Clinics and Research in Hepatology and Gastroenterology (2019) 43, 436—445

Available online at ScienceDirect www.sciencedirect.com

Elsevier Masson France EM|consulte www.em-consulte.com/en

ORIGINAL ARTICLE

Long non-coding RNA LINC00472 suppresses hepatocellular carcinoma cell proliferation, migration and invasion through miR-93-5p/PDCD4 pathway

Changyu Chen, Qiang Zheng, Weibiao Kang, Changjun Yu*

Department of Gastrointestinal Surgery, Department of General Surgery, First Affiliated Hospital of Anhui Medical University, 218, Jixi avenue, Hefei 230022, Anhui, PR China

Available online 3 December 2018

KEYWORDS
LINC00472;
Proliferation;
Migration;
Invasion;
Hepatocellular carcinoma

Summary Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and the second leading cause of cancer-related deaths. In the present study, we have demonstrated that long non-coding RNA (lncRNA) LINC00472 was low expressed in human HCC tissues and cell lines compared with adjacent non-tumor liver tissues and normal liver cell lines respectively. LINC00472 was also low expressed in HCC tissues from patients with metastasis compared with tissues from patients without metastasis. Expression level of LINC00472 was positively correlated with patient overall survival (OS) rate. Forced expression of LINC00472 suppressed cell proliferation, migration, invasion and promoted cell apoptosis in HCC cells Huh-7 and SMMC-7721. MiR-93-5p was a direct target of LINC00472, and miR-93-5p directly targeted PDCD4. The miR-93-5p/PDCD4 pathway mediated the suppressing role of LINC00472 in HCC cells. Therefore, LINC00472 was an important tumor suppressor in human HCC, which could be used as a bio-marker for HCC therapy.
© 2018 Elsevier Masson SAS. All rights reserved.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and the second leading cause of cancer-related deaths [1—3]. Hepatitis B and C virus infection contributes to the raised incidence of HCC [1,4]. Surgery, chemotherapy, radiotherapy, locoregional therapies, and liver transplantation remain main treatments for HCC these years [5—7]. However, patients with HCC always suffer from carcinoma recurrence and treatment resistance, and their mean five-year survival rate is no more than 50% [1]. Lack of efficient targets retarded the development of targeted therapy for HCC. Although numerous studies attempted to unveil the mechanisms related with tumor development and

* Corresponding author: Department of Gastrointestinal Surgery, Department of General Surgery, First Affiliated Hospital of Anhui Medical University, 218, Jixi avenue, Hefei 230022, Anhui, PR China.
E-mail address: 842749628@qq.com (C. Yu).

https://doi.org/10.1016/j.clinre.2018.11.008
2210-7401/© 2018 Elsevier Masson SAS. All rights reserved.

metastasis of HCC, the underlying mechanisms and key factors involved in HCC remain poorly understood. It is desirable and urgent to perform further study for the exact underlying mechanisms of HCC, which will benefit the development of new clinical therapeutic methods for HCC.

Long non-coding RNAs (lncRNAs) (more than 200 nucleotides in size) belonging to non-coding RNAs, commonly do not encode proteins [8,9]. In former studies, few functions of lncRNAs were unveiled, and some of them were considered to have no value in human physiological processes [10]. Recently, more and more studies reported the important role of lncRNAs in many physiological processes, especially many lncRNAs acted as important tumor promoters or tumor suppressors in various kinds of human cancers [1,11]. For example, long non-coding RNA MT1JP acted as a tumor suppressor in human gastric cancer [12]; high level of long non-coding RNA AFAP1-AS1 predicts a poor prognosis in non-small cell lung cancer patients [13]; long non-coding RNA GAS5 suppresses triple negative breast cancer progression [14]. Another kind of non-coding RNA, microRNA (miRNA) (20—25 nucleotides in size), was critically studies in many publications and was documented to directly regulate the expression of their target genes, contributing to tumor initiation, development and metastasis of human cancers [15,16]. LncRNAs could act as competing endogenous RNAs and sponge miRNAs, indirectly regulate the downstream target genes of these miRNAs [10,17]. The important roles and the complex mechanisms of LncRNAs are desirable for further investigation. As reported previously, lncRNA LINC00472 suppressed cell proliferation and migration in human breast cancer cells; low expression of LINC00472 was associated with worse clinicopathological features of human breast cancer patients and epithelial ovarian cancer [18—20]. Moreover, LINC00472 was documented to play important roles in human colorectal cancer [21]. However, the role of LINC00472 in human HCC remains unknown.

In this study, we have determined that LINC00472 was low expressed in human HCC tissues and cell lines compared with adjacent non-tumor liver tissues and normal liver cell lines respectively. LINC00472 was also low expressed in HCC tissues from patients with metastasis compared with tissues from patients without metastasis. Low level of LINC00472 was associated with low overall survival (OS) rate in HCC patients. Moreover, LINC00472 was examined to suppress cell proliferation, migration and invasion of human HCC cells. LINC00472 directly targeted miR-93-5p, and miR-93-5p directly targeted PDCD4. In HCC tissues, LINC00472 and miR-93-5p were negatively correlated; LINC00472 and PDCD4 were positively correlated; miR-93-5p and PDCD4 were negatively correlated. The miR-93-5p/PDCD4 pathway mediated the suppressing role of LINC00472 in cell proliferation, migration and invasion of human HCC cells. Therefore, LINC00472 could be used as a potential target for human HCC treatment.

Materials and methods

Tissue samples and patients

We collected 35 fresh adjacent non-tumor liver tissues and 109 fresh HCC tissues from patients who underwent surgery in the First Affiliated Hospital of Anhui Medical University (Hefei, China) between 2011 and 2014. Patients with other diseases or had underwent special therapies before surgery were excluded. For OS study, the 109 HCC patients were followed up for more than 5 years. We had got local approval from the Institutional Review Boards of Southeast University and the signed informed consent from every patient before the patient-related work. We performed these work according to The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Cell culture

Human liver cell line LO2 and HCC cell lines HepG2, BEL-7404, Hep3B, SMMC-7721, Huh-7 were purchased from ATCC (the American Type Culture Collection) (Rockville, MD). As recommended, these cell lines were cultured in a humidified atmosphere at 5% CO2 and 37 °C.

RT-quantitative PCR (RT-qPCR)

RT-quantitative PCR (RT-qPCR) was performed as described in previous studies [22]. We examined expression levels of LINC00472, miR-93-5p and PDCD4 mRNA in this study, GAPDH and U6 were also detected as control respectively.

RNA oligonucleotide and plasmid transfection

LINC00472 sequence (2958bp) was cloned into mammalian expression plasmid pcDNA3.1 (Invitrogen) [HindIII (splicing at AAGCTT) and XhoI (splicing at CTCGAG) were used] (designated as pcDNA-LINC00472) for LINC00472 over-expressing, the vector plasmid pcDNA3.1 (designated as pcDNA) was used as control. MiR-93-5p/miR-Control mimics and shLINC00472/shControl (shCtrl) were synthesized in GenePharma (Shanghai, China). We carried out RNA oligonucleotide and plasmid transfection in this study using lip2000 (QIAGEN) as recommended and essentially as described earlier [23,24]. Cells were treated with 400 μg/mL G418 (Sangon Biotech, Shanghai, China) after pcDNA-LINC00472/pcDNA transfection for constructing stably-transfected cells as recommended.

