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Original Article

Prognostic roles of miR-124-3p and its target ANXA7 and their effects on cell migration and invasion in hepatocellular carcinoma

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Abstract: Recent studies have indicated that ANXA7 promotes progression and metastasis of hepatocellular carcinoma (HCC). In this study we found a significant negative correlation between the levels of miR-124-3p and ANXA7 protein in HCC. Level of miR-124-3p in tumor tissues was negatively correlated, while ANXA7 protein was positively correlated, with TNM stage and tumor metastasis. Furthermore, we confirmed ANXA7 was a target gene of miR-124-3p by a dual luciferase reporter assay. In vitro, up-regulation of miR-124-3p promotes apoptosis and inhibits migration and invasion of Hca-F. Bcl-2 correlates X protein (Bax) protein level was up-regulated, while ANXA7, B-cell lymphoma-2 (Bcl-2), Matrix metalloproteinase (MMP-9) and C-X-C motif chemokine 12 (CXCL12) protein levels were suppressed relative to miR-124-3p over-expression. In vivo, up-regulation of miR-124-3p suppresses lymph node metastasis (LNM) and tumorigenicity of Hca-F cells. The expression of ANXA7, MMP-9, and CXCL12 protein in transplanted tumors was suppressed relative to miR-124-3p overexpression. In addition, we found the levels of Bcl-2, MMP-9, and CXCL12 in Hca-F cells decreased significantly after transfection of shRNA-Anxa7 in vitro. In conclusion, our study revealed miR-124-3p inhibits tumor growth, invasion, and lymphatic metastasis in HCC by down-regulation of ANXA7 gene, thereby reducing the expression of Bcl-2, MMP-9, and CXCL12.

Keywords: MiR-124-3p, ANXA7, hepatocellular carcinoma, invasion, lymphatic metastasis

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in clinic. It has the characteristics of high invasion, early metastasis, high recurrence and mortality. Tumor metastasis is an important cause of death in patients with HCC [1]. Lymphatic metastasis is an important way of HCC metastasis. The degree and extent of lymphatic metastasis is an important factor determining the prognosis of patients [2]. Studying the mechanism of lymphatic metastasis is of great value for understanding the biological characteristics of HCC and guiding clinical treatment.

Annexin A7 (ANXA7) is an important member of the Annexin family in vertebrate cells [3]. It plays an important role in cytoskeleton activity, membrane phospholipidization, membrane receptor regulation, and mitosis [4, 5]. Recent studies have found that ANXA7 plays an important role in tumorigenesis, development, invasion and metastasis [6, 7]. In the past, our team has found ANXA7 gene is one of the important differentially expressed genes that affect the lymphatic metastasis potential of tumors which differs between Hca-F cells (lymph node metastasis rate > 70%) and Hca-P cells (lymph node metastasis rate < 30%) by gene chip, protein spectrum, and suppression subtraction [8, 9]. Up-regulation of ANXA7 gene expression enhanced the proliferation and migration of Hca-F cells, and down-regulation of ANXA7 gene expression reduced the proliferation and migration of Hca-F cells, suggesting

that ANXA7 plays a role in promoting lymphatic metastasis of HCC [10, 11].

MicroRNAs (miRNAs) are 18-24 nucleotide endogenous non-coding small RNAs [12]. Previous studies have shown that miRNAs have significant effects on signal transduction pathways, cell apoptosis and metabolism, myogenesis, cancer, viral infection, and body development [13-15]. miRNAs may interact with ANXA7 and play an important role in tumorigenesis, development, metastasis, and invasion. However, the interaction between ANXA7 gene and microRNAs has not yet been described.

Therefore, we aimed to explore the effects of miR-124-3p and ANXA7 in HCC, in the hope of providing possible indicators for clinical diagnosis of hepatocellular carcinoma and a potential molecular target for HCC treatment.

Materials and methods

Patients and specimens

60 tumor tissues were obtained from patients with HCC. The patients with HCC underwent surgical resection at the First Affiliated Hospital of Dalian Medical University from August 2017 to July 2019. The specimens were collected with the consent of the patients. These patients did not have history of radiotherapy or chemotherapy before collecting specimens. The experiment was approved by the Ethics Committee of First Affiliated Hospital of Dalian Medical University.

Cell culture

The Hca-F cells (Mouse hepatocellular carcinoma cells with high lymphatic metastasis potential) were established in the Department of Pathology, Dalian Medical University. NCTC-1469 cell and Human embryonic kidney cell line HEK-293T was obtained from American Type Culture Collection (ATCC). Cells were cultured in 90% RPMI 1640 (Gibco, Grand Island, NY, USA) which was supplemented with streptomycin/penicillin (Gibco, USA) and 10% fetal bovine serum (Gibco, USA). The cells were cultured in a humidified incubator with 5% CO_2 at 37°C.

MiRNA prediction

ANXA7 was input into NCBI GENE (https://www.ncbi.nlm.nih.gov/gene/) as a search term to query the Ensembl sequence of ANXA7 (species: mice). The miRNAs that could regulate ANXA7 were retrieved from TargetScan (http://www.targetscan.org/mmu_72/) (species: mice), and a number of miRNAs were predicted by the database. According to the basic principles of bioinformatics prediction of target genes: high degree of conservativeness of target gene "seed sequence" in humans, mice, squirrels, rabbits and other species, low binding free energy between microRNAs and target genes, and closely relation to cell proliferation, candidate microRNAs were selected.

Cell transfection and grouping

MiRNA vectors, miR-124-3p mimics, precursor miR-124-3p scrambled control clones (N-control), and miR-124-3p inhibitors were purchased from GenePharma (Hangzhou, China). The lentiviral packaging kit (Open Biosystems, Huntsville, AL, USA) was used to package the lentiviruses that carried the compounds mentioned above, in accordance with the manual. The shRNA-ANXA7 (hairpin sequence: 5'-GTCAGAATTGAGTGG-GAATT-3') and negative control shRNA (hairpin sequence: 5'-GTTCTCCGAACGTGTCACGT-3') were transfected the unmanipulated Hca-F cells.

Dual-luciferase reporter gene assay

The miRNAs that could regulate ANXA7 were retrieved from TargetScan (http://www.targetscan.org/). "ANXA7" served as the keyword, and a number of miRNAs were predicted in the database. Wild-type pGL3-ANXA7-3'UTR (WT-ANXA7-3'UTR) and mutant pGL3-ANXA7-3'UTR (MUT-ANXA7-3'UTR) plasmids were obtained from Shanghai Genechem Co. Ltd. (Shanghai, China). After 48 h transfection, Passive Lysis Buffer (Promega, USA) was used to collect the lysates of HEK-293t cells from the treatment groups with its protocols. Dual-Luciferase Reporter Assay System (Promega, USA) was used to analyze the relative luciferase activity relative to the Renilla luciferase activity in the same sample.

