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Original Articles

The μ-opioid receptor (MOR) promotes tumor initiation in hepatocellular carcinoma

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ABSTRACT

Hepatocellular carcinoma (HCC) is the most prevalent subtype of liver cancer. Anesthetic regimens possibly influence cancer development. Exploration of novel, effective targets for liver cancer is the current hotspot in cancer treatment. A previous study conducted by us has demonstrated that enhanced expression of the μ-opioid receptor (MOR) promotes cell proliferation, adhesion, migration, and tumorigenesis. The current study investigates whether MOR regulates self-renewal of hepatocellular carcinoma stem cells (HCSCs). We utilize cell function assays, siRNA, shRNA, flow cytometry sorting, and other molecular biology techniques for this purpose. The results indicate that MOR expression is positively related to hepatocarcinoma progression. Silencing MOR greatly reduce HCC-related tumorigenesis both *in vitro* and *in vivo* and significantly extend the survival of tumor-bearing mice. Moreover, MOR silencing will greatly reduce colony formation by HCC cells, indicating down-regulation of cancer initiation. In conclusion, these results establish that MOR can be a novel and reliable HCSC marker and a potential therapeutic target against HCC via MOR-NFAT signaling.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth common malignant tumor and the third leading cause of death among all cancer victims [1]. Owing to recent progress in early diagnosis methods and surgical techniques, the incidence and death rates associated with HCC have declined slightly, but issues related to postoperative recurrence and metastasis continue to be problematic.

Opioids are typically used in cancer pain management, including pain associated with tumor resection and preoperative analgesia. Opioids work on receptors, blocking pain conduction in the nervous system. Generally, opioid receptors are categorized into 3 subtypes; μ, κ, and δ (MOR, KOR, DOR), which can modulate pain relief, cognition, and emotion, respectively [2]. Many studies have indicated that opioid receptors also exist in cancer cells of humans and other animals [3–6]. The Mu opioid receptor, MOR, a G protein-coupled receptor found in squamous cells of lung, breast, colon, and prostate cancer cells, is among the most widely used opioid receptors [7–9]. A previous study of ours has demonstrated that MOR is specifically expressed in HCC tissues, whereas it shows no or very low expression in paracancerous tissues. We tested the hypothesis that MOR is associated with HCC, with a view to elucidating the mechanism underlying MOR-facilitated cancer development.

Recent studies have demonstrated the critical role played by cancer stem cells (CSCs) or cancer-initiating cells in tumorigenesis of many cancers [10,11], including hepatocellular carcinoma [12,13]. CSCs display the capacity to self-renew, differentiate, and give rise to new tumors [14]. In HCC, EpCAM, CD133, CD13, CD90 and CD44 have been defined as CSC surface markers [15–18]. However, molecular therapeutic targets for CSC of HCC remain unestablished. The complicated nature of HCC treatment requires an interdisciplinary approach to ensure optimal outcomes. In a previous study, we sorted $CD133^+$ and $CD133^-$ populations in Huh7 and HepG2 cells and evaluated MOR expression using flow cytometry, revealing that $CD133^+$ subsets expressed higher levels of MOR than the $CD133^-$ subsets in both HCC cell lines, which predicates that MOR is associated with CSC.

We reported for the first time that MOR + cells have CSC properties in HCC and investigated whether MOR + cells offered a new

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therapeutic target. Furthermore, we explored possible signaling pathways of MOR in HCC. We found that downregulation of MOR inhibits the proliferation of hepatocarcinoma cells and can significantly retard tumor growth. Therapy targeting MOR can provide a new and promising approach to HCC treatment.

2. Materials and methods

2.1. Animal models

Nude mice used in this study were purchased from the Animal Center of the Chinese Academy of Medical Science (Beijing, China). Six-week-old female nude mice, weighing approximately 15 g, were used in the current study. The mice were fed under specific-pathogen-free (SPF) conditions. Xenografts of tumor cells were generated via subcutaneous injection of tumor cells (1×10^6 cells suspended in 200 μl PBS) into the dorsal skin of nude mice. Tumor sizes were monitored twice a week. Animal studies were approved by Institutional Animal Care and Use Committee (IACUC) of the Institute of Biophysics (IBP), Chinese Academy of Sciences (CAS) (Beijing, China), and were conducted in accordance with its recommendations and ethical regulations. All experimental protocols were approved by IACUC, IBP and CAS. The mice were maintained under standard conditions according to the institutional guidelines for animal care.

2.2. Cell lines and hepatocellular carcinoma tissues

The cell lines, HepG2, Huh7, HLE and PLC, were obtained from American Type Culture Collection (Rockville, US). Hepatocellular carcinoma tissues were obtained from Chinese PLA General Hospital (Beijing, China) with informed consent. All human studies were reviewed and approved by Institutional Review Board (IRB) of Institute of Biophysics, Chinese Academy of Sciences and conducted according to the stipulations of Helsinki Declaration of the World Medical Association. Cell lines were used for less than 6 months following restoration and were routinely screened for mycoplasma every 4 weeks. No genotypic authentication was conducted. Each cell line was used during the early passages.

2.3. Real-time PCR

Total RNA from either the cell lines or primary patient tumor samples was extracted with an RNA isolation kit (QIAGEN, Germany). RNA was subjected to cDNA synthesis with a PrimeScript RT reagent kit (Takara Bio, Japan). The cDNA was used as the template for real-time PCR analysis on an ABI 7200 analyzer (Applied Biosystems, US) with the fluorescent probe SYBR Green I (Tiangen, China). Relative expression levels of the genes were normalized to the housekeeping gene GAPDH. Each experiment was independently repeated at least 4 times.

2.4. [^{3}H]-TdR incorporation assay

HCC cancer cells were seeded on 96-well culture plates and cultured until the cells reached 70–80% confluence, upon which they were serum-starved in DMEM (Invitrogen, US) for 24 h. Then the medium was replaced with DMEM containing 10% FBS. After 72 h, the cells were pulsed with [^{3}H]-thymidine for 4 h and their [^{3}H]-thymidine incorporation was measured in the liquid scintillation counter LKB1219.

