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*Original Article*

# Sanguinarine promotes apoptosis of hepatocellular carcinoma cells via regulating the miR-497-5p/CDK4 axis

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**Abstract:** Objective: To determine the effect of sanguinarine on the biological behavior of hepatocellular carcinoma (HCC) cells via regulating the miR-497-5p/cyclin-dependent kinase 4 (CDK4) axis. Methods: Swiss Target Prediction was used for target prediction of sanguinarine. The targets were analyzed with KEGG enrichment analysis, and CDK4 was included in this study. Target prediction website, Diana tools enrichment analysis, and dual-luciferase reporter assay were adopted to identify the target miRNAs for CDK4. We measured expression levels of CDK4 and miR-497-5p in cancerous tissues, normal liver L02 cells, HepG2 HCC cells and sanguinarine-treated HepG2 cells. The expression of CDK4/miR-497-5p in HCC cells was intervened by treating HCC cells with sanguinarine. Cell proliferation, invasion and apoptosis were measured with CCK8, Transwell and flow cytometry, respectively. Results: CDK4 was shown to be a target for sanguinarine. Compared with L02 cells, CDK4 expression in HCC cells was significantly increased, but sanguinarine inhibited the CDK4 expression in HCC cells. The proliferation and invasion of HCC cells were inhibited, and the apoptosis was promoted by sanguinarine, but these effects were reversed by CDK4 overexpression (both $P<0.05$). miR-497-5p was confirmed to be a target miRNA for CDK4, and its expression was decreased in HCC cells but could be promoted by sanguinarine. The effect of miR-497-5p knockdown on HCC cells was partially reversed by si-CDK4. Conclusion: Sanguinarine inhibits the proliferation and invasion of HCC cells, and induces the apoptosis of HCC cells by regulating the expression of miR-497-5p/CDK4.

**Keywords:** Sanguinarine, hepatocellular carcinoma cells, miR-497-5p, CDK4

## Introduction

Hepatocellular carcinoma (HCC) is a malignant liver tumor that occurs in the liver [1]. It is more difficult to treat patients with liver cancer in middle and advanced stages. Thus, finding new targets and treatments is essential [2]. Traditional Chinese medicine (TCM) therapy combines TCM theories and applies them to the clinical treatment of a variety of diseases. In recent years, the efficacy of TCM used as adjuvant treatment for cancer has been verified [3]. Besides, the inhibitory effects of Chinese herbal medicine on cancer were confirmed by cell experiments [4]. Sanguinarine, whose molecular formula is C20H14NO4, is an isolation of a nitrogen-containing alkaloid from the Plume poppy. It has been reported that sanguinarine inhibits ulcerative colitis by inhibiting NLRP3, activating and modulating the intestinal flora of mice [5]. Sanguinarine has been confirmed to affect cervical cancer by influencing the ferroptosis of cancer cells [6]. Moreover, sanguinarine regulates the effect of tumor-associated macrophages on angiogenesis in lung cancer [7]. Studies on HCC have shown that sanguinarine influences HCC by modulating ROS-dependent mitosis and apoptosis [8]. However, there are still few studies that have explored the mechanism of it in HCC from a target perspective.

Cyclin-dependent kinase 4 (CDK4) is a tremendous regulator involved in cell senescence, apoptosis and histone regulation [9]. CDK4

inhibits cells from the G1-S transition phase and thus affects the progression of cancer [10]. CDK4 inhibitors are also increasingly being used in cancer treatment [11]. Studies on colon cancer have found that miR-486-5p participates in the regulation of 5-FU resistance in colorectal cancer cells as a competitor of PVT1 and CDK4 to bind miRNAs [12]. It has been confirmed that CDK4 affects the proliferation and apoptosis of HCC [13]. In this study, an association between sanguinarine and CDK4 was found from predicted potential targets of sanguinarine in bioinformatics databases. Therefore, the possible mechanism of sanguinarine to influence HCC by regulating CDK4 was further explored. miR-497-5p is one of many miRNAs that have previously been shown to be involved in the progression of non-small cell lung, prostate and thyroid cancers [14-16]. In addition, it was demonstrated that miR-497-5p played a role in cancer suppression [17].

From the perspective of influencing the progression of HCC by regulating molecular targets, the mechanism of sanguinarine in HCC was explored in this study. We hope to further clarify the pathogenesis of HCC and the role of sanguinarine.

## Materials and methods

### *Bioinformatics analysis*

The information of sanguinarine was searched in PubChem (https://pubchem.ncbi.nlm.nih.gov) and then imported in Swiss Target Prediction database (http://www.swisstargetprediction.ch/) to obtain potential targets. DAVID database (https://david.ncifcrf.gov/tools.jsp) was adopted for Kyoto genomics and genomics encyclopedia (KEGG) pathway enrichment analysis. The Venn Database (http://bioinformatics.psb.ugent.be/webtools/Venn/) was used for the analysis of overlapped genes in cancer pathways and HCC pathways, as well as for overlapped target miRNAs in multi-relational databases. The gene expression of HCC was predicted in GEPIA database (http://gepia.cancer-pku.cn/detail.php). The Targetscan database (https://www.targetscan.org/vert_72/), Starbase database (https://starbase.sysu.edu.cn) and miRWalk database (http://mirwalk.umm.uni-heidelberg.de) were used to predict target miRNAs for CDK4. In addition, miRNA functional enrichment analysis was performed with Diana tools (https://dianalab.e-ce.uth.gr/html/mirpathv3/index.php?r=mirpath).

### *Collection of clinical samples*

The cancerous tissues and para-carcinoma tissues were collected from 56 HCC patients in Hwa Mei Hospital, Zhejiang Province, China from August 2020 to June 2022. Inclusion criteria: (1) Patients were diagnosed with HCC by clinical and pathological examinations. (2) Patients were not given any preoperative treatment, including chemoradiation, drug therapy and immunotherapy, etc. (3) Patients did not have other tumors. Exclusion criteria: (1) Patients had endocrine and immune system diseases. (2) Patients had liver, kidney or heart dysfunction. (3) Patients had a history of other malignancies or liver metastases. After excision, all specimens were quickly stored at -80°C for later use. This study was approved by the Ethics Committee of Hwa Mei Hospital. All patients signed informed consent.