Flow cytometry

The transfected cells were centrifuged and washed by cold PBS for 2 times. 10 μL Annexin V-FITC and 10 μL PI (Invitrogen; Thermo Fisher Scientific, Inc.) were employed to incubate the

harvested cells (3×10^5) at room temperature for 15 min in dark room. A flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure the apoptotic cells.

Transwell assay

The transwell assay detected the cell motility capacities through the Corning transwell chambers with the 8-μm pore size (Corning Co, Corning, USA) with/without matrigel. 4×10^3 cells in serum-free media were put in the upper chamber after 48 h of transfection. The cells in upper membrane were wiped off 48 h after the incubation. The migrated cells were stained with 0.1% crystal violet. A microscope was used to count the cells. A total of three independent experiments were conducted.

Mouse xenograft mode

Fifteen 8-week-old male 615 mice were obtained from Institute of Genome Engineered Animal Models for Human Disease of Dalian Medical University (China). The mice were randomly divided into NC group, miR-124 mimics group, miR-124 inhibitor group, five mice in each. Transfected Hca-F cell suspension 0.05 ml (containing 2×10^6 cells) was injected into each mouse's right foot pad. Tumor growth was monitored and measured every 4 days, and the tumor volume was calculated through the formula [(length (mm) × width (mm)) 2]/2. After 28 days, the mice were euthanized by cervical dislocation under CO_2 anesthesia and the tumor was dissected and weighed. The regional lymph nodes (inguinal and axillary) were dissected to analyze the lymph node metastatic rate. The transplanted tumors were washed with sterile PBS, and dried with filter paper for western blot. Part of lymph node metastases of transplanted tumors was fixed with 10% formalin for immunohistochemical stains. All of the procedures were performed by the Animal Ethics Committee of the Dalian Medical University.

Immunohistochemical analysis

The tumor tissues were sliced into sections of 4 μm, and then they underwent deparaffination and rehydration. The anti-ANXA7 (1:200, Santa Cruz Biotechnology Inc.) and anti-MMP-9 (1:200, Santa Cruz Biotechnology Inc.) was used as the primary antibody at 4°C overnight. The following day, peroxidase conjugated secondary antibody (ZSGB-BIO, Beijing, China) was used to incubate the slides for 30 min. Signal development was done with DAB solution, a peroxidase-labeled polymer, for 5 min. After counterstaining the sections with hematoxylin, dehydrating and mounting were carried out. Immunohistochemistry stain was analyzed by Image-Pro-Plus (IPP) 6.0 software.

RNA extraction and real-time quantitative PCR (q-PCR) analysis

The isolation of total RNA from cells was realized through TRIzol reagent (Invitrogen) in accordance with the protocol of the manufacturer. The primers of genes were as below: miR-124-3p forward, 5'-CGGGTAGCAGGCTTCTGAGT-3' and reverse, 5'-AAACCCCTCTCTGTCGGTAGCT-3'; U6 forward, 5'-CTTTGCCGCACGGCACA-3' and reverse, 5'-AAGCAACTTYCACGYCGT-3'. ANXA7 forward, 5'-AAUGACCUGCAUGCTTGACT-3' and reverse, AGCUAACCGAGAAAGAGCTT; β-actin, forward, 5'-AGCGCCATCCGCAACAAGTT-3'; and reverse, 5'-GGCACGGGCTCAAGATCATT-3'. qRT-PCR was analyzed through Bio-Rad CFX Manager 2.1 software. All of the experiments were performed in triplicate. The $2^{\Delta\Delta Ct}$ was used as the analytic formula.

Western blot analysis

For western blot, the proteins were transferred electrophoretically to PVDF membranes (Merck KGaA, Darmstadt, Germany), which were blotted with antibody overnight through anti-ANXA7 (1:500; ab-63842; Abcam, Cambridge, MA), anti-Bax (1:500, sc-6106, Santa Cruz Biotechnology Inc, CA.), anti-Bcl-2 (1:500, sc-58012, Santa Cruz Biotechnology Inc.), anti-MMP-9 (1:500, SC-6840, Santa Cruz Biotechnology Inc.), anti-CXCL12 (1:500, SC-6372, Santa Cruz Biotechnology Inc.), GAPDH (1:1000, 21872-1-AP, Wuhan Sanying Biotechnology, Wuhan, China) or β-tubulin (1:500, sc-63410, Santa Cruz Biotechnology Inc.) at 4°C. The membranes were washed in TBST and incubated with anti-mouse horseradish peroxidase-conjugated secondary antibody (1:1000, Biosynthesis Biotechnology, China) at room temperature for 2 h. Chemiluminescence (GE, USA) was used to visualize the immunocomplexes according to the manufacturer's protocol.

Statistical analysis

The mean ± standard deviation (SD) expressed the continuous variables. GraphPad Prism soft-

