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microRNA-2117 Negatively Regulates Liver Cancer Stem Cells Expansion and Chemoresistance Via Targeting SOX2

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Keywords: hepatocellular carcinoma | liver cancer stem cell | miR-2117 | SOX2 | TACE

ABSTRACT

Cancer stem cells (CSCs) are involved in the regulation of tumor initiation, progression, recurrence, and chemoresistance. However, the role of microRNAs (miRNAs) in liver CSCs has not been fully understood. Here we show that miR-2117 is downregulated in liver CSCs and predicts the poor prognosis of hepatocellular carcinoma (HCC) patients. Biofunction studies found that knockdown miR-2117 facilitates liver CSCs self-renewal and tumorigenesis. Conversely, forced miR-2117 expression suppresses liver CSCs self-renewal and tumorigenesis. Mechanistically, we find that transcription factor SOX2 is required for miR-2117-mediated liver CSCs expansion. The correlation between miR-2117 and SOX2 was confirmed in human HCC tissues. More importantly, miR-2117 overexpression HCC cells are more sensitive to CDDP treatment. Analysis of patients' cohort further demonstrates that miR-2117 may predict transcatheter arterial chemoembolization benefits in HCC patients. Our findings revealed the crucial role of miR-2117 in liver CSCs expansion, rendering miR-2117 as an optimal therapeutic target for HCC.

1 | Introduction

Hepatocellular carcinoma (HCC) is the most common liver cancer in adults and a deadly disease with a poor prognosis [1]. Most HCC patients are diagnosed at a late stage due to inconspicuous symptoms [2]. Transcatheter arterial chemoembolization (TACE) or targeted drugs are the main choice for these advanced HCC patients [3, 4]. However, only a few patients are benefited from TACE or targeted drug treatment. So, it is urgent to explore the underlying mechanism of HCC tumorigenesis, recurrence, and chemoresistance.

Accumulating evidence shows that there is a distinct subpopulation of cancer cells called cancer stem cells (CSCs) or

Abbreviations: 3′-UTR, 3′-untranslated region; CSCs, cancer stem cells; HCC, hepatocellular carcinoma; miRNA, microRNA; TACE, transcatheter arterial chemoembolization.

Qing Xia and Guanghua Liu contributed equally to this study.

tumor-initiating cells (T-ICs) [5, 6]. CSCs exhibit extended self-renewal ability and tumorigenesis capacity. They are involved with the regulation of tumor initiation, progression, recurrence, and chemoresistance [7, 8]. It was reported that the chemoresistance and poor prognosis of HCC are closely associated with liver CSCs [9]. Therefore, identification of the underlying mechanisms governing liver CSCs propagation may lead to the discovery of promising therapeutic strategies for HCC patients.

microRNAs (miRNAs) are small, endogenous, noncoding RNAs that regulate RNA silencing and posttranscriptional of gene expression by binding the 3′-untranslated region (3′-UTR) of target mRNAs [10]. miRNAs play an important role in the regulation of cellar proliferation, migration, apoptosis, and differentiation [11, 12]. Deregulation of miRNAs is also involved in human disease, especially human cancers. For instance, miR-613 is downregulated in HCC tissues and inhibits the growth and invasiveness of HCC via targeting DCLK1 [13]. miR-552 is upregulated in liver T-ICs and promotes liver T-ICs expansion by regulating the PTEN/AKT pathway [14]. miR-2117 is a newly discovered miRNA, and its function and mechanism of action in biological processes and diseases are not completely understood. Previous studies found that miR-2117 was upregulated in Diabetic Kidney Disease patients [15]. However, the biological function of miR-2117 in liver CSCs remains unknown.

In the present study, we demonstrate that miR-2117 expression is downregulated in liver CSCs and predicts the poor prognosis of HCC patients. Functional tests indicate that miR-2117 knockdown leads to upregulation of liver CSCs self-renew and tumorigenesis. Conversely, overexpression of miR-2117 inhibits liver CSCs self-renew and tumorigenesis. Further mechanism study reveals that SOX2 is a direct target of miR-2117 in liver CSCs. More importantly, we find that miR-2117 overexpression HCC cells are more sensitive to CDDP treatment. Analysis of patients' cohorts further demonstrates that miR-2117 may predict TACE benefits in HCC patients. Taken together, our findings demonstrate that the critical role of the miR-2117 in liver CSCs expansion and TACE response.

2 | Materials and Methods

2.1 | HCC Patients' Tissues

HCC samples were collected from patients who underwent the resection of their primary HCC in the Eastern Hepatobiliary Surgery Hospital (EHBH). A total of 120 patients were followed for 5 years, and recurrence-free survival (DFS) and overall survival (OS) analyses were performed using the Kaplan–Meier method. OS was defined as the interval between the dates of surgery and death. DFS was defined as the interval between the date of surgery and recurrence. Kaplan–Meier analyses evaluating DFS and OS were performed on the expression of miR-2117 (i.e., using a cutoff of 5%, then 10%, up to 95%) [16]. Assessment of these results revealed the natural split in the data. Detailed clinicopathological features of the patients are in Table S1. A total of 60 patients received adjuvant TACE therapy after surgery for primary HCC at EHBH from 2014 to 2017. Detailed clinicopathological features of these patients are described in Table S2. Fifty HCC patients' tissues were used for analysis the relationship between miR-2117 and SOX2. The level of miR-2117 and SOX2 was determined by real-time PCR analysis. Data were normalized to β-actin as DCt and analyzed by Spearman's correlation analysis. Patient informed consent was also obtained and the procedure of human sample collection was approved by the Ethics Committee of EHBH.

2.2 | Cell Culture

The patient-derived primary HCC cultures of tumor cells were obtained from fresh tumor specimens of HCC patients described previously [17]. The human primary hepatoma cells were isolated by collagenase perfusion and centrifugation. Briefly, the liver cancer tissues were washed several times in pre-cooled sterile PBS buffer containing double antibodies to remove blood and connective tissue, GBSS mixed enzyme solution was used for digestion, centrifugation, and the supernatant was discarded, cell activity was detected by trypanosoma blue staining with cell filtrate, with complete medium heavy suspension inoculation after cell count after the package is the cultivation of the bottle, at 37°C and 5% CO_2 environment culture; and then identification of cell morphology.

