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MOR/ERK Signalling Pathway Promotes the Malignant Progression of Residual Hepatocellular Carcinoma Cells After Insufficient Radiofrequency Ablation

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Abstract

Objective: Radiofrequency ablation (RFA) is the first-line of treatment for unresectable early hepatocellular carcinoma (HCC). However, the accelerated malignancy progression of residual HCC cells post-RFA pose a major obstacle to its clinical application. The mechanisms underlying the increased malignancy of residual HCC cells following RFA require further investigation.

Methods: In the present study, HepG2 cells, an established human HCC cell line, were subjected to repeated heat treatment to simulate residual HCC cells after RFA, surviving cells were designated as HepG2-H cells. Malignancy parameters and μ-opioid receptor (MOR) expression levels were compared between HepG2 and HepG2-H cells. RNA sequencing was subsequently performed to identify differentially expressed genes (DEGs) between the two groups of cells, and pathways enrichment analysis was conducted to investigate signaling pathways potentially driving HepG2-H cells malignancy.

Results: HepG2-H cells exhibited significantly elevated malignancy compared to HepG2 cells, as evidenced by enhanced proliferation, migration, and colony formation. MOR expression was significantly upregulated in HepG2-H cells. RNA sequencing revealed that DEGs between the two groups were predominantly enriched in cancer pathways. Furthermore, phosphorylated ERK1/2 (P-ERK1/2) levels were 1.18-fold higher in HepG2-H cells, and the inhibition of MOR activity could reduce both the level of P-ERK1/2 and the malignancy of HepG2-H cells.

Conclusion: Our findings suggest that the activation of MOR/ERK signaling pathway contributes to the malignant progression of residual HCC cells post-RFA. Targeting MOR may offer a novel therapeutic strategy to mitigate tumor recurrence following RFA.

Keywords: μ-opioid receptor, hepatocellular carcinoma, radiofrequency ablation, ERK

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third-leading cause of cancer-related mortality in the world.[1] While advancements in early detection and therapeutic strategies have improved curative potential for early-stage HCC, postoperative outcomes remain suboptimal, primarily due to persistently high rates of metastasis and recurrence.

Radiofrequency ablation (RFA) has become a favourable treatment modality for small HCCs because of its minimal invasiveness, low postoperative pain, fast recovery, and short hospitalization.[2] Local control rates equivalent to hepatic resection can be reached by RFA alone when treating small HCCs (<3 cm) in favourable locations. However, local tumour progression and recurrence rates with RFA monotherapy increase sharply when treating larger lesions (>3 cm).[3,4] Relevant research results indicate that the recurrence rate of HCC after RFA can be as high as 25%.[5] Furthermore, incomplete ablation may exacerbate the aggressive progression of residual liver tumor.[6,7]

Opioids are widely used for the management of cancer and surgical pain in the perioperative period. Opioids and opioid peptides selectively bind to the opioid receptors. Classical opioid receptors are seven transmembrane G protein-coupled receptors (GPCRs) and have three major receptor subtypes: μ (MOR), δ (DOR) and κ (KOR). They have roles in pain relief, cell proliferation, immune function, and emotional modulation.[8] While MOR overexpression has been linked to tumorigenesis in cancers like lung, breast, and colon,[9–13] its specific contribution to HCC recurrence after RFA has not been systematically investigated. Previous studies on post-RFA malignancy largely focused on pathways such as PI3K/AKT, HIF-1α/VEGF, or autophagy,[6,14] leaving MOR-mediated mechanisms unaddressed.

GPCRs and growth factor receptor pathways lead to activation of the ERK/MAP kinase phosphorylation cascade.[15] In pancreatic ductal adenocarcinomas cells, Joran et al. found that somatostatin receptor 2 and MOR were significantly co-localized and the heterodimer activated EGFR-1 and ERK1/2 and then promoted cell metastasis.[16] In HCC, ERK hyperactivation is a hallmark of aggressive phenotypes.[17] However, it remains to be further studied whether the accelerated malignancy of residual HCC cells after RFA is related to the upregulation of MOR and subsequent the activation of ERK pathway.

In the present study, HCC cells survived from heat treatment were used to simulate the residual cells after RFA, untreated HCC cells were used as control. The malignancy of two groups of cells and the level of MOR were determined. Subsequently, RNA sequencing was used to detect differentially expressed genes (DEGs) in the two groups of cells,

predicting the signaling pathways that may be involved in the accelerated malignancy of HCC cells. Finally, the mechanism of accelerated malignancy of residual HCC cells after RFA was clarified.

Materials and Methods

Cell Culture and Heat Treatment

The HepG2 cells, a well-established human HCC cell line, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in high-glucose DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco), 100 IU/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. When the cells reached 80% confluence, trypsin was used to digest the cells for passaging or cryopreservation.

Heat treatment was used to mimic insufficient RFA to prepare residual HepG2 cells after RFA, as described previously.[14,18] Briefly, HepG2 cells was seeded into 6-well plates at a density of 5×10^4 cells/well. After 24 hr, the plates were sealed and submerged in a 47°C water bath for 5 min, and surviving cells were cultured *in vitro* into 80% confluence. Then, they were seeded into 6-well plates again and exposed to above heat treatment for 10 min and recovered again. Subsequently, the cells were sequentially exposed to above heat treatment for 15, 20, and 25 min. Cells survived after last heat treatment were used as residual cells after insufficient RFA and designated as HepG2-H cells.

Cell Viability Assay

Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. HepG2 and HepG2-H cells were seeded in 96-well plates at 1×10^4 cells/well. Following 24, 48, and 72 hr of incubation, 10 μl of MTT reagent (coolaber technology Co., Ltd., Beijing, China), 5 mg/ml dissolved in phosphate-buffered saline (PBS), was added to each well, with subsequent incubation for 4 hr at 37°C. The resulting formazan crystals were dissolved by adding 100 μl DMSO to each well, followed by gentle agitation for 10 min. Optical density (OD) of each well at 490 nm was recorded by a microplate reader (Cytation 5 Imaging Reader, BioTek Instruments, Inc., Winooski, VT, USA). Cell viability (%) was calculated as: (OD_{490} of HepG2-H/OD_{490} of HepG2) × 100%.