2.5. Cell attachment and migration assay

Cell-substrate attachment assays and cell migration assay were performed using a previously described method with modification [19]. Firstly, 96-well plates were incubated with matrigel (BD Biosciences) and blocked with heat-denatured BSA (Sigma) for 1 h. Near confluent cells were harvested with trypsin (Life Technologies), resuspended in DMEM with 1% FBS (Life Technologies), and recovered at 37 °C for 15min. Attached cells were fixed and stained with crystal violet (Sigma). The absorbance of each well was measured at 575 nm with an ELISA reader. A migration assay was performed via a modified Boyden chamber (8 μm pore size; Costar, Corning, New York, USA). Tumor cells were trypsinized, washed, and resuspended in serum-free medium containing 1% BSA. Following incubation for 4 h at 37 °C, cells remaining at the upper surface of the membrane were removed, whereas the cells migrating to the lower surface were fixed with ethanol and stained with Giemsa solution.

2.6. BrdU and Ki-67 analysis

Cells grown as nonadherent spheres were treated with 30 μM BrdU for 4 h. Next, the cells were fixed, permeabilized, treated with DNase and stained with anti-BrdU antibodies according to the manufacturer's instructions (BD Pharmingen). Cells were then stained with 7-AAD and analyzed using a BD LSR II flow cytometer. For Ki67 (51–36525X, BD) analyses, cells were fixed and permeabilized prior to intracellular staining. Cells were analyzed using either a BD LSR II or a FACSCanto II flow cytometer.

2.7. Oncosphere assay and colony formation assay

A single-cell suspension of hepatocellular cancer cells was seeded at a density of 3×10^3 cells/well in 6-well plates with ultra-low attachment surfaces (Corning, US). Cells were cultured in DMEM/F12 media supplemented with 20 ng/ml bFGF, 20 ng/ml EGF, 1% N2, 2% B27 (Invitrogen, US) and 100 mg/mL streptomycin (Invitrogen, US). The number of spheres was calculated 2 weeks after seeding. Each assay was repeated at least 3 times. HCC cancer cells were suspended in soft agar and culture media in 6-well plates at a density of 1000 cells/well. After 2–3 weeks, the number of colonies ($\geq$10 cells) in 5 microscope fields per well was counted and photographed. Each experiment was independently repeated at least 3 times.

2.8. Silencing of MOR

To construct a MOR knockdown lentivirus, target sequences were designed as follows: shMOR-1: 5′-CACGAACGCCAGCAATTGCACT GAT-3′, shMOR-2: 5′-ACTGATGCCTTGGCGTACTCAAGTT-3'; and shMOR-3: 5′-GATGCCTTGGCGTACTCAAGTTGCT-3'. In brief, both the shMOR and control hairpins were cloned into the pSICO-R vector. Production of lentiviral particles and transduction of CSCs was performed according to protocols from the RNAi consortium at MIT. HCC cancer cells were transfected with lentiviral constructs expressing either shRNA against MOR or shCtrl for 24 h. Positive cells were selected at 2 weeks following puromycin treatment. Cell lines expressing MOR were identified using real-time PCR.

2.9. Statistical analysis

Kaplan–Meier analysis was used to estimate cumulative cause-specific survival rates and differences in survival between mice receiving shMOR xenografts and mice receiving shCtrl xenografts. The effect of MOR on sphere and colony formation capacities was analyzed using a two-tailed Student's t-test. For all statistical analyses, statistical significance was set at $P \leq 0.05$.

3. Results

3.1. MOR is generally expressed in the hepatocellular carcinoma cell lines, human tumor tissues and MOR + hepatocellular carcinoma cancer cells resembled cancer stem cells