HCC cell lines Huh7, HCCLM3, and HepG2 were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine and 25 μg/mL gentamicin and maintained at 37°C in a 5% CO_2 incubator. Huh7 and HepG2 were infected with miR-2117 sponge (5′-GUACCCCU GGAGAUUCUGAUAA-3′) or miR-2117 mimic (5′-UUAUCAG AAUCUCCAGGGGUAC-3′) lentivirus and their control lentivirus (Ribobio, Shanghai, China) and the stable infectants were screened by puromycin. HepG2 cells has been performed authenticated using Short Tandem Repeat (STR) analysis on 2020 in Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. Huh7 cells have been performed authenticated using STR analysis in 2017 in Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. HCCLM3 cells has been performed authenticated using STR analysis on 2021 in Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd.

2.3 | RNA Interference

Small interference RNAs (siRNAs) against SOX2 and NC (NC, negative control) siRNA were synthesized by Ribobio (Shanghai, China). SOX2 siRNA target sequences are 5′-CCAUGGAUUUA UUCCUAAATT-3′. The siRNAs were transfected into the HCC cells at a final concentration of 200 nM using an siRNA transfection reagent according to the manufacturer's instructions (Polyplus, Illkirch, France). Gene knockdown was validated by western blot analysis. Huh7 miR-2117 sponge and HepG2 miR-2117 sponge or their control HCC cells were transfected with SOX2 siRNA or negative control, and then subjected to spheroids formation, in vitro and in vivo limiting dilution assay.

2.4 | Spheroid Assay

The HCC cells were seeded in 96-well ultra-low attachment culture plates (Corning Incorporated Life Sciences) (300 cells per well) and cultured in DMEM/F12 (Gibco) supplemented with 1% FBS, 20 ng/mL bFGF, and 20 ng/mL EGF for 1 week.

The number of spheroids was counted and representative views were shown. The results were repeated for three times.

2.5 | In Vitro Limiting Dilution Assay

The HCC cells were seeded in 96-well ultra-low attachment culture plates (Corning Incorporated Life Sciences) (2, 4, 8, 16, 32, 64 cells per well [$n = 10$]) and cultured in DMEM/F12 (Gibco) supplemented with 1% FBS, 20 ng/mL bFGF, and 20 ng/mL EGF for 1 week. The proportion of CSCs was assessed using ELDA software (http://bioinf.wehi.edu.au/software/elda/index.html) [18]. The results were repeated for three times.

2.6 | Animal Models

All mouse experiments were performed according to the guidelines of the animal care and use committees at Hua Mei Hospital (University of Chinese Academy of Sciences, Ningbo, China). Four- to 6-week-old male NOD-SCID mice (SIPPR-BK Experimental Animal Co., China) were housed and fed in standard pathogen-free conditions.

For in vivo limiting dilution assay, hepatoma cells were diluted serially to the indicated doses (1×10^3, 5×10^3, 1×10^4, 5×10^4), and were mixed with 100 μL matrigel gel (1:1). Then, the mixed cells were injected subcutaneously into NOD-SCID mice ($n = 8$ for each group, randomized allocated). After 2 months, the mice were killed, and the number of tumors was counted.

2.7 | Flow-Cytometric Analysis

For CD24 and EpCAM positive cells sorting, primary HCC patients' cells and HCC cells were incubated with the primary anti–CD24 (Cat. no. ab202073; Abcam) or anti–EpCAM (Biolegend Inc., San Diego, CA) for 30 min at room temperature. The cells were then subjected to flow cytometry using a MoFlo XDP cell sorter from Beckman Coulter (Indianapolis, IN, USA) according to the manufacturer's instructions. The sorted cells from three independent experiments were subjected to Real-time PCR assay. The results were repeated for three times.

2.8 | Apoptosis Assay

Huh7 miR-2117 mimic and HepG2 miR-2117 mimic or their control HCC cells were treated with CDDP (4 μg/mL) for 48 h, followed by staining with Annexin V and 7-AAD for 15 min at room temperature in the dark. Apoptotic cells were determined by an Annexin VFITC Apoptosis Detection Kit I (BD Pharmingen, San Diego, CA) and flow cytometer according to the manufacturer's instructions. The results were repeated for three times.

2.9 | Luciferase Reporter Assay

A 400-bp fragment of the SOX2 3′-UTR containing the conserved miR-2117-binding sites was inserted into a luciferase reporter plasmid. The SOX2 3′-UTR mutant luciferase plasmid contained changes in potential miR-2117-binding base sequence "AGAGAACA" to "UCGAUUGC" Then, the 400-bp fragment of the SOX2 mutant 3′-UTR fragment was inserted into a luciferase reporter plasmid. For the luciferase reporter assay, Huh7 miR-2117 sponge and HepG2 miR-2117 sponge or their control HCC cells were seeded on 24-well plates and cotransfected using Lipofectamine 2000 (Invitrogen) with 100 ng per well of the resulting luciferase UTR-report vector, 2 ng per well of pRLCMV vector (internal control, Promega), and 20 ng per well of miR-2117 precursor molecules or control precursor (Applied Biosystems) following the manufacturer's instructions. After 24 h, the cells were lysed and the relative luciferase activity was assessed with the Dual-Luciferase Assay Reporter System (Promega). The results were repeated for three times.

2.10 | Real-Time PCR

Total RNA was isolated from cells or tissues using TRIZOL (Invitrogen) according to the manufacturer's instructions. The purity of RNA was measured with a UV spectrophotometer (NanoDrop ND-1000), and RNA integrity was validated with agarose gel electrophoresis. The extracted RNA was then reverse-transcribed to cDNA with the M-MLV RTase cDNA Synthesis Kit (Promega). Real-time PCR analysis was performed using a SYBR Green PCR Kit (Roche) and LightCycler 480 System (Roche). PCR conditions included 1 cycle at 95°C for 5 min, followed by up to 40 cycles of 95°C for 15 s (denaturation), 60°C for 30 s (annealing), and 72°C for 30 s (extension). The specificity of primers was confirmed by melting curves following the reaction. Each sample was measured in triplicate biological replicates. Hsa-RNU6B and β-actin were used as endogenous controls for miRNAs and mRNAs expression, respectively. The primer sequences were shown in Table S2. The results were repeated for three times.

2.11 | Western Blot Analysis Assay

Samples were obtained with cell lysis buffer and disposed as we described before [19]. After quantification with bicinchoninic acid assay (Weiao, Shanghai, China), we separated each protein through 10% SDS-PAGE and then moved them onto PVDF membranes (Millipore, USA). Then, samples were blocked with 5% nonfat milk. After incubation with primary antibodies and secondary antibodies, protein levels were detected with Image Quant LAS 4000 (GEHealthcare Life Sciences). The antibodies were showed in Table S3.

