, cell number was counted using an inverted light microscope.

## Flow Cytometry

The Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, USA) was used to estimate the apoptosis ability of MHCC97H and HCCLM3 cells according to the manufacturer's protocol. Simply, the transfected MHCC97H and HCCLM3 cells were harvested, washed with PBS and resuspended in



**Figure 1** The expression of ANCR was lowly expressed in both HCC tissues and HCC cells. (A) The expression of ANCR was detected by qRT-PCR in HCC tissue and adjacent nontumor tissue. (B) The expression of ANCR was detected by qRT-PCR in THLE-3, Huh7, Hep-3B, MHCC97H and HCCLM3 cells. (C) The expression of ANCR was detected by qRT-PCR in transfected MHCC97H and HCCLM3 cells. \*\* $P < 0.01$ , vs Adjacent nontumor tissue group (A); \*\* $P < 0.01$ , vs THLE-3 cells group (B); \*\* $P < 0.01$ , vs Control and Vector groups, # $P < 0.05$ , ## $P < 0.01$ , vs Control and si-NC groups (C).

**Abbreviations:** ANCR, anti-differentiation noncoding RNA; HCC, hepatocellular carcinoma; qRT-PCR, quantitative real-time polymerase chain reaction.

Binding buffer. After that, Annexin V-FITC and propidium iodide (PI) were added to the cell suspension and maintained 15 min. Finally, apoptotic cells were observed by flow cytometry.

## qRT-PCR

Total RNA from HCC tissues and HCC cells was extracted according to the instructions of TRIZOL (Invitrogen, USA). The cDNA was synthesized using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Subsequently, the total RNA was analyzed by qRT-PCR (Bio-Rad, USA) with SYBR green qPCR Master Mix (Thermo Scientific, USA). Primer sequences were listed as follows: ANCR (forward): 5'-GACATTCCTGAGTCG TCTTCGAACGGAC-3', (reverse): 5'-TAGTGCGATTGA GAGCTGTACAAGTTTC-3'; GAPDH (forward): 5'-CGA GCCACATCGCTCAGACA-3', (reverse): 5'-GTGGTGA AGACGCCAGTGGA-3'.

## Western Blot Analysis

MHCC97H and HCCLM3 cells were lysed with RIPA buffer (Thermo Fisher, USA) containing protease inhibitors. A total of 50µg protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene fluoride membrane. After blocked in 5% skimmed milk, the membranes were incubated in the primary antibodies (GSK-3β, 1:1000, #8480, β-catenin, 1:1000, #8480, cleaved caspase-3, 1:1000, #9661, Bax, 1:1000, #5023, Bcl-2, 1:1000, #4223, Vimentin, 1:1000, #5741, E-cadherin, 1:1000, #3195, N-cadherin, 1:1000, #13116, β-actin, 1:2000, #4967, Cell Signaling, USA; Wnt1, 1:1000, ab15251, Abcam, UK) overnight at 4°C. Then, the membranes were incubated in the horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. At last, the protein bands were visualized with ECL system (Thermo, USA).

## Statistical Analysis

All statistical analyses were performed using GraphPad Prism 8 software. Data were presented as mean ± standard deviation. Student's *t*-test or one-way ANOVA was used for analysis of significant differences.  $P < 0.05$  was considered to be statistically significant.

## Results

### The Expression of ANCR is Downregulated and Related to the Clinical Parameters in HCC

As shown in Figure 1A, the expression of ANCR in HCC tissues was significantly lower than that in adjacent non-tumor tissues ( $P < 0.01$ ). Similarly, the expressions of ANCR in THLE-3 cells were also markedly higher than those in Huh7 ( $P < 0.01$ ) and Hep-3B cells ( $P < 0.01$ ), especially in MHCC97H ( $P < 0.01$ ) and HCCLM3 cells ( $P < 0.01$ ) (Figure 1B). Therefore, MHCC97H and HCCLM3 cells were selected for the subsequent experiments. The results of qRT-PCR (Figure 1C) showed that ANCR expression in MHCC97H and HCCLM3 cells was significantly increased in ANCR group compared with Control and Vector group ( $P < 0.01$ ). Meanwhile, when compared with Control and si-NC group, ANCR expression in MHCC97H and HCCLM3 cells was dramatically reduced in si2-ANCR group ( $P < 0.01$ ), especially in si1-ANCR group ( $P < 0.01$ ) (Figure 1C). Thus, si1-ANCR was selected for the following experiments. In addition, the results of Table 1 revealed that ANCR expression was closely

**Table 1** Association Between ANCR Expression and Clinical Parameters in Patients with HCC

Variable	Total	ANCR Expression		P-value
		Low	High	
Age				0.544
<60	31	14	17	
≥60	44	23	21	
Gender				0.571
Male	43	20	23	
Female	32	17	15	
Tumor size				0.017*
<5cm	24	7	17	
≥5cm	51	30	21	
Cirrhosis				0.891
Present	31	15	16	
Absent	44	22	22	
TNM stage				0.034*
I+II	25	8	17	
III+IV	50	29	21	
Vascular invasion				0.006**
Present	47	29	18	
Absent	28	8	20	