Cell proliferation, migration and invasion assays

MTT assay, cell counting assay, cell colony formation assay, cell migration assay and cell invasion assay were carried out in this study to examine cell proliferation and metastasis of human HCC cells essentially as described in previous studies [23,24]. Briefly, for MTT assay, stably-transfected cells or cells after transient-transfection for 24 hours were seeded into 96-well cell plates (2000 per well). After another 72 hours, MTT evaluation was carried out. For cell counting assay, stably-transfected cells or cells after transient-transfection for 24 hours were seeded into 6-well cell plates (10,000 per well). Cell total number was documented everyday in the next 5 days, and cell growth curves were analyzed. For cell colony formation assay, stably-transfected cells or cells after transient-transfection for 24 hours were seeded into 6-well cell plates (1000 per

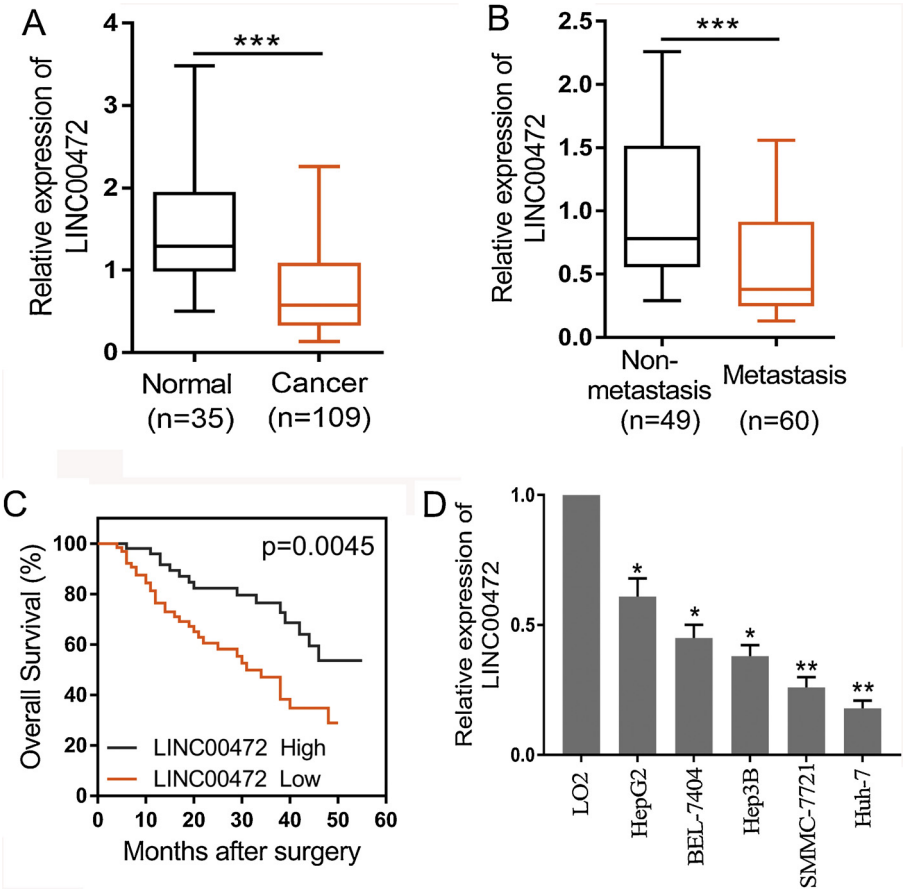

Fig. 1 Expression of LINC00472 in human tissues from HCC patients and in HCC cell lines. A. Expression levels of LINC00472 in human adjacent non-tumor liver tissues (Normal) and HCC tissues (Cancer) were examined using RT-qPCR. B. Expression levels of LINC00472 in human HCC tissues with or without metastasis were examined using RT-qPCR. GAPDH was also examined as control. C. OS rates between LINC00472 high group and LINC00472 low group in 109 HCC patients were analyzed by Kaplan-Meier curves. D. Expression levels of LINC00472 in normal liver cell line LO2 and HCC cell lines HepG2, BEL-7404, Hep3B, SMMC-7721, and Huh-7 were detected using RT-qPCR. GAPDH was also detected as control. $^{*}P < 0.05$; $^{**}P < 0.01$.

well). Status of cell colony formation was analyzed after 10—15 days. For cell migration and invasion, cells were seeded into Transwell chambers (migration assay without Matrigel (BD Biosciences, San Diego, CA) and invasion assay with Matrigel). Cell migration was examined after 18 hours and cell invasion was examined after 36 hours.

Flow cytometry

Flow cytometry was carried out in Huh-7 and SMMC-7721 cells to evaluate cell apoptosis and cell cycle condition. Stably-transfected cells or cells after transient-transfection for 72 hours were harvested, fixed and then incubated in Annexin V antibody-FITC and PI or Rnase A and propidium iodide for 0.5 hour at room temperature. Cells were analyzed in flow cytometer.

Luciferase reporter assay

Luciferase reporter plasmids including PsiCHECK2-LINC00472-WT, PsiCHECK2-LINC00472-MUT, PsiCHECK2-PDCD4-3'-UTR-WT and PsiCHECK2-PDCD4-3'-UTR-MUT were constructed and luciferase reporter assay was carried out essentially as described earlier by using a Dual Luciferase Reporter Assay Kit (Promega Corp.) [23,24].

Western Blot

Western blot was performed to examine protein levels of PDCD4 in transfected cell lines as described earlier [23,25]. PDCD4 Mouse Monoclonal antibody (Proteintech Group, Inc., Chicago, USA) and β-actin Mouse Monoclonal antibody (Sigma) were used. β-actin was examined as control.

Statistical analyses

Three independent repeating experiments were carried out for every examination and each figure represented the average. For RT-qPCR, cell functional experiments, flow cytometry and luciferase reporter assay, two-tailed t test was used in statistical analyses. For patient OS analyses, Kaplan-Meier curves and log-rank test were used. It was considered statistically significant when $P < 0.05$.