2.12 | Statistical Analysis

All experiments were performed at least three times. Data were presented as the mean ± SD. GraphPad Prism (GraphPad Software Inc., La Jolla, USA) was used for all statistical analyses. Statistical analysis was carried out using t test or Bonferroni Multiple Comparisons Test: $^*p < 0.05$. A p value of less than 0.05 was considered statistically significant.

3 | Results

3.1 | The Level of miR-2117 in Liver CSCs Is Downregulated

It was well accepted that EpCAM and CD24 are liver CSCs markers [20, 21]. Then, we isolated $EpCAM^+$ and $CD24^+$ cells from patient-derived primary HCC cells and HCC cell lines by flow cytometry sorting. As shown in Figure 1A,B, the expression of miR-2117 was significantly decreased in sorted $EpCAM^+$ or $CD24^+$ primary HCC cells. Compared with adherent cells, miR-2117 expression was decreased in HCC spheres derived from human primary HCC cells (Figure 1C). Moreover, we also found that miR-2117 expression was downregulated in sorted $EpCAM^+$ or $CD24^+$ HCC cell lines (Figure 1D,E). The level of miR-2117 was reduced in HCC spheres derived from HCC cell lines (Figure 1F). Notably, miR-2117 level was partially restored to origin level when the spheres were reattached (Figure 1G).

To investigate the clinical significance of miR-2117, we checked miR-2117 expression in a total of 120 HCC tissues. Clinical association studies found that downregulation of miR-2117 was significantly associated with HBsAg ($p < 0.05$), AFP ($p < 0.05$), tumor size ($p < 0.05$), portal vein tumor thrombus ($p < 0.05$), and TNM ($p < 0.05$) (Table S1). The average level of miR-2117 was lower in HCC tumors than adjacent nontumor tissues determined by real-time PCR (Figure 1H). Moreover, patients with lower miR-2117 levels exhibited worse OS and shorter time to recurrence (Figure 1I,J).

3.2 | miR-2117 Knockdown Promotes Liver CSCs Expansion

To explore the potential function of miR-2117 in liver CSCs, we checked miR-2117 expression in numbers of liver cancer cells. The results showed that miR-2117 expression in HepG2 was higher than other liver cancer cells, while in Huh7 was lower than other liver cancer cells (Figure S1A). So, we choose these two liver cancer cell lines for further analysis. Huh7 and HepG2 cells were infected with miR-2117 interference virus and the miR-2117 knockdown effect was determined by real-time PCR assay (Figure 2A). Spheroids formation was used to check the self-renewal ability. As shown in Figure 2B, miR-2117 knockdown HCC cells formed much more spheres compared with negative control cells. Moreover, we also found that the expression of CSC markers and stemness-associated genes was upregulated in miR-2117 knockdown HCC cell (Figure 2C,D). In vitro limiting dilution assay was used to determine the proportion of CSCs between miR-2117 knockdown and control HCC cells. The result showed that the proportion of CSCs in miR-2117 knockdown HCC cells was dramatically increased (Figure 2E). Then, we checked the tumorigenesis capacity of miR-2117 knockdown and control HCC cells by in vivo limiting assay. The result demonstrated that miR-2117 knockdown markedly upregulated the tumorigenesis capacity in hepatoma cells (Figures 2F,G and S1B).

3.3 | miR-2117 Overexpression Suppresses Liver CSCs Expansion

To further explore the biological function of miR-2117 in liver CSCs, Huh7 and HepG2 cells were transfected with miR-2117 overexpression virus. The miR-2117 overexpression effect was determined by real-time PCR assay (Figure 3A). Spheroids formation was used to check the self-renewal ability of miR-2117 overexpression and control HCC cells. As shown in Figure 3B, miR-2117 overexpression HCC cells formed fewer spheres compared with negative control cells. Moreover, we also found that the expression of CSC markers and stemness-associated transcription genes was decreased in miR-2117 overexpression HCC cells compared with control cells (Figure 3C,D). In vitro limiting dilution assay was used to determine the proportion of CSCs between miR-2117 overexpression and control HCC cells. The result showed that the proportion of CSCs in miR-2117 overexpression HCC cells was dramatically reduced (Figure 3E). Then, we checked the tumorigenesis capacity of miR-2117 overexpression and control HCC cells by in vivo limiting assay. The result demonstrated that miR-2117 overexpression markedly downregulated the tumorigenesis capacity in hepatoma cells (Figures 3F,G and S2A). Taken together, the above results indicated that miR-2117 inhibits liver CSCs self-renew and tumorigenesis.

3.4 | SOX2 Is Required for miR-2117 Mediated Liver CSCs Expansion

1To explore the downstream molecular of miR-2117 in liver CSCs, we used TargetScan to predict the direct targets and found that SOX2 harbored potential miR-2117 binding site (Figure 4A). To verify that SOX2 is a direct target of miR-2117, the full-length 3′-UTR of SOX2 gene was cloned into the downstream of the Renilla luciferase gene. As shown in the Figure 4B, miR-2117 knockdown led to an enhancing of luciferase activity when the reporter construct contained the wild-type 3′-UTR. However, mutation of the miR-2117 target site abrogated miR-2117-mediated reduction in luciferase activity. Conversely, the luciferase activity was decreased by overexpression of miR-2117 in reporter gene construction containing wild-type 3′-UTR, but not in construction containing mutant 3′-UTR (Figure S3A). Moreover, we found that SOX2 mRNA and protein expression was upregulated in miR-2117 knockdown liver CSCs (Figure 4C,D). In addition, SOX2 mRNA and protein expression was downregulated in miR-2117 overexpression liver CSCs (Figure S3C,D). There was a significant negative correlation between miR-2117 and SOX2 mRNA expression in human HCC tissues (Figure 4E).

Next, we explore whether SOX2 was required for miR-2117 mediated liver CSCs expansion. The special SOX2 siRNA was transfected to miR-2117 knockdown and control HCC cells. The SOX2 interference effect was determined by western blot assay (Figure 4F). As expected, special SOX2 siRNA abrogated the discrepancy of self-renewal ability, proportion of CSCs and tumorigenesis capacity between miR-2117 knockdown and control HCC cells (Figure 4G–J). Collectively, these results demonstrated that SOX2 was required for miR-2117-mediated liver CSCs expansion.