**Notes:** \* $P < 0.05$ , \*\* $P < 0.01$ .

**Abbreviations:** ANCR, anti-differentiation noncoding RNA; HCC, hepatocellular carcinoma; TNM, tumor-node-metastasis.

associated with tumor size ( $P < 0.05$ ), tumor-node-metastasis (TNM) stages ( $P < 0.05$ ) and vascular invasion ( $P < 0.01$ ) in HCC. However, other clinical parameters such as age, gender and cirrhosis had no significant association with *ANCR* expression ( $P > 0.05$ ).

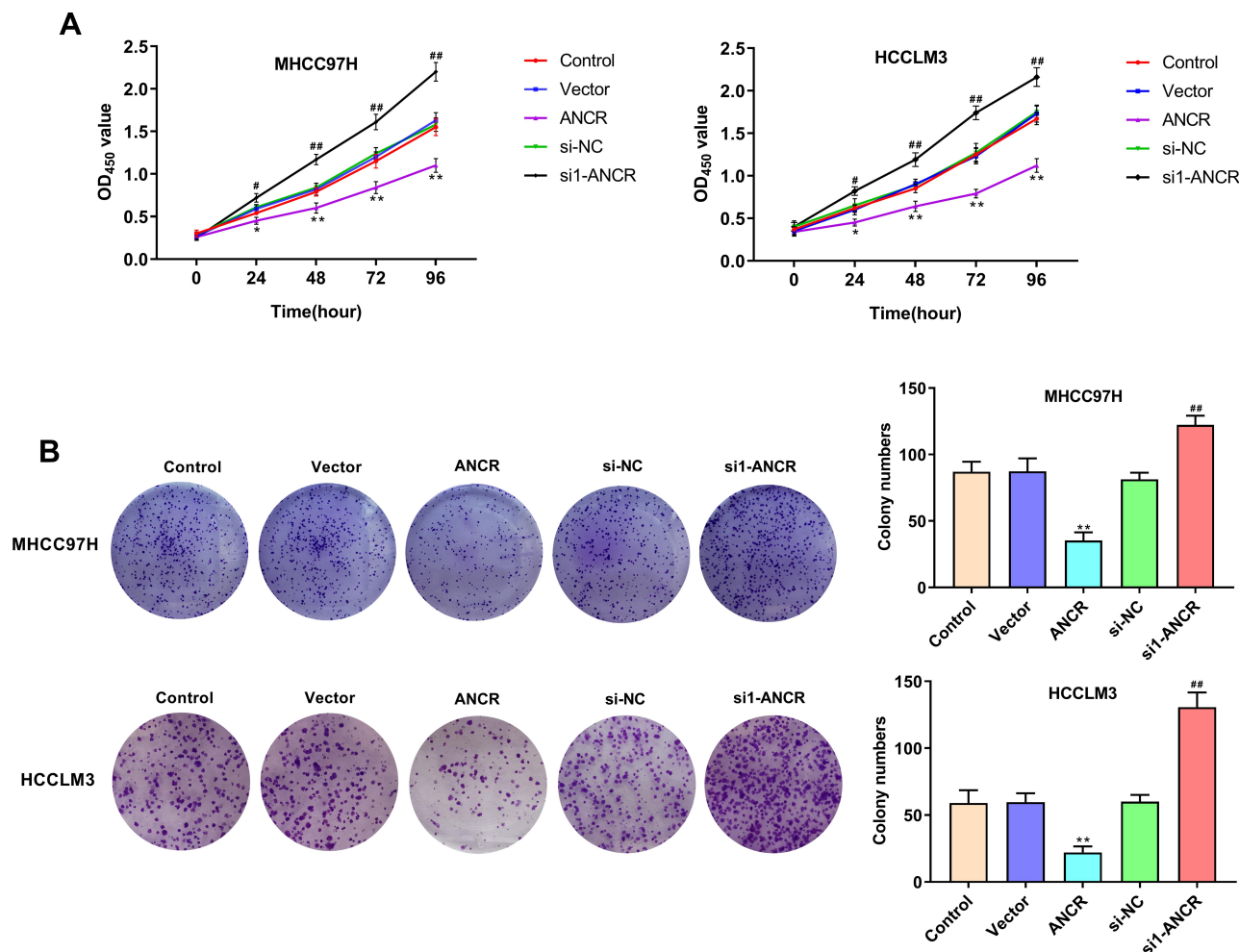
## ANCR Inhibits Cell Proliferation in MHCC97H and HCCLM3 Cells

The results of MTT (Figure 2A) showed that the ANCR overexpression significantly inhibited the proliferation at 24 ( $P < 0.05$ ), 48 ( $P < 0.01$ ), 72 ( $P < 0.01$ ) and 96 h ( $P < 0.01$ ) in both MHCC97H and HCCLM3 cells. On the contrary, silencing *ANCR* markedly elevated cell proliferation at 24 ( $P < 0.05$ ), 48 ( $P < 0.01$ ), 72 ( $P < 0.01$ ) and 96 h ( $P < 0.01$ ). Colony formation assay also indicated that *ANCR*

overexpression prominently suppressed the growth of MHCC97H and HCCLM3 cells ( $P < 0.01$ ), while silencing *ANCR* dramatically promoted their growth ( $P < 0.01$ ) (Figure 2B). Altogether, these results suggested that *ANCR* could inhibit cell proliferation in MHCC97H and HCCLM3 cells.