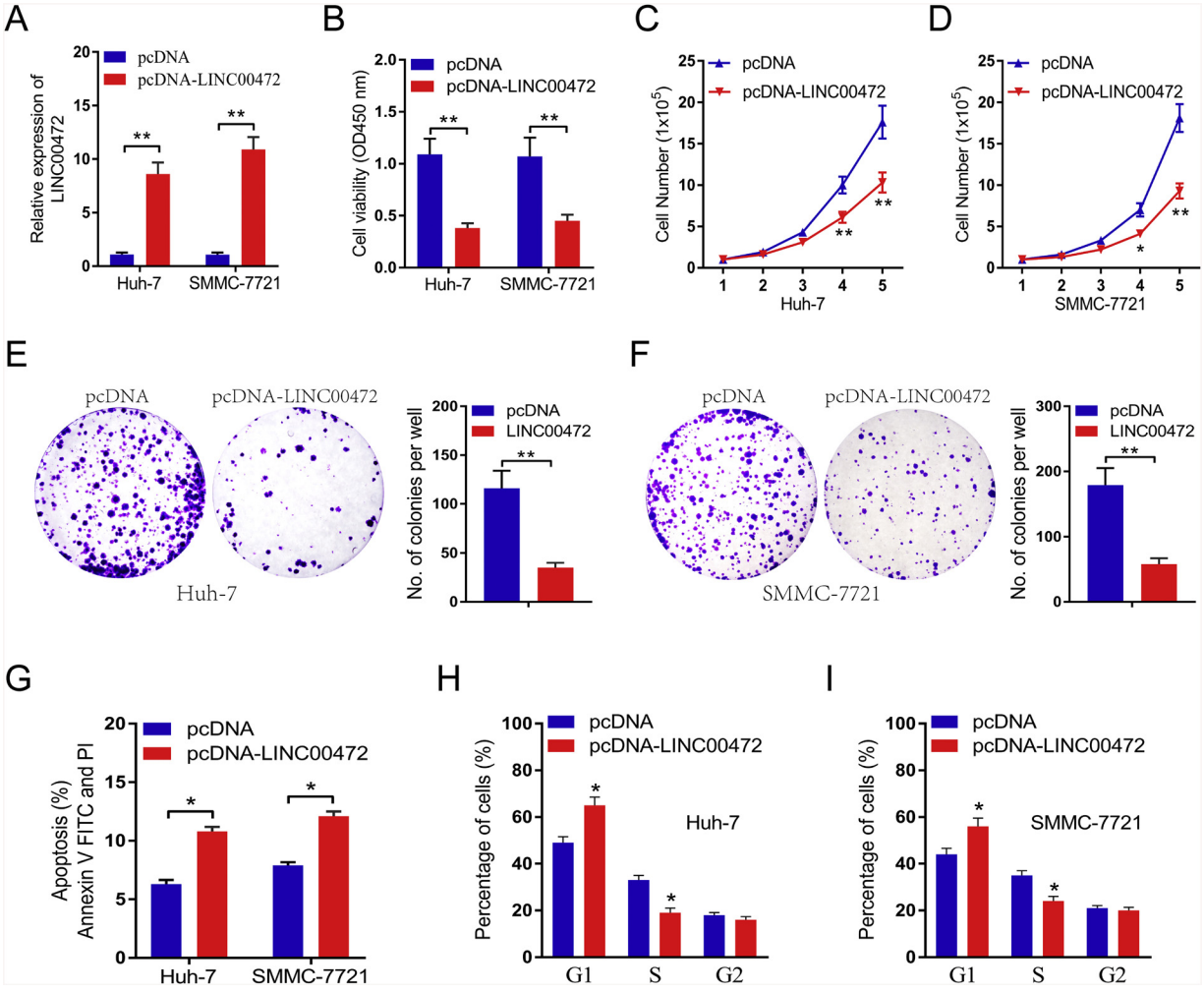

Fig. 2 LINC00472 suppressed cell growth of human HCC cells. A. Expression levels of LINC00472 in Huh-7 and SMMC-7721 cells stably transfected with pcDNA-LINC00472 or control pcDNA plasmid were detected by RT-qPCR. GAPDH was also detected as control. B. MTT assay, C, D. cell counting assay. E, F. Cell colony formation assay were performed in Huh-7 and SMMC-7721 cells stably transfected with pcDNA-LINC00472 or control pcDNA plasmid. G. Flow cytometry (incubating with Annexin V antibody-FITC and PI) was carried out to evaluate cell apoptosis in Huh-7 and SMMC-7721 cells stably transfected with pcDNA-LINC00472 or control pcDNA plasmid. H, I. Flow cytometry was performed to evaluate the percentages of cells in G1, S, and G2 phases of the cell cycle in Huh-7 and SMMC-7721 cells stably transfected with pcDNA-LINC00472 or control pcDNA plasmid. $^{*}P < 0.05$; $^{**}P < 0.01$.

Results

Expression of LINC00472 in human tissues and the correlation between LINC00472 expression and overall survival (OS) rates in HCC patients

Thirty-five normal human liver tissues and 109 (49 without metastasis and 60 with metastasis) human HCC tissues were collected and the expression level of LINC00472 were examined using RT-qPCR. As shown in Fig.1A and Fig.1B, the expression level of LINC00472 was significantly lower in human HCC tissues compared with adjacent non-tumor liver tissues and was significantly lower in HCC tissues from patients with metastasis compared with HCC tissues from patients without metastasis.

For further study, we followed these HCC patients for more than 5 years and the correlation of LINC00472 expression with patient overall survival (OS) rates was analyzed by using Kaplan-Meier curves. The OS rates was dramatically lower in HCC patients with low level of LINC00472 compared with patients with high level of LINC00472 ($P = 0.0045$) (Fig.1C).

Therefore, LINC00472 was low-expressed in HCC tissues compared with adjacent non-tumor liver tissues and was low-expressed in HCC tissues with metastasis compared with tissues without metastasis; low level of LINC00472 was associated with poor prognosis in human HCC patients.

LINC00472 inhibited cell growth of human HCC cells

Normal liver cell line LO2 and HCC cell lines HepG2, BEL-7404, Hep3B, SMMC-7721, and Huh-7 were cultured and the expression levels of LINC00472 in these cell lines were examined using RT-qPCR. Concordant with former