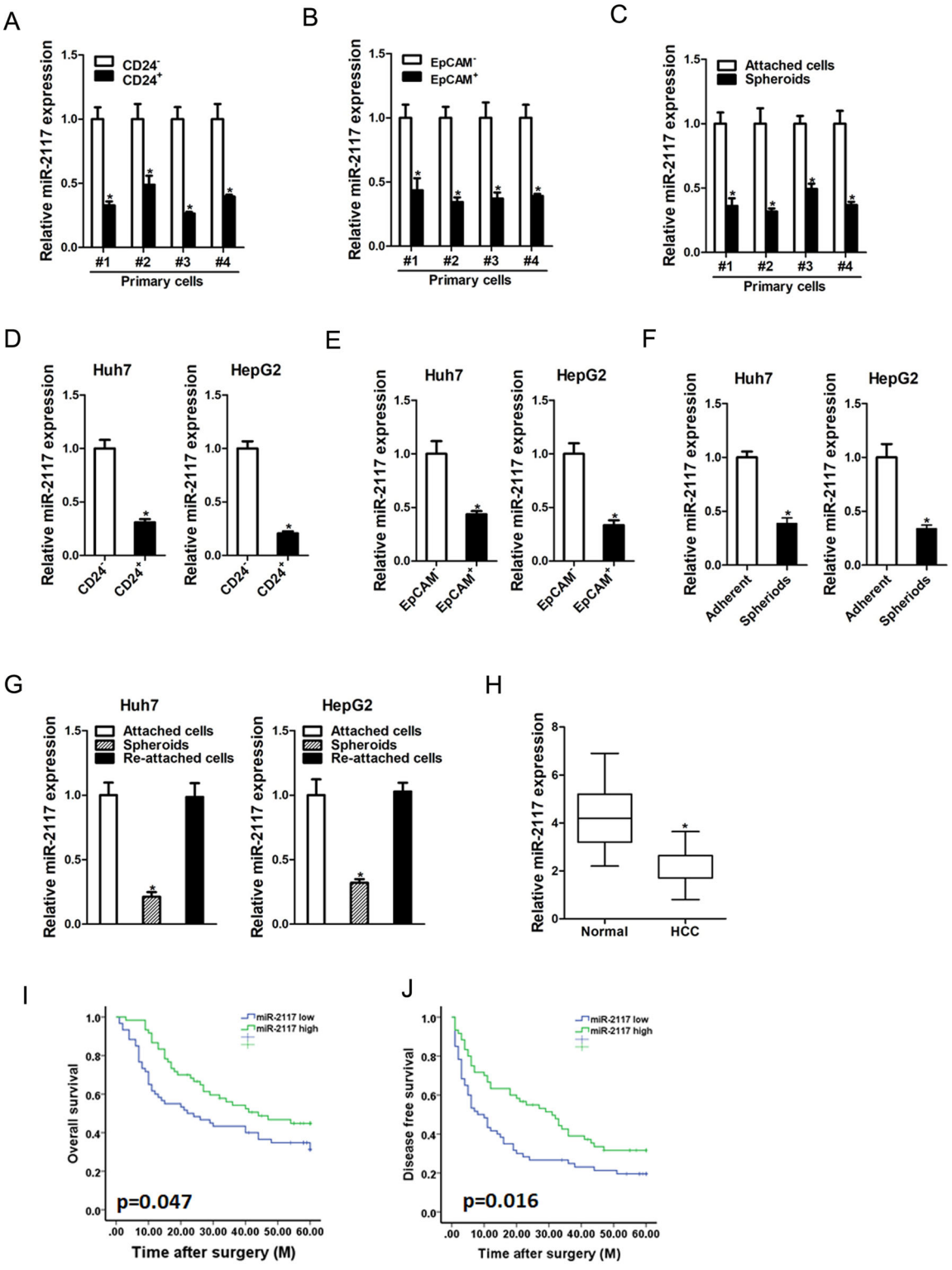
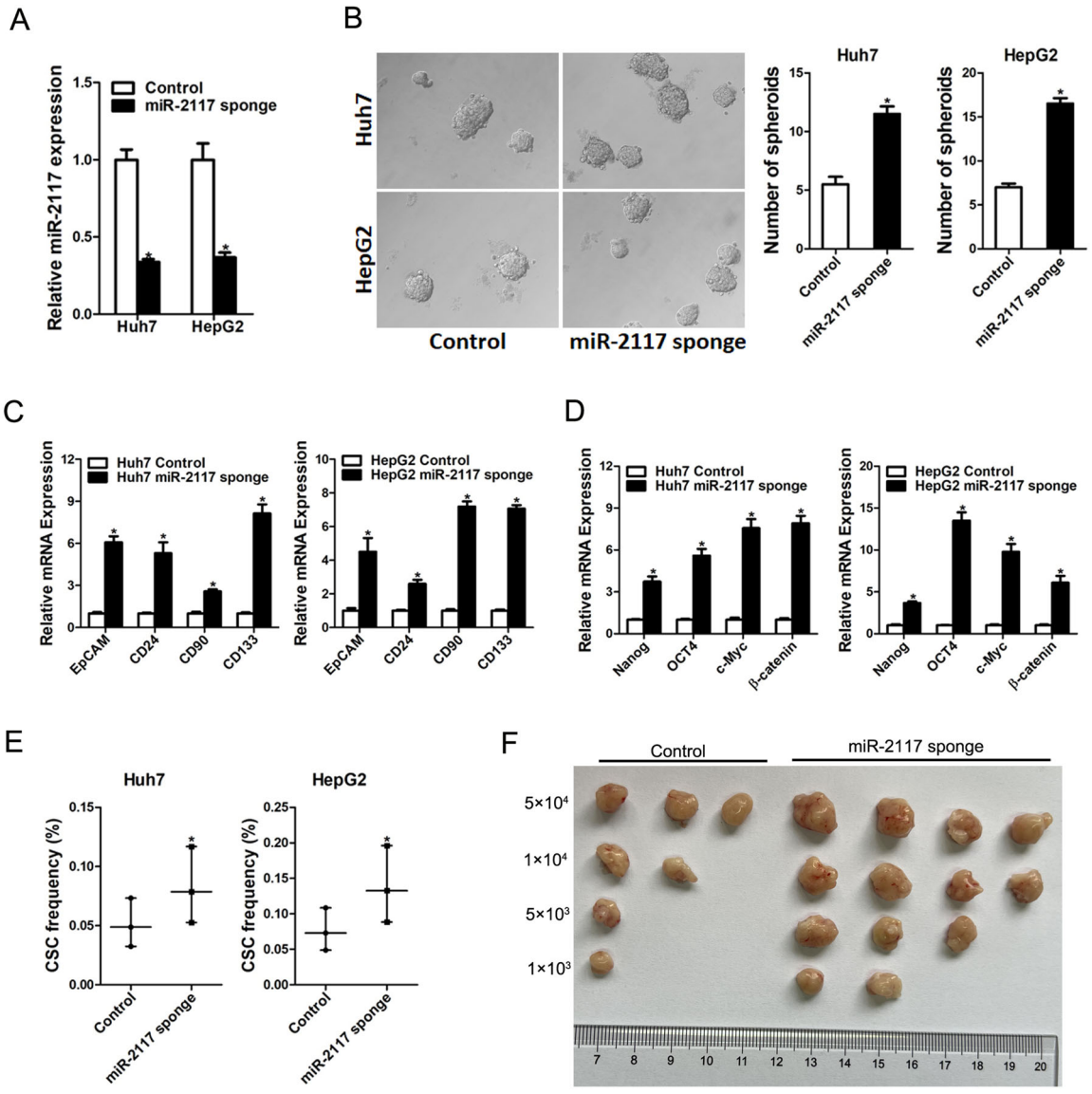