### *Cell culture and transfection*

Human normal liver L02 cells and HepG2 HCC cells were purchased from Fenghui Biology (Hunan, China). Sanguinarine (HS026156198) was purchased from Baoji Kerui Biotechnology Co., Ltd. (Shaanxi, China). First, the cells were cultured in DMEM medium (Sigma, USA) at 5% $CO_2$ and 37°C. The transfected plasmid was designed and synthesized by Shanghai Jima Co., Ltd. (Shanghai, China). Then, the HCC cells were divided into 8 groups, control group (HCC cells + 0.1% DMSO), sanguinarine group (HCC cells treated with Sanguinarine), sanguinarine + pcDNA-NC group (HCC cells transfected with negative control vector for oe-CDK4 and treated with sanguinarine), sanguinarine + oe-CDK4 group (HCC cells transfected with CDK4-overexpressing vectors and treated with sanguinarine), sanguinarine + inhibitor NC group (HCC cells transfected with inhibitor NC and treated with sanguinarine), sanguinarine + miR-497-5p inhibitor group (HCC cells transfected with miR-497-5p inhibitor and treated with sanguinarine), sanguinarine + miR-497-5p inhibitor + siRNA-NC group (HCC cells transfected with miR-497-5p inhibitor and siRNA-NC and treated with sanguinarine), sanguinarine + miR-497-5p inhibitor + siRNA-CDK4 group (HCC cells transfected with miR-497-5p inhibitor and siRNA-CDK4 and treated with sanguinarine). The transfection sequence is shown in **Table 1**.

**Table 1.** Transfection sequences

| | Transfection sequence |
|---|---|
| pcDNA-NC | CGCTAGCGGACTAGCTAGCAGTCGGTTGAT |
| oe-CDK4 | TGCGTAGCGGCGATGGTTGCAACGTGTACT |
| inhibitor NC | TAGAAGCTGATTCGGAGCTCCGCTTGTA |
| miR-497-5p inhibitor | TGCAGGTTACGCTAACATCGAACACTG |
| siRNA-NC | GACATACATTCACTGCTGACGCCTAGATA |
| siRNA-CDK4 | CCGATCTGGGTGCGATTGCTGTAACGTCC |

**Table 2.** qRT-PCR primer sequences

| Gene | Sequences (5'-3') | |
|---|---|---|
| | Forward | Reverse |
| miR-497-5p | GCCGATGGCAAGGCGTTTGCTCT | TTTACGTAAGCTCAAGAGCAGC |
| miR-326 | CATGCACGCGGTGAGTATCAGCG | TCAACTCTGAACAGGCGTGTAC |
| miR-744-5p | CGGAAGTCCGAGTTCGACAA | TCACTTCCACAGCTCGAGCA |
| miR-330-5p | TCTGGAGTGACTGAGGATGC | GTGTGTAGGTGCTGGAAGTCC |
| CDK4 | ACCTCATGTCAGAATGACTTG | CATCGAGCCTTCACGAATCC |
| U6 | CCTCTAGAGAGAGTCCGAAAT | TAGATCACTCGAATTCTAGG |
| GAPDH | TCGTGTAGCTTGGATGGAGCGC | CTCGATAGTCCGCTCTGACGA |

HepG2 cells were treated with different concentrations of sanguinarine (0.5 μM, 1 μM, 2 μM). The HepG2 cells not treated with sanguinarine were seen as controls. The proliferation of HepG2 cells were measured according to the following CCK8 assay. The concentration with the best inhibitory effect on cell viability was selected for subsequent experiments. Equal concentrations of 0.1% DMSO solution were set as control. In each group, $1\times10^4$ cells were transfected with Lipofectamine 3000 (L3000015, Invitrogen, USA). The transfection procedures were in accordance with the kit instruction.

*qRT-PCR*

Total RNA was extracted using Trizol reagent (15596-026, Invitrogen, USA). Reverse transcription kit (18080-044, Invitrogen, USA) was used to synthesize cDNA. qRT-PCR and quantification of mRNA were performed with SYBR Premix Ex Taq II kit (RR820A, Takara, Hanpan) and Real-Time PCR system (7500, ABI, USA). There was a total of 20 μL PCR reaction system, including SYBR Premix Ex Taq II (10 μL), forward primer (0.8 μL), reverse primer (0.8 μL), DNA template (2 μL) and $dH_2O$ (6.4 μL). PCR was programmed as pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 60°C for 34 s and elongation at 72°C for 1 min for 40 cycles, with a final 7-min extension at 72°C. U6 and GAPDH were used as internal references for miR-497-5p and CDK4, respectively. The relative expression of mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences are shown in **Table 2**.

*Western blot*

RIPA lysis buffer (P0013B, Beyotime Biotechnology, Shanghai, China) was applied for cell lysis. Protein concentration was measured with BCA kit (P0010, Beyotime Biotechnology, Shanghai, China). Protein was separated by SDS-PAGE, transferred to a PVDF membrane and then washed with TBST. The membrane was blocked with 5% skim milk for 1 h. After that, the PVDF membrane was washed, followed by incubation with primary antibodies CDK4 (1:1000, ab68266, ABCAM, UK) and GAPDH (1:20000, ab128915, ABCAM, UK) overnight at 4°C. Then, the PVDF membrane was washed with TBST, and incubated with secondary antibody goat anti-rabbit IgG (1:1000, ab133470, ABCAM, UK) at room temperature for 1 h. Enhanced chemiluminescence was added for development, and then the bands were observed under a light microscope. GAPDH was used as a control, and the bands were visualized using Bio-Rad. Quantifications of protein expressions were performed with ImageJ.

*Dual-luciferase reporter assay*

The wild-type (WT) or mutant (MUT) fragments of CDK4 synthesized by Shanghai Jima Co., Ltd. (Shanghai, China) were cloned into the pmiR-GLO luciferase vector. Lipofectamine 3000 kit (L3000001, Invitrogen, USA) was used to transfect WT/MUT and control/miR-497-5p mimic for 48 h. The cells were then collected, and the luciferase activity was determined by dual luciferase kit (RG009, Beyotime Biotechnology, Shanghai, China).

*CCK8*

CCK-8 solution (10 μL, C0038, Beyotime Biotechnology, Shanghai, China) was added to the