After HepG2-H cells was treated with 10 μM Morphiceptin, a MOR agonist, or 25 μM Naloxone, a MOR inhibitor (all purchased from MedChemExpress, Shanghai, China) for 12, 24, and 48 hr, the proliferation of HepG2-H cells was determined using the same methods as described above.

Colony Formation Assay

HepG2 and HepG2-H cells were plated in 6-well plates at 1×10^3 cells/well for adherent culture. Following 10–14 days incubation until visible colony formation, cells were fixed with 4% (w/v) paraformaldehyde (Solarbio, Beijing, China) for 30 min and stained with 0.1% (w/v) crystal violet for 20 min at room temperature. After washing three times with PBS, the stained colonies were imaged using an inverted microscope (Ti-U, Nikon, Tokyo, Japan) and quantified by manual counting. Colony formation efficiency (%) was calculated as: (number of colonies/number of seeded cells) × 100%.

Scratch Wound Healing Assay

HepG2 and HepG2-H cells were seeded in 35 mm culture dishes at a density of 10^4 cells/dish. When the cells reached 90% confluence, a straight scratch was made with a 10 μl sterile tip. Subsequently, the cells were cultured *in vitro*, and cells spreading into the wound area were photographed per 24 hr until the scratch wound was completely healed.

During scratch wound healing assay, HepG2-H cells were treated with 10 μM Morphiceptin, or 25 μM Naloxone, the effect of these agonists or inhibitors on the migration of HepG2-H cells was determined.

Western Blot

HepG2 and HepG2-H cells were cultured in 35 mm culture dishes for 24 hr, then, culture medium was discarded, and 100 μl radio immunoprecipitation assay lysis buffer (RIPA) containing a protease inhibitor (YangGuangBio, Beijing, China) was added into the culture dishes to lyse cells. After cells lysate incubated at 80°C for 10 min, total proteins from cells were separated by SDS-PAGE and electrotransferred onto a polyvinylidene fluoride membrane. The membrane was blocked with 5% skim milk and incubated overnight with rabbit anti-human polyclonal antibodies to MOR (Abmart, Shanghai, China), and mouse anti-human monoclonal antibodies to Tubulin (Quayad, Beijing, China) at 4°C. After washed three times with Tris buffered saline with Tween-20 (TBST) (YangGuangBio), the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (H&L) (Quayad) at room temperature for 1 hr. Immunoreactive protein bands on the membrane were developed with ECL Plus (YangGuangBio) according to the manufacturer's protocols, and the band density was analyzed using the ImageJ software.

After HepG2-H cells were treated with 10 μM Morphiceptin or 25 μM Naloxone for 24 hr, western blot was used to determine the level of ERK1/2 and Phospho-Erk1 (Thr202/Tyr204)/Erk2 (Thr185/Tyr187) (P-ERK). Rabbit anti-human monoclonal antibodies to ERK1/2, to P-ERK1/2 were purchased from Beyotime (Shanghai, China), and Tubulin was also used as endogenous control protein.

Immunocytochemistry

HepG2 and HepG2-H cells were cultured overnight on glass coverslips (14 mm in diameter), which were placed at the bottom of 24-well culture plates. Then, cells were washed with PBS and fixed with 4% (v/v) paraformaldehyde (Solarbio) at room temperature for 10 min. After washed thrice with cold PBS, the cells were blocked with 10% (v/v) goat serum (dissolved in PBS) (ZSGB-BIO, Beijing, China) at room temperature for 1 hr, then, incubated overnight with rabbit anti-human monoclonal antibody to MOR (Affinity Biosciences, Changzhou, China) (1:250) at 4°C. Subsequently, the coverslips were washed thrice with Phosphate Buffered Saline with Tween 20 (PBST), each for 5 min. The cells were stained in the dark with Alexa Fluor 594 -conjugated goat anti-rabbit IgG (H&L) (Quayad) at room temperature for 1 hr, washed thrice with PBS, and stained with 1 μg/ml DAPI (Abcam, Shanghai, China) for 1 min. After washed with PBS, the cells were mounted under a coverslip in antifade mounting medium

(YangGuangBio), and allowed to dry overnight at room temperature in the dark. Fluorescence was observed under an A1R confocal laser-scanning microscope (Nikon, Tokyo, Japan).

RNA Sequencing

Total RNA was isolated from HepG2 and HepG2-H cells using the Trizol Reagent (Takara, Beijing, China), delivered to Major Bio (Shanghai, China) for RNA sequencing on NovaSeq X Plus platform. DEGs analysis was conducted using the 'DESeq2' R package, with a cutoff criteria of $|\log_2$FoldChange ($\log_2$FC)$| > 1.0$ and P-value < 0.05.

Statistical Analyses

All experiments were repeated at least three times and values are shown as mean ± standard deviation (SD). The single factor variance analysis was used to compare the significance between groups, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. $P < 0.05$ was considered statistically significant.

Results

RFA Increased Malignancy of Residual HepG2 Cells

Both HepG2 and HepG2-H cells are epithelial-like cells when cultured *in vitro*, with no significant differences in size and morphology (Fig. 1A).

