Upregulation of the δ opioid receptor in liver cancer promotes liver cancer progression both in vitro and in vivo

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Abstract. The δ opioid receptor (DOR), is the first cloned G protein-coupled receptor. Many recent studies on DOR functions have determined that the DOR is involved in the regulation of malignant transformation and tumor progression in multiple cancers. However, it is still unclear if the DOR is involved in the regulation of malignant transformation and tumor progression in hepatocellular carcinoma (HCC). The purpose of our study was to investigate the expression of the DOR in HCC and to determine its effect on progression to further understand the influence of the DOR on the biological characteristics of HCC. Higher expression of DOR was observed in liver tumor tissue/cells compared to normal liver tissue/cells. When DOR gene expression was silenced or inhibited, the proliferation of HCC cells was inhibited, and tumor cells underwent apoptosis, the cell cycle was arrested and tumor cell invasion and migration were significantly decreased. Nude mice inoculated with cells stably expressing low levels of DOR displayed reduced tumor formation rates and reduced tumor growth. In conclusion, DOR is highly expressed in HCC and is involved in HCC progression, suggesting that DOR is a potential target for HCC treatment.

Introduction

Hepatocellular carcinoma (HCC) is a highly malignant disease with extremely poor prognosis. Due to its difficult early diagnosis, high malignancy, and most importantly the inefficiveness of treatments using radiotherapy and chemotherapy, HCC is the third leading cause of cancer deaths worldwide, with more than 600,000 deaths each year (1-3). Conventional surgical resection is still the major treatment strategy for HCC (4,5), however, the 5-year overall survival rate after hepatic resection remains low. A single treatment method cannot satisfy clinical needs (6). Therefore, understanding the molecular mechanisms underlying liver tumor formation, cancer progression, recurrence, and metastasis may contribute to the discovery of more effective intervention methods and liver cancer treatment targets.

The opioid receptor family members are all G protein-coupled receptors (7). The activation of opioid receptors can promote intracellular signal transduction via different pathways to regulate a variety of physiological body functions. Among the three classic opioid receptors, δ opioid receptor (DOR) was the first to be cloned (8,9). The human DOR gene is located on chromosome 6q24-25. The coding region of DOR is 1,119 bp and encodes for 372 amino acid residues (10,11). DOR protein is widely distributed throughout the human body. According to the literature, DOR is present in various human cancers (12-15). Moreover, DOR is involved in malignant transformation or tumor progression. It was shown that the activation of the RTK/PI3K/Akt signaling pathway through DOR can increase the survival rate of NG108-15 cells, which is a neuroblastoma-glioma hybrid cell line (16). The direct activation of DOR affects the invasive ability of the HCT-8/E11 colon cancer cell line (17).

Functional studies of DOR further demonstrated that DOR plays important roles in the formation and progression of other liver diseases, including hepatitis, hepatic fibrosis, liver damage, and cholestatic liver disease (18-20). Our previous study (21) determined that the specific DOR agonist, DADLE, via the activation of DOR on the liver cell membrane, plays a role in protecting liver cells from apoptosis through the mitochondrial apoptotic pathway.

This study aims to investigate DOR expression in HCC and its involvement in tumor progression. Our data suggest that DOR is widely expressed in human HCC tissues and cells and may promote cancer cell growth. The downregulation of DOR can significantly inhibit HCC progression. Our long-term research goal is to use DOR as a diagnostic, prognostic, and treatment response marker for HCC.

Materials and methods

Samples. The surgical specimens from 41 patients who had primary surgical treatment of HCC at Guilin Medical
University Affiliated Hospital between 2009 and 2010 were studied. These surgical specimens included the primary HCC lesion and its corresponding adjacent tissue (2 cm away from the edge of the tumor lesion). All cases were pathologically confirmed, and the patients had no prior treatment before surgery. Forty cases of normal adjacent liver tissues from liver trauma and hepatic hemangioma were used as the control tissues. All of the samples were collected with approval from the Medical Ethics Committee at Guilin Medical University Affiliated Hospital.