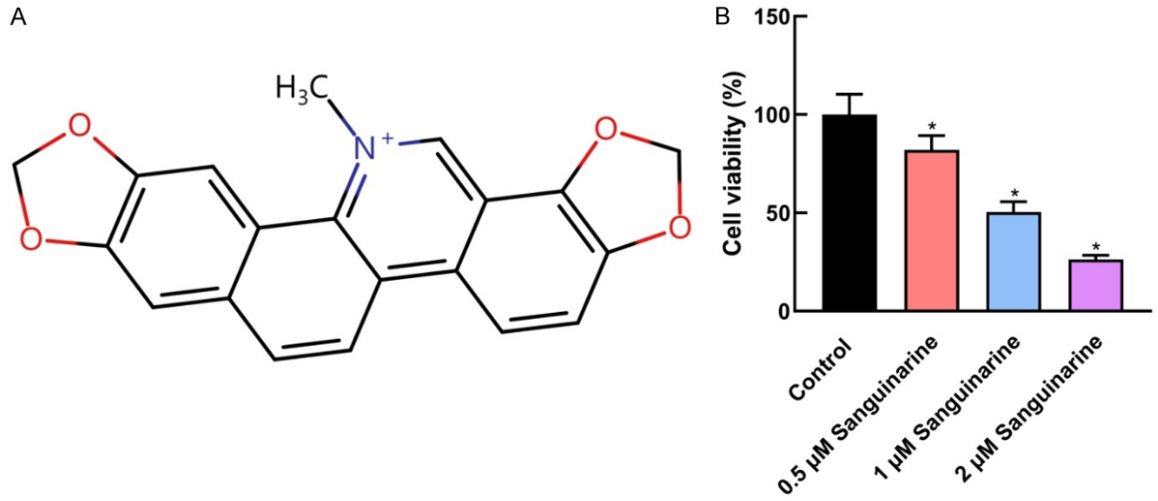


**Figure 1.** The molecular formula of sanguinarine and its effect on the proliferation of HCC cells. A: The molecular formula of sanguinarine (C20H14NO4); B: The proliferation of HCC cells after 48 h of sanguinarine intervention. Compared with the control group, *P<0.05. HCC: hepatocellular carcinoma.

HCC cells that were transfected with plasmid and treated with sanguinarine for 48 h. Then these cells were incubated for 1 h at 5% $CO_2$ and 37°C. The absorbance at a wavelength of 450 nm was measured using a microplate reader (Synergy HT, Biotech, USA).

*Transwell*

In the Transwell upper chamber (3422, Corning, USA), the mixture of serum-free medium (17502-048, Gibco, USA) with Matrix gel (356234, Corning, USA) as well as $1\times10^5$ HCC cells were placed for incubation. In the lower chamber, medium was mixed with 10% fetal bovine serum. After being incubated for 24 h, the cells in the upper chamber were removed. Crystal violet staining was used on membrane-penetrating cells in the lower chamber. The cell invasion was observed with a microscope (DM2000, Leica, China), after the cells were fixed with neutral gum.

*Flow cytometry*

A total of $5\times10^4$ cells in logarithmic growth phase were added to EP tubes, centrifuged at 3500 rpm, re-suspended with PBS and blocked with serum. Annexin V-FITC (5 μL) and PI staining solution (10 μL, C1062S, Beyotime Biotechnology, Shanghai, China) were added and mixed. Then, the cells were washed with PBS and incubated in the dark for 20 min. The cell apoptosis rate was determined using a flow cytometer (FACSCanto II, BD Bioscience, USA).

*Statistical analyses*

All data were statistically analyzed using SPSS 23.0. Measurement data were expressed as mean ± standard deviation ($\bar{x}$ ± sd). Independent sample t-test was used to assess the differences between two groups, and the differences among groups were compared by one-way analysis of variance followed by LSD test. Spearman correlation analysis was adopted for the comparisons of miR-497-5p and CDK4 levels. Statistical significance level was set at P<0.05.

## Results

*Sanguinarine inhibited the proliferation of HCC cells in a dose-dependent manner*

The molecular formula of sanguinarine was shown in Pubchem (**Figure 1**). HepG2 cells were treated with different concentrations of sanguinarine (0.5 μM, 1 μM, and 2 μM) for 48 h. The higher concentration of the sanguinarine led to the lower viability of HepG2 cells (**Figure 1B**). In the subsequent experiments, HepG2 cells were treated with 2 μM of sanguinarine, because this concentration had the best inhibitory effect on HCC cells.

*Analysis of targets of sanguinarine*

The "SMILES" of sanguinarine retrieved from Pubchem was (C(N+)1=C2C(=C3C=CC4=C(C3=C1)OCO4)C=CC5=CC6=C(C=C52)OCO6).