The proliferative capacity, clone-formation and migration ability of HepG2 and HepG2-H cells were evaluated using MTT assay, clone formation assay and scratch wound healing assay, respectively. As shown in Fig. 1B, HepG2-H

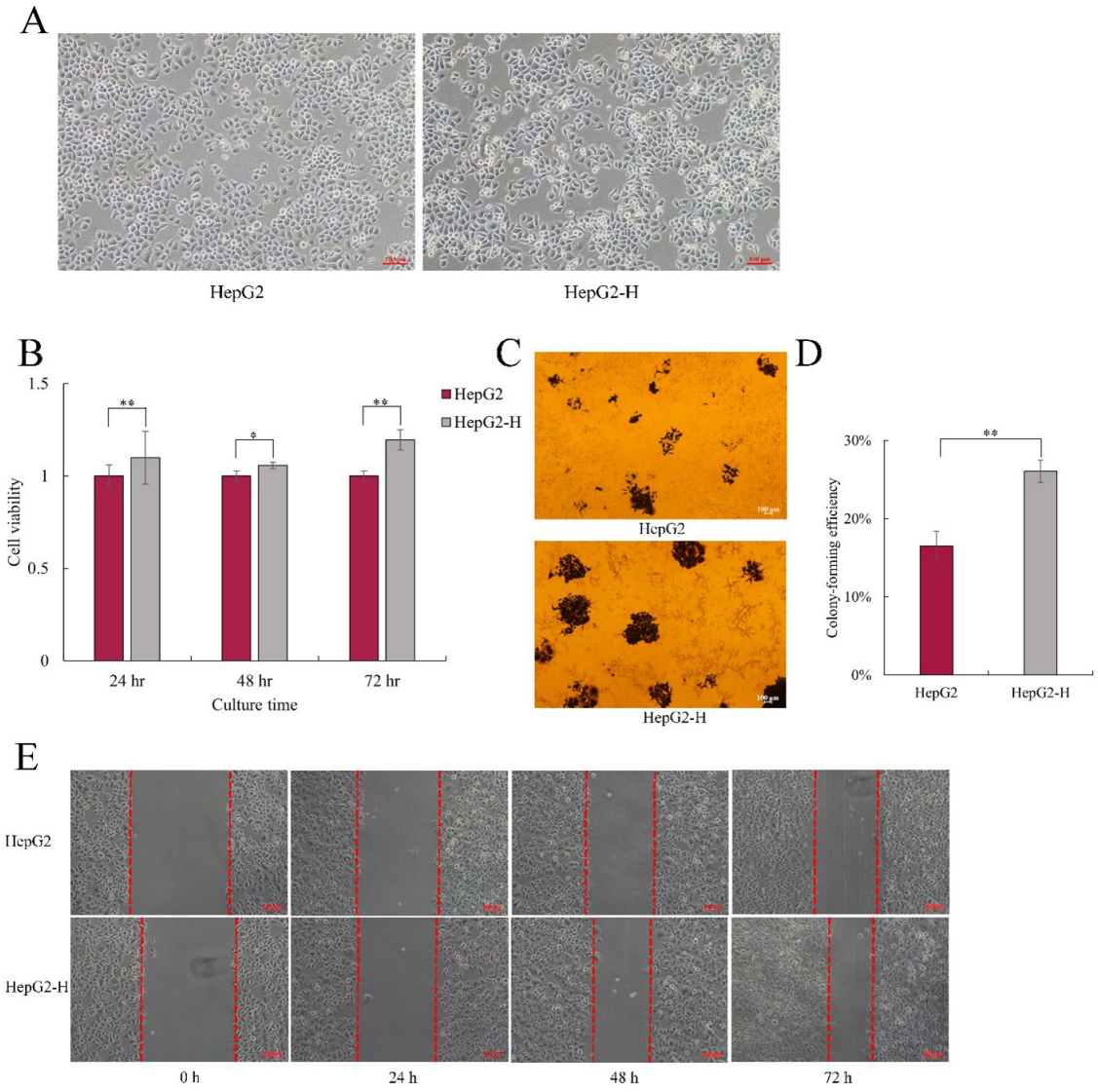

Fig. 1 **Cell viability, clone formation and migration ability of HepG2 and HepG2-H cells. (A: Morphological features of two groups of cells under *in vitro* culture. B: The viability of two groups of cells was determined by MTT assay. Viability was calculated as (OD_{490} of HepG2-H cells/OD_{490} of HepG2 cells) × 100%. C and D: Cells were stained using crystal violet, the number of clones was counted, and the clone formation rate was calculated as: (the number of clones/the number of seeded cells) × 100%. E: The migration of two groups of cells was determined by scratch wound healing assay. All experiments were carried out in triplicates, and the data are shown as the mean ± SD. The single factor variance analysis was used to compare the significance between groups: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Difference with $P < 0.05$ was considered statistically significant.)**

cells exhibited significantly enhanced viability compared to HepG2 cells at 24 hr (1.1-fold, P = 0.007), 48 hr (1.06-fold, P = 0.01) and 72 hr (1.20-fold, P = 0.002) of *in vitro* culture, suggesting that insufficient RFA promotes residual HCC cell proliferation. Colony formation assays revealed a striking increase in the number of HepG2-H colonies (P = 0.001), indicative of superior clonogenic survival (shown in Fig 1C and D). Furthermore, scratch wound healing assays demonstrated accelerated wound closure in HepG2-H cells, with significantly reduced scratch widths observed at 72 hr compared to controls (Fig. 1E), highlighting RFA-induced migration enhancement. Collectively, these findings demonstrate that insufficient RFA exacerbates the malignant potential of residual HCC cells.

RFA Elevated the Level of MOR in Residual HepG2 Cells

Proteins were extracted from the two groups of cells, western blot was used to determine the amount of MOR. As shown in Fig. 2A and 2B, MOR expression was 1.6-fold higher in HepG2-H cells compared to HepG2 controls (P = 0.024), as quantified by densitometric analysis of western blot bands.