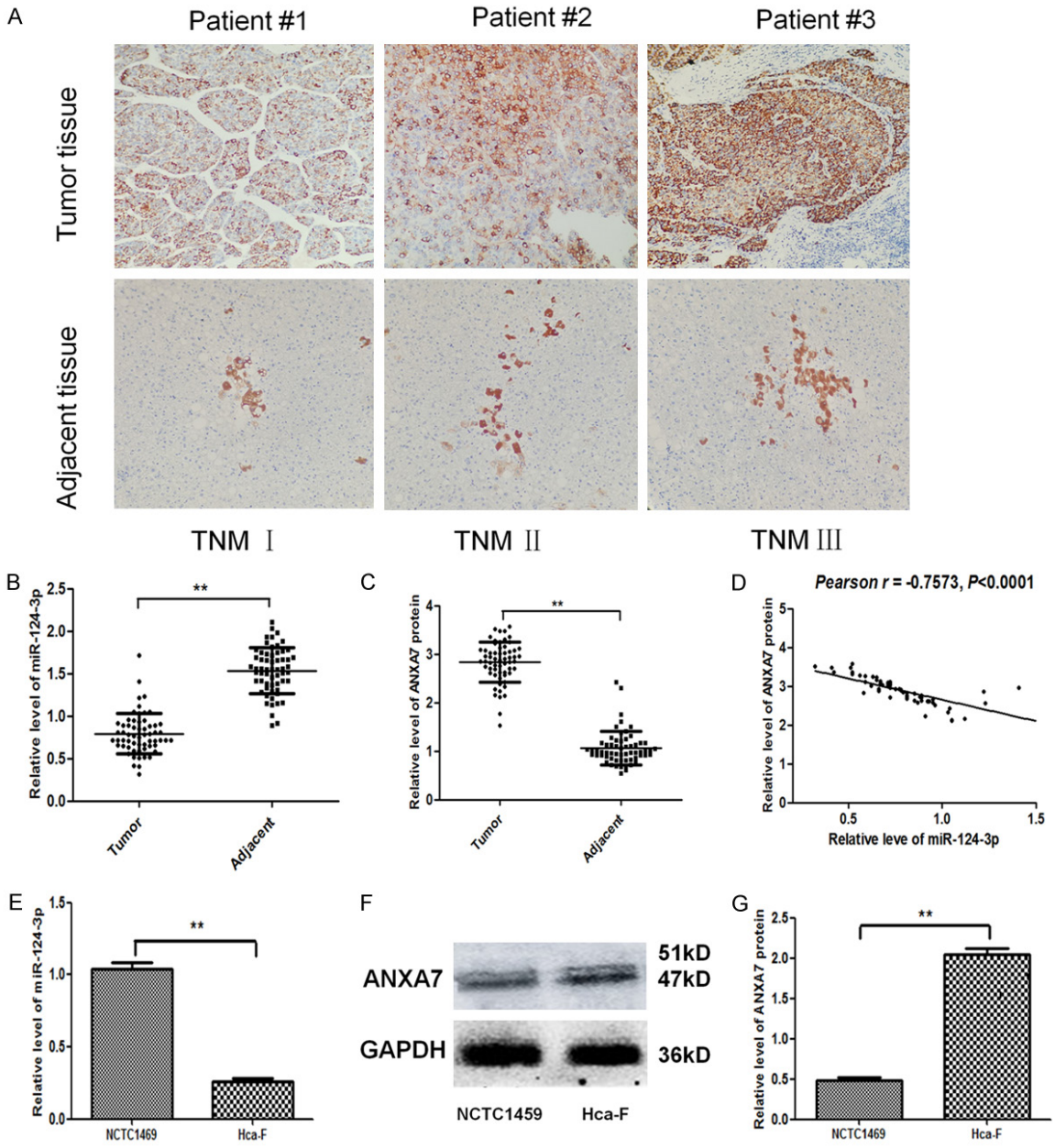

Figure 1. miR-124-3p was down-regulated while ANXA7 was up-regulated in Hca-F cells and HCC. A. Protein expression of ANXA7 in tumor tissues and related adjacent tissues from HCC patients by immunohistochemical stain revealed that miR-124-3p was down-regulated while ANXA7 protein was up-regulated in tumor tissue. B. miR-124-3p level in tumor tissues and related adjacent tissues from HCC patients by q-PCR. C. Protein expression of ANXA7 in tumor tissues and related adjacent tissues from HCC patients. D. Pearson analysis between level of ANXA7 protein and level of miR-124-3p. There was a significant negative correlation. E. miR-124-3p level in Hca-F cells, compared with a normal hepatic cell line NCTC1469 by q-PCR. F, G. Protein expression of ANXA7 in Hca-F cells, compared with a normal hepatic cell line NCTC1469 by western blot. The error bar represents the SD (n = 3). **P < 0.01.

ware, version 5.0 (GraphPad, La Jolla, CA, USA) was used to perform one-way ANOVA for multiple comparisons. A significant difference was indicated by *P*-values < 0.05 (*P < 0.05). All data are representative of at least three different experiments.

Results

miR-124-3p and ANXA7 are involved in HCC

A total of 5 ANXA7-related miRNAs, namely, miR-19-3p, miR-124-3p, miR-135-5p, miR-190-

Table 1. Clinicopathologic characteristics of HCC patients according to miR-124-3p expression and ANXA7 protein expression

Characteristic	n	miR-124-3p expression	*P* value	ANXA7 protein expression	*P* value
Age (years)			0.8171		0.8954
≥ 60	42	0.78±0.12		2.89±0.38	
< 60	18	0.80±0.07		2.87±0.32	
Gender			0.9721		0.9921
male	38	0.79±0.11		2.88±0.36	
female	22	0.79±0.09		2.88±0.33	
TNM stage			0.0021*		0.0010*
I-II	46	0.85±0.13		2.77±0.41	
III	14	0.62±0.07		3.27±0.30	
AFP level			0.1026		0.1973
≥ 400	45	0.77±0.13		2.91±0.35	
< 400	15	0.85±0.10		2.82±0.27	
Alcohol consumption			0.6842		0.8362
Yes	25	0.77±0.09		2.90±0.33	
No	35	0.80±0.12		2.88±0.35	
HBV/HCV infection			0.9183		0.9072
Yes	39	0.79±0.12		2.89±0.38	
No	21	0.80±0.08		2.88±0.34	
Tumor metastasis			0.0013*		0.0001*
Yes	12	0.60±0.07		3.35±0.28	
No	48	0.84±0.14		2.77±0.41	

*p < 0.05 indicates a significant association among the variables.

5p, miR-323-3p were retrieved from TargetScan. It was found that the position 155-162 of ANXA7 3'UTR segment was consequentially pairing to target region of miR-124-3p. Sequence alignment showed that the conserved sequence of ANXA7 in human, mouse, squirrel, rabbit and other species was strictly conserved, while the free energy was low.

miR-124-3p was down-regulated while ANXA7 was up-regulated in Hca-F cells and HCC

We tested tumor and related adjacent tissues from 60 HCC patients by qPCR and immunohistochemical analysis. The results revealed that miR-124-3p was down-regulated while ANXA7 protein was up-regulated in tumor tissues (**Figure 1A-C**). Pearson analysis showed that the expression level of ANXA7 protein decreased significantly with the increase of the expression of miR-124-3p. There was a significant negative correlation ($r = -0.7573$, $P < 0.05$, **Figure 1D**). Using q-PCR, we detected the miR-124-3p was down-regulated in Hca-F cells, compared with a normal hepatic cell line NCTC1469 ($P < 0.05$) (**Figure 1E**). Using western blot, we detected the ANXA7 protein expression was higher in Hca-F cells, compared with NCTC1469 ($P < 0.05$) (**Figure 1F** and **1G**).

Level of miR-124-3p and ANXA7 was associated with HCC progression

We collected clinicopathological data and conducted an analysis. The results showed that the level of miR-124-3p in tumor tissues was negatively correlated, while ANXA7 protein was positively correlated with TNM stage and tumor metastasis ($P < 0.05$). There was no significant difference in the expression of miR-124-3p and ANXA7 between different age, gender, AFP level, alcohol consumption, and HBV/HCV infection groups (**Table 1**).

ANXA7 is a target gene of miR-124-3p

The luciferase activity of WTANXA7-3'UTR was inhibited by miR-124-3p ($P < 0.05$), while that of MUT-ANXA7-3'UTR did not exhibit the same effect ($P > 0.05$) (**Figure 2B**). ANXA7 protein expression level was down-regulated relative to miR-124-3p over-expression, However, miR-124-3p down-regulation had the opposite effect ($P < 0.05$) (**Figure 2C** and **2D**). These findings indicated that miR-124-3p may specifically bind to ANXA7-3'UTR and down-regulate ANXA7 gene expression at a post-transcriptional level. The aforementioned results suggest that ANXA7 is a target gene of miR-124-3p.