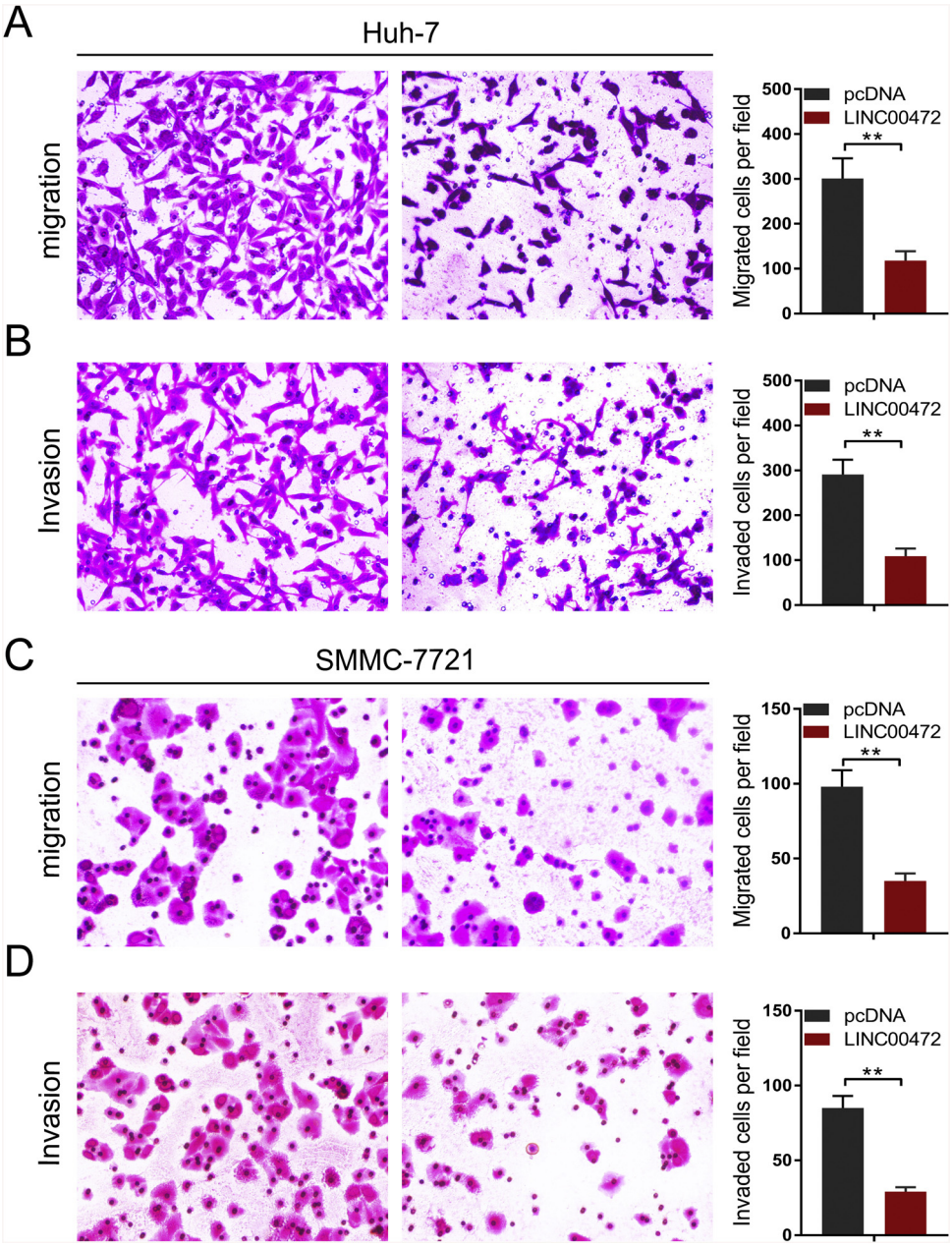

Fig. 3 LINC00472 suppressed migration and invasion of human HCC cells. A, C, Cell migration assay and B, D, cell invasion assay were performed to evaluate metastasis in Huh-7 and SMMC-7721 cells stably transfected with pcDNA-LINC00472 or control pcDNA plasmid. $^{**}P < 0.01$.

tissue results, the expression level of LINC00472 was significantly lower in these 5 HCC cell lines compared with normal liver cell line LO2 (Fig.1D). Huh-7 and SMMC-7721 cells were used for next cell functional studies. As shown in Fig.2A, Huh-7 and SMMC-7721 cells were stably transfected with pcDNA3.1 plasmid containing LINC00472 sequence (designated as pcDNA-LINC00472), and therefore these cells expressed higher level of LINC00472 compared with cells transfected with control pcDNA3.1 plasmid (designated as pcDNA) respectively as determined by RT-qPCR. As determined by MTT assay, cell viabilities of both Huh-7 and SMMC-7721 cells decreased significantly after transfected with pcDNA-LINC00472 compared with control pcDNA plasmid (Fig.2B). Concordantly, pcDNA-LINC00472 dramatically decreased cell total number in both Huh-7 and SMMC-7721 cells over a period 5 days as determined by cell counting assay (Fig.2C, D). In addition, as determined by cell colony formation assay, pcDNA-LINC00472 also dramatically reduced cell colony formation of both Huh-7 and SMMC-7721 cells compared with control pcDNA plasmid (both $P < 0.01$) (Fig.2E, F). Moreover, cell apoptosis was determined using flow cytometry by incubating with Annexin V FITC and PI. As shown in Fig.2G, pcDNA-LINC00472 significantly increased cell apoptosis in both Huh-7 and SMMC-7721 cells compared with control. Also examined by flow cytometry, pcDNA-LINC00472 significan-

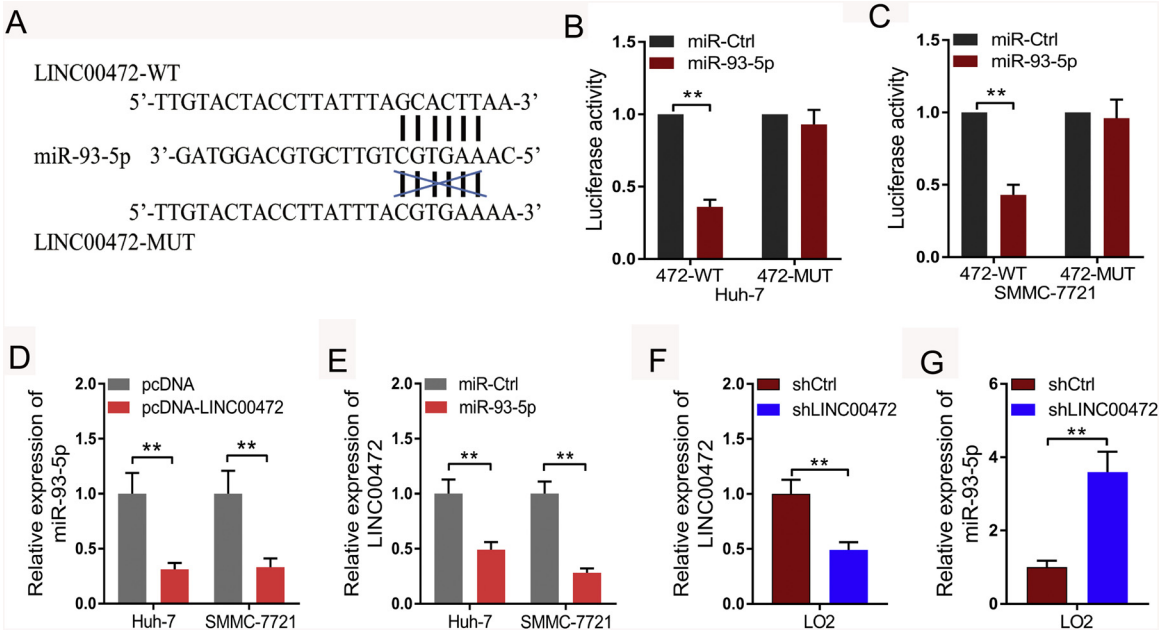

Fig. 4 LINC00472 directly targeted miR-93-5p. A. Predicted consequential pairing of LINC00472 and miR-93-5p; the mutant LINC00472 sequence. B, C. Luciferase assay of Huh-7 and SMMC-7721 cells co-transfected with miR-93-5p mimics/miR-Control mimics, and luciferase reporter plasmid PsiCHECK2-LINC00472-WT/PsiCHECK2-LINC00472-MUT. D. Expression levels of miR-93-5p in Huh-7 and SMMC-7721 cells transfected with pcDNA-LINC00472 or control pcDNA plasmid were detected by RT-qPCR. U6 was also detected as control. E. Expression levels of LINC00472 in Huh-7 and SMMC-7721 cells transfected with miR-93-5p mimics or miR-Control mimics were detected by RT-qPCR. GAPDH was also detected as control. F, G. Expression levels of LINC00472 and miR-93-5p in LO2 cells transfected with shLINC00472 or shCtrl were detected by RT-qPCR. GAPDH and U6 were also detected as control respectively. $^{**}P < 0.01$.

tly increased the G1 phase percentage and decreased the S phase percentage in both Huh-7 and SMMC-7721 cells (Fig.2H, I). As a result, LINC00472 dramatically suppressed cell proliferation and promoted cell apoptosis in human HCC cells.

LINC00472 inhibited migration and invasion of human HCC cells

Cell metastasis assays including cell migration assay and invasion assay were performed to evaluate the role of LINC00472 in metastasis of human HCC cells. As shown in Fig.3A—D, both of cell migration and invasion decreased dramatically in both Huh-7 and SMMC-7721 cells after transfection of pcDNA-LINC00472 compared with control pcDNA plasmid respectively (all $P < 0.01$). As a result, LINC00472 suppressed migration and invasion of human HCC cells.