The increased expression of MOR in HepG2-H cells was further validated by immunocytochemistry. As shown in

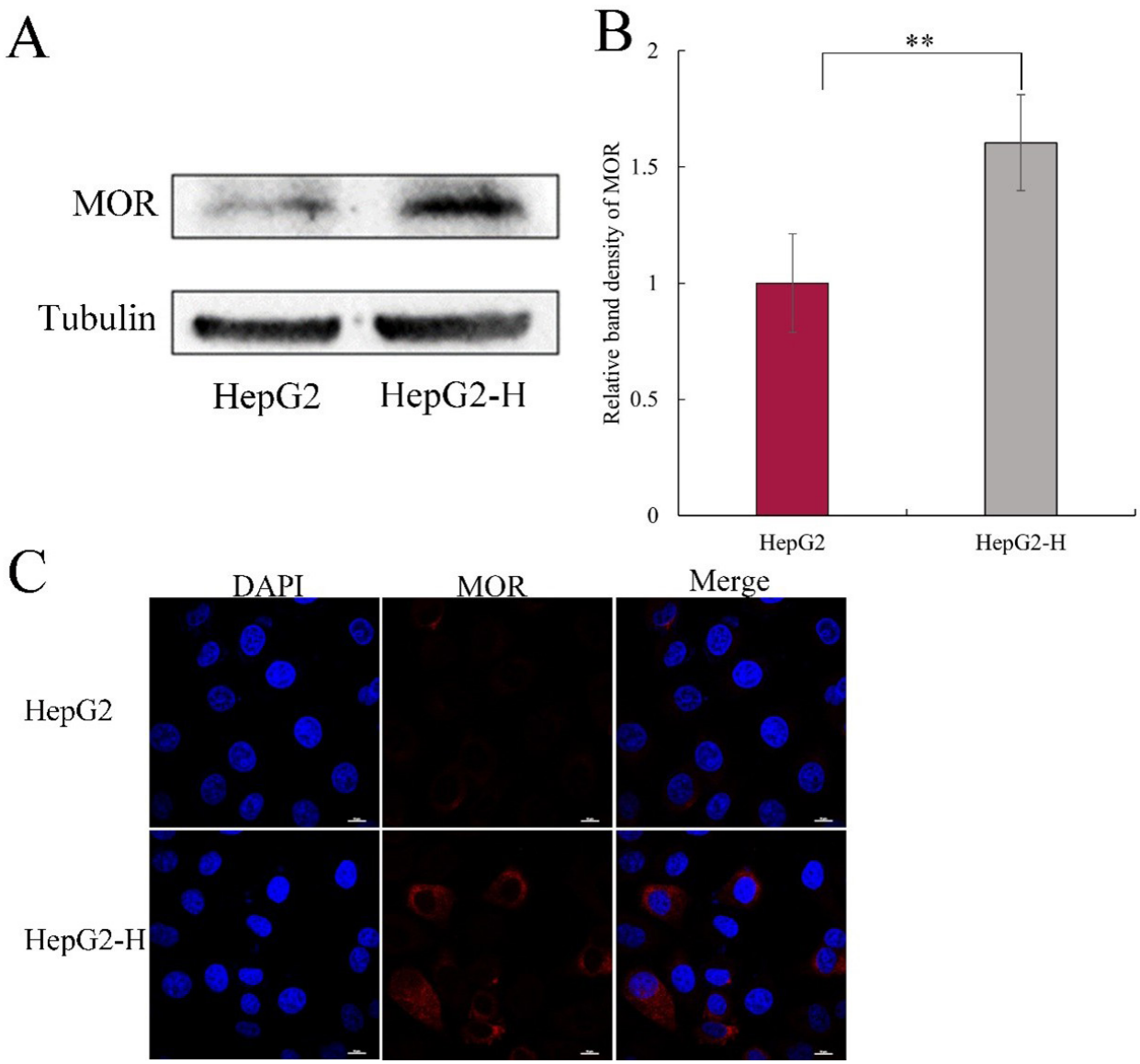

Fig. 2 **The amount of MOR in HepG2 and HepG2-H cells. (A and B: The amount of MOR in two groups of cells was determined by western blot, Tubulin was used as endogenous control protein, and the band density was analyzed by ImageJ software. The band density of MOR was firstly compared with that of β-Tubulin, then, the ratio was normalized to HepG2 group. C: The amount of MOR in two groups of cells was determined by immunocytochemistry, bar = 25 μm. All experiments were carried out in triplicates, and the data are shown as the mean ± SD. The single factor variance analysis was used to compare the significance between groups: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Difference with $P < 0.05$ was considered statistically significant.)**

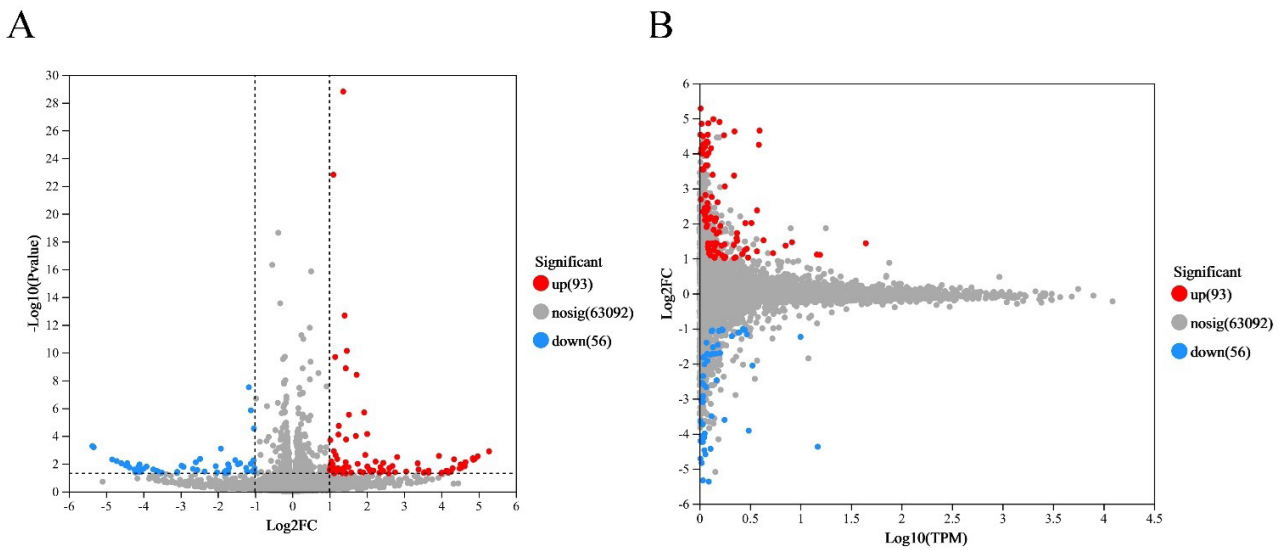

Fig. 3 **RNA Sequencing was used to determine DEGs in HepG2 and HepG2-H cells. (A. Volcano plot of DEGs, red and blue dots indicate significantly up- and down-regulated genes, respectively (|log2FC| > 1.0 and *P*-value < 0.05), and gray dots indicate genes without significant differences in expression. B. MA plot of DEGs.)**