MOR transcription was measured via qPCR in a normal liver cell

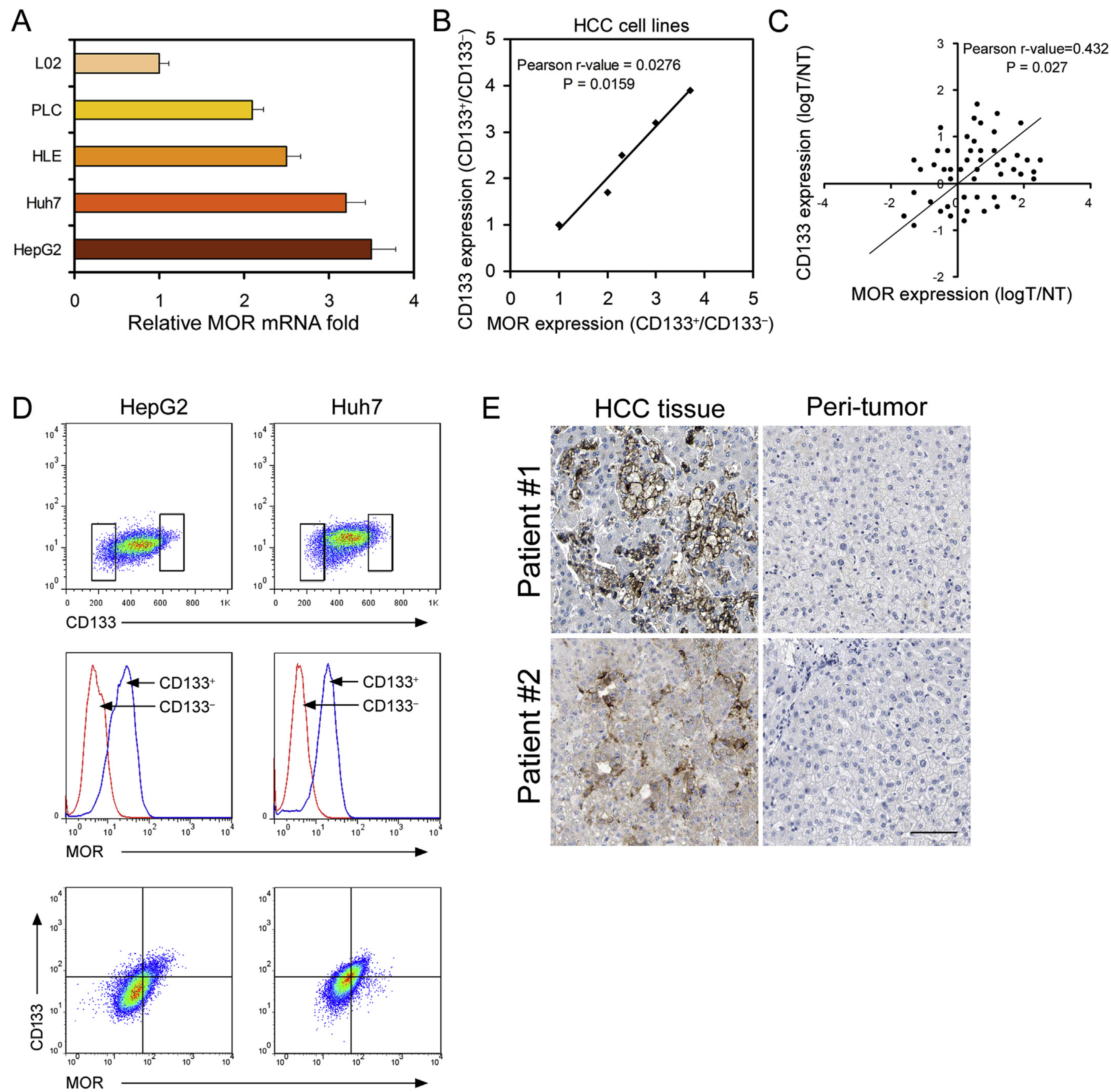


Fig. 1. MOR is highly expressed in HCC cell lines and human tumor tissue. (A) Relative MOR mRNA expression levels among the liver cell line L02 and various HCC cancer cell lines. ** = P < 0.01. (B) Positive correlation between MOR and CD133 expression in a control liver cell line (L02) and in HCC cell lines (HepG2, Huh7, HLE and PLC). (C) Positive correlation between MOR and CD133 expression in tumors from HCC patients. (D) FACS histograms showing separate gating for the isolation of CD133 and in MOR HepG2 and Huh7 cells. (E) Immunohistochemical staining of MOR expression in HCC tissues and paracancerous tissues. (scale bar = 50 μm).

line (L02) and several HCC cell lines (PLC, HLE, Huh7 and HepG2). MOR expression was the lowest in L02, higher in HCC cell lines at different levels. But the highest expression of MOR was observed in Huh7 and HepG2 (Fig. 1A).

CD133 is a surface marker of CSC. In order to analyze whether MOR is a potential marker for HCC CSCs, we sorted both CD133$^+$ and CD133$^-$ populations in Huh7 and HepG2 cells, and determined their MOR expression using flow cytometry. Results indicated that CD133$^+$ subsets express higher levels of MOR than CD133$^-$ in both HCC cell lines, which showed that MOR should be associated with CSC (Fig. 1B, C and D). Then, we checked MOR expression in samples from HCC patients. Immunohistochemistry (IHC) results from two HCC patients show that MOR is mainly expressed in HCC tissue, but very low or none in paracancerous tissues (Fig. 1E).

3.2. MOR expression is positively correlated with hepatocarcinoma progression

Having identified that MOR expression was specific to HCC rather than to paracancerous tissues, we explored the correlation between MOR expression levels and tumor progression in HCC patients. MOR expression was analyzed with IHC in tumor samples from 215 HCC

Table 1
Relationship between the expression levels of MOR and clinicopathological features of HCC patients.

Total		Patients	Expression of aberrantly MOR		
			High	Low	P
Age	Mean	62.8	63.7	61.9	0.427
Sex	Male	123	62	61	0.635
	Female	92	40	52	
Tumor stage	T1	53	19	34	< 0.001
	T2	76	28	48	
	T3	72	43	29	
	T4	12	12	2	
Grade	G1 or 2	127	47	80	< 0.001
	G3	88	55	33	
Number of tumors	Solitary	132	69	63	0.063
	Multiple	83	33	50	
Lymphatic invasion	Negative	76	23	53	< 0.005
	Positive	82	60	22	
	Unknown	57	19	38	
Follow-up (month)	Mean	85.7	87.6	83.8	0.537

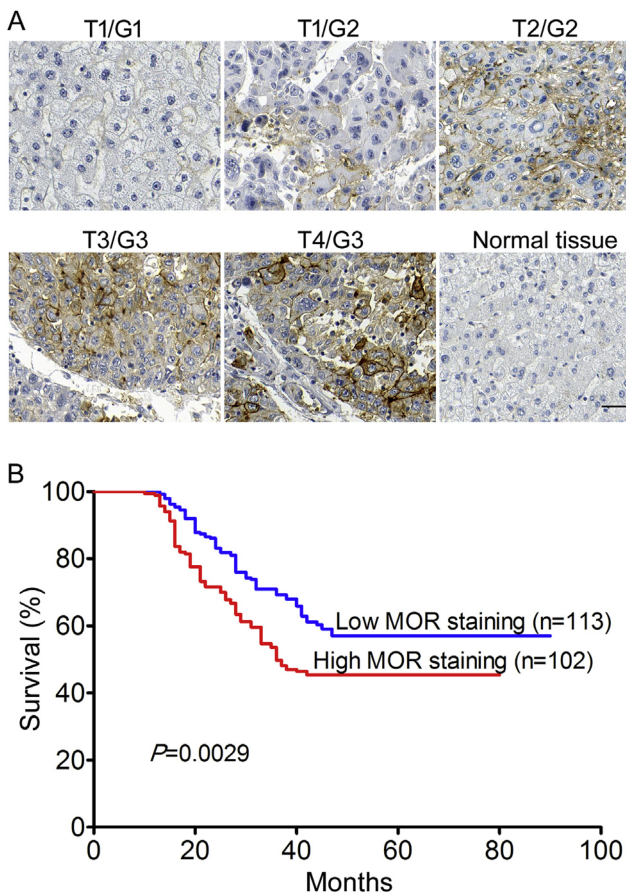


Fig. 2. MOR expression is consistent with clinical severity and prognosis of patients with HCC. (A) Expression level of MOR is in agreement with clinical severity. Serial sections of HCC biopsies were stained with anti-MOR antibodies via immunohistochemistry analysis. Normal liver biopsy from a healthy person not stained with anti-MOR antibodies. (B) The relationship between MOR expression and clinicopathologic features of patients. Patients with high MOR expressions had significantly worse prognoses than those with low MOR expression ($P < 0.0029$); n = patient number.