Up-regulation of miR-124-3p promotes Hca-F cell apoptosis

In order to evaluate whether miR-124-3p regulated HCC cell apoptosis, apoptosis assay was conducted by flow cytometry. There was an increased apoptotic population after miR-124-3p mimic transfection in Hca-F cells, but the apoptosis rate of Hca-F cells decreased as miR-124-3p expression was inhibited (**Figure 3A**). Furthermore, we detected the protein expression of ANXA7, Bax, and Bcl-2 in Hca-F cells. As

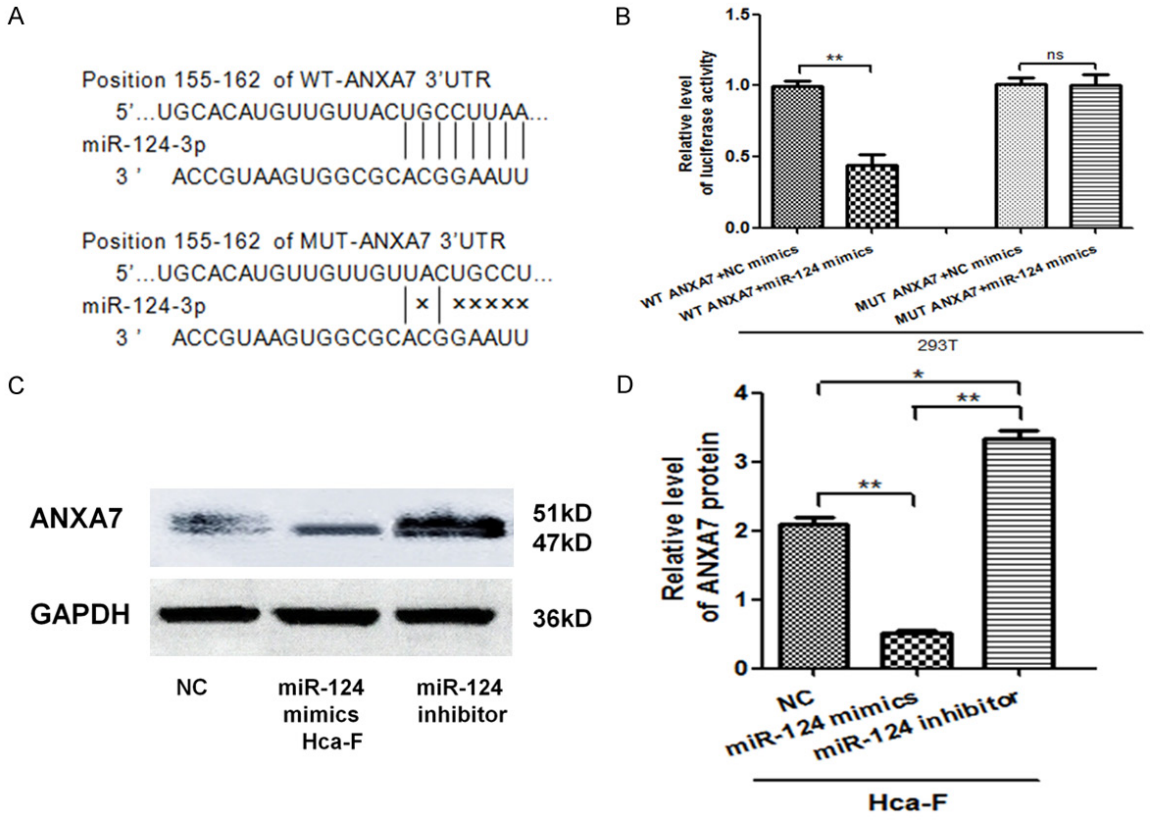

Figure 2. ANXA7 is a target gene of miR-124-3p. A. Sequencing results of WT ANXA7 3'-UTR gene and MUT ANXA7 3'-UTR gene. The sequences were consistent with miRanda, Pubmed, and Targetscans databases and there were binding sites with miR-124-3p in the 155-162 bp sites, while the MUT ANXA7'-UTR 155-162 sites had no miR-124-3p binding sites. B. miR-124-3p binding site in WT ANXA7 3'-UTR and MUT ANXA7 3'-UTR mutation site. C. Dual-luciferase reporter gene assay. D. Comparison of ANXA7 protein levels in Hca-F cells after transfection of miR-124-3p mimics, and miR-124-3p inhibitor. The error bar represents the SD (n = 3). *P < 0.05, **P < 0.01.

the data show (**Figure 3B** and **3C**), Bax protein expression was up-regulated relative to miR-124-3p over-expression, while ANXA7 and Bcl-2 protein expression were suppressed. However, miR-124-3p down-regulation had an opposite effect.

Up-regulation of miR-124-3p inhibits Hca-F cell migration and invasion

To detect the correlation of miR-124-3p level with the migration and invasion of HCC progression, Hca-F cells were infected with a miR-124-3p mimic or inhibitors. Fewer migrated miR-124-3p over-expressing cells were seen on the insert membrane than control cells. In contrast, silencing miR-124-3p in HCC cells significantly increased cell invasion and metastasis compared to the control group (**Figure 4A** and **4B**). To further assess the contribution of miR-124-3p on cell invasion, we investigated the protein expression of ANXA7, MMP-9, and CXCL12 in Hca-F cells by western blot. As the results showed, the expression of MMP-9 and CXCL12 in HCC cells with stable over-expression of miR-124-3p inhibitors was higher than in the other control cells. However, miR-124-3p down-regulation had an opposite effect (**Figure 4C** and **4D**).

Up-regulation of miR-124-3p suppresses LNM and tumorigenicity in 615 mice with Hca-F cells

Hca-F cells were chosen as the LNM model for in vivo study. As the figure shows (**Figure 5A**), in comparison with the NC group, growth was significantly increased in the miR-124-3p inhibitor group. The tumor volume and weight from miR-124-3p mimics cells decreased at day 28 days post-injection (**Figure 5B** and **5D**). Moreover, we observed the LNM rate in different groups. The result showed that there were more visible metastases in the axillary and