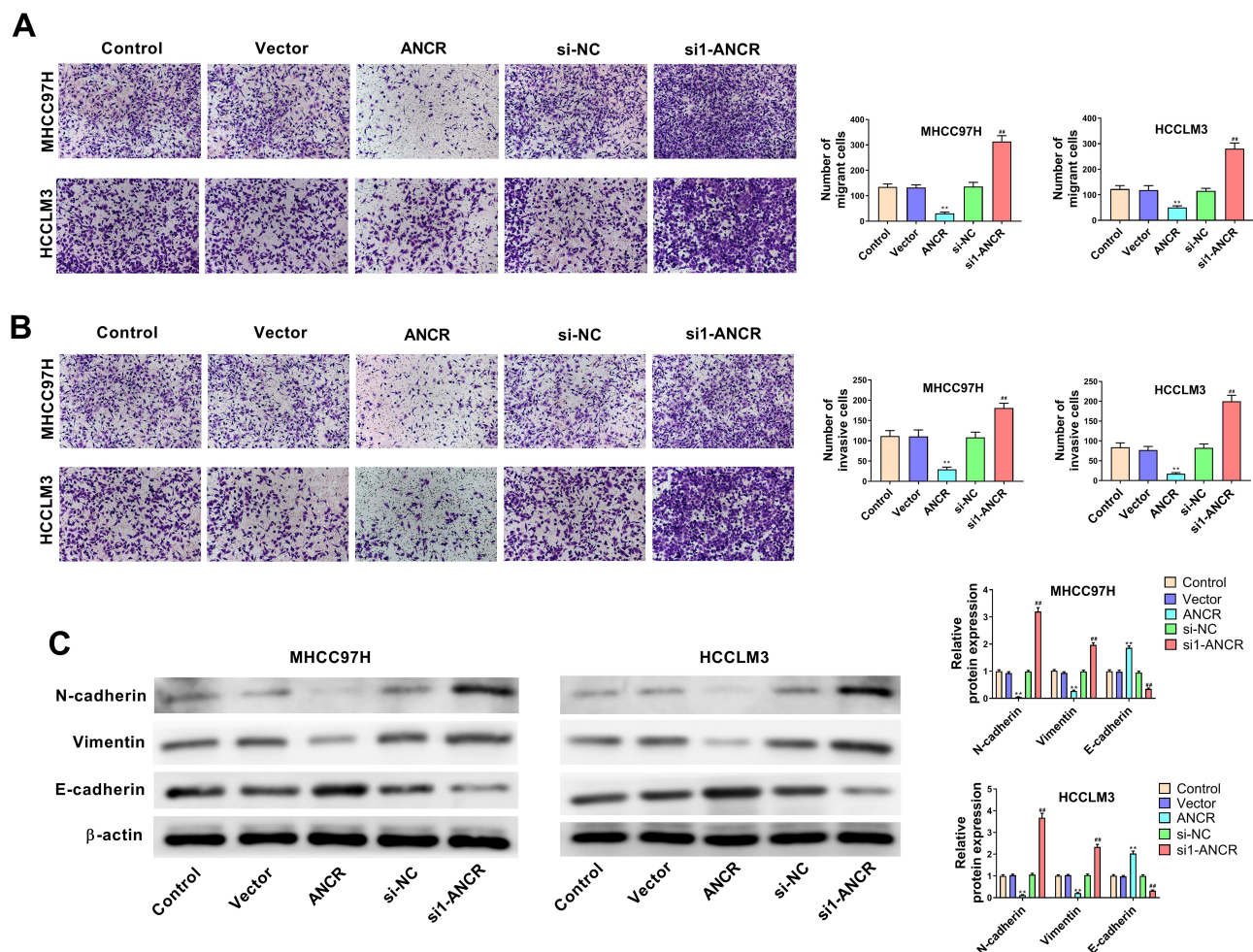
## ANCR Inhibits Cell Migration and Invasion in MHCC97H and HCCLM3 Cells

The migration and invasion abilities of MHCC97H and HCCLM3 cells were detected by transwell assay (Figure 3A and B). The results indicated that *ANCR* overexpression significantly inhibited the migration and invasion of MHCC97H and HCCLM3 cells ( $P < 0.01$ ). Conversely, silencing *ANCR* markedly promoted the migration and invasion of MHCC97H and HCCLM3 cells ( $P < 0.01$ ). It is reported that the epithelial to mesenchymal transition (EMT) is closely related to the cancer



**Figure 2** ANCR inhibited cell proliferation in MHCC97H and HCCLM3 cells. (A) The cell viability of MHCC97H and HCCLM3 cells was measured by MTT assay. (B) Cell clones number of MHCC97H and HCCLM3 cells was measured by colony formation assay. \* $P < 0.05$ , \*\* $P < 0.01$ , vs Control and Vector groups, # $P < 0.05$ , ## $P < 0.01$ , vs Control and si-NC groups.