**Cell culture.** LO2, HepG2, and Hep3B cells (American Type Culture Collection, USA) were cultured in RPMI-1640 medium (Gibco-BRL, NY, USA) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., UT, USA) and 100 U/ml of penicillin plus 100 U/ml of streptomycin in a 37°C incubator with 5% CO₂ and 95% air. MHCC97-H and MHCC97-L cells (American Type Culture Collection) were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (Hyclone Laboratories) and 100 U/ml of penicillin plus 100 U/ml of streptomycin in a 37°C incubator with 5% CO₂.

**Immunohistochemistry staining.** The paraffin sections were dewaxed with xylene and rehydrated in descending concentrations of ethanol. The endogenous peroxidase was inhibited, and the slides were incubated with antibodies against DOR (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and incubated at 4°C overnight in a humidified container. After washing with PBS three times, the tissue slides were treated with a non-biotin horseradish peroxidase detection system according to the manufacturer's instructions (Dako).

**Immunofluorescence.** Cells in the logarithmic growth phase were digested with 0.25% trypsin. The cells were seeded on coverslips inside the wells at a concentration of 1x10⁵ cells/ml and incubated for 24 h. After the incubation, the old medium was discarded, and the cells were washed three times in PBS. Paraformaldehyde (4%) was used to fix the cells for 30 min, followed by three washes in PBS to remove the excess fixative. Cells were then incubated with 0.5% Triton X-100 for 20 min at room temperature (RT), washed three times with PBS, and blocked using 5% goat serum at RT for 30 min. After removing the serum, a rabbit anti-human DOR monoclonal antibody (1:500 dilution) was added and incubated at 37°C for 2 h. The cells were washed three times in PBS for 5 min each before incubating with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (1:200 dilution) at 37°C for 30 min. After three washes in PBS for 5 min each, all of the coverslips were collected and mounted onto glass slides using glycerol. All of the samples were analyzed using fluorescent microscopy. The primary antibody was omitted in the negative control.

**Cell viability.** Cells during the logarithmic growth stage were digested using 0.25% trypsin. Cells were seeded into a 96-well plate at a concentration of 1x10⁴ cells/ml and incubated at RT for 24 h. After the cells attached, different doses of naltrindole (Sigma, USA) were added to 6-wells for each dose. The negative control group had no naltrindole treatment. The plate was incubated in a 5% CO₂ incubator for 48 h and then 20 µl of MTT (5 mg/ml) was added and incubated in a CO₂ incubator for 4 h at 37°C. After the incubation, the excess liquid was discarded and the cells were incubated with 150 µl of DMSO at RT for 10 min on a shaker. The OD₅₇₀ value was measured using a microplate reader.

**Cell cycle analysis.** Cells were digested in trypsin and centrifuged to collect the cell pellets. The cell pellets were washed three times in PBS, fixed in pre-chilled 70% ethanol and then stored at 4°C overnight. The next day, the cells were washed three times with PBS and resuspend into 100 µl of PBS at a final concentration of 1x10⁶ cells/ml. Comprehensive DNA Stain (500 µl) (RNase 50 mg/ml, propidium iodide (PI) 100 mg/l and Triton X-100 1 ml/l) was added to the cell solution followed by light protected incubation at RT for 15 min. The labeled cells were analyzed using flow cytometry.

**Apoptosis detection.** During the early stage of apoptosis, the loss of cell membrane symmetry causes phosphatidylserine (PS) to be exposed on the outside of the cell membrane. Annexin V-FITC can bind specifically with PS on the intact cell membrane. Therefore, Annexin V-FITC staining can be used to detect early stage apoptotic cells in a faster and more sensitive way. The cells were digested in 0.25% trypsin and then incubated at a concentration of 1x10⁶ cells/ml with 5 µl of Annexin V-FITC and PI. The cells were further incubated protected from light at 37°C for 15 min and analyzed using flow cytometry.