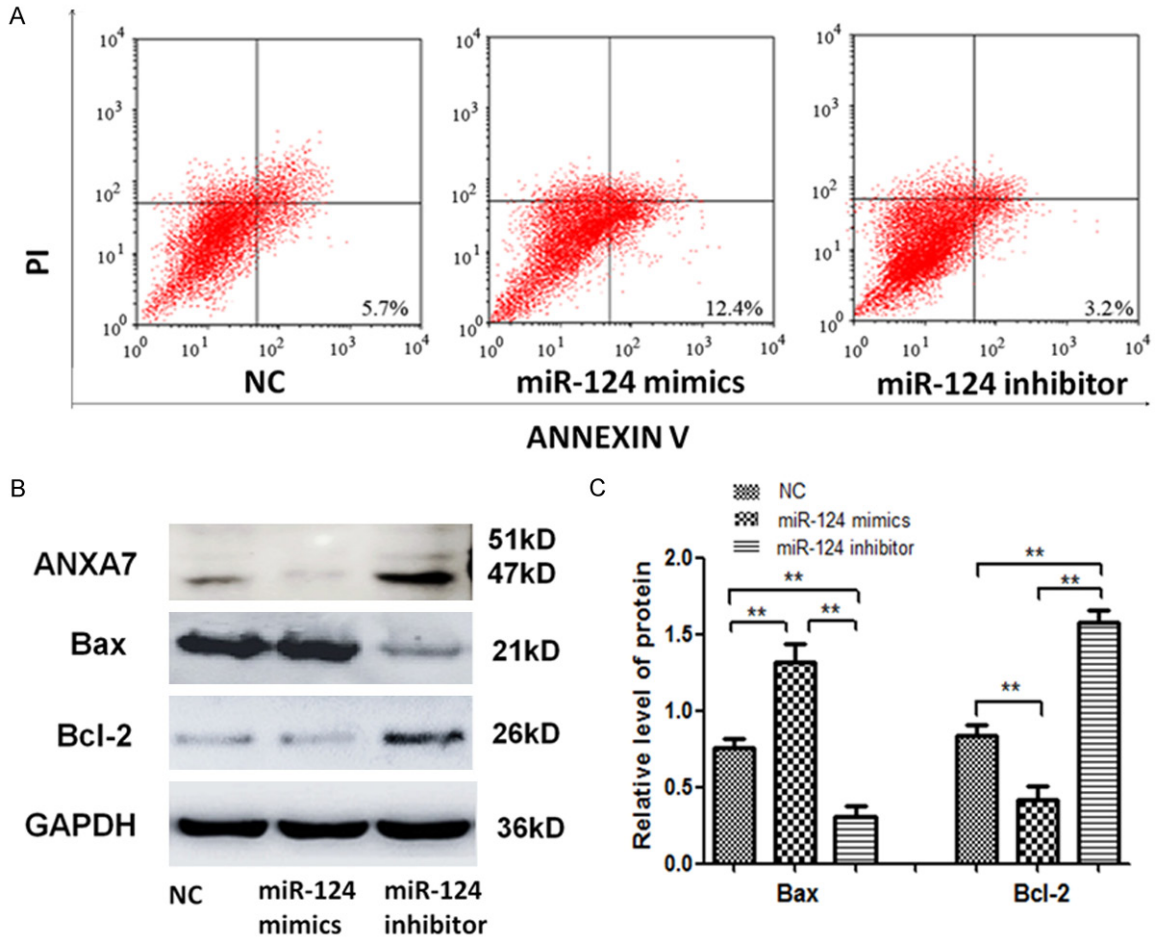

Figure 3. Upregulation of miR-124-3p promotes Hca-F cell apoptosis. A. Annexin V-FITC/PI flow cytometry was used to detect the apoptosis of Hca-F cells, miR-124-3p mimic cells, and miR-124-3p inhibitor cells. B, C. The levels of ANAX7, Bax, and Bcl-2 proteins in Hca-F cells, miR-124-3p mimic cells, and miR-124-3p inhibitor cells were detected by western blot. The error bar represents the SD (n = 3). **P < 0.01

inguinal lymph nodes (**Figure 5C** and **Table 2**). The LNM rate in the miR-124-3p mimics group was lower compared with the NC group and the miR-124-3p inhibitor group (30% vs. 50.0%, 30% vs. 80.0%). The axillary and inguinal lymph node volume from miR-124-3p mimic cells decreased at day 28 days post injection (**Figure 5E**). The expression levels of ANXA7, MMP-9, and CXCL12 protein in transplanted tumors in the miR-124 mimics group were significantly lower than those in the NC group, while the expressions of ANXA7, MMP-9 and CXCL12 protein in the miR-124 inhibitor group were significantly higher than those in the NC group (P < 0.05) (**Figure 5F** and **5G**). The expressions of ANXA7, MMP-9, and CXCL12 were detected by immunohistochemical assay. **Figure 6A** and **6B** show the levels of ANXA7, MMP-9, and CXCL12 protein in lymph node metastases of the miR-124-3p mimics group were significantly lower than those of the NC group, and the levels of ANXA7, MMP-9, and CXCL12 protein in lymph node metastases of the miR-124-3p inhibitor group were significantly higher than those of the NC group (P < 0.05).

Silencing ANXA7 affects miR-124-3p related protein in Hca-F cells

The expression vector ANXA7 and its unrelated sequence were transfected into Hca-F cells by Lipofectamine 2000. The green fluorescent protein was observed under fluorescence microscope after incubation at 37°C for 24-48 hours (**Figure 7A**). qPCR showed no significant difference in the level of ANXA7 gene between transfected unrelated sequence and Hca-F