LINC00472 directly targeted miR-93-5p

For further study, we searched for potential miRNAs that might be direct targets of LINC00472 in human HCC cells using DIANA TOOL. MiR-93-5p (several candidate miRNAs were selected at first but through the next luciferase reporter assay and RT-qPCR examination, miR-93-5p was examined to change most, so miR-93-5p was focused for further study) was found to be a candidate target of LINC00472 and the binding site in miR-93-5p was 5'-CGTGAA-3' (Fig.4A). Both of wild type LINC00472 (designated as LINC00472-WT) and mutant LINC00472 (binding site was changed from 5'-GCACTT-3' into 5'-CGTGAA-3') (designated as LINC00472-MUT) were cloned into psiCHECK-2 luciferase reporter plasmids, and luciferase reporter assay was carried out (Fig.4A). As shown in Fig.4B and C, the luciferase activities decreased significantly in both Huh-7 and SMMC-7721 cells after co-transfected with PsiCHECK2-LINC00472-WT and miR-93-5p mimics compared with co-transfected with PsiCHECK2-LINC00472-WT and miR-Control mimics respectively. There were no differences of the luciferase activities between co-transfection with PsiCHECK2-LINC00472-MUT/miR-93-5p mimics and co-transfection with PsiCHECK2-LINC00472-MUT/miR-Control mimics in both Huh-7 and SMMC-7721 cells. Moreover, as determined by RT-qPCR, expression level of miR-93-5p decreased significantly after stably transfected with pcDNA-LINC00472 compared with control pcDNA plasmid in both Huh-7 and SMMC-7721 cells (Fig.4D); expression level of LINC00472 decreased significantly after transfected with miR-93-5p mimics compared with miR-Control mimics in both Huh-7 and SMMC-7721 cells (Fig.4E). In addition, knockdown of LINC00472 by shLINC00472 significantly decreased the LINC00472 level and increased the miR-93-5p level in normal liver cell LO2 (Fig.4F, G). As a result, miR-93-5p was a direct target of LINC00472.

PDCD4 was a direct target of miR-93-5p

We next searched for potential targets of miR-93-5p by using online TargetScan. PDCD4 was predicted as a candidate

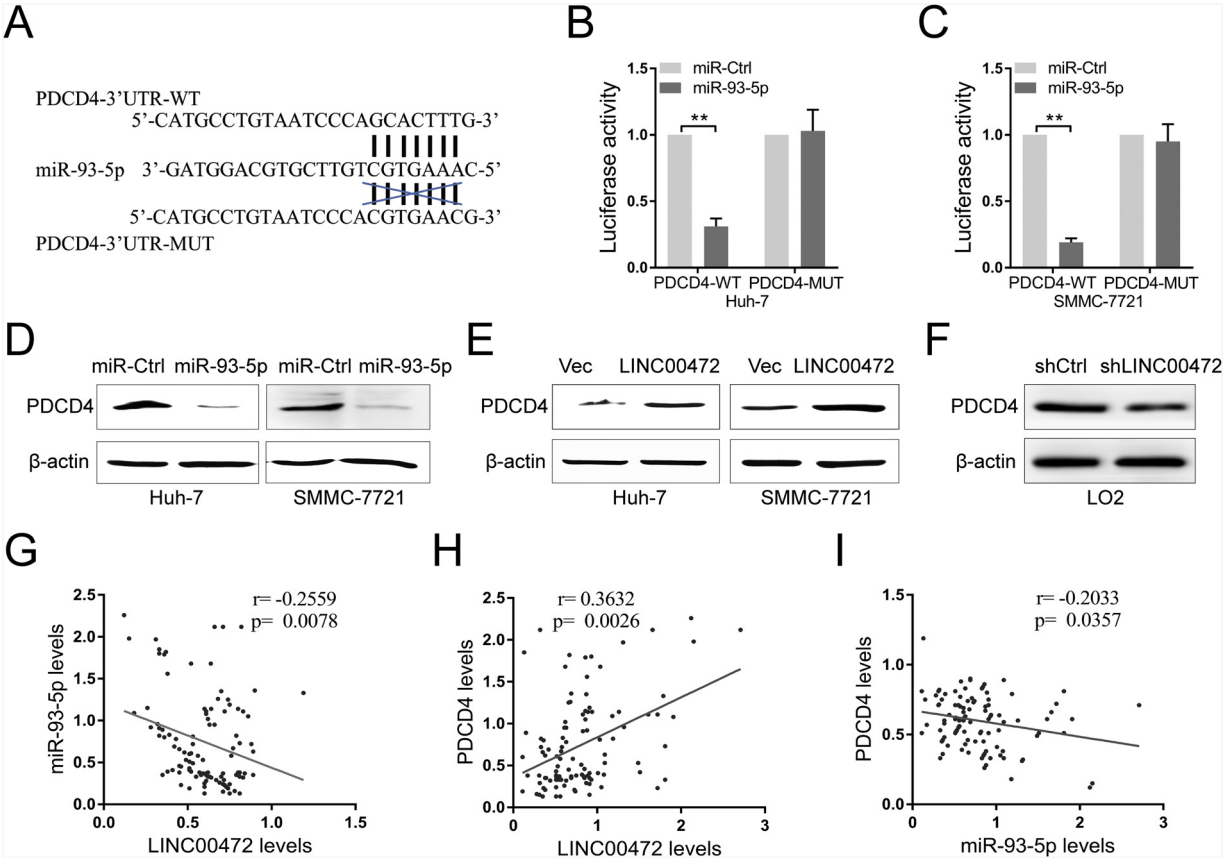

Fig. 5 PDCD4 was a direct target of miR-93-5p. A, Predicted consequential pairing of miR-93-5p and the wild type (WT) or mutant (MUT) 3'-UTR of PDCD4. B, C, Luciferase assay of Huh-7 and SMMC-7721 cells co-transfected with miR-93-5p mimics/miR-Control mimics, and luciferase reporter plasmid PsiCHECK2-PDCD4-3'-UTR-WT/PsiCHECK2-PDCD4-3'-UTR-MUT. D, Protein levels of PDCD4 in Huh-7 and SMMC-7721 cells transfected with miR-93-5p mimics or miR-Control mimics were examined by western blot. β-actin was also examined as control. E, Protein levels of PDCD4 in Huh-7 and SMMC-7721 cells stably transfected with pcDNA-LINC00472 or control pcDNA plasmid (Vec) were examined by western blot. β-actin was also examined as control. F, Protein levels of PDCD4 in LO2 cells transfected with shLINC00472 or shCtrl were examined by western blot. β-actin was also examined as control. G, H, I, LINC00472, miR-93-5p and PDCD4 mRNA levels in HCC tissues were examined by RT-qPCR, and the correlations of LINC00472/miR-93-5p, LINC00472 / PDCD4 mRNA, miR-93-5p/PDCD4 mRNA were analyzed respectively. $^{**}P < 0.01$.

target of miR-93-5p and the miR-93-5p-binding site with the 3'-UTR of PDCD4 mRNA was 5'-GCACTTT-3' (Fig.5A). Luciferase reporter assay was carried out to examine the interaction of miR-93-5p and PDCD4. The luciferase activity decreased significantly after co-tranfected with luciferase reporter plasmid PsiCHECK2-PDCD4-3'-UTR-WT and miR-93-5p mimics in both Huh-7 and SMMC-7721 cells (Fig.5B, C). However, there was no significant change of the luciferase activity after co-transfected with luciferase reporter plasmid PsiCHECK2-PDCD4-3'-UTR-MUT and miR-93-5p mimics in both Huh-7 and SMMC-7721 cells (Fig.5B, C). Moreover, protein levels of PDCD4 dramatically decreased after transfected with miR-93-5p mimics compared with miR-Control mimics in both Huh-7 and SMMC-7721 cells as determined by western blot (Fig.5D). Forced expression of LINC00472 significantly increased the protein level of PDCD4 in both Huh-7 and SMMC-7721 cells (Fig.5E). In addition, knockdown of LINC00472 by shLINC00472 significantly decreased the protein level of PDCD4 in normal liver cell line LO2 (Fig.5F). Therefore, PDCD4 was a direct target of miR-93-5p, and the LINC00472/miR-93-5p pathway regulated the expression of PDCD4.