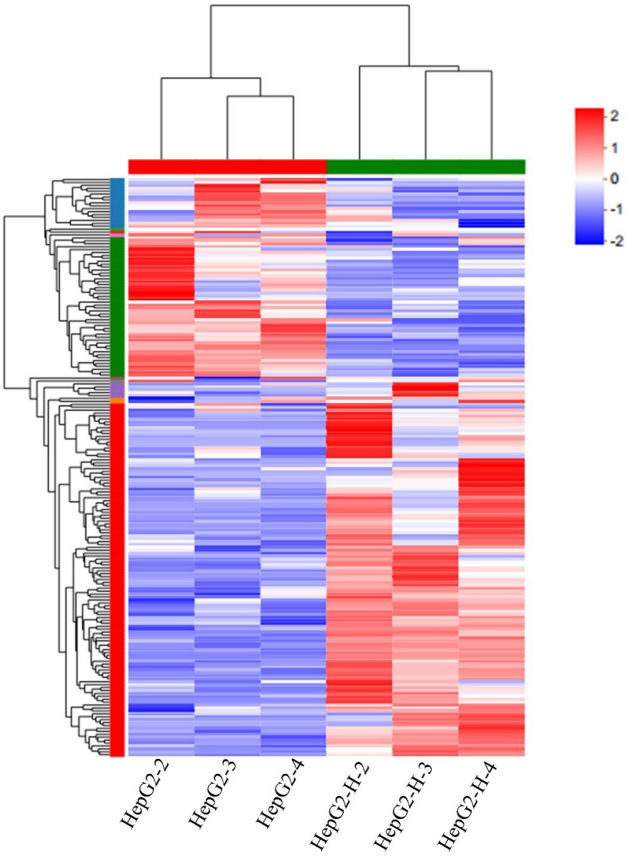

Fig. 4 **Heatmap was generated by bi-directional clustering analysis of DEGs between HepG2 and HepG2-H cells.**

Fig. 2C, fluorescence signals were predominantly localized to the cell membrane, consistent with the membrane-bound nature of MOR as a GPCR. Notably, fluorescence intensity in HepG2-H cells was apparently stronger compared to HepG2 controls, corroborating MOR upregulation. These findings collectively suggest that insufficient RFA enhances MOR expression, which may mechanistically contribute to the augmented malignancy of residual HCC cells.

RFA Activated Cancer-Related Signal Pathways

RNA sequencing was performed to investigate the impact of insufficient RFA on global genes expression profiles in HepG2 cells. A Total of 63,092 genes were analyzed, with 149 DEGs identified between HepG2 and HepG2-H cells (93 genes were significantly up-regulated and 56 genes were significantly down-regulated ($|\log_2 FC| > 1.0$ and p-value < 0.05), as shown in Fig. 3A and 3B). Bi-directional hierarchical clustering analysis showed that the DEGs were similar to each other among three biological duplicates, while different between HepG2 and HepG2-H cells (Fig. 4). GO enrichment analysis showed that DEGs were predominantly localized to the plasma membrane, related to cell-cell adhesion and involved in the proliferation of fibroblasts (Fig. 5A). KEGG pathway analysis showed that DEGs were prominently enriched in cancer pathways (Fig. 5B).

MOR Activated ERK Signaling Pathway

Previous studies showed that RFA activates multiple signaling pathways, including MAPK, PI3K-AKT, STAT and TGF-β.[19–21] Our above results showed that MOR was overexpressed in HepG2-H cells. Therefore, we further explored whether the overexpression of MOR could activate these signal pathways. Phosphorylation of ERK can lead to the activation of Ras-Raf-MEK-ERK signal pathway, a classic MAPK signal pathway.[17,22] We quantified ERK1/2 and P-ERK1/2 levels via western blot analysis. As shown in Fig. 6A–6C, the levels of P-ERK1/2 and ERK1/2 were 1.18- (P = 0.04) and 1.29-fold (P = 0.004) in HepG2-H cells compared to HepG2 controls. This result indicated that the overexpression of MOR activated ERK1/2, this maybe contribute to increased malignancy of HepG2-H cells.