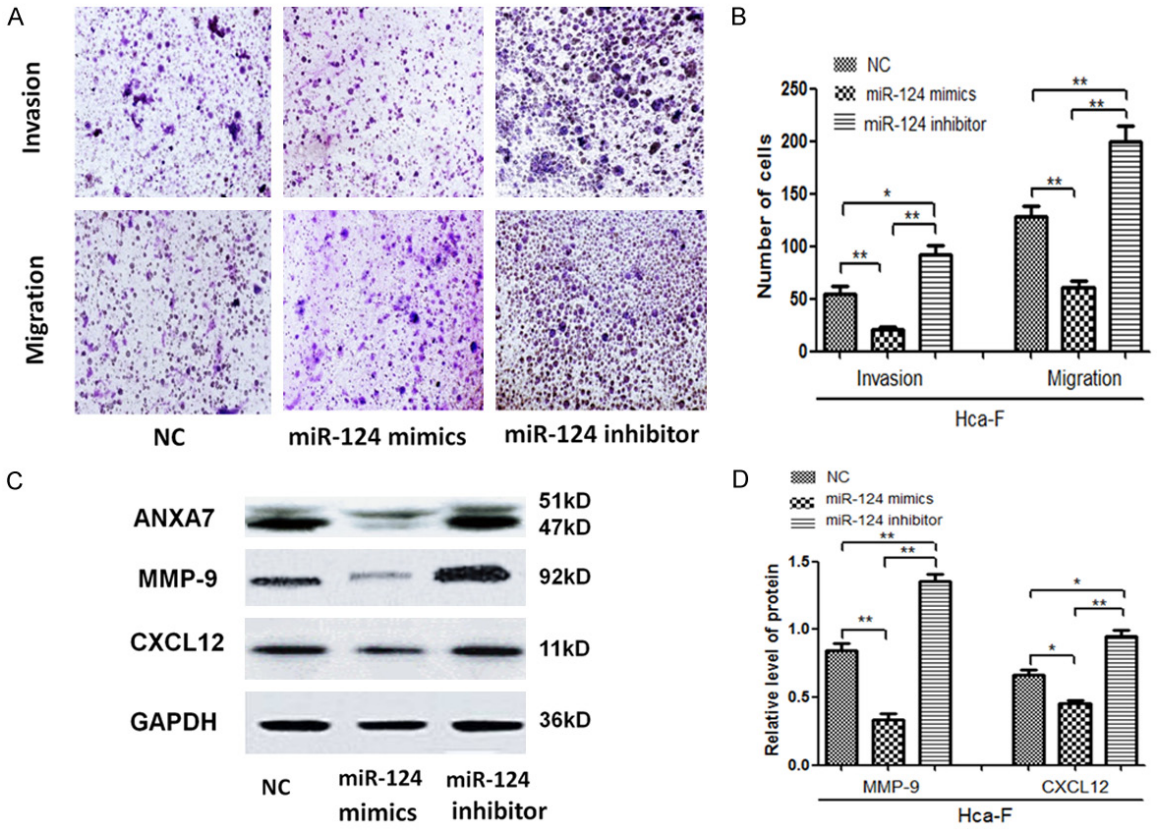

Figure 4. Upregulation of miR-124-3p promotes Hca-F cell apoptosis. A. Transwell migration and invasion experiment in Hca-F cells, miR-124-3p mimic cells, and miR-124-3p inhibitor cells. B. The number of cells on the insert membrane after transwell migration and invasion experiments in Hca-F cells, miR-124-3p mimic cells, and miR-124-3p inhibitor cells. C, D. The levels of ANXA7, MMP-9, and CXCL12 protein in Hca-F cells, miR-124-3p mimic cells, and miR-124-3p inhibitor cells were detected by western blot. The error bar represents the SD (n = 3). *P < 0.05, **P < 0.01.

cells (P > 0.05). The level of ANXA7 gene in transfected expression vector was significantly lower than that in Hca-F cells and transfected unrelated sequence (P < 0.05) (**Figure 7B**). Western blot showed that there was no significant difference between the level of ANXA7 protein in transfected unrelated sequence and Hca-F cells (P > 0.05). The level of ANXA7 protein in transfected expression vector was significantly lower than that in Hca-F cells and transfected unrelated sequence (P < 0.05) (**Figure 7C** and **7D**). The levels of Bax, Bcl-2, MMP-9, and CXCL12 in Hca-F cells did not change significantly after transfection of unrelated sequence (P > 0.05), but the levels of Bcl-2, MMP-9, and CXCL12 in Hca-F cells decreased significantly after transfection of shRNA-Anxa7 (P < 0.05), while the levels of Bax and protein did not change significantly (P > 0.05) (**Figure 7E-G**).

Discussion

ANXA7 protein is one of the Annexin family in vertebrate cells which plays an important role in cytoskeleton activity, membrane phospholipidization, membrane receptor regulation, and mitosis [16, 17]. In the past, our team has found that ANXA7 plays a role in promoting lymphatic metastasis of HCC. Up-regulation of ANXA7 gene expression enhanced the proliferation and migration of Hca-F cells, and down-regulation of ANXA7 gene expression reduced the proliferation and migration of Hca-F cells [18, 19].

In this study, miR-124-3p was selected as a candidate according to the basic principles of bioinformatics prediction. miR-124-3p is one of the important members of the miR-124 family that was first extracted from mouse brain by

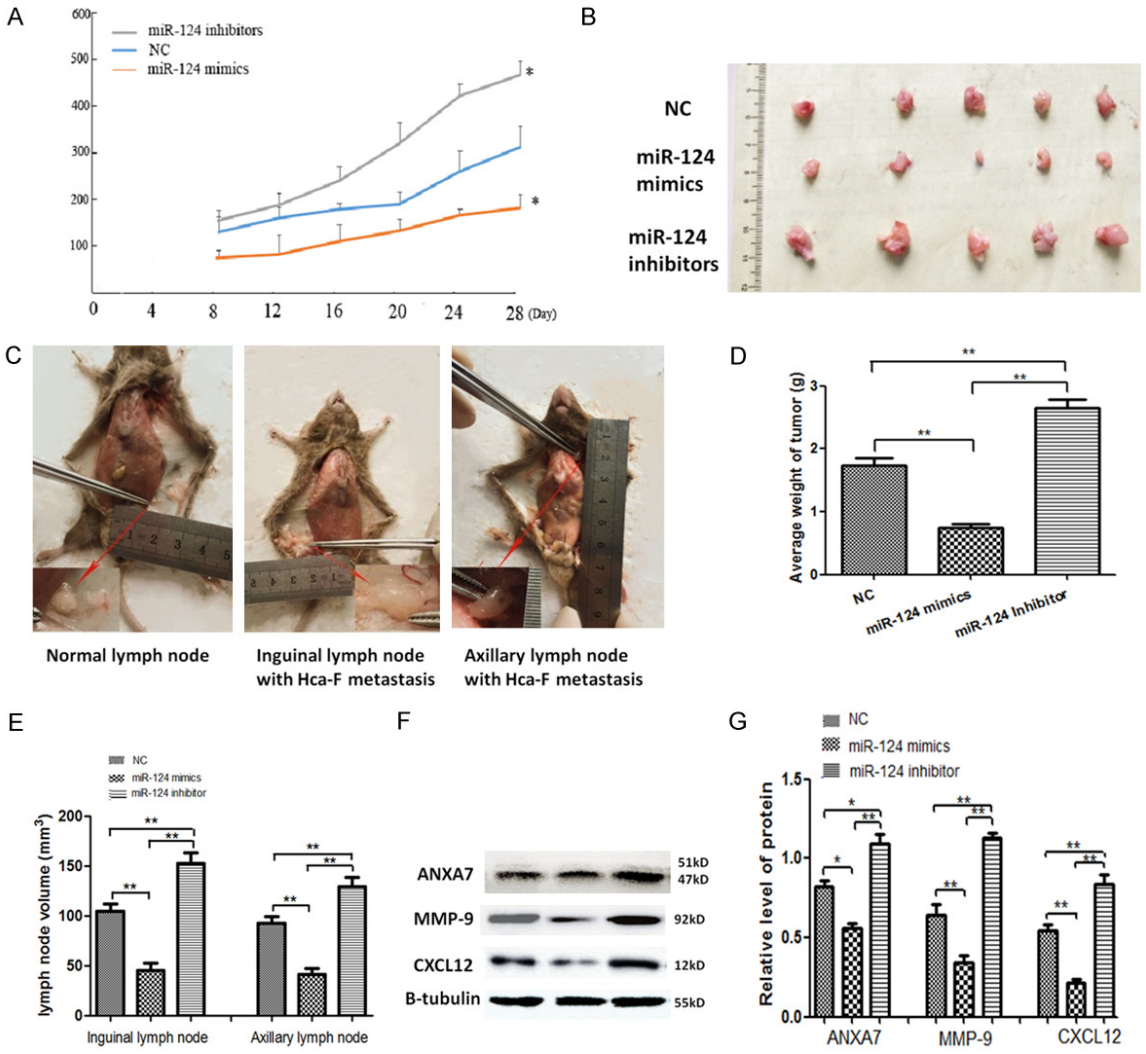

Figure 5. Upregulation of miR-124-3p suppresses LNM and tumorigenicity in 615 mice with Hca-F cells. A. The volume of transplanted tumors on the footpad of mice in each group at different time points. B. The transplanted tumors at the footpad of mice in each group at 28 days. C. The volume of normal lymph nodes smaller than the nodes infected with Hca-F cells. D. The average weight of transplanted tumors on the footpad of mice in each group at 28 days. E. The axillary and inguinal lymph node volume in each group on 28 days. F, G. The expression levels of ANXA7, MMP-9, and CXCL12 proteins in transplanted tumors in each group. The error bar represents the SD (n = 3). *P < 0.05, **P < 0.01.