patients being divided into three grades (1–3) based on MOR staining. These MOR expression grades were further analyzed together with HCC stages (T1-T4) and grades (G1-G3) (Table 1). The results manifest that MOR expression is low in HCC patients with low grades (G1, G2) or low stages (T1, T2), but higher in patients with high grades (G3) or high stages (T3, T4), which demonstrates a positive correlation between MOR expression and HCC progression (Fig. 2A, Table 1). We further performed a prognostic analysis of 215 HCC patients, the results of which indicate a significant difference in survival rates between patients with high *MOR* expression and those with low *MOR* expression ($p = 0.0029$; Fig. 2B, Table 1). These findings demonstrate a negative correlation between MOR expression and HCC patient prognosis.

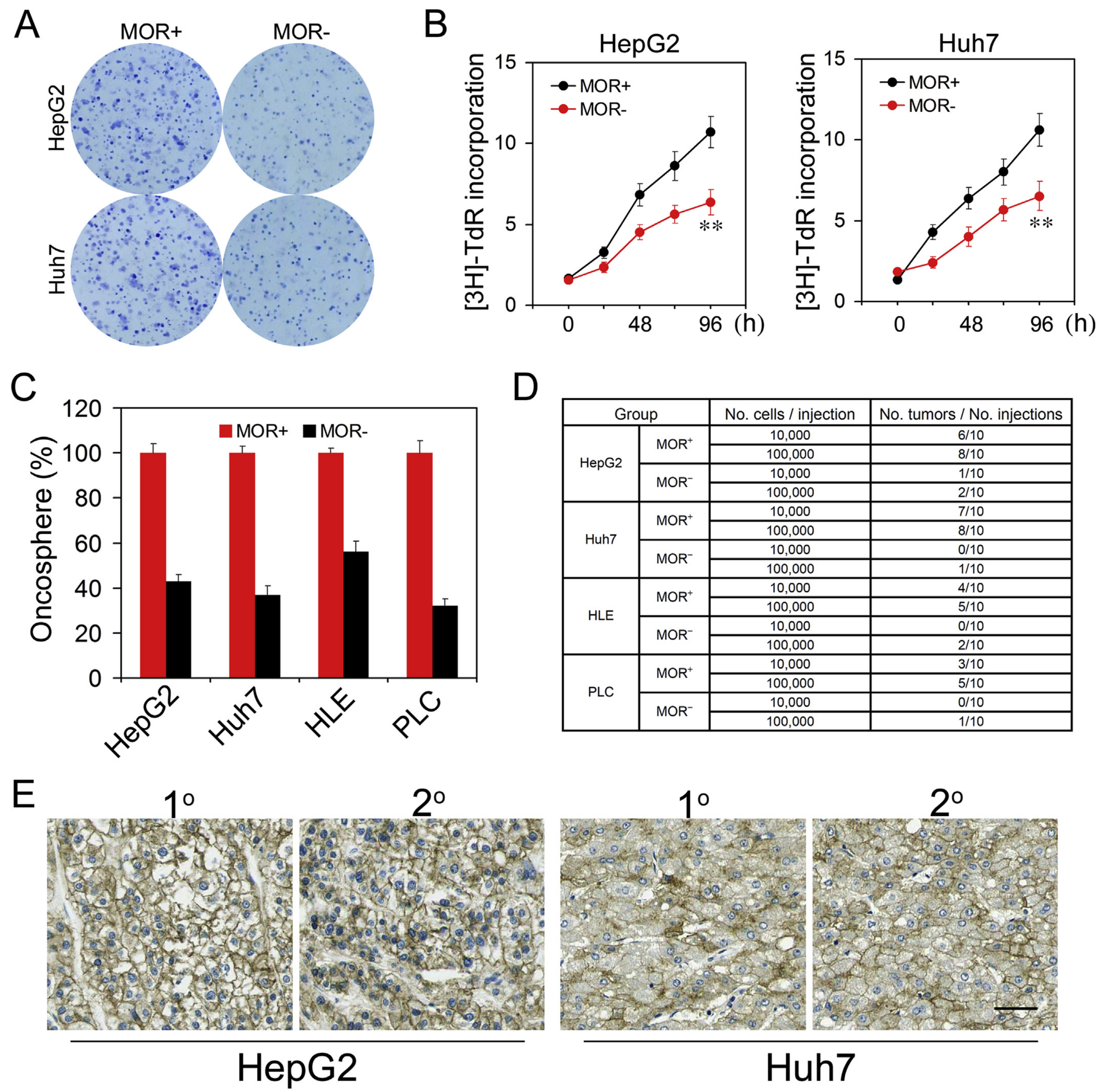
3.3. MOR gene expression promote cancer cell proliferation and tumor formation

To explore whether MOR expression could affect HCC tumor proliferation, we isolated MOR^+ and MOR^- cells from Huh7 and HepG2 lines via flow cytometry, and we used the isolated cells in 2 proliferation assays, the crystal violet assay and the 3H-Thymidine (3H-TdR) assay. Violet staining indicates that MOR^+ cells grew significantly faster than MOR^- cells in both cell lines (Fig. 3A). The 3H-TdR assay also yielded consistent results (Fig. 3B) demonstrating that MOR expression facilitated HCC proliferation. Sphere-forming capacity is an important characteristic of CSCs. We compared the tumorigenic capacity of MOR^+ and MOR^- cells from 4 HCC lines via an oncosphere formation assay, which has shown that MOR^+ HCC cells carry a stronger capacity to form oncospheres in all tested HCC lines (Fig. 3C). These data suggest that high MOR expression can promote HCC proliferation and tumorigenesis.