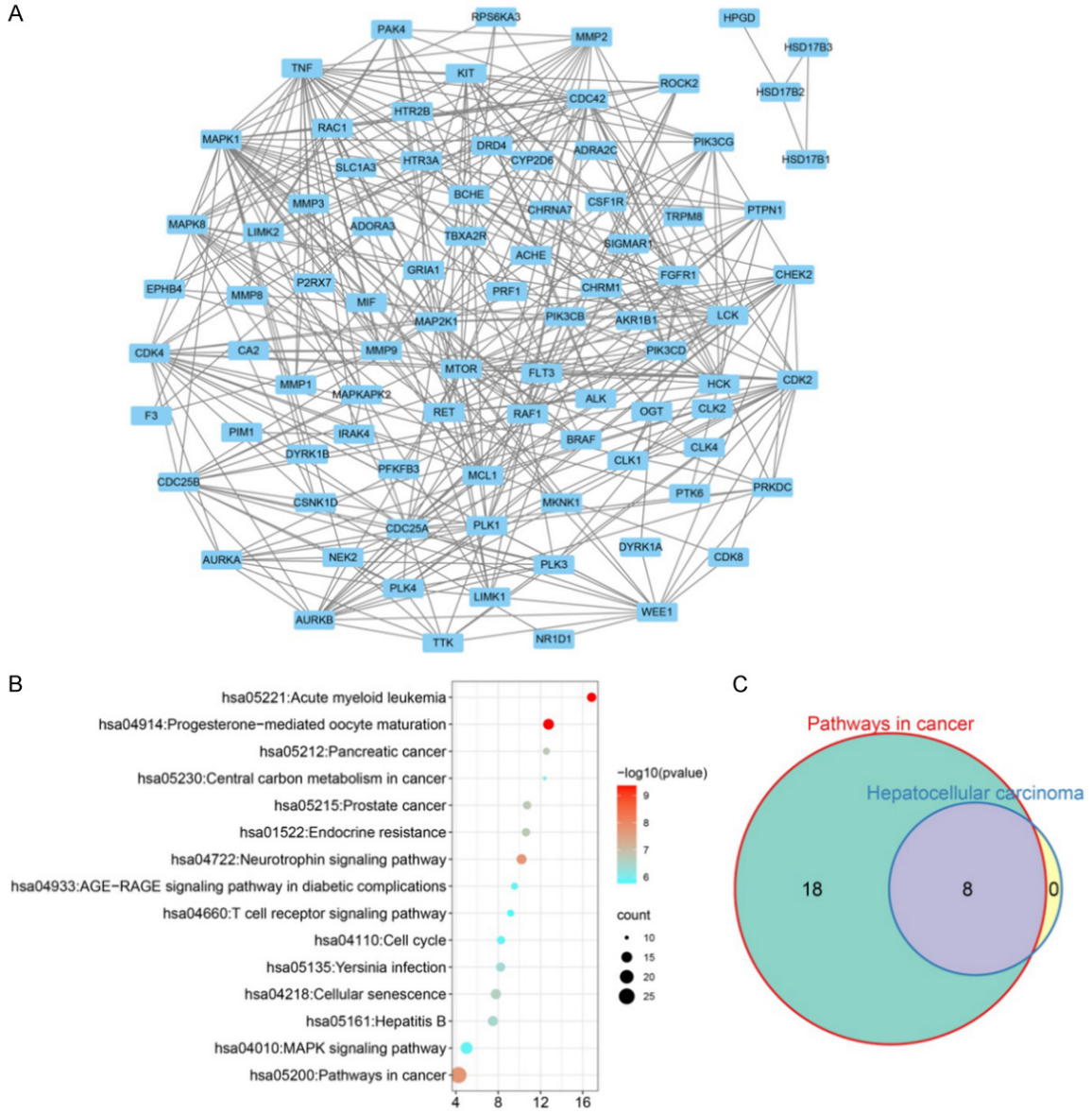


**Figure 2.** Analysis of targets of sanguinarine. A: Targets of sanguinarine; B: KEGG enrichment analysis of targets of sanguinarine; C: Overlapped genes in cancer pathways and HCC cell pathways. HCC: hepatocellular carcinoma; KEGG: Kyoto genomics and genomics encyclopedia.

The "SMILES" was input into Swiss Target Prediction to predict potential target genes which sanguinarine may bind to, and 100 target genes were found (**Figure 2A**). These 100 genes were put into the DAVID database for KEGG analysis. This indicated that the targets of the sanguinarine were enriched in multiple pathways such as the cancer pathway, cell cycle and HCC pathway. Only the first 15 pathways are listed in **Figure 2B**. It was suggested that sanguinarine participated in the regulation of multiple biological behaviors. In a follow-up experiment, overlapped genes were collected from enriched cancer and HCC cell pathways. It was found that the genes in the HCC cell pathway were all enriched in the cancer pathway at the same time, and the overlapped genes included MAP2K1, CDK4, MAPK1, PIK3CD, BRAF, PIK3CB, RAF1, and MTOR (**Figure 2C**).

The GEPIA database was used for expression prediction of the above 100 genes, which showed that CDK4 had the most significant differential expression and was highly expressed in HCC tissues (**Figure 3A**). Differential expression of CDK4 in HCC cells and its correlation

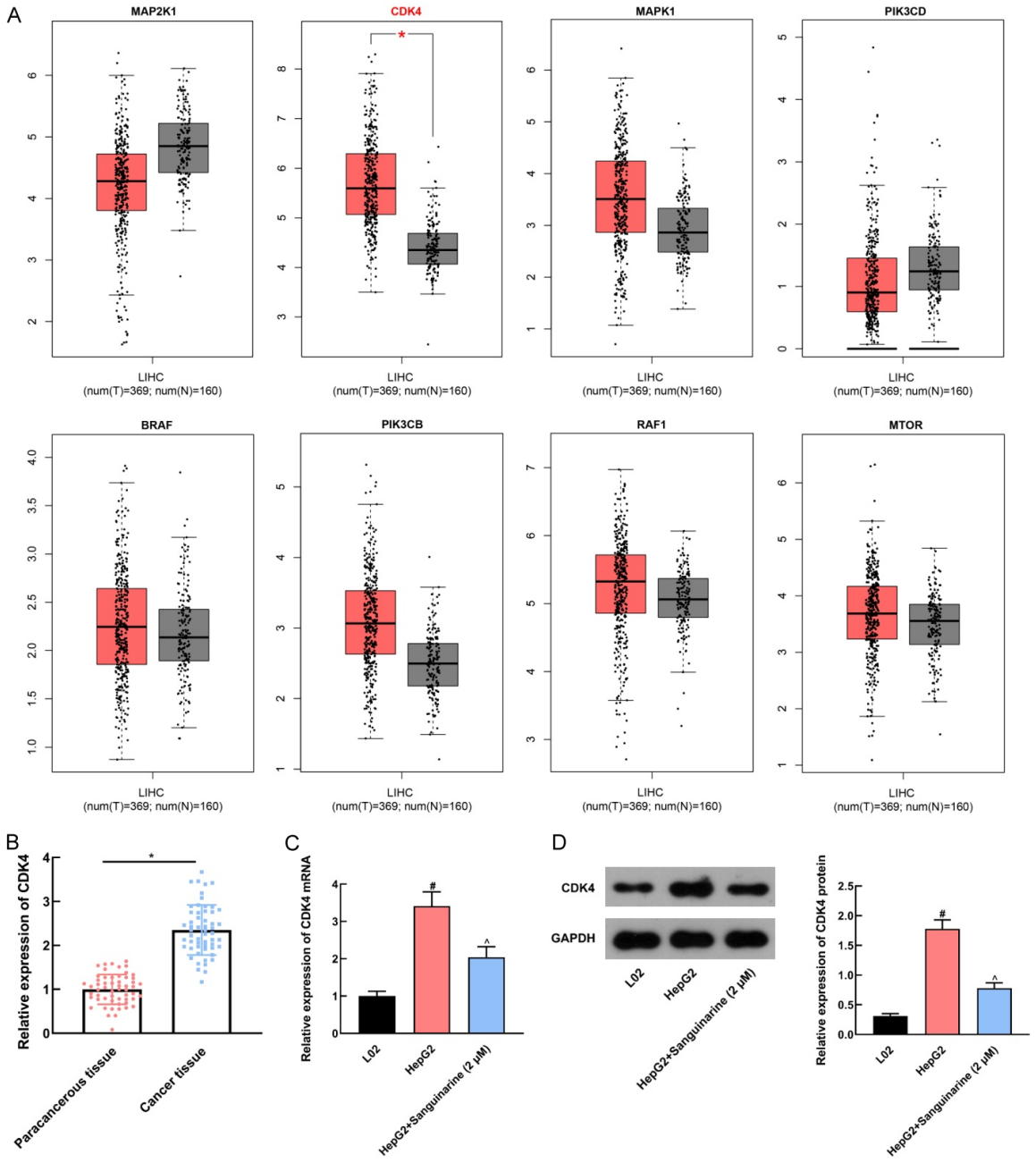


**Figure 3.** Verification of CDK4 expression in HCC cells and the effect of sanguinarine (2 μM) on its expression. A: Results from GEPIA database; B: Expression of CDK4 in tissue specimens of HCC patients (n=56); C, D: CDK4 expression was enhanced in HCC cells, and sanguinarine (2 μM) inhibited the expression of CDK4 in HCC cells. Compared with para-carcinoma, *P<0.05; compared with L02 cells, #P<0.05; compared with HepG2 cells, ^P<0.05. HCC: hepatocellular carcinoma; CDK4: cyclin-dependent kinase 4.

with prognosis have been partially revealed [18, 19]. The expression of CDK4 in the tissue samples included in this study was measured by qRT-PCR, and the expression of CDK4 in the HCC tissue was found to be increased compared to that in the para-carcinoma tissue (**Figure 3B**). The effect of sanguinarine (2 μM) on CDK4 expression was measured in cell experiments. It was found that the expression of CDK4 in HepG2 cells was increased compared with that in L02 cells, while the expression of CDK4 was inhibited after the addition of sanguinarine (2 μM) (all P<0.05; **Figure 3C** and **3D**).