**Hoechst 33342 staining.** The cells were cultured for 24 h in a 6-well plate with poly-L-lysine pre-coated coverslips. After apoptosis induction, the medium from the cell culture was removed. The cells were fixed in 4% paraformaldehyde for 30 min. The fixing solution was then incubated at RT for 10 min on a shaker. The OD₅₇₀ value was measured using a microplate reader. The cells were washed with PBS twice for 3 min each. After washing, 5 µg/ml of Hoechst 33342 dye was added to each sample. The plate was incubated at 37°C for 10 min. The staining solution was removed followed by two washes in PBS for 3 min each. One drop of fluorescence quencher was added to the slide. The coverslip with the cells attached was exposed to the fluorescence quencher and mounted onto the slide carefully to avoid bubbles. The slides were observed and documented using fluorescent microscopy.

**Total RNA extraction and real-time PCR (RT-PCR) analysis.** Total RNA was extracted as previously described (22) and the total RNA concentration was measured. The primer sequences for DOR and β-actin are listed in Table I. RT-PCR was performed according to the RT-PCR kit manual (Takara Bio Inc.). The PCR products were analyzed using 10% agarose gel electrophoresis and a gel imaging system.

**RNA interference (RNAi).** Cells at a concentration of 5x10⁴ cells/ml were seeded into a 6-well plate. After reaching 70% confluence, the cells were transfected with lentiviral vectors according to the transfection agent manual. The short interfering RNA (siRNA) sequences targeting DOR (synthesized by Invitrogen) are listed in Table II. The RNA interference efficiency was tested using RT-PCR and western blotting.
Western blot analysis. Cells were harvested and lysed in 2 ml of lysis buffer [50 mM Tris-HCl, 137 mM NaCl, 10% glycerol, 100 mM sodium orthovanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40 and 5 mM cocktail solution (pH 7.4)] to extract the proteins. The extracted proteins were quantified using the BCA method and analyzed using SDS-PAGE. The extracted proteins were transferred onto a PVDF membrane by semi-dry transfer. The membrane was blocked in 5% non-fat milk overnight at 4˚C. The membrane was washed using TBST and incubated with primary antibody at 37˚C for 1 h, washed with TBST and then incubated with secondary antibody at 37˚C for 1 h. The membrane was washed with TBST and developed for 5 min using autoradiography. Quantity One software was used to analyze the optical density. The results are displayed as the ratio of the optical density value/internal reference optical density value.

Scratch test. Cells were incubated in a 6-well plate to 100% confluence. A sterilized tip was then used to scratch a line in the center of the monolayer cells. The dead cells were removed by washing and cell growth was observed and documented at different time-points (0, 24, 48 and 72 h) under a microscope. Image-pro Plus software was used to measure the distance between the scratch edges at different time-points (0, 24, 48 and 72 h). The cell migration distance at different time-points was measured based on the following equation: Distance at 0 h / Distance at 24, 48, or 72 h. The results were analyzed using statistical software.

Cell invasion test. Transwell migration chambers (Corning, NY, USA) were used for the cell invasion test. Different groups of MHCC97-H cells were digested by trypsin. Cell counting was performed and the cells were then resuspended to a final concentration of 1x10^5 cells/ml in DMEM media. Resuspended cells (200 µl) were added to the upper chamber in each Transwell (8-µm diameter pore size) and 500 µl of medium supplemented with serum was added to the lower chamber. Transwell migration chambers were incubated in a 5% CO₂ incubator for 24 h, washed three times with PBS and then stained with crystal violet. Five fields were randomly picked and the cell numbers were counted in every field using a light microscope at x200 magnification. The mean value of the cell numbers in the five fields was used as the number of cells that passed through the artificial human basal membrane.

Inoculation of nude mice. The animal experiments were approved by the Medical Ethics Committee at Guilin Medical University. Ten nude mice (male, 6-8-week-old and weighing ~20 g) were purchased from the Animal Center at Guilin Medical University. The mice were randomly divided into two groups with five mice in each group. All of the mice were spleen inoculated with cells (0.5x10^5 cells per mouse) transfected with control oligonucleotides (N-control) or DOR-siRNAs. Animals were sacrificed after four weeks using cervical dislocation and the xenograft tumor was harvested aseptically for testing.