Table 2. Lymph node metastaticsis of LNM model in different groups

Group	LN	Lymph node metastatic rate level		
		LNM rate	% LNM rate	*P* value
NC	Inguinal	3/5	50.00	
	axillary	2/5		
miR-124 mimics	Inguinal	2/5	30.00	< 0.05*
	axillary	1/5		
miR-124 inhibitors	Inguinal	5/5	80.00	
	axillary	3/5		

*p < 0.05 indicates a significant association among the variables.

Mishima in 2007. miR-124 can inhibit the occurrence of colon cancer [20]. Tian et al. found that, miR-124 can inhibit the invasion and metastasis of cholangiocarcinoma [21]. Hu et al. found that, there is an abnormal decrease of miR-124 in nasopharyngeal carcinoma [22]. In this study we demonstrated miR-124-3p was down-regulated while ANXA7 protein was up-regulated in HCC. There was a significant negative correlation between level of ANXA7

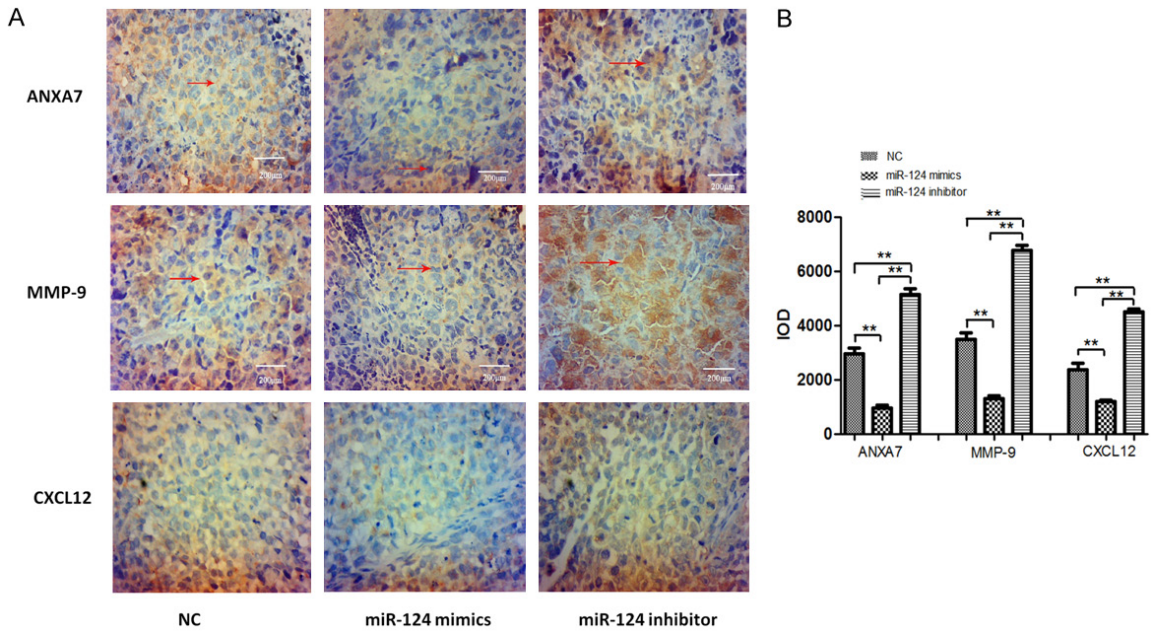

Figure 6. The expression of ANXA7, MMP-9, and CXCL12 detected by immunohistochemical assay. (A) Immunostaining results of ANXA7, MMP-9, and CXCL12 in transplanted tumors of mice in each group, using a magnification of 40 × 10, (B) IOD values of positive results in each group. The error bar represents the SD (n = 3). **P < 0.01.

protein and miR-124-3p. Furthermore, we collected clinicopathologic data and conducted an analysis. The results showed that levels of miR-124-3p in tumor tissues was negatively correlated, while ANXA7 protein was positively correlated with TNM stage and tumor metastasis (**Table 1**). These results suggest that miR-124-3p and ANXA7 play an important role in occurrence and development of HCC, and are closely related to the progression and metastasis of HCC.

The luciferase reporter assay demonstrated that there was a binding site of miR-124-3p in the 3'-UTR of ANXA7, and miR-124-3p decreased the activity of ANXA7 3'-UTR, while no change was seen in the mutant group (**Figure 2**). Then we chose miR-124-3p mimics and inhibitor for further mechanism investigation. We demonstrated the over-expression of miR-124-3p can induce the apoptosis rate in Hca-F cells partly though the mitochondrial apoptotic pathway. miR-124-3p enhanced the Bax level and inhibited the expression of Bcl-2 and ANXA7 (**Figure 3**). Bax and Bcl-2 are two important proteins involved in apoptosis that belong to the Bcl-2 family [23, 24]. Studies have shown that Bcl-2 protein can stabilize the mitochondrial membrane and inhibit the opening of mitochondrial membrane channels, thereby inhibiting the release of apoptosis-related proteins in mitochondria [25]. Bcl-2 can also inhibit the formation of apoptotic bodies by inhibiting the release of cytochrome C and the binding of caspase-9 to cytochrome C [26]. Bax protein is the first confirmed apoptotic protein in the Bcl-2 family [27]. The results in this study showed that miR-124-3p could promote the apoptosis of Hca-F cells by stimulating the expression of Bax and inhibiting the expression of Bcl-2.

Next, the functional studies were performed to reveal the correlation of miR-124-3p level with the migration and invasion of HCC progression. HCC progression was inhibited by up-regulating miR-124-3p through suppression migration and invasion in vitro, whereas the activities were facilitated by miR-124-3p knockdown. As the results showed, the expression of MMP-9 and CXCL12 in HCC cells with stable over-expression of miR-124-3p inhibitors was higher than that of the other control cells (**Figure 4**). MMP-9, also known as gelatinase B, is an important member of the matrix metalloproteinase family [28]. Samad et al. reported that MMP-9 plays an important role in promoting metastasis of hepatocellular carcinoma Hep G2 cells [29]. Frenoux et al. found that serum