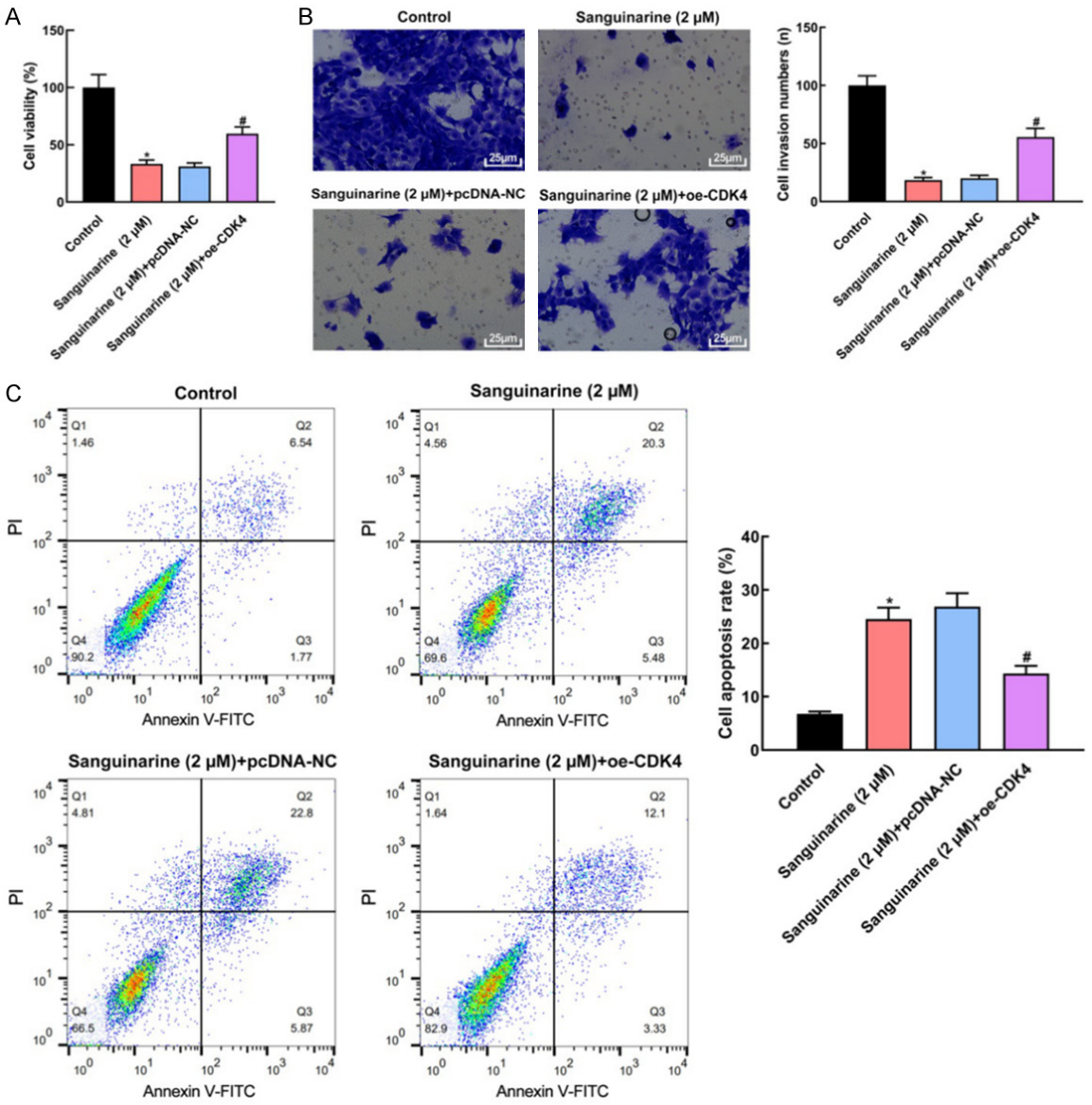


**Figure 4.** Sanguinarine regulated CDK4 to affect liver cancer progression. A: Cell viability; B: Cell invasion; C: Cell apoptosis. Compared with the control group, *P<0.05; compared with the sanguinarine (2 μM) + pcDNA-NC group, #P<0.05. CDK4: cyclin-dependent kinase 4.

*Sanguinarine regulated CDK4 expression to affect liver cancer progression*

HCC cells were treated with sanguinarine (2 μM) and the expression of CDK4 in cells was also intervened. In the cell experiments, the effects of sanguinarine (2 μM)/CDK4 on HCC cells were measured. It was revealed that cell proliferation and invasion were inhibited, and apoptosis was increased in the sanguinarine (2 μM) group (both P<0.05). Compared with the sanguinarine (2 μM) + pcDNA-NC group, the sanguinarine (2 μM) + oe-CDK4 group had increased cell proliferation and invasion, and reduced cell apoptosis (all P<0.05; **Figure 4A-C**). This demonstrated that CDK4 improved the proliferation, invasion and apoptosis of HCC cells processed with sanguinarine.

*Analysis of target miRNAs for CDK4*

Multiple databases were used to predict target miRNAs of CDK4. Overlapped genes were collected with Venn (**Figure 5A**). Enrichment analysis of overlapped miRNAs was performed with Diana tools. Several “hot genes” were enriched