We then investigated the *in vivo* tumor-initiation capacity of HCC cells at different MOR expression levels by injecting 10,000 or 100,000 HCC cells with either high or low MOR expression into the back of nude mice. And tumor formation was monitored 5 days later. In HepG2 and Huh7 cells with higher MOR transcription, isolated MOR^+ cells initiated tumors at a higher rate (60–80%) compared with 0–20% for MOR^- cells (Fig. 3D). In HLE and PLC cells with relatively low MOR transcription, isolated MOR^+ cells still can generate more tumors (30–50%) compared with MOR^- cells (0–20%) (Fig. 3D). Later, we performed continuous passage assays, in which formed tumors from the first generation were processed and injected into new mice, which resulted in the 2nd generation tumors being generated. This shows that high MOR expression in the 2nd generation tumors is consistent with that in the first generation, demonstrating the positive correlation between MOR expression and HCC tumor initiating capacity (Fig. 3E).

3.4. MOR knockdown inhibits hepatocellular cancer stem cell characteristics and attenuates hepatocarcinoma cell proliferation, attachment, migration and promotes apoptosis

Our data indicate that MOR + cells show hepatoma CSC properties. To investigate the cellular function of MOR in hepatoma CSCs, 3 shRNAs, MOR-1, MOR-2 and MOR-3, were used to knock down *MOR* expression in Huh7 and HepG2 cell lines. Compared with control shCtrl, all the 3 shMORs efficiently silenced *MOR* expression, where shMOR-1 was the most potent and therefore used in the cellular assays which followed (Fig. 4A). 3H-TdR assay demonstrates that the proliferation of HCC cells is significantly suppressed by shMOR-1 compared with shCtrl (Fig. 4B). Afterwards, we investigated the role of MOR in cell adhesion, migration, and chemoresistance using HCC cells from 3 patient samples. A cell attachment assay and a transwell assay indicate that HCC adhesion to the extracellular matrix (ECM) as well as its migration capacity is inhibited by MOR knockdown (Fig. 4C and D). Colony-forming ability is an important feature of CSCs. Our results revealed that MOR + HCC cells have greater colony-forming ability than MOR-cells. MOR silencing greatly reduced the colony forming ability of HCC cells, indicating a down-regulation of cancer stemness (Fig. 4E). Both MOR silenced HCC cells and control cells were treated with the commonly-used chemodrug, cisplatin (DDP; 1 μg/ml) and apoptosis was significantly increased with MOR silencing, indicating that MOR



Group		No. cells / injection	No. tumors / No. injections
HepG2	MOR+	10,000	6/10
		100,000	8/10
	MOR−	10,000	1/10
		100,000	2/10
Huh7	MOR+	10,000	7/10
		100,000	8/10
	MOR−	10,000	0/10
		100,000	1/10
HLE	MOR+	10,000	4/10
		100,000	5/10
	MOR−	10,000	0/10
		100,000	2/10
PLC	MOR+	10,000	3/10
		100,000	5/10
	MOR−	10,000	0/10
		100,000	1/10

Fig. 3. A MOR$^+$ subpopulation of cancerous cells is defined as HCSCs. (A) Representative light microscope fields and comparative quantification of crystal violet assay of MOR$^+$ or MOR$^-$ HepG2 and Huh7 cells. (B) MOR$^+$ or MOR$^-$ HepG2 and Huh7 cells were cultured from 24 to 96 h followed by incubation with [^{3}H]-thymidine for 4 h. Data are representative of at least 3 separate experiments. (C) MOR$^+$ or MOR$^-$ HCC cells were detected via oncosphere analysis. (D) Tumor formation frequency of HCC from MOR$^+$ and MOR$^-$ subpopulations derived from HCC cell lines. (E) Immunohistochemical analysis of in situ MOR expression in both primary and secondary tumor samples collected from xenografted MOR$^+$ tumors. H&E-stained tissues are shown as a reference (scale bar = 50 μm). ** = P < 0.01.

knockdown can overcome chemoresistance in HCC stem cells (Fig. 4F).

3.5. MOR knockdown can inhibit the hepatocarcinoma cell cycle and reduce tumorigenic ability

In order to assess the effect of MOR on the cell cycle, MOR-silenced HCC cells from patient samples were utilized in both BrdU and Ki67 staining assays. The BrdU incorporation assay show that knockdown of MOR significantly can reduce cells in the S phase (Fig. 5A) and the Ki67 staining data further indicate that MOR silencing can significantly increase the number of cells in the G0 phase (Fig. 5B). These data collectively demonstrate that MOR knockdown can lead to HCC cell cycle arrest. Moreover, MOR silencing can significantly inhibit oncosphere formation by HCC cells (Fig. 5C). To test tumorigenesis *in vivo*, MOR-1 silenced and control HCC cells from a patient were injected into nude mice and the number of mice lacking tumor generation was counted. These results indicate that MOR knockdown can significantly reduce HCC tumorigenesis at multiple starting cell numbers *in vivo* (Fig. 5D).