Moreover, the correlations of LINC00472/miR-93-5p, LINC00472/PDCD4 mRNA, miR-93-5p/PDCD4 mRNA were analyzed in the 109 human HCC tissues. As shown in Fig.5G, LINC00472 and miR-93-5p expression levels were negatively correlated in HCC tissues ($P = 0.0078$, Pearson's correlation coefficient: −0.2559). In addition, there was a significant positive correlation between LINC00472 and PDCD4 expression levels in HCC tissues ($P = 0.0026$, Pearson's correlation coefficient: 0.3632) (Fig.5H). There was a statistical negative correlation between miR-93-5p and PDCD4 expression levels in HCC tissues ($P = 0.0357$, Pearson's correlation coefficient: −0.2033) (Fig.5I).

LINC00472 suppressed proliferation, migration and invasion of human HCC cells through PDCD4

To examine whether LINC00472 suppressing proliferation, migration and invasion of human HCC cells was mediated by PDCD4, Huh-7 and SMMC-7721 cells were co-transfected with pcDNA-LINC00472 or control pcDNA plasmid and PDCD4-siRNA (designated as si-PDCD4) or NC-siRNA (designated

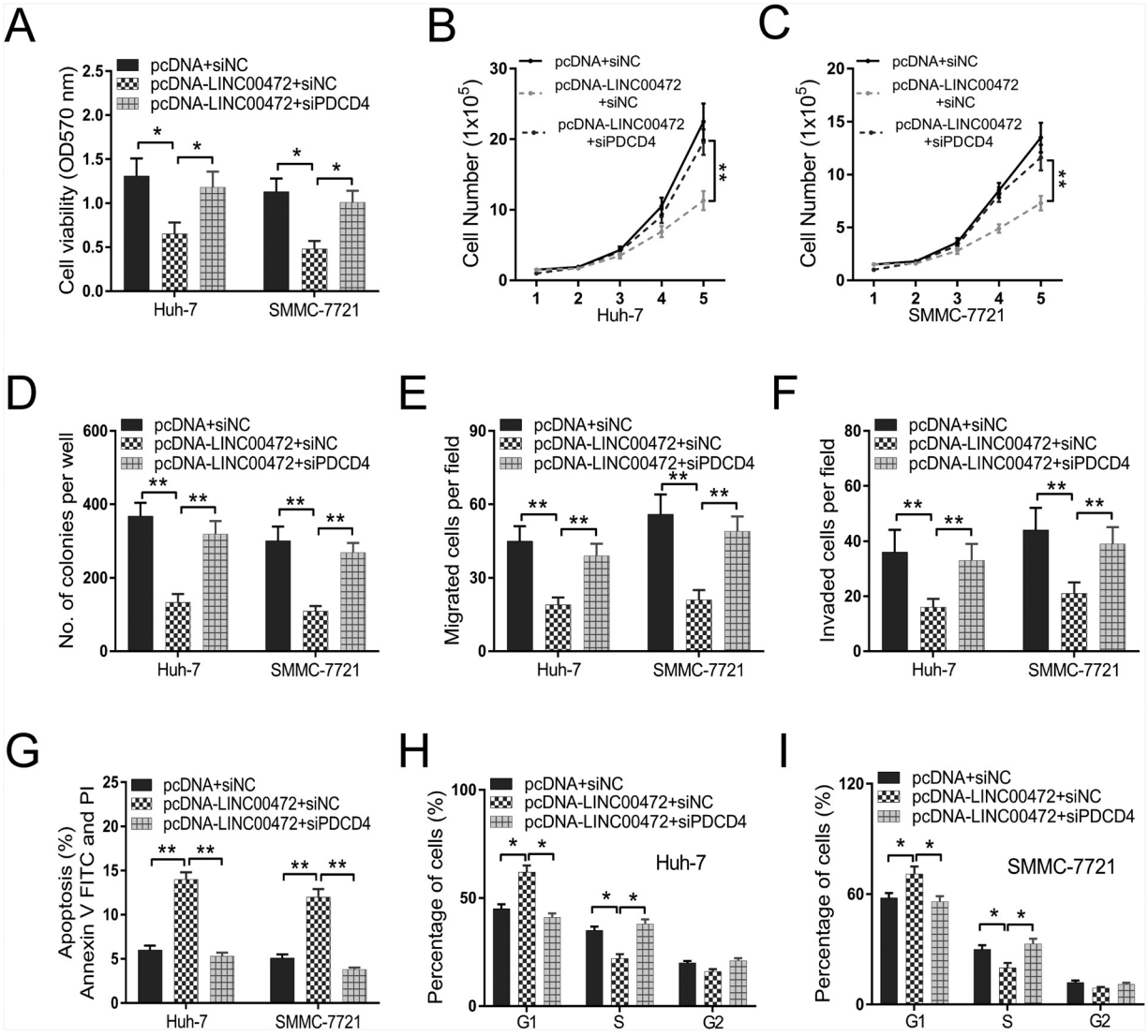

Fig. 6 LINC00472 suppressed proliferation, migration and invasion of human HCC cells through PDCD4. Huh-7 and SMMC-7721 cells were co-transfected with pcDNA-LINC00472 or control pcDNA plasmid and si-PDCD4 or si-NC. A. MTT assay. B, C. Cell counting assay. D. Cell colony formation assay. E. Cell migration assay. F. Cell invasion assay. G. Flow cytometry to evaluate cell apoptosis. H, I. Flow cytometry to evaluate cells in G1, S, and G2 phases were carried out to examine cell proliferation, migration and invasion. $^{*}P < 0.05$; $^{**}P < 0.01$.

as si-NC) and cell functional experiments were carried out. Concordant with former results, forced expression of LINC00472 dramatically decreased cell viability (evaluated by MTT assay) (Fig.6A), cell total number (Fig.6B, C), cell colony formation (Fig.6D), cell migration (Fig.6E) and cell invasion (Fig.6F). However, these decreases were abolished by transfection with siPDCD4 (Fig.6A—F). Moreover, forced expression of LINC00472 increased cell apoptosis, increased G1 phase cells and decreased S phase cells in Huh-7 and SMMC-7721 cells; these changes were all abrogated by co-transfection with siPDCD4 (Fig.6G—I). Therefore, LINC00472 suppressing proliferation, migration and invasion of human HCC cells was mediated by PDCD4.