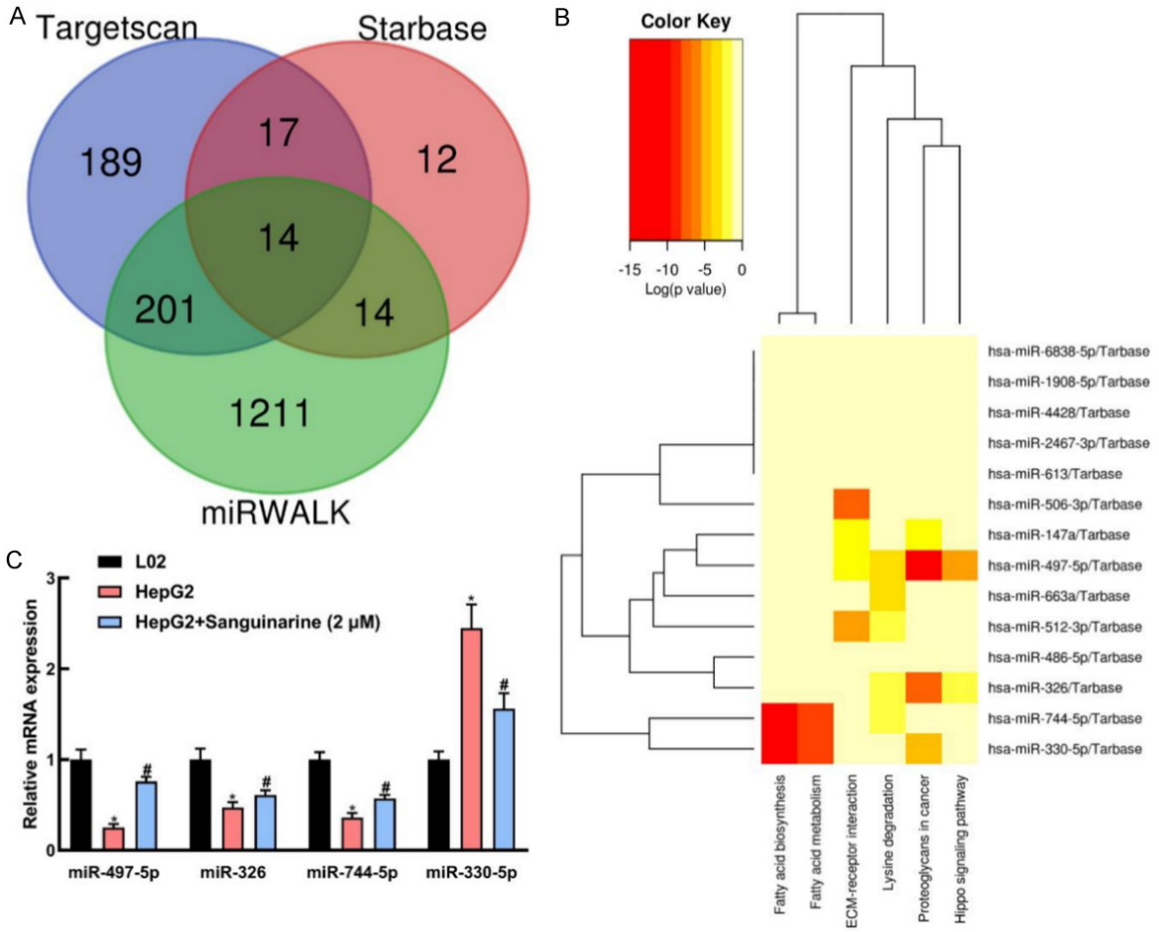


**Figure 5.** Analysis of target miRNAs for CDK4. A: The Targetscan, Starbaseand miRWalk databases were used to predict target miRNAs for CDK4; B: miRNA functional enrichment analysis was performed with Diana tools; C: The expression of target miRNAs for CDK4 in HCC cells and the effect of sanguinarine (2 μM) on its expression. CDK4: cyclin-dependent kinase 4. Compared with L02 cells, *P<0.05; compared with HepG2 cells, #P<0.05.

in many pathways, such as miR-326, miR-744-5p and miR-330-5p, suggesting that they may be involved in the regulation of multiple biological processes (**Figure 5B**). The expressions in L02 cells, HepG2 cells, and HepG2 + sanguinarine (2 μM) cells were measured. It was found that compared with that in L02 cells, miR-330-5p had enhanced expression in HepG2 cells, and sanguinarine (2 μM) could inhibit its expression in HepG2 cells. Since miRNAs generally negatively regulate the expression of target genes, this study focused on miR-497-5p, miR-326 and miR-744-5p. Compared with those in L02 cells, the expression of miR-497-5p, miR-326 and miR-744-5p in HepG2 cells was downregulated, and sanguinarine (2 μM) promoted their miRNA expression. The downregulation of miR-497-5p was the most significant in HepG2 cells and had the most pronounced effects by sanguinarine (2 μM) than others (**Figure 5C**). Meanwhile, miR-497-5p has been found to be involved in the progression of HCC [20, 21]. Therefore, miR-497-5p was included in this study to explore its association with sanguinarine.

*Validation of targeted binding between CDK4 and miR-497-5p*

The miR-497-5p expression in HCC tissue was significantly inhibited as compared with that in paracancerous tissue (t=11.14, P<0.0001, **Figure 6A**). In addition, we found that the expression levels of miR-497-5p and CDK4 in HCC tissue were inversely correlated (r=-0.4117, P=0.0016, **Figure 6B**). Cell experimental results revealed that the expression of miR-497-5p in HepG2 cells was reduced compared