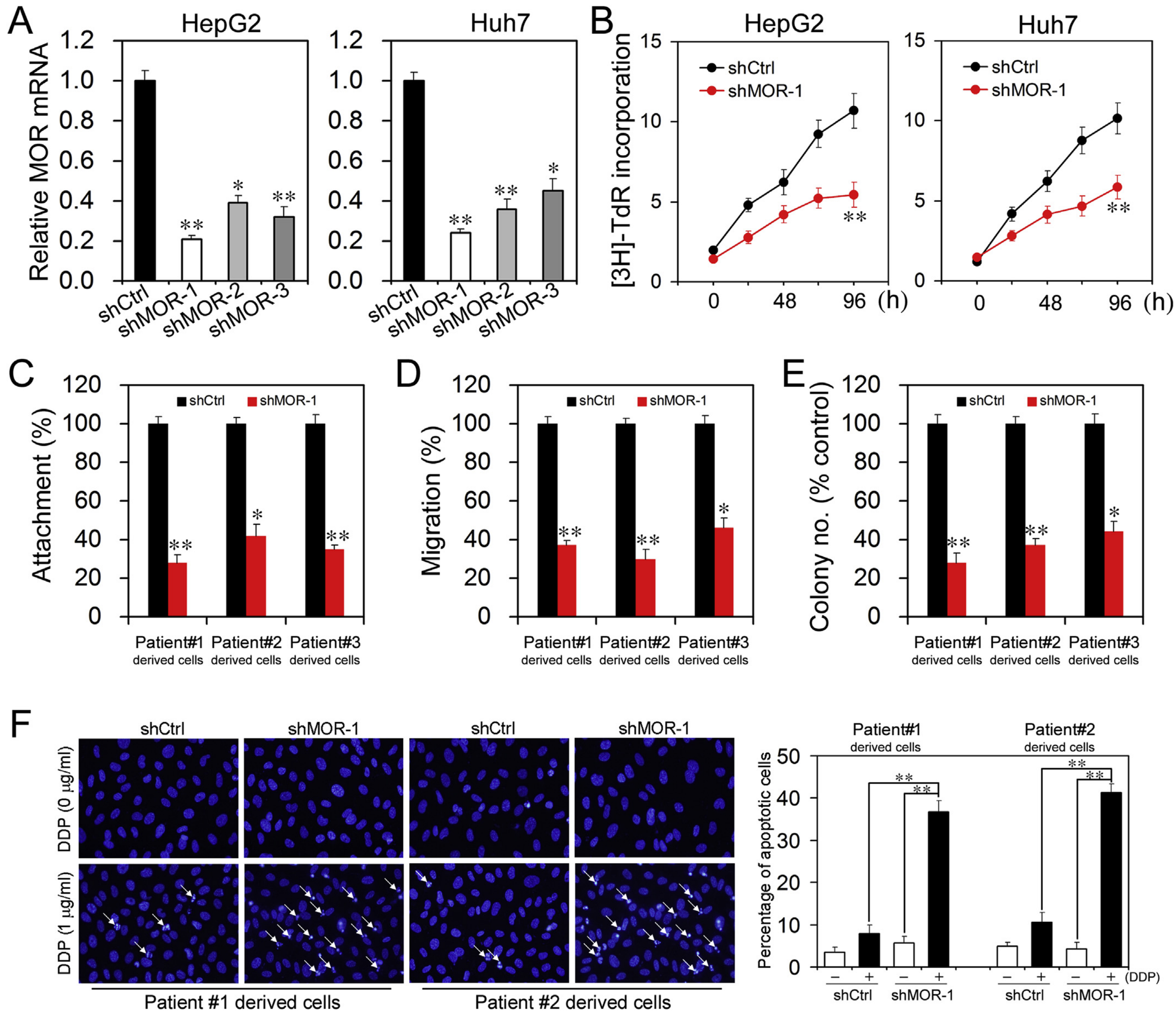


Fig. 4. Attenuated proliferation and enhanced apoptosis due to MOR knockdown. (A) Real-time PCR analysis of relative MOR mRNA expression in HepG2 and Huh7 cells following transfection with control shRNA (shCtrl) or shRNAs targeting MOR (shMOR-1, shMOR-2 and shMOR-3). (B) MOR-silenced HepG2 cells and Huh7 cells were cultured from 24 to 96 h followed by incubation with [^{3}H]-thymidine for 4 h. Data are representative of at least 3 separate experiments. (C) Reduction in cancer cell attachment to matrigel due to MOR knockdown. (D) Cancer cell migration through transwell was suppressed by MOR knockdown. (E) Inhibition of cancer cell colony formation via MOR knockdown. (F) Representative microscope fields and comparative quantification of apoptosis in vehicle-treated or cisplatin (DDP)-treated HCC cells transfected with shCtrl or shMOR-1. Arrows indicate apoptotic cells.

3.6. Experiments in vivo show that MOR gene knockdown can significantly inhibit tumor growth, prolonging life of tumor-bearing mice and that MOR affect CSC tumorigenesis by regulating the NFAT signal pathway

An amount equivalent to 1×10^6 of MOR silenced or control CSCs from 2 patients were injected subcutaneously into nude mice and tumor sizes were measured for 30 d. Obtained data show that MOR knockdown can significantly suppress tumor growth (Fig. 6A) and reduce the tumor weight on day 30 (Fig. 6B). Moreover, mice bearing MOR-silenced tumors can exhibit better survival than those bearing control HCC tumors (Fig. 6C).

Then, the transcriptome was compared between MOR-silenced and control HCC tumors from these patients. The analysis indicates that the expression of AP-1, NF B, NFAT, STAT, SMAD, PI3K, MAPK and NOTCH are increased in the control tumors compared to MOR-silenced tumors, among which NFAT expression levels are elevated most dramatically (Fig. 6D). We further examined the downstream target genes of NFAT and found that the expression of MAPK, COX2, c-Myc and WNT were significantly inhibited by MOR knockdown (Fig. 6E), demonstrating that MOR can regulate HCC proliferation and migration via the NFAT pathway (Fig. 6F).