**Abbreviations:** ANCR, anti-differentiation noncoding RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.



**Figure 3** ANCR inhibited cell migration and invasion in MHCC97H and HCCLM3 cells. **(A)** The migration ability of MHCC97H and HCCLM3 cells was measured by transwell assay. **(B)** The invasion ability of MHCC97H and HCCLM3 cells was measured by transwell assay. **(C)** The expression of N-cadherin, vimentin and E-cadherin in MHCC97H and HCCLM3 cells was detected by Western blot. \* $P < 0.01$ , vs Control and Vector groups, \*\* $P < 0.01$ , vs Control and si-NC groups. **Abbreviation:** ANCR, anti-differentiation noncoding RNA.

cell metastasis.<sup>18</sup> The results of Western blot (Figure 3C) showed that ANCR overexpression significantly decreased the expression of N-cadherin ( $P < 0.01$ ) and Vimentin ( $P < 0.01$ ), and increased E-cadherin ( $P < 0.01$ ) expression in MHCC97H and HCCLM3 cells. On the contrary, silencing ANCR prominently promoted the expression of N-cadherin ( $P < 0.01$ ) and Vimentin ( $P < 0.01$ ), and suppressed E-cadherin expression ( $P < 0.01$ ) in MHCC97H and HCCLM3 cells. All data cumulatively suggested that ANCR could inhibit the migration and invasion ability of MHCC97H and HCCLM3 cells.

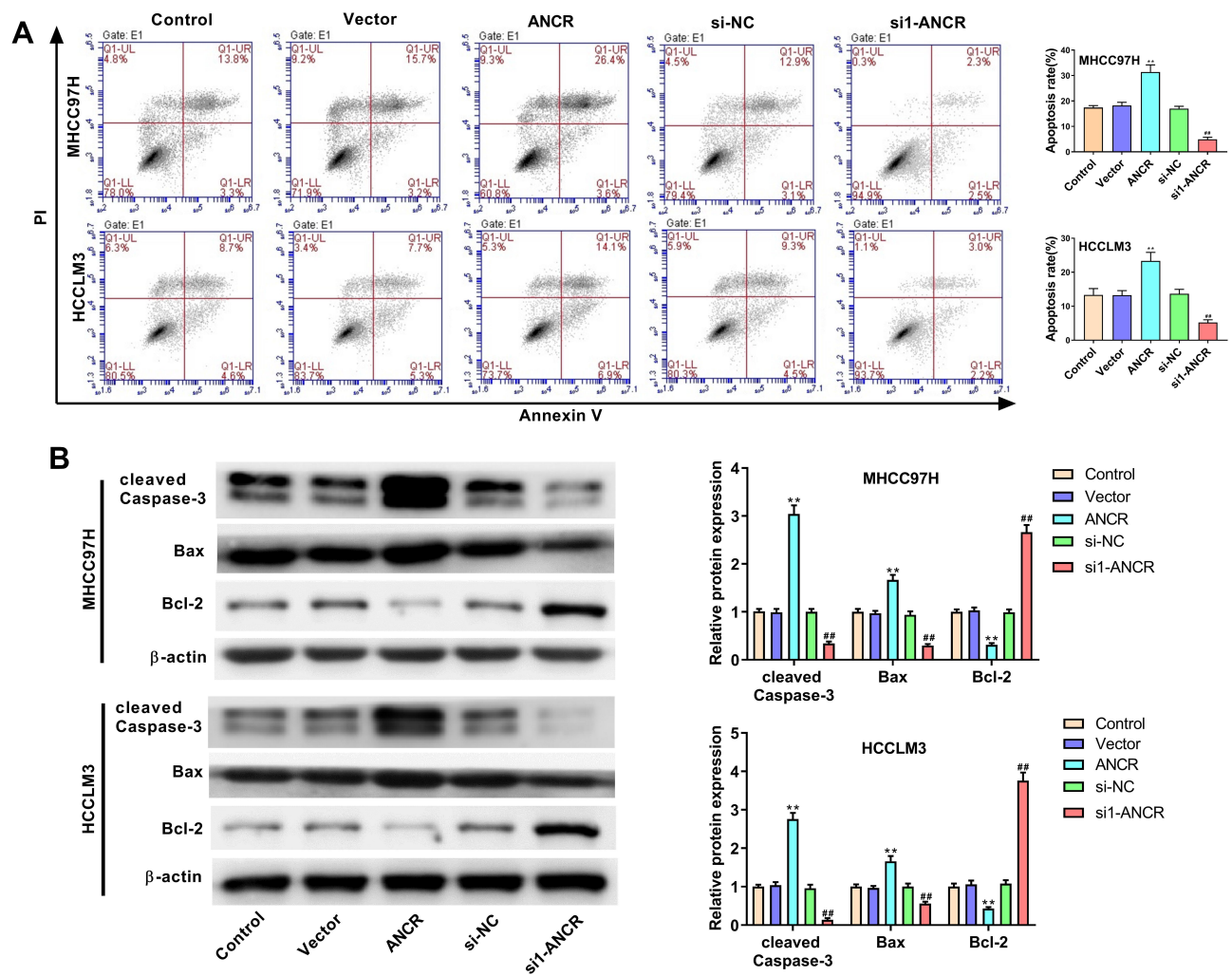
### ANCR Promotes Cell Apoptosis in MHCC97H and HCCLM3 Cells