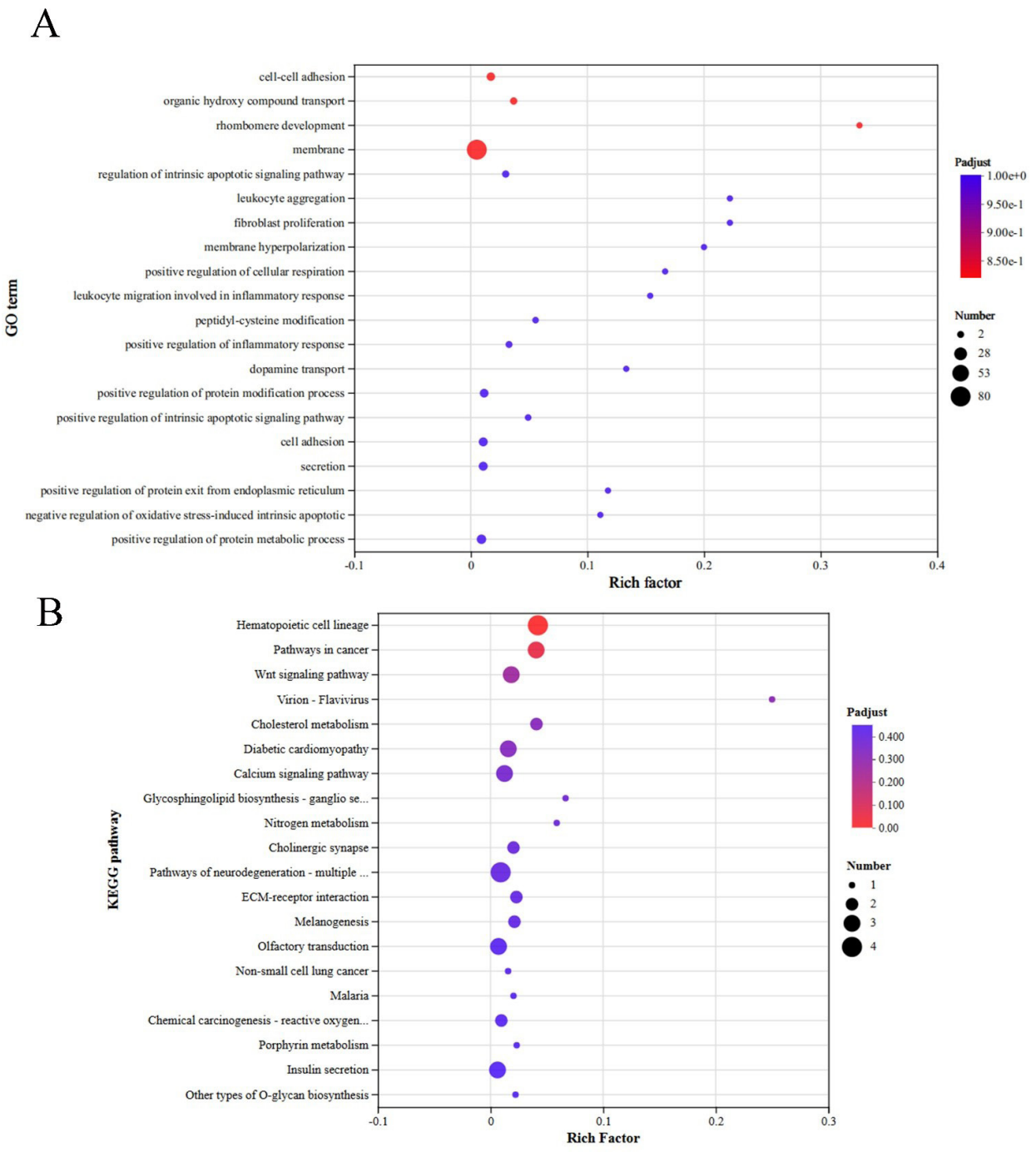

Fig. 5 **GO and KEGG enrichment analysis of DEGs. (A. GO enrichment analysis, B. KEGG signal pathway enrichment analysis.)**

To delineate the role of MOR activity in ERK1/2 activation, HepG2-H cells were treated with the MOR agonist Morphiceptin (10 μM) or the MOR antagonist Naloxone (25 μM) for 24 hr. As shown in Fig. 6D–6F, Naloxone treatment significantly decreased P-ERK levels by 52% (P = 0.000), without altering total ERK1/2 expression, confirming MOR activity is essential for ERK1/2 phosphorylation. In contrast, Morphiceptin treatment for 24 hr did not further elevate ERK1/2 or P-ERK1/2 levels in HepG2-H cells (Fig. 6G–I). This result may be due to the fact that the ERK signal pathway was already activated in HepG2-H cells, and further activation of MOR could not lead to a further increase in the activity of ERK.

Inhibiting MOR Activity Decreased the Malignancy of Residual HepG2 Cells

Morphiceptin and Naloxone were used to treat HepG2-H cells, viability and migration was used to evaluate the malignancy of HepG2-H cells, the effect of overexpression of MOR on the malignancy of HepG2-H cells was determined.