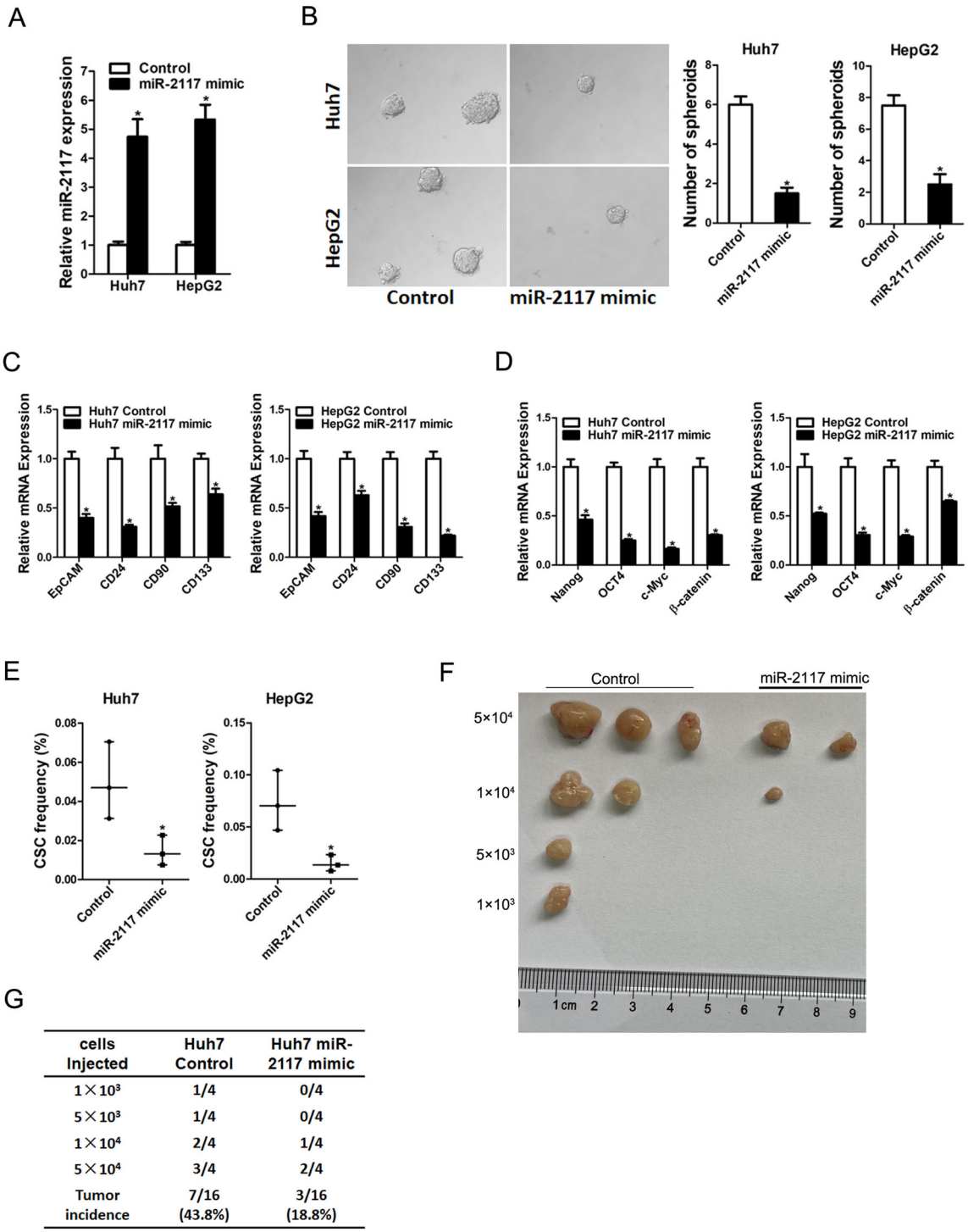
FIGURE 1 | miR-2117 expression is reduced in liver CSCs. (A) The expression of miR-2117 in EpCAM-positive primary HCC cells and EpCAM-negative control HCC cells was checked by real-time PCR assay ($n = 3$). (B) The expression of miR-2117 in CD24 positive primary HCC cells and CD24 negative control HCC cells was determined by real-time PCR assay ($n = 3$). (C) The expression of miR-2117 in primary HCC spheroids cells and primary HCC adherent cells was determined by real-time PCR assay ($n = 3$). (D) Real-time PCR analysis of miR-2117 in EpCAM positive HCC cells and its negative control HCC cells ($n = 3$). (E) Real-time PCR analysis of miR-2117 in CD24 positive HCC cells and its negative control HCC cells ($n = 3$). (F) Real-time PCR analysis of miR-2117 in HCC spheroids cells and HCC adherent cells was determined by real-time PCR assay ($n = 3$). (G) The expression of miR-2117 in attached cells, spheroids, and reattached hepatoma cells and was checked by real-time PCR assay ($n = 3$). (H) Real-time PCR analysis of miR-2117 in HCCs and peri-tumor normal tissues from 120 patients. (I) and (J) Real-time PCR and scoring of miR-2117 expression were performed in 120 human HCC samples. Overall survival time and disease-free survival after surgery of the patients were compared between the "miR-2117 low" ($n = 60$) and "miR-2117 high" ($n = 60$) groups, $p < 0.05$. (Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's *t*-test.).