As shown in Figure 4A, ANCR overexpression significantly promoted the apoptosis of MHCC97H HCCLM3 cells ( $P < 0.01$ ), but silencing ANCR markedly inhibited the apoptosis ( $P < 0.01$ ). To further explore the pro-apoptotic mechanism of

ANCR, apoptosis-related protein expression was measured by Western blot (Figure 4B). ANCR overexpression significantly increased the expression of cleaved caspase-3 ( $P < 0.01$ ) and Bax ( $P < 0.01$ ), and decreased Bcl-2 expression ( $P < 0.01$ ) in MHCC97H and HCCLM3 cells. On the contrary, silencing ANCR prominently reduced the expression of cleaved caspase-3 ( $P < 0.01$ ) and Bax ( $P < 0.01$ ), and elevated Bcl-2 expression ( $P < 0.01$ ) in MHCC97H and HCCLM3 cells. These results indicated that ANCR could promote cell apoptosis in MHCC97H and HCCLM3 cells.

### ANCR Inhibits Wnt/ $\beta$ -Catenin Signaling Pathway in MHCC97H and HCCLM3 Cells

The results of Western blot showed that the overexpression of ANCR could obviously reduce the expression of Wnt1 ( $P < 0.01$ )



**Figure 4** ANCR promoted cell apoptosis in MHCC97H and HCCLM3 cells. **(A)** The apoptosis ability of MHCC97H and HCCLM3 cells was measured by flow cytometry. **(B)** The expression of cleaved caspase-3, Bax and Bcl-2 in MHCC97H and HCCLM3 cells was detected by Western blot. \*\* $P < 0.01$ , vs Control and Vector groups, ### $P < 0.01$ , vs Control and si-NC groups.

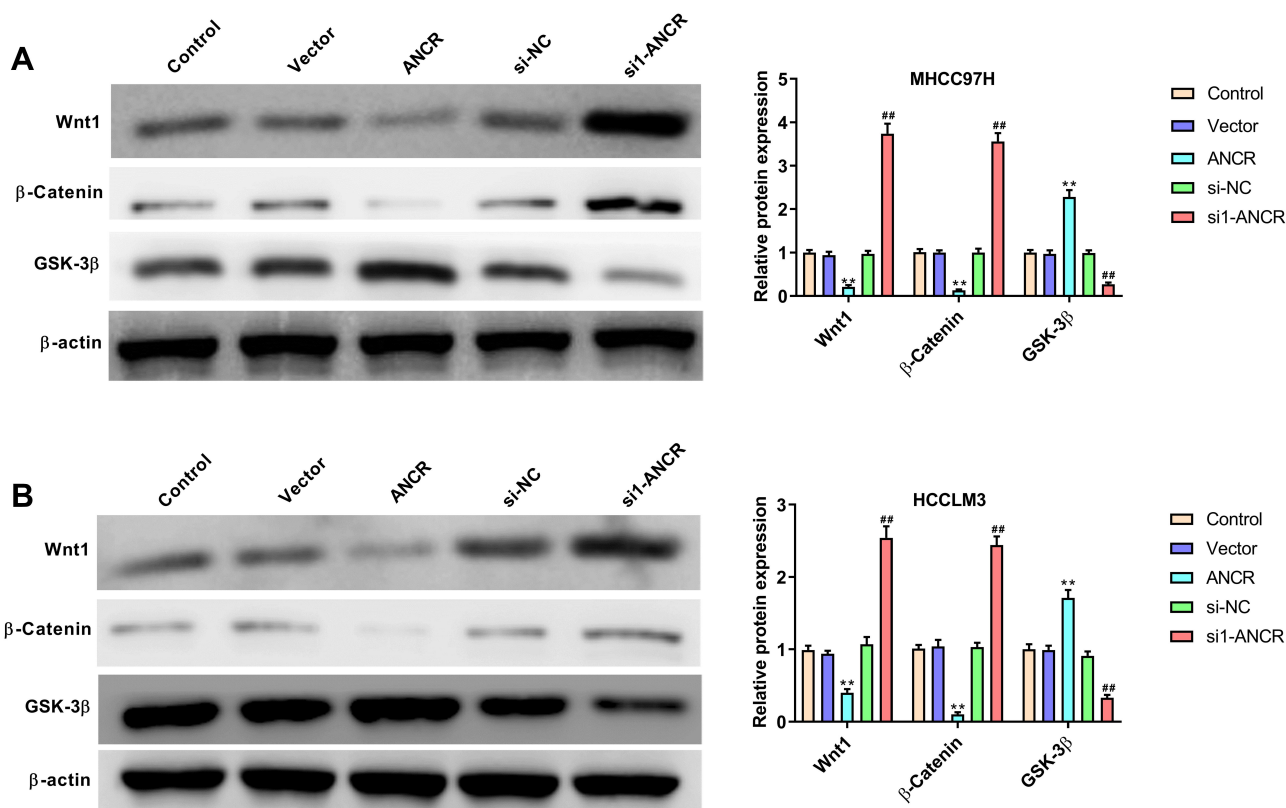
**Abbreviations:** ANCR, anti-differentiation noncoding RNA; Bax, B cell lymphoma/leukemia-2 associated X protein; Bcl-2, B cell lymphoma/leukemia-2.