4. Discussion

All opioid receptor family members are G protein-coupled receptors. The μ-opioid receptor (MOR) is the main member of the opioid receptor super family, which plays an important role in relieving acute post-operative pain and chronic pain. MOR agonists are commonly used not only as analgesics during cancer surgery but also to relieve cancer pain in patients with late malignant tumors. Besides, opioid receptors have been reported to affect cardiovascular and immune systems significantly [20]. MOR is expressed not only in the nervous system, but

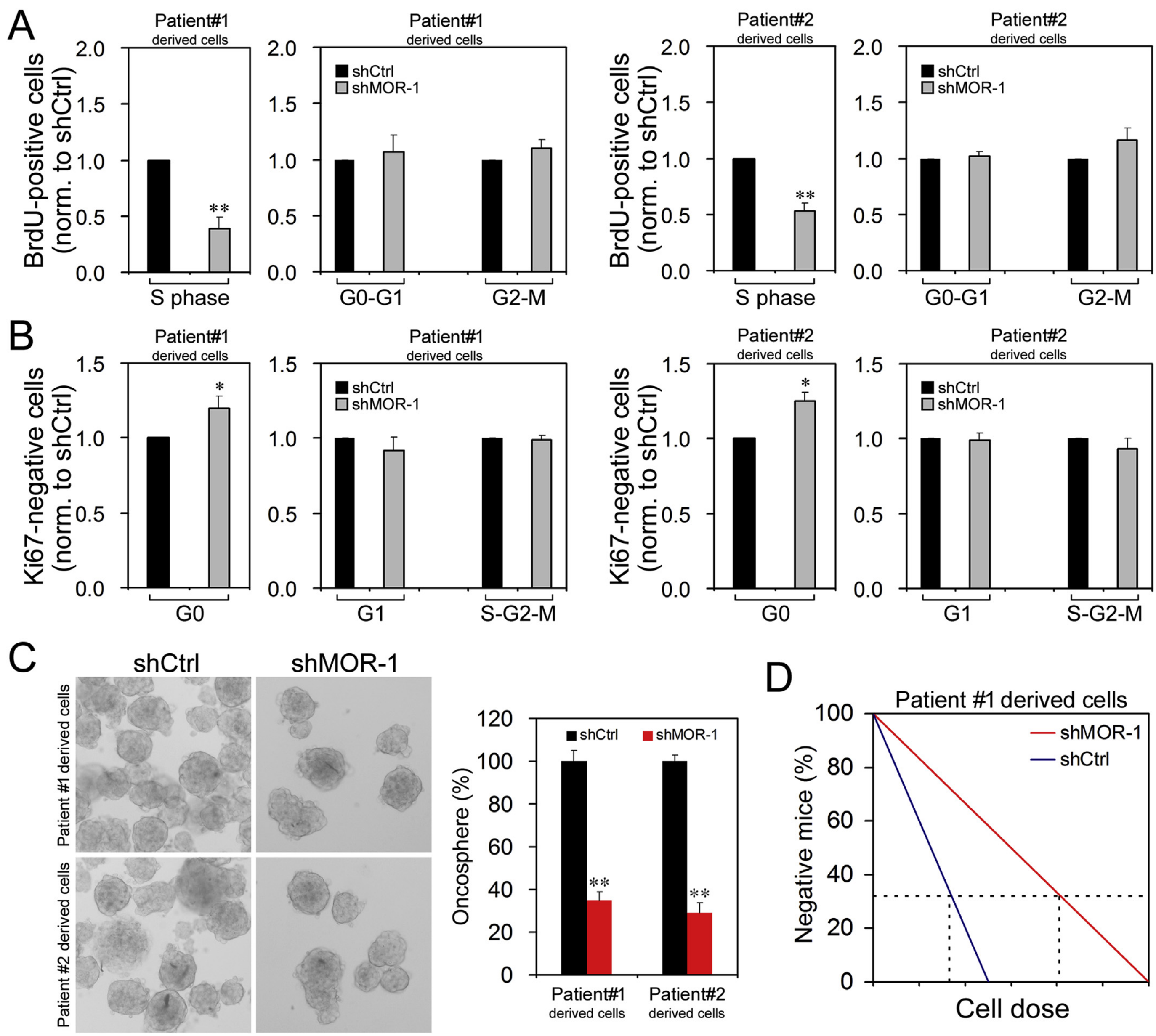


Fig. 5. Impairment of cancer cell self-renewal by MOR knockdown. (A) Quantification of BrdU staining in 3 biological samples with two replicate experiments each. (B) Flow cytometric profile of Ki67 expression in control and shMOR-1 cells (n = 4 biological samples with 2–3 replicates each). (C) Oncosphere numbers were decreased following MOR-silencing. (D) Tumor formation capability was attenuated following MOR-silencing.

also in peripheral tissues, such as squamous cells of the lung, colon, prostate, and breast [7–9]. The role of MOR in tumorigenesis has drew a great deal of attention in recent years.

CSCs have been identified in many solid tumors, including lung, colon, prostate, liver and breast cancers [21–23]. CSCs display stem characteristics such as self-renewal and differentiation. These cancer-initiating cells are key to promoting tumor growth [10,24]. Several surface makers have been identified in liver CSCs. Our results revealed that MOR may serve as a marker of CSC in liver cancer. Single-cell culture analyses showed that MOR + cells had self-renewal ability and differentiation potency. Cell proliferation revealed the higher malignant potency of MOR + cells. These findings strongly suggest that MOR + cells in HCC possess CSC characteristics.

CD133 has been identified as a specific surface marker of stem cells and cancer stem cells (CSC), and thus CD133 can be used to isolate stem cells and precursor cells [25,26]. We found that CD133 was positively correlated with the amount of MOR in cell lines and human tissues. The positive correlation between CD133 and MOR demonstrated that MOR promotes HCC progression by CSC regulation. Analysis of the relationship between opioid receptors and CSC is a future research objective of our team.

Our results indicate that NFAT signaling is activated in MOR + cells and suppressed by MOR knockdown. Moreover, MOR knockdown can also suppress tumor growth and reduce tumor weight. Mice bearing MOR-silenced tumors exhibited better survival than those bearing control HCC tumors. These results indicate that targeting MOR-NFAT signaling can be a potential and novel strategy for future clinical hepatocarcinoma treatment.