G

cells Injectedx	Huh7 Control	Huh7 miR-2117 sponge
1×10³	1/4	2/4
5×10³	1/4	3/4
1×10⁴	2/4	4/4
5×10⁴	3/4	4/4
Tumor incidence	7/16 (43.8%)	13/16 (81.2%)

FIGURE 2 | miR-2117 knockdown promotes liver CSCs expansion. (A) Huh7 and HepG2 cells were infected with miR-2117 sponge virus and control virus. The miR-2117 knockdown effect was determined by real-time PCR assay ($n = 3$). (B) The self-renewal ability of miR-2117 knockdown and control HCC cells was compared by spheroids formation assay ($n = 3$). Representative images of spheres are shown. (C) The mRNA expression of liver CSCs markers in miR-2117 knockdown and control HCC cells was checked by real-time PCR assay ($n = 3$). (D) The mRNA expression of stemness-associated genes in miR-2117 knockdown and control HCC cells was checked by real-time PCR assay ($n = 3$). (E) In vitro limiting dilution assay of miR-2117 knockdown and control HCC cells. The results were shown as natural logarithm of the proportion of CSCs ($n = 10$). (F) and (G) The tumorigenicity of liver CSCs in miR-2117 knockdown and their control cells was compared by in vivo limiting dilution assay. Tumors were observed over 2 months; $n = 8$ for each group. (Data are represented as mean ± SD; $^*p < 0.05$; two-tailed Student's *t*-test.).



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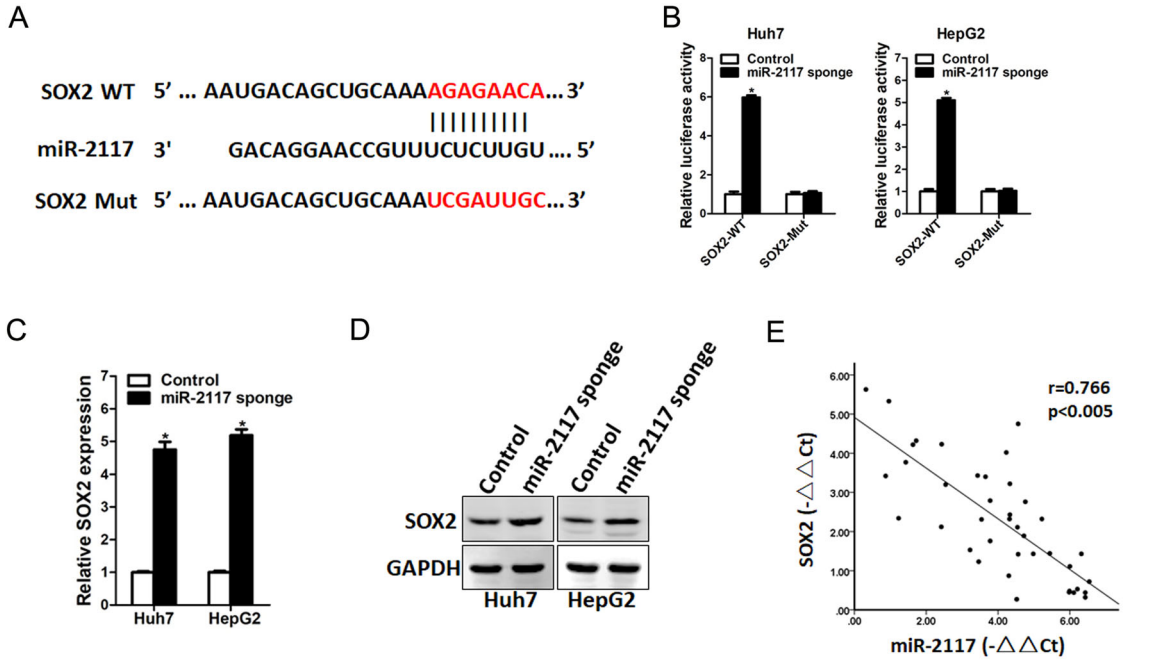
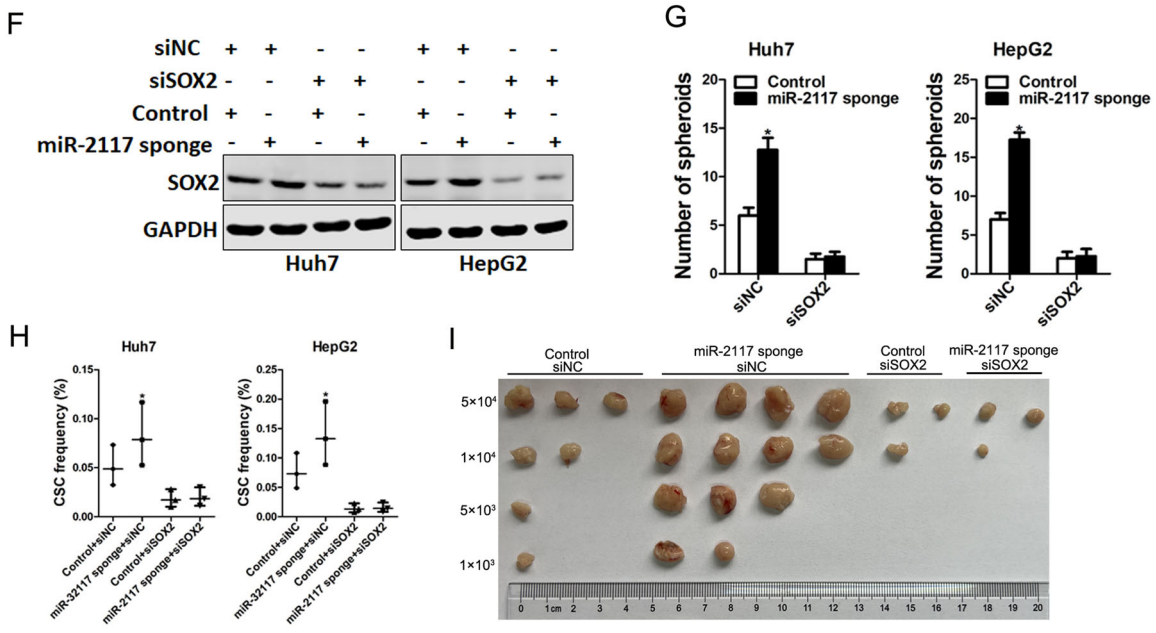
cells Injected	Huh7 Control	Huh7 miR-2117 mimic
1×10³	1/4	0/4
5×10³	1/4	0/4
1×10⁴	2/4	1/4
5×10⁴	3/4	2/4
Tumor incidence	7/16 (43.8%)	3/16 (18.8%)

FIGURE 3 | miR-2117 overexpression inhibits liver CSCs expansion. (A) Hepatoma cells were infected with miR-2117 mimic virus and control virus. The miR-2117 overexpression effect was determined by real-time PCR assay ($n = 3$). (B) The self-renewal ability of Huh7 miR-2117 mimic or HepG2 miR-2117 mimic and their control HCC cells was compared by spheroids formation assay ($n = 3$). Representative images of spheres are shown. (C) The mRNA expression of liver CSCs markers in Huh7 miR-2117 mimic or HepG2 miR-2117 mimic and their control HCC cells was checked by real-time PCR assay ($n = 3$). (D) The mRNA expression of stemness-associated genes in Huh7 miR-2117 mimic or HepG2 miR-2117 mimic and their control HCC cells was checked by real-time PCR assay ($n = 3$). (E) In vitro limiting dilution assay of Huh7 miR-2117 mimic or HepG2 miR-2117 mimic and their control HCC cells. The results were shown as natural logarithm of the proportion of CSCs ($n = 10$). (F) and (G) The tumorigenicity of liver CSCs in miR-2117 mimic and theri control cells was compared by in vivo limiting dilution assay. Tumors were observed over 2 months; $n = 8$ for each group. (Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's *t*-test.).