and  $\beta$ -catenin ( $P < 0.01$ ) in MHCC97H and HCCLM3 cells, but significantly elevate GSK-3 $\beta$  level ( $P < 0.01$ ) (Figure 5A). In addition, silencing ANCR could prominently increase the expression of Wnt1 ( $P < 0.01$ ) and  $\beta$ -catenin ( $P < 0.01$ ), but decrease GSK-3 $\beta$  expression ( $P < 0.01$ ) in MHCC97H and HCCLM3 cells (Figure 5B), suggesting that ANCR could inhibit Wnt/ $\beta$ -catenin signaling pathway in MHCC97H and HCCLM3 cells.

## ANCR Suppresses Cell Proliferation, Migration and Invasion, as Well as Promotes Apoptosis Through Inhibiting Wnt/ $\beta$ -Catenin Signaling Pathway in HCC

To further verify the effect of Wnt/ $\beta$ -catenin pathway in ANCR-mediated progression of HCC, HCCLM3 cells

which transfected with si1-ANCR were treated with IWP-2. As shown in Figure 6A, the expressions of Wnt1 and  $\beta$ -catenin in si1-ANCR group were higher than those in si-NC group ( $P < 0.01$ ), but the expression of GSK-3 $\beta$  was lower ( $P < 0.01$ ). When compared with si1-ANCR group, the expressions of Wnt1 ( $P < 0.05$ ) and  $\beta$ -catenin ( $P < 0.01$ ) were significantly decreased in si1-ANCR + IWP-2 group, while the expression of GSK-3 $\beta$  was increased ( $P < 0.05$ ). Colony formation and transwell assays indicated that the treatment of IWP-2 could partially reverse the facilitating effect of silencing ANCR on the proliferation ( $P < 0.05$ ), migration ( $P < 0.01$ ) and invasion abilities ( $P < 0.01$ ) of HCCLM3 cells (Figure 6B–D). In addition, flow cytometry also confirmed that IWP-2 treatment could partially reverse the inhibitory effect of silencing ANCR on



**Figure 5** ANCR inhibited Wnt/ $\beta$ -catenin signaling pathway in MHCC97H and HCCLM3 cells. **(A)** The expression of Wnt1,  $\beta$ -catenin and GSK-3 $\beta$  in MHCC97H cells was measured by Western blot. **(B)** The expression of Wnt1,  $\beta$ -catenin and GSK-3 $\beta$  in HCCLM3 cells was measured by Western blot. \*\* $P < 0.01$ , vs Control and Vector groups, ### $P < 0.01$ , vs Control and si-NC groups.

**Abbreviations:** ANCR, anti-differentiation noncoding RNA; GSK-3 $\beta$ , glycogen synthase kinase 3 $\beta$ .

the apoptosis ability of HCCLM3 cells ( $P < 0.01$ ) (Figure 6E). These results further demonstrated that ANCR could suppress cell proliferation, migration and invasion, as well as promote apoptosis through inhibiting Wnt/ $\beta$ -catenin signaling pathway in HCC.