Statistical analyses. SPSS 16.0 statistical software was used to perform all of the statistical analyses. The data are the mean ± standard deviation. Both a one-way ANOVA and an LSD-t test were used to compare the differences among the different groups. The difference between two groups was considered to be statistically significant at p<0.05.

Results

High expression of DOR in HCC tissues and cells. The RT-PCR results demonstrated variable levels of DOR expression in 41 cases of HCC samples. The DOR mRNA levels in HCC lesions were higher than in the adjacent tumor tissues and normal liver tissues (p<0.05) (Fig. 1A). The DOR mRNA was expressed in different types of HCC cells at levels that were significantly higher than in normal liver cells (p<0.05) (Fig. 1B). Western blot analysis determined that DOR protein expression in HCC tissue was higher than in the adjacent tumor tissues and normal liver tissues (p<0.05) (Fig. 1C). Moreover, DOR protein expression in different types of HCC cells was higher than in normal liver cells (p<0.05) (Fig. 1D).

Immunohistochemical detection of DOR expression and distribution of human hepatocellular carcinoma were carried out. The results show that the DOR positive staining in HCC are mainly located in the cell membrane, a small amount of

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staining in the cytoplasm and none in the nucleus (Fig. 1E). Immunofluorescence using FITC-conjugated antibodies was used to detect DOR expression and its distribution in HCC and normal cells. The results from the HCC cells showed that the green fluorescence FITC signal was distributed evenly on the cancer cell membrane, was weak in the cytoplasm and was absent in the nucleus (Fig. 1F), indicating that DOR was mainly expressed on the HCC cell membrane.

**Activation of DOR promotes HCC cell proliferation.** HepG2 and Hep3B HCC cell lines were employed to study the effect of DOR on HCC cell proliferation. An MTT assay was used to quantify the cell proliferation rate after DADLE treatment. When the DADLE concentration was increased from 10 nM to 10 µM, the OD_{570} values of the DADLE-treated HepG2 and Hep3B cells also increased, but there was no increase in the control groups (without DADLE treatment). However, when the concentration of DADLE was above 1.0 µM, the OD_{570} values of HepG2 and Hep3B cells did not increase further, indicating that the activation of DOR can promote HCC cell proliferation and that 1.0 µM of DADLE induces maximal cell proliferation (Fig. 2).

**Silencing DOR expression with RNAi.** Three siRNAs (DOR-siRNA-1, DOR-siRNA-2 and DOR-siRNA-3) and the control siRNA (N-control) were used to transfected HCC cells. Post-transfection, the total RNA was extracted for use in the RT-PCR analysis of DOR expression in HCC cells. Upon siRNA transfection, DOR expression decreased relative to cells transfected with the control siRNA. DOR-siRNA-1 gave the most obvious silencing effect (Fig. 3A). Western blot
analysis further illustrated that DOR protein expression also decreased after DOR-siRNA-1 transfection (Fig. 3B).

**Downregulation of DOR inhibits HCC cell growth.** OD\(_{570}\) values of DOR-siRNA-transfected HepG2 and Hep3B cells were significantly reduced relative to those from the HepG2 and Hep3B cells transfected with the control siRNA (p<0.05). Similar results were observed in naltrindole (specific DOR inhibitor) treated cells (p<0.05) (Fig. 4), suggesting that the downregulation of DOR can significantly suppress HCC cell proliferation.

**Downregulation of DOR promotes HCC cellular apoptosis.** To study the function of DOR in HCC cellular apoptosis, Hoechst 33342 staining was used to measure the effect of the downregulation of DOR on apoptotic HCC cell morphology using fluorescence microscopy. The HCC cell nucleus in the N-control group was circular or oval in shape with the chromatin evenly distributed in light blue fluorescence. The silencing of the DOR gene or the inhibition of DOR using the specific antagonist naltrindole led to a significant increase in the number of apoptotic cells. The chromatin in the apoptotic HCC cells was condensed and unevenly distributed. Chromatin accumulation near the nuclear membrane, chromatin condensation, increased intensity of fluorescence staining, nuclear condensation and apoptotic cell bodies were also detected when DOR was downregulated (Fig. 5A).