Recent epidemiologic studies have indicated that opioid receptors shall promote tumor formation and shall be associated with neoplasm recurrence and metastasis. Opioid receptors can promote HCC progression, both directly and indirectly. Activation of opioid receptors will directly regulate the function of hepatic stellate cells, leading to liver cirrhosis and play an important role in cholestasis, which

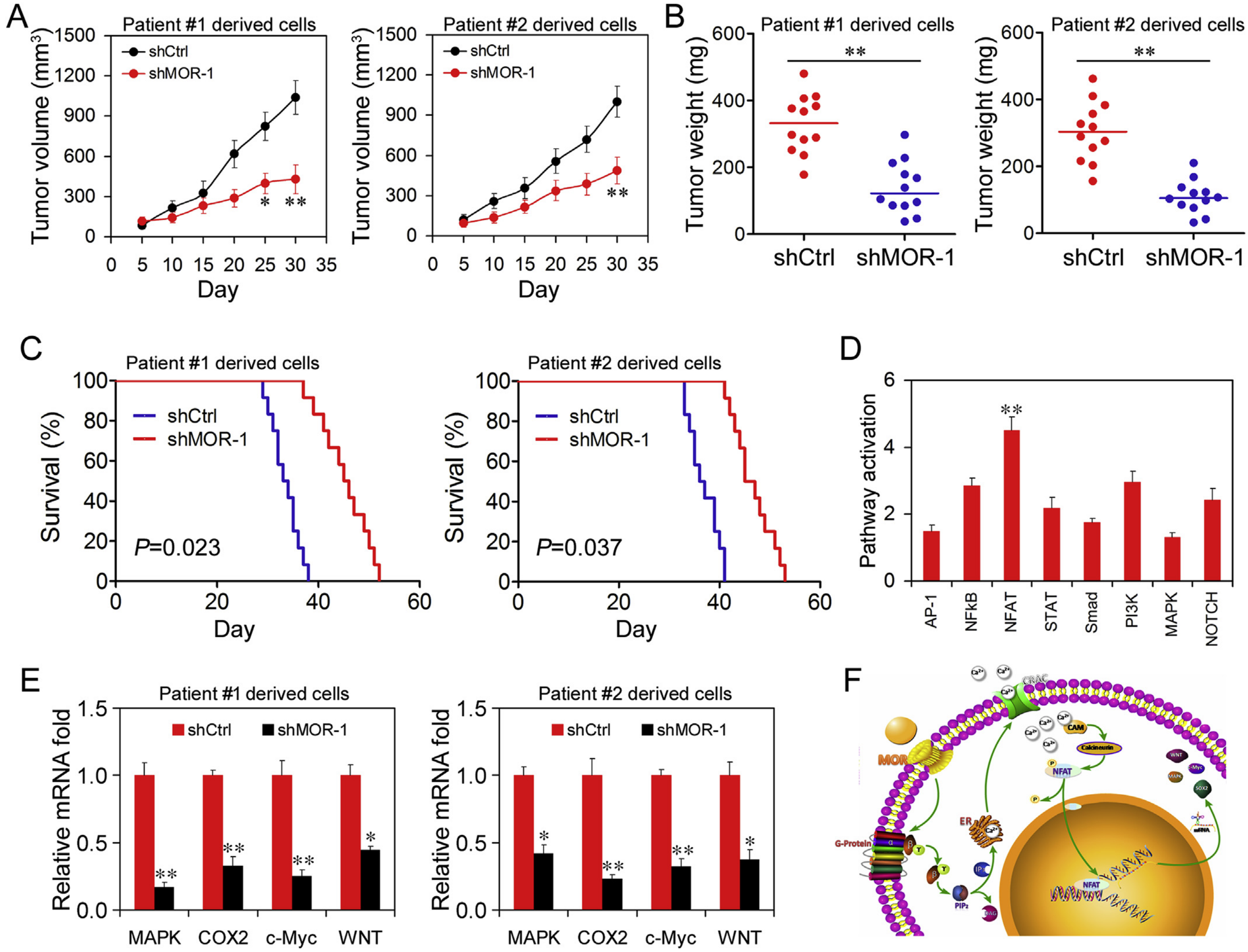


Fig. 6. Tumor growth of HCC cells was inhibited by MOR knockdown. (A–B) Tumor growth (A) and weight (B) were reduced by MOR knockdown *in vivo*. Tumor cells from 2 patients were inoculated to nude mice (n = 12). Tumor growth was monitored and tumor weight was determined on day 30. (C) Survival of mice was increased by MOR knockdown. (D) Pathway Array analysis of gene expression from shCtrl and shMOR-1 patient #1 tumor cells in each group. (E) NFAT downstream target genes were detected via Q-PCR in MOR-silenced patient #1 and patient #2 tumor cells. (F) A model chart described the interaction between MOR and the NFAT pathway.

indirectly promotes HCC formation [27,28]. As indicated by the current study, MOR silencing can significantly reduce HCC cell proliferation, attachment, migration, and cloning ability, which, in turn, promotes the survival of tumor-bearing mice. Besides, shMOR-1 increases the HCC apoptosis rate and enhances the efficacy of chemotherapy. A previous study has shown that reduced *MOR* expression in HCC cells inhibited tumor growth via the MKK7-JNK signal pathway [29]. Reducing *MOR* expression in lung cancer cells by siRNA prevent lung metastasis in 75% of tumor-bearing mice, and treatment with opioid receptor inhibitor MNTX in mice with lung cancer cells can inhibit cancer cell proliferation [30]. These studies predict that MOR present the potential of a new target in cancer therapy.

Methylnaltrexone (MNTX) is a selective peripheral μ-type opioid receptor antagonist. It counters the peripheral effects of opiates while preserving centrally mediated analgesia [31]. Studies investigating the effect of MOR in cancers can help clinicians to use opioids more appropriately. We can use opiates to relieve cancer pain, while using MNTX to inhibit opiate-induced cancer progression. Currently, research studies aiming to reduce side effects of opioids promoting tumor growth by using opioids in combination with MNTX are underway [32]. This will also be a main focus of our future studies which will be directed at using medication effectively.

Conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

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