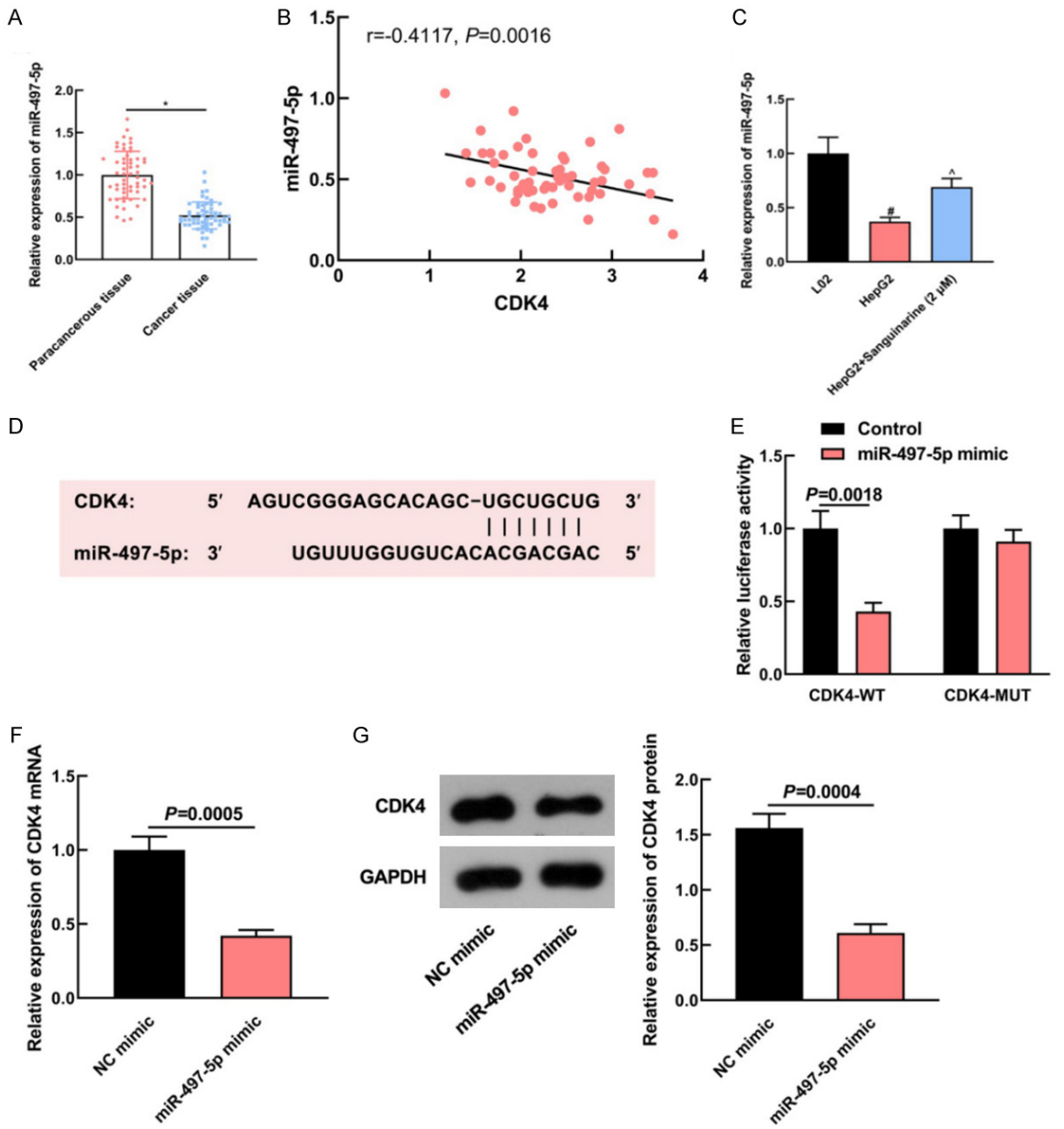


**Figure 6.** Validation of targeted binding between CDK4 and miR-497-5p. A: Expression level of miR-497-5p in HCC tissue (n=56); B: The correlation of expression levels of miR-497-5p and CDK4 in HCC tissue (n=56); C: Expression of miR-497-5p in HCC and effect of sanguinarine (2 μM) on its expression; D: Binding site of CDK4 and miR-497-5p; E: Results from dual-luciferase reporter assay; F, G: Effect of miR-497-5p on CDK4 expression. Compared with paracarcinoma, *P<0.05; compared with L02 cells, #P<0.05; compared with HepG2 cells, ^P<0.05. HCC: hepatocellular carcinoma; CDK4: cyclin-dependent kinase 4.

to that in L02 cells, but sanguinarine (2 μM) increased the expression of miR-497-5p in HCC cells (t=6.197, P=0.0034, **Figure 6C**). Predicted binding site of CDK4 and miR-497-5p was shown in **Figure 6D**. The targeted relationship between miR-497-5p and CDK4 was verified by dual luciferase analysis (t=7.359, P=0.0018, **Figure 6E**). Furthermore, the mRNA expression (t=10.20, P=0.0005) and protein expression (t=10.78, P=0.0004) of CDK4 in the miR-497-5p group were suppressed compared to those in the NC mimic group (**Figure 6F** and **6G**). The above experimental results revealed that miR-497-5p expression was deregulated in HCC cells, but the expression was upregulated by sanguinarine (2 μM).

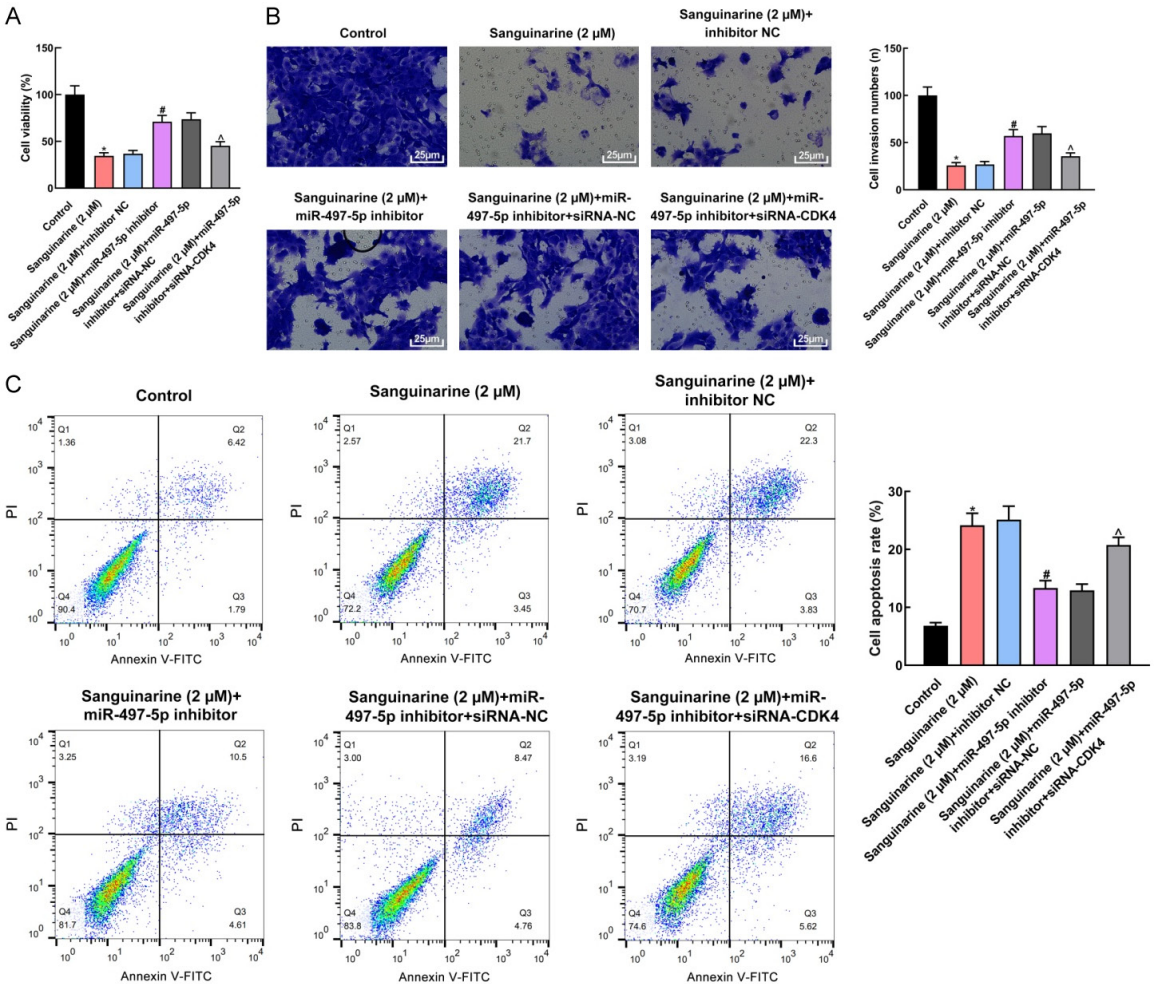


**Figure 7.** Sanguinarine influenced the biological behavior of HCC cells via the miR-497-5p/CDK4 axis. Note: (A) Cell viability; (B) Cell invasion; (C) Cell apoptosis. Compared with the control group, *P<0.05; compared with the sanguinarine (2 μM) + inhibitor NC group, #P<0.05; compared with the sanguinarine (2 μM) + miR-497-5p inhibitor + siRNA-NC group, ^P<0.05. HCC: hepatocellular carcinoma; CDK4: cyclin-dependent kinase 4.