The Annexin V-FITC/PI double-labeling method was used to study the effect of DOR downregulation on the HCC cell apoptotic rate. DOR silencing increased the rate of early apoptosis in HepG2 and Hep3B cells relative to the N-control group (p<0.05). We also observed that the rate of early apoptosis in the HepG2 and Hep3B cells increased with naltrindole treatment (p<0.05) (Fig. 5B and C). These results suggest that the downregulation of DOR can promote apoptosis in HCC cells.

**Downregulation of DOR causes liver cancer cell cycle arrest.** Flow cytometry analysis was used to determine whether DOR affected the cell cycle in HCC cells. When the DOR gene was silenced using siRNAs or the cells were treated with naltrindole, the cell cycle of HepG2 and Hep3B cells was arrested at G0/G1 (p<0.05), suggesting that the downregulation of DOR increased the percentage of HCC cells in G0/G1. This downregulation also inhibited HCC cell growth (Fig. 6).

**Downregulation of DOR inhibits HCC cell invasion and migration.** To determine if the downregulation of DOR can affect HCC cell invasion and migration, we compared the number of cells passing through an artificial human basal membrane before and after siRNA transfection. Five fields of cells were randomly chosen for this analysis. The downregulation of DOR decreased the invasion ability of HCC cells and significantly reduced the number of cells passing through the artificial human basal membrane (p<0.05). Moreover, the downregulation of DOR also reduced the migration of HCC cells (p<0.05) (Fig. 7A and B).

The cell monolayer was scratched and the distance between scratch margins was measured at 0, 24, 48 and 72 h. The downregulation of DOR led to a significant decrease in the migration of HCC cells at the four different time-points (p<0.05), which suggested that the downregulation of DOR led to a significant reduction in liver cancer cell migration (Fig. 7C and D).

**Effect of DOR downregulation on cancer progression in nude mice.** After four weeks of inoculation, the tumor formation rates reached 100% in all nude mice (n=5) in the N-control group and 80% in the nude mice inoculated with the DOR-siRNA-transfected cancer cells. Tumor progression in the nude mice of the N-control group was more rapid than in the nude mice inoculated with DOR-siRNA-transfected cancer cells (Fig. 8A). We also found that the tumor volumes in the DOR-siRNA group were significantly smaller than in the N-control group (p<0.05) at all time-points (Fig. 8B). The tumor weights of the DOR-siRNA group were significantly smaller than the weights of the N-control group (Fig. 8C). These results suggested that the downregulation of DOR in vivo can significantly decrease cancer progression.
Discussion

Since the discovery of the opioid receptor and opioid receptor drugs, researchers have studied their roles in neurological diseases and pain control. DOR, a key member of the opioid receptor superfamily and a G protein-coupled receptor, is mainly expressed in the central nervous system (CNS) and is involved in the functional regulation of the CNS and pain control (23-25). Studies have shown that DOR is also expressed in the circulatory system (26), digestive system (27,28), reproductive system (29,30) and immune system (31), suggesting that apart from the CNS, DOR may play different roles in these peripheral tissues.

In addition to the being expressed in the stomach, small intestine, large intestine and pancreas, DOR was reported to be expressed in the liver (27,28). Our previous studies (21) determined that DADLE (a specific DOR agonist) had a dose-dependent protective effect on human liver cells by suppressing liver cell apoptosis. However, naltrindole (a highly selective DOR antagonist) can suppress the function of DOR, which suggests that DOR is expressed and regulates function in normal human livers, consistent with previous studies.
It was reported that DOR is widely expressed in multiple human malignant cancers (12-15,16,17). DOR is also involved in malignant transformation and cancer progression. However, there is not enough evidence on the functional effects of DOR in liver cancers. The aim of this study was to investigate DOR expression in human liver cancers and its effects on human liver cancer progression. First, we found that DOR is expressed in human HCC tissues and cells. The expression level of DOR in human HCC tissues and cells was significantly higher than in normal liver. Our immunofluorescent data for the intracellular localization of DOR determined that DOR is mostly localized to the liver cell membrane. These results suggest that DOR may be involved in HCC formation and tumor progression. It may also be involved in other physiological functions in the human body.