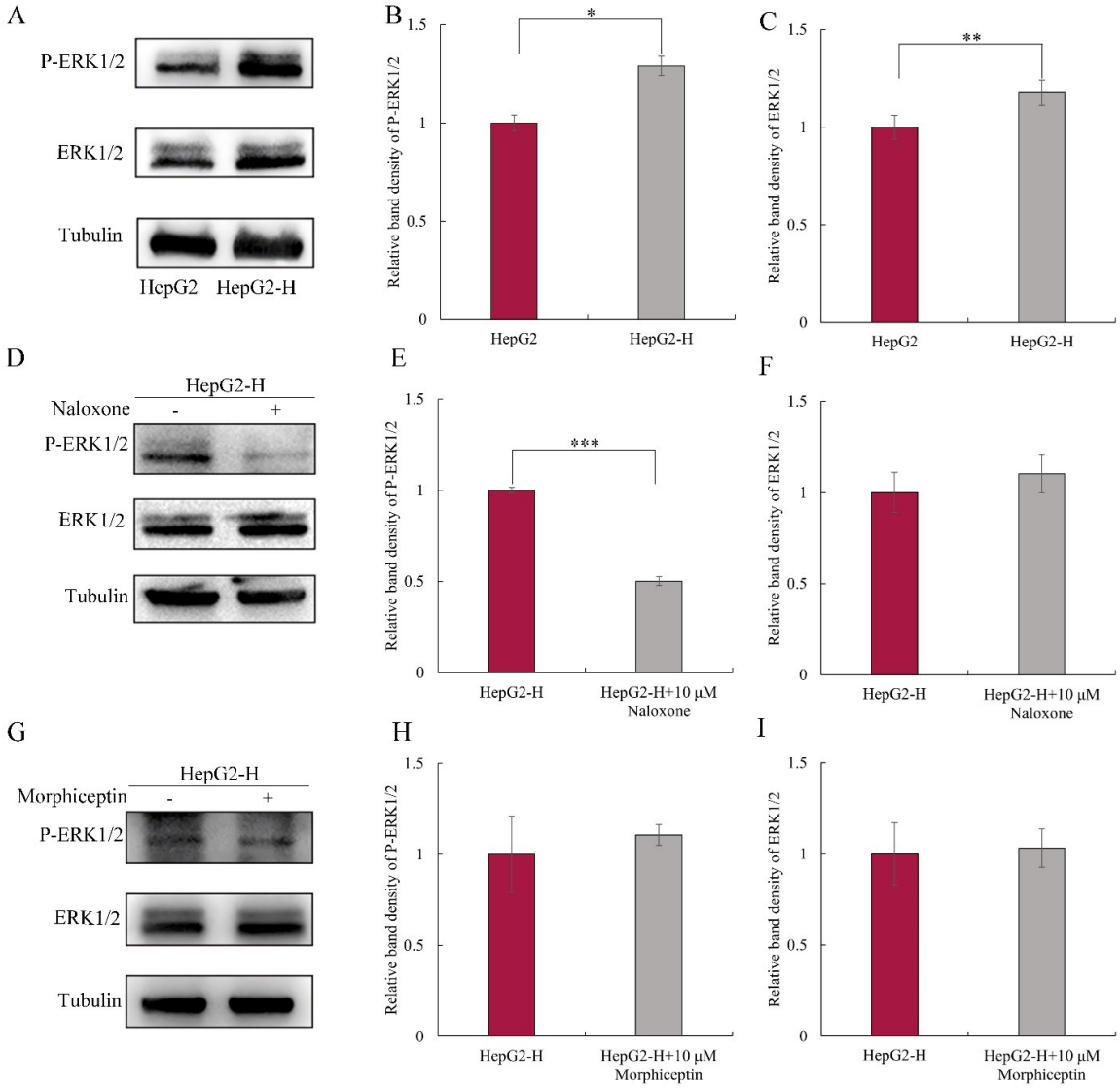

Fig. 6 **The amount of ERK1/2 and P-ERK1/2 in HepG2 and HepG2-H cells treated with MOR agonist/inhibitor (Morphiceptin/Naloxone). (A. Western blot was used to determine the amount of ERK1/2 and P-ERK1/2, and Tubulin was used as endogenous control protein. (B and C) Bands density in (A) were analyzed by ImageJ software. The band density of P-ERK1/2 or ERK1/2 was firstly compared with that of ERK1/2 or β-Tubulin, respectively, then, the ratio were normalized to HepG2 group. D and G: Western blot was used to determine the effect of MOR inhibitor/agonist on the amout of ERK1/2 and P-ERK1/2 in HepG2-H cells, and Tubulin was used as endogenous control protein. E, F, H and I: Bands density in (D) and (G) were analyzed by ImageJ software. The band density of P-ERK1/2 or ERK1/2 was firstly compared with that of ERK1/2 or β-Tubulin, respectively, then, the ratio were normalized to untreated groups. All experiments were carried out in triplicates, and the data are shown as the mean ± SD. The single factor variance analysis was used to compare the significance between groups: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Difference with $P < 0.05$ was considered statistically significant.)**

As shown in Fig. 7A, HepG2-H cells exhibited 1.15-fold ($P = 0.007$) and 1.36-fold ($P = 0.002$) higher viability compared to HepG2 cells at 24 and 48 hr post-treatment, respectively, consistent with our earlier findings. Pharmacological inhibition of MOR with Naloxone (25 μM) significant decreased viability of HepG2-H cells in 24 hr (0.88-fold, $P = 0.004$) and 48 hr (0.72-fold, $P = 0.000$). On the contrary, treatment with a MOR agonist, Morphiceptin, led to a significant increase in the viability of HepG2-H cells in 24 hr (1.31-fold, $P = 0.008$) and 48 hr (1.45-fold, $P = 0.02$). This result indicated that the overexpression of MOR contributed to the increase in the viability of HepG2-H cells.

Naloxone treatment markedly attenuated HepG2-H cells migration (Fig. 7B), underscoring the critical role of MOR in promoting residual HCC cell motility. Morphiceptin treatment, however, could not significantly affect the migration of HepG2-H cells, indicating no effect of further activation of MOR on the migration of HepG2-H cells.

Collectively, these results indicated that MOR overexpression contributes significantly to the enhanced malignancy