3.5 | miR-2117 Overexpression HCC Cells Are More Sensitive to CDDP Treatment

Accumulating evidence shows that liver CSCs are closely associated with the resistance of cancers to targeted drugs or chemotherapeutic drugs [22]. Then, we checked miR-2117 expression in CDDP-resistant HCC xenografts and HCC cell lines. As expected, the expression of miR-2117 was significantly reduced in both CDDP-resistant HCC xenografts and HCC cell lines (Figure 5A,B). Next, we found that the proportion of





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cells Injected	Huh7 Control siNC	Huh7 miR-2117 sponge siNC	Huh7 Control siSOX2	Huh7 miR-2117 sponge siSOX2
1×10³	1/4	2/4	0/4	0/4
5×10³	1/4	3/4	0/4	0/4
1×10⁴	2/4	4/4	1/4	1/4
5×10⁴	3/4	4/4	2/4	2/4
Tumor incidence	7/16 (43.8%)	13/16 (81.2%)	3/16 (18.8%)	3/16 (18.8%)

FIGURE 4 | Legend on next page.

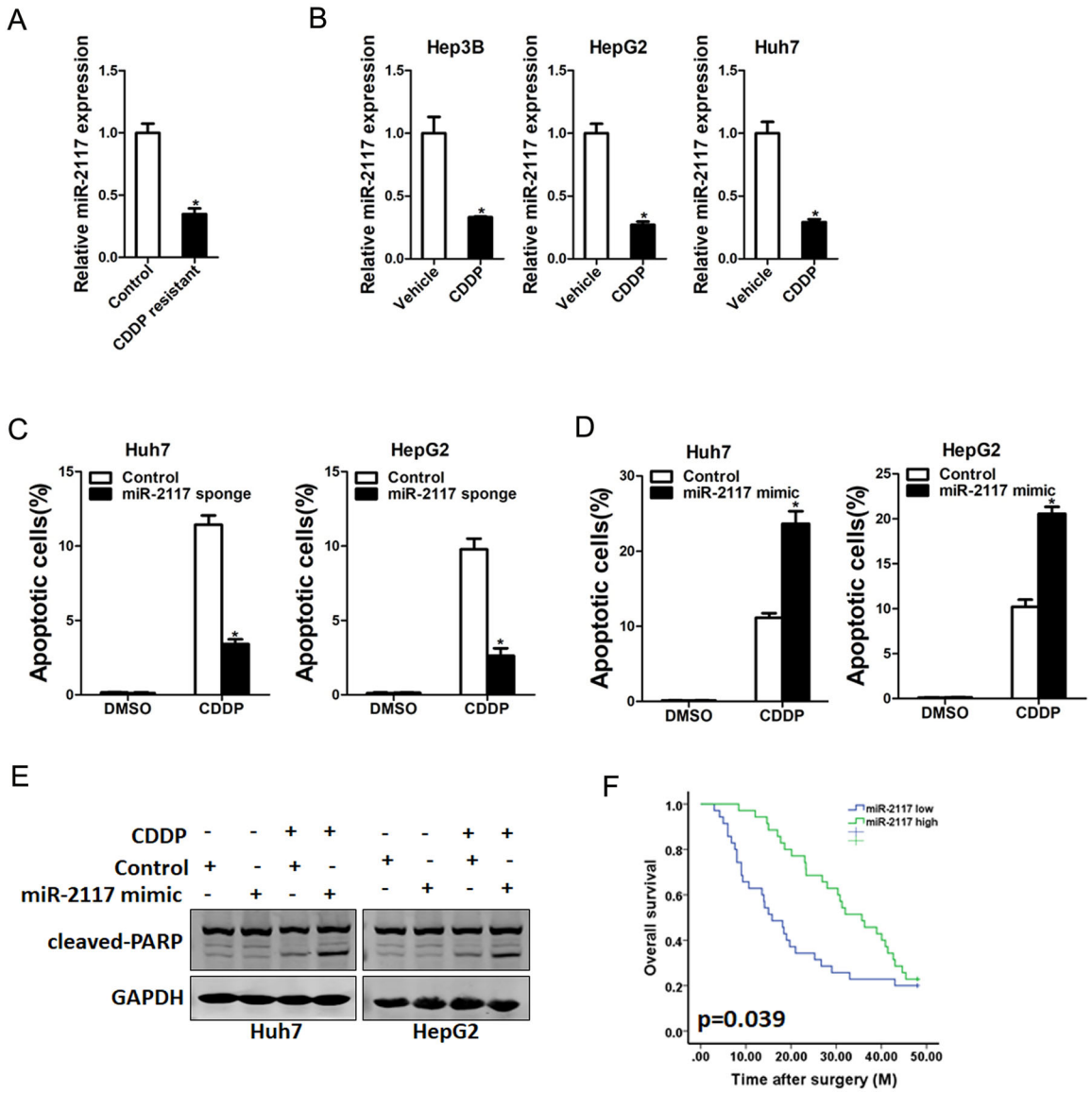

FIGURE 5 | miR-2117 overexpression HCC cells are more sensitive to CDDP treatment. (A) The expression of miR-2117 in CDDP-resistant HCC xenografts was determined by real-time PCR assay ($n = 3$). (B) The expression of miR-2117 in CDDP-resistant HCC cells and control cells was determined by real-time PCR assay ($n = 3$). (C) miR-2117 overexpression and control HCC cells were treated with CDDP (4 μg/mL) for 48 h, and then the apoptotic cells were determined by flow cytometry assay ($n = 3$). (D) miR-2117 knockdown and control HCC cells were treated with CDDP (4 μg/mL) for 48 h, and then the apoptotic cells were determined by flow cytometry assay ($n = 3$). (E) miR-2117 overexpression and control HCC cells were treated with CDDP (4 μg/mL) for 48 h and subjected to western blot assay. GAPDH acted as a loading control. (F) The overall survival of patients between miR-2117-high ($n = 30$) or miR-2117-low ($n = 30$) groups was evaluated by Kaplan–Meier analysis in HCC patients treated with TACE. ($p < 0.05$). (Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's *t*-test.).