Among the members of the opioid receptor superfamily, DOR is most closely involved in cell survival and proliferation (32,33). Studies have shown that DOR can protect liver damage in cholestatic liver disease by promoting liver generation and liver cell proliferation (18,34). The protective effect in the liver was due to the activation of DOR on the liver cell membrane. We speculated that DOR may have some effects on liver cancer progression. Further studies are needed to investigate this effect of DOR. Surprisingly, the activation of DOR significantly increased HCC cell proliferation and promoted cancer cell growth in our study. We suggest that DOR may also play an important role in HCC cell proliferation. This finding may provide an effective method to suppress HCC progression in clinical practice.

Kuniyasu et al suggested that methionine-enkephalin can suppress colorectal cancer (CRC) cell growth and invasion based on their finding from a model of the liver metastasis of CRC (35), which is contradictory to our results. We found that the downregulation of DOR suppressed HCC cell proliferation and that the tumor cells underwent apoptosis. We also found that the HCC cell cycle was arrested at G0/G1. In addition, our

![Figure 6](image_url)

Figure 6. The effect of the downregulation of DOR on the cell cycle of HCC. (A) Flow cytometry analysis of cell cycles. (B) Histogram displaying the percentage of cells in different phases of the cell cycle. The data were collected from three independent experiments. *p<0.05 represents a significant difference relative to the control group.
Figure 7. Effect of the downregulation of DOR on liver cancer cell invasion and migration. (A) Transwell migration chamber experiment of cell invasion and migration under the microscope (x200 magnification). (B) Histogram of cell invasion and migration. (C) Cell scratch experiment to observe cell migration (x100 magnification). (D) Histogram of cell migration from the scratch experiment. The data were collected from three independent experiments. *p<0.05 represents a statistically significant difference relative to the control group.

Figure 8. Downregulation of DOR suppressed cancer progression in nude mice. (A) Surgically removed tumor tissues from nude mice 21 days post-inoculation. (B) The measurement of tumor volume at different time-points after inoculation. (C) Tumor weights of the two groups 21 days post-inoculation.
results were contradictory to results from Hatzoglou et al (36). This group showed that opioid receptor agonists can suppress cancer cell proliferation. This controversy could be caused by the differences between liver cancers and other cancers as well as the multiple subtypes of DOR. Whether the downregulation of DOR in vivo can suppress proliferation in other cancer cell types still needs to be tested.

Although progress has been made in the study of HCC formation and progression, the 5-year survival rate of liver cancer is still very low, which may be due to the high invasion and metastasis rates of HCC (37). In the present study, we established that DOR expression in human HCC cells (MHCC97-H) with a high metastatic rate was higher than in human HCC cells (MHCC97-L) with a low metastatic rate, which suggests that DOR might be closely involved in cancer cell malignancy and invasion. However, in this previous study, there was no evidence that demonstrated that DOR plays a functional role in human HCC invasion and metastasis. In the present study, we found that silencing the DOR gene decreased liver cancer cell invasion and migration. Nude mice inoculated with cells stably expressing low levels of DOR displayed low tumor formation rates and reduced cancer progression. These results suggest that DOR may play some role in HCC cell invasion and migration. This can provide a new theoretical basis for the prevention of HCC invasion and migration.

In conclusion, high expression levels of DOR were observed in HCC tissues and cells and DOR was mainly localized to the tumor cell membrane. Our results demonstrated that downregulation of DOR can suppress liver cancer progression. Therefore, DOR may become a new marker and target for HCC treatment and provide a potential treatment method to reduce or inhibit tumor malignancy.

Acknowledgements

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