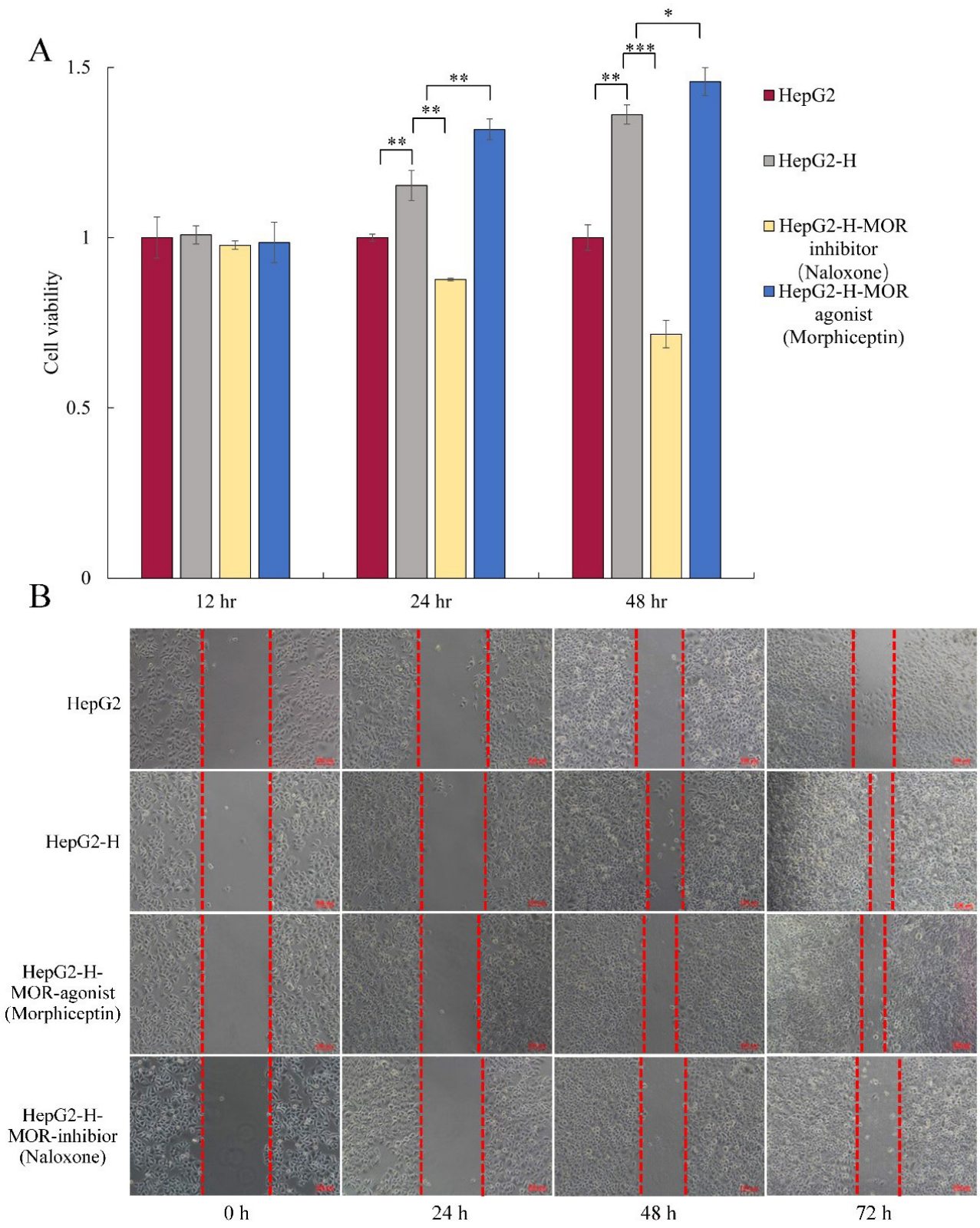

Fig. 7 **The effects of MOR agonist/inhibitor (Morphiceptin/Naloxone) on the proliferation and migration of HepG2-H cells. (A. The effect of MOR agonist/inhibitor on the viability of HepG2-H cells was determined by MTT assay and calculated as: cell viability (%) = (OD_{490} of treated HepG2-H cells/OD_{490} of untreated HepG2 cells) × 100%. B. The effect of MOR agonist/inhibitor on the migration of HepG2-H cells was determined by scratch wound healing assay. All experiments were carried out in triplicates, and the data are shown as the mean ± SD. The single factor variance analysis was used to compare the significance between groups: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Difference with $P < 0.05$ was considered statistically significant.)**

of residual HCC cells post-RFA, at least partially through ERK-dependent proliferative and migratory mechanisms.

Discussion

RFA demonstrates excellent local tumor control and is widely recognized as a minimally invasive, safe and clinically advantageous modality. It plays an increasingly important role in the comprehensive treatment strategy of HCC.[23] However, accumulating experimental and clinical evidence highlights that incomplete RFA inevitably promotes residual tumour recurrence and metastasis.[24] Therefore, it is of great significance to further investigate the mechanism of increased malignancy of residual HCC cells after RFA. In the present study, after HepG2 cells, an established human HCC cell line, repeatedly experienced heat treatment, survived cells, named HepG2-H cells, were used to simulate residual cells after HCC cells experienced RFA.[25] The proliferation, colony formation and migration of HepG2 and HepG2-H cells, which are three important cancer cell malignant characteristics, were compared to evaluate the effect of RFA on the malignant of HCC cells. The results showed that HepG2-H cells was significantly higher than HepG2 cells in proliferation, colony formation and migration, which were consistent with previous reports,[26–28] and confirmed that RFA could promote the malignant of residual HCC cells.

Preclinical studies have associated opioids with cancer progression and overall survival.[13,29] The three main brain receptors that opioids bind to are the MOR, KOR, and DOR in the central nervous system and peripheral organs, which are responsible for a plethora of physiological functions, such as analgesia, respiration, and hormonal regulation. MOR agonists (MORAs) are the most effective analgesics to date and are widely used all over the world. In cancer patients, MORAs are indispensable to pain control. Emerging evidence suggests that MOR overexpression promotes tumorigenic behaviors such as proliferation, adhesion, migration, and tumorigenesis. For example, Lennon et al. found in their studies that the overexpression of MOR could promote the activation of Akt and mTOR, resulting in the tumor growth and metastasis.[30] Mathew et al. found that MOR was highly expressed in human lung cancer tissues and promoted tumor progression.[31] In the present study, we observed membrane-localized MOR overexpression in HepG2-H cells, which mechanistically aligns with their enhanced malignant potential. Therefore, these findings suggest that MOR serves as a critical mediator of residual HCC aggressiveness following RFA.

The MAPK signal pathway responds to both exogenous and endogenous stimuli.[32] There are at least three different MAPK signal pathways, including ERK1/2, c-Jun NH2-terminal kinase (JNK)1/2/3, and p38, that modulate and transduce extracellular signals into the nucleus to induce response genes in mammalian cells.[33,34] For over two decades, the effective inhibition of the ERK has remained a key focus in cancer research and targeted therapy efforts.[35,36] We hypothesized that MOR-mediated ERK activation drives the aggressive phenotype of post-RFA residual HCC cells. The results showed that HepG2-H cells exhibited significant MOR upregulation concurrent with elevated ERK1/2 and P-ERK1/2 levels. This mechanistic link suggests that insufficient RFA promotes residual HCC malignancy through MOR-dependent ERK activation. Indeed, it was found that MOR can stimulate ERK phosphorylation via a CaM- and PKC-dependent pathway.[37]

While our *in vitro* model provides mechanistic insights, the lack of *in vivo* validation remains a limitation. Animal studies are critical to confirm the role of MOR/ERK signaling pathway in residual HCC progression within a physiologically relevant tumor microenvironment. Additionally, we proposed that RFA followed by MOR inhibitor intake maybe is a better treatment protocol for HCC. However, it needs to be clinically evaluated.

Conclusion

Our findings indicate that insufficient RFA promotes residual HCC cell malignancy via MOR/ERK signaling activation. Targeting MOR with pharmacological inhibitors may represent a novel therapeutic strategy to improve post-RFA outcomes and reduce recurrence rates in HCC patients.

Acknowledgments

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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