FIGURE 4 | SOX2 is a direct target of miR-2117 in liver CSCs. (A) TargetScan of miR-2117 potential binding sites at the 3′-UTR of SOX2 and the nucleotides mutated in the SOX2-3′-UTR mutant. (B) Luciferase reporter assays in miR-2117 knockdown and control HCC cells. Cells were transfected with SOX2 wild-type or mutant 3′-UTR-reporter ($n = 3$). (C) The mRNA expression of SOX2 in miR-2117 knockdown and control liver CSCs was determined by real-time PCR assay ($n = 3$). (D) The protein expression of SOX2 in miR-2117 knockdown and control liver CSCs was checked by western blot assay. GAPDH acted as a loading control. (E) The correlation between the transcription level of miR-2117 and SOX2 in 50 HCC tissues was determined by real-time PCR analysis. Data were normalized to U6 or β-actin as ▵Ct and analyzed by Spearman's correlation analysis. (F) Huh7 miR-2117 sponge and HepG2 miR-2117 sponge or their control HCC cells were transfected with SOX2 siRNA or negative control, and then subjected to western blot assay. GAPDH acted as a loading control. (G) Huh7 miR-2117 sponge and HepG2 miR-2117 sponge or their control HCC cells were transfected with SOX2 siRNA or negative control, and then subjected to spheroids formation ($n = 3$). (H) Huh7 miR-2117 sponge and HepG2 miR-2117 sponge or their control HCC cells were transfected with SOX2 siRNA or negative control, and then subjected to in vitro limiting dilution assay ($n = 10$). (I) and (J) Huh7 miR-2117 sponge and its control cells were transfected with siSOX2 or siNC, and then subjected to in vivo limiting dilution assay. Tumors were observed over 2 months; $n = 8$ for each group. (Data are represented as mean ± SD; *$p < 0.05$; two-tailed Student's *t*-test.).

apoptotic cells in miR-2117 overexpression HCC cells was much higher than control HCC cells when these cells were treated with same dose CDDP (Figure 5C). Conversely, the proportion of apoptotic cells in miR-2117 knockdown HCC cells was much less than control HCC cells when these cells were treated with same dose CDDP (Figure 5D). The protein level of cleaved-PARP in miR-2117 overexpression HCC cells was significantly increased compared with control HCC cells when they were exposed to the same doses of CDDP (Figure 5E). More importantly, we used Kaplan–Meier analysis of HCC patients who received TACE treatment after surgery and found that HCC patients with high miR-2117 expression displayed longer survival time after TACE therapy (Figure 5F). Taken together, our results demonstrated that miR-2117 might serve as a reliable predictor for TACE treatment.

4 | Discussion

Increasing evidence shows that liver CSCs are responsible for the liver cancer therapies fail [23, 24]. However, the understanding of regulatory mechanisms for liver CSCs is unclear. In the present study, we demonstrated the critical role of miR-2117 in liver CSCs and the underlying mechanism. We also found that the miR-2117 predicted the poor prognosis of HCC. To our knowledge, this is the first report for miR-2117 in the regulation of liver CSCs.

miR-2117 is a newly discovered miRNA, and its function and mechanism of action in biological processes and diseases are not completely understood. Its biological role in liver CSCs still unknown. EpCAM and CD24 are well-accepted liver CSCs marks. Our result showed that the expression of miR-2117 was reduced in sorted EpCAM and CD24 positive primary HCC cells as well as primary HCC spheres. Liver CSCs exhibit extended self-renewal ability and tumorigenesis capacity. Our data also showed that knockdown miR-2117 in HCC cells upregulated liver CSC markers and promoted the self-renewal capacity and tumorigenicity of liver CSCs. On the contrary, overexpress miR-2117 in HCC cells downregulated liver CSC markers and inhibited the self-renewal capacity and tumorigenicity of liver CSCs.

Sox2, a protein that belongs to the family of high-mobility group transcription factors, is pivotal for the early development and maintenance of undifferentiated embryonic stem cells [25]. It was reported that dysregulated SOX2 expression contributed to the growth and metastasis of numerous cancers, including squamous-cell carcinoma, gastric cancer, lung cancer, and bladder cancer [26–29]. In addition, SOX2 was reported to be involved in the modulation of CSCs or T-ICs [30–32]. In this study, we found that miR-2117 downregulates SOX2 expression through binding to its 3′-UTR in liver CSCs. Furthermore, As expected, special SOX2 siRNA abrogated the discrepancy of self-renewal ability, proportion of CSCs and tumorigenesis capacity between miR-2117 knockdown liver CSCs and control CSCs cells. The correlation between miR-2117 and SOX2 is further validated in human HCC tissues.

TACE is the preferred treatment for advanced HCC patients, which is suitable for primary or metastatic HCC and postoperative recurrence of HCC, but the survival improvement of patients receiving TACE treatment is limited [33, 34]. Therefore, increasing studies have concentrated on the quest for biomarkers of TACE response and patient outcome. In this study, we find that miR-2117 overexpression HCC cells are more sensitive to CDDP induced apoptosis. Furthermore, Kaplan–Meier analysis of HCC patients who received TACE treatment after surgery and found that HCC patients with high miR-2117 expression displayed longer survival time after TACE therapy.

Taken together, we demonstrate that miR-2117 is downregulated in liver CSCs, which in turn suppresses the self-renewal and tumorigenicity of liver CSCs. In addition, miR-2117 inhibits liver CSCs expansion through directly regulating SOX2. In conclusion, our findings provide insight into the miR-2117/SOX2 axis as potential therapeutic target against liver CSCs and a potential predictor for TACE treatment of HCC patients.

Author Contributions

Q.X. and J.Z. conceived the research. Q.X. and G.H.L. conducted the experiments and wrote the manuscript. Q.X., W.B.L., and G.H.L. contributed to the cell culture and proliferation assays. Q.X. and G.H.L. contributed to western blot and RT-PCR assays. Q.X., W.B.L., and G.H.L. contributed to immunohistochemistry assays. J.Z. contributed to the statistical analysis.

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Ethics Statement

The tissue samples used in this study were approved by the Ethics Committee of EHBH (Shanghai, China), and written informed consent of all the patients was obtained before taking these clinical data for research purposes.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data used to support the findings of this study are available from the corresponding author upon request.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.