Discussion

In the present study, we systematically examined that LncRNA LINC00472 suppressed cell proliferation (as determined by MTT assay, cell counting assay, cell colony formation assay and flow cytometry), migration and invasion (as determined by cell migration assay and invasion assay) of human HCC cells. LINC00472 was found to express lower in HCC tissues compared with adjacent non-tumor liver tissues, in HCC tissues from patients with metastasis compared with tissues from patients without metastasis, in HCC cell lines compared with normal liver cell lines. In addition, low level of LINC00472 was associated with low OS rate in HCC patients. As reported previously, forced expression of LINC00472 suppressed cell proliferation and migration in human breast cancer cells [20]. Breast cancer patients with high expression of LINC00472 was associated with better outcomes and had better responses to adjuvant chemo- or hormonal therapy [20]. Shen Y et al. also reported that LINC00472 is functionally a tumor suppressor in human breast cancer; expression level of LINC00472 was positively correlated with disease-free survival rates in patients with

grade 2 breast cancer [19]. Moreover, LINC00472 was also examined to be a tumor suppressor in human epithelial ovarian cancer and colorectal cancer [18,21]. These results were concordant with ours. Therefore, LINC00472 was an important tumor suppressor in various human cancers including HCC. As reported in Shen Y et al.'s study and Chen L et al.'s study, the promoter methylation induced the low-expression of LINC00472 in human breast cancer and colorectal cancer [19], therefore the mechanisms leading to LINC00472 downregulation in HCC might be also DNA methylation which should be examined in the future work.

For mechanism study, LINC00472 was determined to directly target miR-93-5p and could sponge miR-93-5p. Forced expression of LINC00472 dramatically decreased the level of miR-93-5p, and transfection with miR-93-5p mimics dramatically decreased the level of LINC00472 in HCC cells Huh-7 and SMMC-7721. Knockdown of LINC00472 by shLINC00472 significantly increased the miR-93-5p level in normal liver cell LO2. It was logical that miR-93-5p should perform tumor promoting role in human HCC cells. As reported in Wang X. et al.'s study, miR-93-5p was determined to promote cell proliferation through down-regulating PPARGC1A in HCC cells [26]. This data supported our present results. Meanwhile, Li L. et al. reported that miR-93-5p promoted gastric cancer-cell progression via inactivation of the Hippo signaling pathway [27]. Ma DH. et al. reported that miR-93-5p/IFNAR1 axis promoted gastric cancer metastasis through activating the STAT3 signaling pathway [28]. Moreover, miR-93-5p was also documented to play promoting role in human non-small cell lung cancer, esophageal cancer, lacrimal gland adenoid cystic cancer, etc. [29—31] Therefore, miR-93-5p played tumor promoting roles in many kinds of human cancers, and miR-93-5p might mediate the suppressing role of LINC00472 in HCC cells.

We next examined that PDCD4 was a direct target of miR-93-5p. Expression level of PDCD4 was dramatically suppressed by miR-93-5p, and was dramatically promoted by LINC00472. Combinational experiments showed that PDCD4 mediated the tumor suppressing role of LINC00472 in HCC cells. Therefore, PDCD4 was a critical tumor suppressor in HCC cells. As reported previously, PDCD4 was determined to be negatively regulated by miR-93 and miR-21, promoting cell apoptosis and suppressing tumor proliferation and metastasis in human HCC cells [32—35]. These results were concordant with ours. Furthermore, PDCD4 also played tumor suppressing role in human colon cancer [36], bladder cancer [37], epithelial ovarian cancer [38], pancreatic cancer [39], breast cancer [40], gastric cancer [41], and so on. As a result, PDCD4 was a well received tumor suppressor in nearly all kinds of human cancers and herein mediated the tumor suppressing role of LINC00472 in HCC cells. The LINC00472/miR-93-5p/PDCD4 pathway contributed to cell proliferation, apoptosis, migration and invasion of human HCC.

In summary, the present study systematically examined the tumor suppressing role of LINC00472 in human HCC cells. The expression level of LINC00472 was low in HCC tissues/cell lines compared with adjacent non-tumor liver tissues/normal liver cell lines, was negatively correlated with metastasis and positively correlated with patient OS rates. LINC00472 suppressed proliferation, migration and invasion of HCC cells. MiR-93-5p was a direct target of LINC00472, and miR-93-5p directly targeted PDCD4. The miR-93-5p/PDCD4 pathway mediated the suppressing role of LINC00472 in HCC cells. As an important tumor suppressor, LINC00472 could be used as a bio-marker for HCC therapy.

Authors' contributions

CC and QZ maintained all of the cell cultures, designed and performed shRNA experiment, and ran qRT-PCR and Western blots. QZ helped with data collection and drafted statistical methods. CC and QZ wrote the manuscript. CY conceived of the ideas of the manuscript and provided funding for the experiments performed in the manuscript. All authors read and approved the manuscript for publication.

Disclosure of interest

The authors declare that they have no competing interest.

Acknowledgments

This work was supported by Anhui Provincial Education Department Natural Science Foundation KJ2018ZD017.

References

[1] Zhao L, Zhang Y. Long non-coding RNA CASC2 regulates hepatocellular carcinoma cell oncogenesis through miR-362-5p/Nf-kappaB axis. J Cell Physiol 2018 [Epub 2018/01/11].
[2] Ding W, Tan H, Li X, Zhang Y, Fang F, Tian Y, et al. MicroRNA-493 suppresses cell proliferation and invasion by targeting ZFX in human hepatocellular carcinoma. Cancer biomarkers : section A of Disease markers. 2018. Epub 2018/05/16.
[3] Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global Cancer Statistics, 2012. Ca-Cancer J Clin 2015;65(2):87—108.
[4] Lau WY, Lai ECH. Hepatocellular carcinoma: current management and recent advances. Hepatob Pancreat Dis 2008;7(3):237—57.
[5] El-Serag HB, Rudolph L. Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. Gastroenterology 2007;132(7):2557—76.
[6] Burkhart RA, Ronnekleiv-Kelly SM, Pawlik TM. Personalized therapy in hepatocellular carcinoma: molecular markers of prognosis and therapeutic response. Surg Oncol 2017;26(2):138—45.
[7] Luo X, Liu Y, Ma S, Liu L, Xie R, Li M, et al. STIP1 is over-expressed in hepatocellular carcinoma and promotes the growth and migration of cancer cells. Gene 2018;662:110—7 [Epub 2018/03/30].
[8] Li Q, Zhang C, Chen R, Xiong H, Qiu F, Liu S, et al. Disrupting MALAT1/miR-200c sponge decreases invasion and migration in endometrioid endometrial carcinoma. Cancer Lett 2016;383(1):28—40 [Epub 2016/10/30].
[9] Maher CA, Kumar-Sinha C, Cao XH, Kalyana-Sundaram S, Han B, Jing XJ, et al. Transcriptome sequencing to detect gene fusions in cancer. Nature 2009;458(7234):U97—9.
[10] Liu LF, Chen X, Zhang Y, Hu YR, Shen XQ, Zhu WP. Long non-coding RNA TUG1 promotes endometrial cancer development via inhibiting miR-299 and miR-34a-5p. Oncotarget 2017;8(19):31386—94.
[11] Cao YJ, Xu RF, Xu XL, Zhou YJ, Cui L, He XZ. Downregulation of lncRNA CASC2 by microRNA-21 increases the proliferation