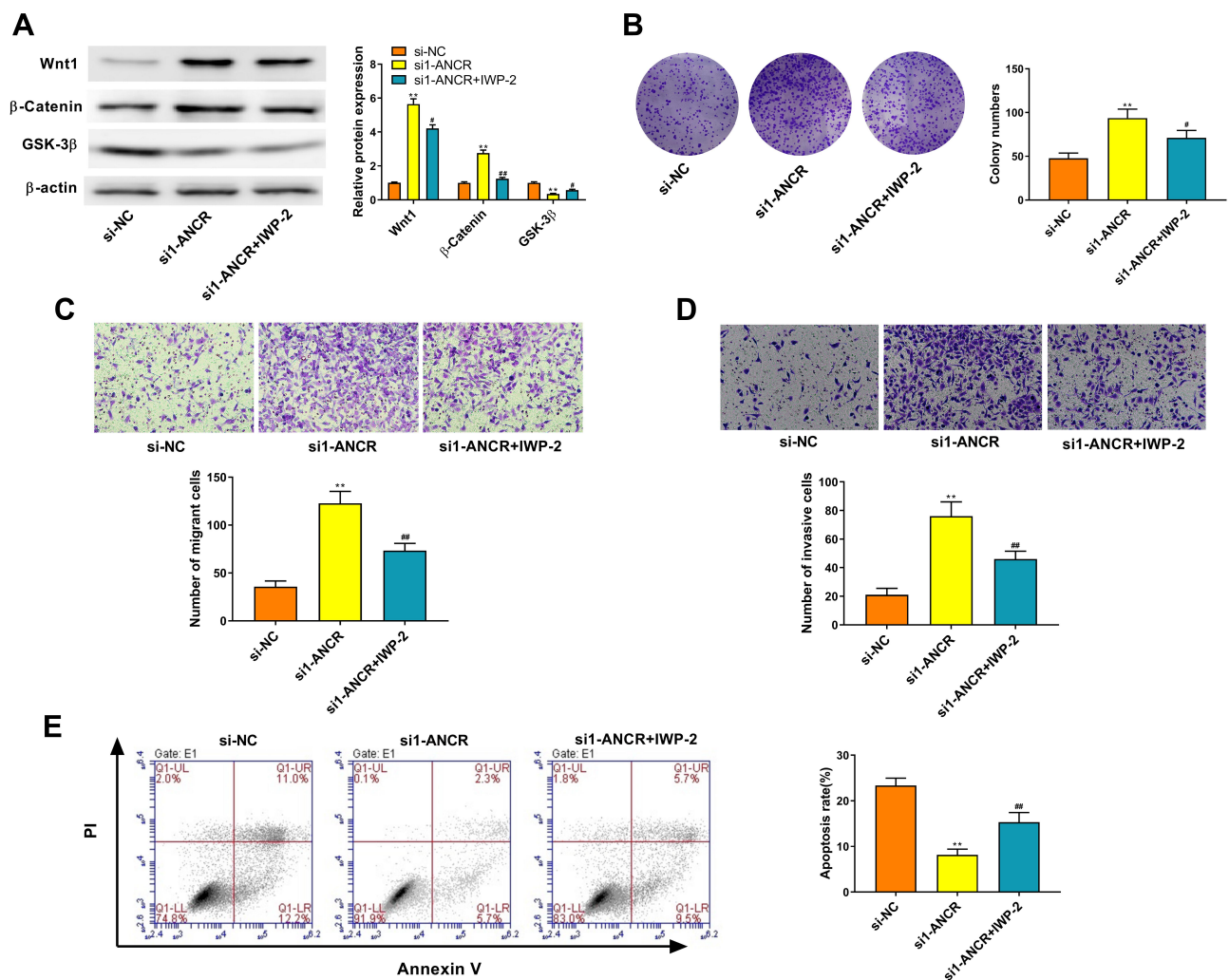
## Discussion

In recent years, the incidence rate of HCC is increasing all over the world<sup>19</sup> and more than 780,000 cases are reported every year.<sup>20</sup> However, the etiology and pathogenesis of HCC are still unclear. It is urgent to explore new molecular mechanism and therapeutic targets to better treat HCC. In our study, we confirmed that ANCR could suppress cell proliferation, migration and invasion, as well as promote apoptosis through inhibiting Wnt/ $\beta$ -catenin signaling pathway in HCC.

Recently, more and more studies have suggested that lncRNAs can participate in the pathophysiological processes of HCC.<sup>9</sup> Our results demonstrated that ANCR expression was closely associated with some clinical parameters including tumor size, TNM stages and vascular

invasion in HCC, which confirms the views of previous researches. The abnormal expressions of lncRNAs have been frequently observed in malignancies.<sup>21</sup> A study of Chen et al<sup>22</sup> has confirmed that the expression of lncRNA *SNHG16* is significantly upregulated in both HCC tissues and HCC cells. Kong et al<sup>23</sup> report that lncRNA *MIR4435-2HG* is highly expressed in HCC and facilitates HCC cell proliferation via modulating *miRNA-487a*. In our study, the expression of ANCR was markedly downregulated in HCC tissues and HCC cells (Huh7, Hep-3B, MHCC97H and HCCLM3 cells), which is in line with previous studies. Moreover, accumulating evidences have indicated that lncRNAs play a regulatory role in the proliferation, apoptosis, migration and invasion of HCC cells. For example, lncRNA *MAFG-AS1* is reported to accelerate the proliferation, migration and invasion of HCC cells by regulating *miR-6852*.<sup>24</sup> Xiao et al<sup>25</sup> have confirmed that LINC00339 could facilitate the proliferation and invasiveness of HCC cells through the *miR-1182/SKAI* pathway. Previous research has reported that lncRNA *IHS* could promote cell proliferation and metastasis in HCC through