*Sanguinarine influenced the proliferation and invasion of HCC cells via the miR-497-5p/CDK4 axis*

The levels of miR-497-5p and CDK4 in HCC cells were intervened upon. Cell experiments were performed to determine the regulatory effects via the miR-497-5p/CDK4 axis on proliferation and invasion of HCC cells. It was revealed that the proliferation and invasion of HCC cells were inhibited in the sanguinarine (2 μM) group, and the apoptosis was increased, as compared with those in the control group (all P<0.05). Compared with the sanguinarine (2 μM) + inhibitor-NC group, cell proliferation and invasion were increased, and the apoptosis was decreased in the sanguinarine (2 μM) + miR-497-5p inhibitor group (all P<0.05). As compared with those in the sanguinarine (2 μM) + miR-497-5p inhibitor + siRNA-NC group, the effect of sanguinarine (2 μM) and miR-497-5p inhibitor on HCC cells was partially reversed by siRNA-CDK4 (**Figure 7A-C**). This further suggested that sanguinarine influenced the progression of HCC cells by regulating miR-497-5p/CDK4.

## Discussion

Effects of sanguinarine on breast, thyroid papillary and lung cancers have been reported [22-24]. This study revealed the effect of sanguinarine on HCC. Previous studies on HCC cells have found that sanguinarine is involved in the progression of HCC cells by influencing mitochondrial apoptosis and cell death [25]. In this

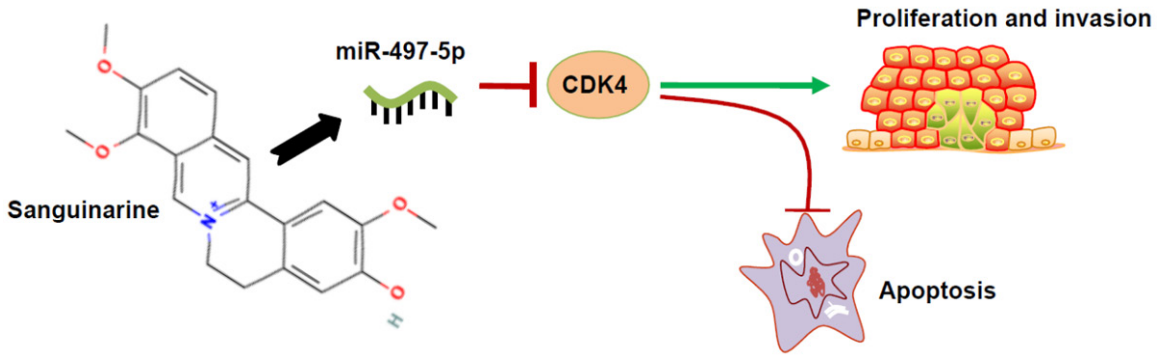


**Figure 8.** Mechanistic diagram of the influence of sanguinarine on the biological behavior of hepatocellular carcinoma cells through the miR-497-5p/CDK4 axis. CDK4: cyclin-dependent kinase 4.

study, the role of sanguinarine in regulating the proliferation, invasion and apoptosis of HCC cells was further revealed.

Drug targets refer to the binding sites of drugs and biological macromolecules of the body, including biological macromolecules such as nucleic acids, gene loci and ion channels [25]. In recent years, it has gradually become an important trend to explore the impact of different drugs on disease progression from a drug target perspective [26, 27]. In Yu's study, the effect of sanguinarine on reducing neuroinflammation-driven neuropathic pain might be achieved by influencing the activation of the p38 MAPK pathway [28]. Additionally, Zhong et al. concluded that sanguinarine upregulated CDKN1A and was effective in treating small cell lung cancer after screening the natural compounds [29]. In this study, Swiss Target Prediction was used for target prediction of sanguinarine, and CDK4 was then included in our study. Zhou et al. found that VPS9D1-AS1 intervenes in apoptosis of HCC cells by regulating the HuR/CDK4 axis [30]. Highly expressed CDK4 was also observed in HCC tissue and cells in this study. Functional experiments demonstrated that sanguinarine played an active role in inhibiting the malignant biological behaviors of HCC cells, but this effect was partially reversed by CDK4 overexpression, which further confirmed that the role of sanguinarine on the malignant progression of HCC cells may be achieved by regulating CDK4, a member of the cell cycle-dependent kinase family. CDK4 tends to regulate cell proliferation and apoptosis by influencing cell cycle blockade [31]. In this study, it was speculated that sanguinarine regulated CDK4 and thus affected apoptosis of HCC cells via the cell cycle pathway.

The targeted relationship between miR-497-5p and CDK4 was further confirmed. Diana tools analysis found that miR-497-5p was mainly enriched in cancer-related ECM receptor pathways, proteoglycan pathways in cancer and Hippo pathways, etc. Gharib et al. have demonstrated that miR-497-5p inhibits fatty acid metabolism and thus affects the progression of bowel cancer [32]. Besides, miR-497-5p can target insulin-like growth factor 1 to inhibit HCC transfer [33]. In this study, the expression of miR-497-5p in HCC tissue and cells was inhibited, but sanguinarine induced an enhancement of miR-497-5p expression. Further analysis revealed that the inhibition of miR-497-5p reduced the effect of sanguinarine in liver cancer, but the role of miR-497-5p inhibitor after transfection of si-CDK4 was partially revered. This indicated that sanguinarine was involved in regulating miR-497-5p/CDK4 and thus participates in the regulation of the proliferation and apoptosis of HCC cells.

However, there are some limitations in this study. The sample size was limited, and the further exploration of the signaling pathways that may be regulated by sanguinarine were not investigated. In addition, only cell apoptosis but no cell cycle distribution was detected in each group, and *in vivo* experiments were not designed to explore the role of sanguinarine/miR-497-5p/CDK4 in HCC. These will be improved in the future.

In summary, this study confirms the mechanism of sanguinarine influencing HCC cells by regulating the miR-497-5p/CDK4 axis from the perspective of drug targets affecting cancer progression, reveals the role of sanguinarine in HCC, and also provides a new target and experi-

mental basis for the treatment of HCC. The mechanism of this study is shown in **Figure 8**.

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## Disclosure of conflict of interest

None.

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