and migration of renal cell carcinoma cells. Mol Med Rep 2016;14(1):1019—25.
[12] Zhu C, Ma J, Li Y, Zhang Y, Da M. Low expression of long non-coding RNA MT1JP is associated with poor overall survival in gastric cancer patients: Protocol for meta-analysis. Medicine 2018;97(21):e10394 [Epub 2018/05/26].
[13] Yin D, Lu X, Su J, He X, De W, Yang J, et al. Long non-coding RNA AFAP1-AS1 predicts a poor prognosis and regulates non-small cell lung cancer cell proliferation by epigenetically repressing p21 expression. Mol Cancer 2018;17(1):92.
[14] Li S, Zhou J, Wang Z, Wang P, Gao X, Wang Y. Long non-coding RNA GAS5 suppresses triple negative breast cancer progression through inhibition of proliferation and invasion by competitively binding miR-196a-5p. Biomed Pharmacother 2018;104:451—7 [Epub 2018/05/25].
[15] Kasinski AL, Slack FJ. Epigenetics and genetics. MicroRNAs en route to the clinic: progress in validating and targeting microRNAs for cancer therapy. Nat Rev Cancer 2011;11(12):849—64.
[16] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136(2):215—33.
[17] Kong QL, Qiu M. Long non-coding RNA SNHG15 promotes human breast cancer proliferation, migration and invasion by sponging miR-211-3p. Biochem Bioph Res Co 2018;495(2):1594—600.
[18] Fu YY, Biglia N, Wang ZW, Shen Y, Risch HA, Lu LG, et al. Long non-coding RNAs, ASAP1-IT1, FAM215A, and LINC00472, in epithelial ovarian cancer. Gynecol Oncol 2016;143(3):642—9.
[19] Shen Y, Wang ZW, Loo LWM, Ni Y, Jia W, Fei PW, et al. LINC00472 expression is regulated by promoter methylation and associated with disease-free survival in patients with grade 2 breast cancer. Breast Cancer Res Tr 2015;154(3):473—82.
[20] Shen Y, Katsaros D, Loo LWM, Hernandez BY, Chong C, Canuto EM, et al. Prognostic and predictive values of long non-coding RNA LINC00472 in breast cancer. Oncotarget 2015;6(11):8579—92.
[21] Chen L, Zhang W, Li DY, Wang X, Tao Y, Zhang Y, et al. Regulatory network analysis of LINC00472, a long non-coding RNA downregulated by DNA hypermethylation in colorectal cancer. Clin Genet 2018;93(6):1189—98.
[22] Zhang XL, Wang MT, Sun HH, Zhu T, Wang XT. Downregulation of LINC00894-002 Contributes to Tamoxifen Resistance by Enhancing the TGF-beta Signaling Pathway. Biochem Moscow 2018;83(5):603—11.
[23] Ding KH, Wu ZS, Wang NN, Wang XN, Wang YJ, Qiand PX, et al. MiR-26a performs converse roles in proliferation and metastasis of different gastric cancer cells via regulating of PTEN expression. Pathol Res Pract 2017;213(5):467—75.
[24] Ding KS, Tan S, Huang X, Wang XN, Li XC, Fan R, et al. GSE1 predicts poor survival outcome in gastric cancer patients by SLC7A5 enhancement of tumor growth and metastasis. J Biol Chem 2018;293(11):3949—64.
[25] Wu Q, Yan H, Tao SQ, Wang XN, Mou L, Chen P, et al. XIAP 3' -untranslated region as a ceRNA promotes FSCN1 function in inducing the progression of breast cancer by binding endogenous miR-29a-5p. Oncotarget 2017;8(10):16784—800.
[26] Wang XR, Liao ZJ, Bai ZM, He Y, Duan J, Wei LY. MiR-93-5p Promotes Cell Proliferation through Down-Regulating PPARGC1A in hepatocellular carcinoma cells by bioinformatics analysis and experimental verification. Genes Basel 2018;9(1).
[27] Li L, Zhao JG, Huang SS, Wang Y, Zhu LL, Cao Y, et al. MiR-93-5p promotes gastric cancer-cell progression via inactivation of the Hippo signaling pathway. Gene 2018;641:240—7.
[28] Ma DH, Li BS, Liu JJ, Xiao YF, Yong X, Wang SM, et al. miR-93-5p/IFNAR1 axis promotes gastric cancer metastasis through activating the STAT3 signaling pathway. Cancer Lett 2017;408:23—32.
[29] Yang W, Bai JQ, Liu DY, Wang SW, Zhao N, Che RC, et al. MiR-93-5p up-regulation is involved in non-small cell lung cancer cells proliferation and migration and poor prognosis. Gene 2018;647:13—20.
[30] Liu MX, Liao J, Xie M, Gao ZK, Wang XH, Zhang Y, et al. miR-93-5p transferred by exosomes promotes the proliferation of esophageal cancer cells via intercellular communication by targeting PTEN. Biomed Environ Sci 2018;31(3):171—85.
[31] Hao J, Jin X, Shi Y, Zhang H. miR-93-5p enhance lacrimal gland adenoid cystic carcinoma cell tumorigenesis by targeting BRMS1L. Cancer Cell Int 2018;18.
[32] Ji CM, Liu H, Yin Q, Li H, Gao H. miR-93 enhances hepatocellular carcinoma invasion and metastasis by EMT via targeting PDCD4. Biotechnol Lett 2017;39(11):1621—9.
[33] Huang H, Wang X, Wang C, Zhuo L, Luo S, Han S. The miR-93 promotes proliferation by directly targeting PDCD4 in hepatocellular carcinoma. Neoplasma 2017;64(5):770—7.
[34] Zhu Q, Wang ZM, Hu Y, Li JD, Li XY, Zhou LD, et al. miR-21 promotes migration and invasion by the miR-21-PDCD4-AP-1 feedback loop in human hepatocellular carcinoma. Oncol Rep 2012;27(5):1660—8.
[35] Qiu X, Dong S, Qiao F, Lu S, Song Y, Lao Y, et al. HBx-mediated miR-21 upregulation represses tumor-suppressor function of PDCD4 in hepatocellular carcinoma. Oncogene 2013;32(27):3296—305.
[36] Hao Y, Huang J, Ma Y, Chen W, Fan Q, Sun X, et al. Asiatic acid inhibits proliferation, migration and induces apoptosis by regulating Pdcd4 via the PI3K/Akt/mTOR/p70S6K signaling pathway in human colon carcinoma cells. Oncol Lett 2018;15(6):8223—30.
[37] Liu JL, Zhai RR, Zhao JJ, Kong F, Wang J, Jiang W, et al. Programmed cell death 4 overexpression enhances sensitivity to cisplatin via the JNK/c-Jun signaling pathway in bladder cancer. Int J Oncol 2018;52(5):1633—42.
[38] Mahmoud EH, Fawzy A, RA AE. Serum microRNA-21 negatively relates to expression of programmed cell death-4 in patients with epithelial ovarian cancer. Asian Pac J Cancer Prev 2018;19(1):33—8 [Epub 2018/01/28].
[39] Yu G, Jia BL, Cheng YS, Zhou LB, Qian B, Liu ZN, et al. MicroRNA-429 sensitizes pancreatic cancer cells to gemcitabine through regulation of PDCD4. Am J Transl Res 2017;9(11):5048.
[40] Cheng Y, Xiang GX, Meng YB, Dong RZ. MiRNA-183-5p promotes cell proliferation and inhibits apoptosis in human breast cancer by targeting the PDCD4. Reprod Biol 2016;16(3):225—33.
[41] Hu XT, Wang YB, Liang HW, Fan Q, Zhu RC, Cui JY, et al. miR-23a/b promote tumor growth and suppress apoptosis by targeting PDCD4 in gastric cancer. Cell Death Dis 2017;8.