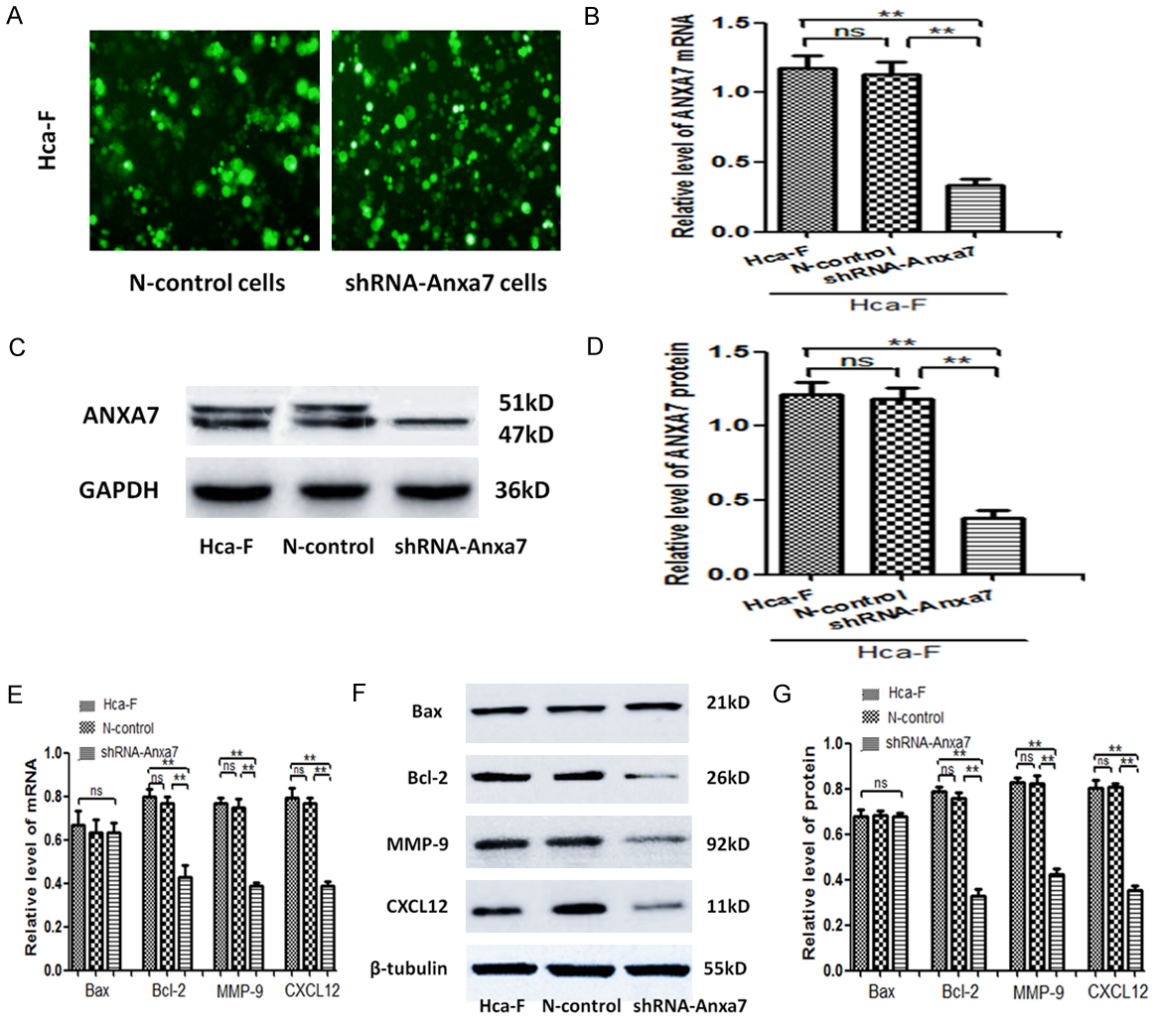

Figure 7. Silencing ANXA7 affects miR-124-3p related protein in Hca-F cells. A. The expression vector ANXA7 and its unrelated sequence were transfected into Hca-F cells by Lipofectamine 2000. The green fluorescent protein was observed under fluorescence microscope after incubation at 37°C for 24-48 hours. B. The levels of ANXA7 gene in Hca-F group, N-control group, and shRNA-Anxa7 group were detected by qPCR. C, D. Western blot was used to detect the levels of ANXA7 protein in Hca-F group, N-control group, and shRNA-Anxa7 group. E. q-PCR was used to detect the levels of Bax, Bcl-2, MMP-9, and CXCL12 gene in the Hca-F group, N-control group, and shRNA-Anxa7 group. F, G. Western blot was used to detect the levels of Bax, Bcl-2, MMP-9, and CXCL12 protein in the Hca-F group, N-control group, and shRNA-Anxa7 group. The error bar represents the SD (n = 3). *P < 0.05, **P < 0.01, ns P > 0.05.

MMP-9 levels were significantly increased in patients with metastasis after radiofrequency ablation of HCC [30]. CXCL12, also known as matrix-derived factor 1, is an important member of the chemokine CXC family. Recent studies have found that CXCL12 is also highly expressed in lung, cervical, esophageal, and hepatocellular carcinomas, and plays an important role in directional migration of cancer cells [31, 33]. Semaan reported that CXCL12 promotes the adhesion of hepatocellular carcinoma cells by binding to its receptor CXCR4 through autocrine or paracrine mechanisms, thus promoting the invasion and metastasis of tumors [34]. The results showed that miR-124-3p could inhibit the migration and invasion of Hca-F cells by inhibiting the expression of MMP-9 and CXCL12.

In vitro, overexpression of miR-124-3p decreased the tumor volume significantly and resulted in fewer lymph node metastases in mice. The opposite effects were yielded by miR-124-3p knockdown (**Figure 5**). In this study the expression of ANXA7, MMP-9, and CXCL12 was detected by immunohistochemi-

cal assay. As the data showed, the levels of ANXA7, MMP-9, and CXCL12 protein in lymph node metastases of the miR-124-3p mimics group were significantly lower than those of the NC group (**Figure 6**). It was further confirmed that the expression of MMP-9 and CXCL12 could be regulated by miR-124-3p and ANXA7 to inhibit lymphatic metastasis of HCC. The expression vector ANXA7 and its unrelated sequence were transfected into Hca-F cells by Lipofectamine 2000. The data demonstrated that silencing ANXA7 affects miR-124-3p related protein on Hca-F cells, such as Bcl-2, MMP-9, and CXCL12 in Hca-F cells (**Figure 7**). As predicted, we believe that miR-124-3p can regulate the expression of Bcl-2, MMP-9, and CXCL12 by targeting the ANXA7 gene, promoting the apoptosis of Hca-F cells, and inhibiting the migration, invasion, and lymphatic metastasis of Hca-F cells.

In summary, miR-124-3p was downregulated while ANXA7 protein was upregulated in HCC, and both are closely related to the progression and metastasis of HCC. Our results revealed miR-124-3p inhibits tumor growth, invasion, and lymphatic metastasis in HCC by down-regulating the ANXA7 gene, thereby reducing the expression of Bcl-2, MMP-9, and CXCL12. The positive expression of miR-124-3p and ANXA7 in primary and metastatic tumors also provides a possible marker for the clinical diagnosis of the occurrence and development of HCC, and might be a potential therapeutic target for HCC.

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Disclosure of conflict of interest

None.

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