**Figure 6** IWP-2 partially reversed the effects of silencing *ANCR* on the proliferation, migration, invasion and apoptosis of HCCLM3 cells. (A) The expression of Wnt1,  $\beta$ -catenin and GSK-3 $\beta$  in HCCLM3 cells was measured by Western blot. (B) Cell clones' number of HCCLM3 cells was measured by colony formation assay. (C) The migration ability of HCCLM3 cells was measured by transwell assay. (D) The invasion ability of HCCLM3 cells was measured by transwell assay. (E) The apoptosis ability of HCCLM3 cells was measured by flow cytometry. \*\* $P < 0.01$ , vs si-NC group, \* $P < 0.05$ , ### $P < 0.01$ , vs si1-ANCR group.

**Abbreviations:** *ANCR*, anti-differentiation noncoding RNA; GSK-3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; IWP-2, Wnt/ $\beta$ -catenin pathway inhibitor.

modulating ERK signaling pathway.<sup>26</sup> Our results indicated that *ANCR* dramatically suppressed HCC cell proliferation, migration and invasion, as well as promoted cell apoptosis. To further confirm the apoptosis and metastasis mechanism influenced by *ANCR*, the expressions of apoptosis-related proteins (cleaved caspase-3, Bax and Bcl-2) and metastasis-related proteins (N-cadherin, vimentin and E-cadherin) were assessed. Western blot results showed that *ANCR* could promote the expression of proapoptotic proteins cleaved caspase-3 and Bax, but inhibit antiapoptotic protein Bcl-2 expression, further revealing that *ANCR* could accelerate the apoptosis of HCC cells. EMT is considered to be the main mechanism of tumor invasion and metastasis.<sup>27</sup> A growing number of researches have

indicated that EMT plays a vital role in multiple malignancies metastasis including HCC, which has significant phenotypic changes through the acquisition of mesenchymal marker proteins (N-cadherin and vimentin) and the loss of epithelial marker protein (E-cadherin).<sup>28,29</sup> Moreover, lncRNAs are also reported to be involved in the process of EMT.<sup>30</sup> Our study confirmed that *ANCR* could promote E-cadherin expression and reduced the expression of N-cadherin and vimentin, revealing that *ANCR* could inhibit HCC cell metastasis by suppressing the process of EMT. Thus, we believed that *ANCR* may play an important role in the pathogenesis of HCC through promoting cell apoptosis and suppressing proliferation, migration and invasion.

Wnt/ $\beta$ -catenin pathway is believed to play an important role in the process of liver development, regeneration and zonation, which is necessary for the spatial separation of different metabolic functions in liver.<sup>31</sup> In normal hepatocytes,  $\beta$ -catenin level is low due to the presence of  $\beta$ -catenin destruction complex, and the complex is composed of adenomatous polyposis coli, glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) and axin.<sup>15</sup> The aberrant activation of the Wnt/ $\beta$ -catenin pathway exerts a vital role in the pathogenesis of various cancers, including HCC.<sup>30</sup> In the recent years, lncRNAs are emerging as a new regulator in regulating the progress of HCC via modulating Wnt/ $\beta$ -catenin pathway.<sup>16</sup> For instance, lncRNA *DUXAP10* can promote the proliferation and metastasis of HCC cells via regulating Wnt/ $\beta$ -catenin pathway.<sup>32</sup> Ma et al<sup>17</sup> have reported that lncRNA *FAM83H-AS1* facilitates the proliferation, migration and invasion of HCC cells via the modulation of Wnt/ $\beta$ -catenin pathway. In this study, our results showed that *ANCR* significantly reduced Wnt1 and  $\beta$ -catenin expression, and elevated GSK-3 $\beta$  level in HCC cells. In addition, the treatment of IWP-2 could partially reverse the effects of silencing *ANCR* on the proliferation, migration, invasion and apoptosis of HCC cells. All our results revealed that *ANCR* could suppress cell proliferation, migration and invasion, as well as promote apoptosis through inhibiting Wnt/ $\beta$ -catenin signaling pathway in HCC.

In conclusion, our work confirmed that *ANCR* was highly expressed in both HCC tissues and HCC cells. In addition, the present study also demonstrated that *ANCR* could suppress cell proliferation, migration and invasion, as well as promote apoptosis through inhibiting Wnt/ $\beta$ -catenin signaling pathway in HCC. Our research provides an innovatively regulatory mechanism about *ANCR* in HCC and points a new way to the treatment of HCC. Of course, there are some limitations in this study. The exact mechanisms of *ANCR* regulating Wnt/ $\beta$ -catenin pathway and other pathways will be further studied.

## Disclosure

The authors report no potential conflicts of interest for this work.

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