

Downregulation of Rap1 promotes 5-fluorouracil-induced apoptosis in hepatocellular carcinoma cell line HepG2

YONG ZHA^{1*}, PING GAN^{1*}, QIAN YAO², FENG-MING RAN³ and JING TAN¹

¹Department of Abdominal Surgery, ²Yunnan Cancer Research Institute, ³Department of Pathology, Yunnan Cancer Hospital (The Third Affiliated Hospital, Kunming Medical University), Kunming, Yunnan 650118, P.R. China

Received December 9, 2013; Accepted January 30, 2014

DOI: 10.3892/or.2014.3033

Abstract. Recent studies have revealed that repressor/activator protein (Rap1) not only protects telomeres from sister chromatid exchange, but also functions in genomewide transcriptional regulation. Knockdown of Rap1 sensitizes breast cancer cells to adriamycin-induced apoptosis. However, little is known about the role of Rap1 in the progression of hepatocellular carcinoma (HCC). The present study aimed to investigate the functions of Rap1 in HCC progression and to determine whether targeting the Rap1 signaling pathway may be of therapeutic value against HCC. We found knockdown of Rap1 by microRNA (miRNA) interference enhanced significantly apoptosis and 5-fluorouracil (5-FU) chemosensitivity in HepG2 cell line. Rap1 miRNA downregulated nuclear factor- κ B p65 (NF- κ B p65) expression, and upregulated inhibitor of NF- κ B (I κ B) expression. *In vivo*, Rap1 miRNA combined with 5-FU treatment led to a significant reduction of tumor growth as compared with 5-FU alone. The results indicate that Rap1 miRNA can effectively enhance sensitivity of HepG2 cell line to 5-FU chemotherapy *in vitro* and *in vivo*.

Introduction

Hepatocellular carcinoma (HCC) is the the fifth most common cancer and the third leading cause of cancer-related death in the world (1). Its annual incidence is estimated at 600,000 new cases, more than half of which come from China (2). Surgical resection is the most effective therapy for HCC. However, most of the patients are not good candidates for surgery because of being diagnosed in later stages. On the other hand, HCC is frequently resistant to conventional chemotherapeutic agent-

and radiation (3). Resistance to apoptosis is one of the reasons that HCC cells are not sensitive to chemotherapy (4,5).

Some studies have revealed that NF- κ B is constitutively activated and plays a vital role in the regulation of genes involved in cell apoptosis in some cancers including HCC (6,7). Inhibition of NF- κ B p65 by miRNA resulted in a significant increased apoptosis in HCC (8). Interestingly, Rap1 (repressor/activator protein), a member of telomeres shelterin complex, has been shown to be an essential modulator of NF- κ B-mediated pathways (9). Human breast cancers with NF- κ B hyperactivity show elevated levels of cytoplasmic Rap1. Knockdown of Rap1 sensitizes breast cancer cells to apoptosis (9). However, to date, few studies have reported the biologic function of Rap1 in HCC progression, the interactive regulation between Rap1 and NF- κ B, and the potential functional contribution of such a network to HCC cancer progression and apoptosis have not been explored yet.

In the present study, to investigate the role of Rap1 in HCC progression, we examined cell survival/growth and apoptosis after downregulation of Rap1 expression by miRNA in HepG2 cells, and explored its mechanisms.

Materials and methods

Cell culture. HepG2, HuH-7, SMMC-7721, QGY-7703 and HL-7702 cell lines were obtained from Shanghai Cell Bank. The cells were maintained under a humidified atmosphere of 5% CO₂ at 37°C in DMEM medium (HyClone), supplemented with 10% fetal bovine serum (FBS; HyClone).

Transient transfection of HepG2 cells and establishment of stable transfectants. Set of four Rap1-miRNA oligonucleotides were designed and synthesized by Invitrogen for Rap1 gene knockdown studies. The Rap1-miRNA expression vector (pcDNATM 6.2-GW/EmGPF-miR; Invitrogen; catalog no. K4936-00) was used to construct a Rap1 miRNA plasmid by inserting the miRNA-coding sequence. HepG2 cells were transfected with selected Rap1-miRNA plasmid or control miRNA plasmid.

HepG2 cells were seeded on 6-well plates at a density of 5x10⁵ cells/well, and grown for 48 h to reach 80% confluence before transfection. Control miRNA and Rap1 miRNA were transfected respectively into cells using Lipofectamine 2000 (Invitrogen; catalog no. 11668-027) according to the manufac-

Correspondence to: Dr Yong Zha, Department of Abdominal Surgery, Yunnan Cancer Hospital (The Third Affiliated Hospital, Kunming Medical University), No. 519 Kunzhou Road, Kunming, Yunnan 650118, P.R. China
E-mail: zhayong888@sina.com

*Contributed equally

Key words: Rap1, hepatocellular carcinoma, NF- κ B, apoptosis

turer's instructions. The cells were then harvested and analyzed by flow cytometry, electron microscopy, reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analysis at 24-72 h after the transfection. All measurements were performed in triplicate.

The transfected cells were cultured in the medium with Blasticidin S HCl (Invitrogen; catalog no. R210-01) at 2-10 $\mu\text{g/ml}$. After 8 weeks, surviving colonies arising from stably transfected cells were selected and individually amplified.

MTT assay. At 48 h after transfection, cell survival was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The transfected and control cells were seeded at a density of 5,000 cells/well in 96-well plates and incubated at 37°C in humidified 5% CO₂ for 24 h. Serially diluted 5-FU (Sigma) was added to give the intended final concentrations. Cells were then incubated an additional 72 h, and the MTT assay was performed according to the manufacturer's instructions. Absorbance values were determined at 570 nm on a SpectraMax 250 spectrophotometer. The assays were performed three times in triplicate. The 50% inhibitory concentration (IC₅₀) values were defined as the drug concentrations required to reduce cellular proliferation to 50% of the untreated control well.

Flow cytometry for apoptosis. The HepG2 cells were harvested for evaluation of apoptosis at 48 and 72 h after the transfection. Firstly, the cells were washed with PBS and fixed in 4% paraformaldehyde for 30 min. Then, the cells were re-suspended in PBS supplemented with 0.1% Triton X-100, and incubated at 4°C for 2 min. TUNEL solution (50 μl) was added to each tube, and incubated in the dark at 37°C for 60 min. After that, the cells were washed with PBS, and re-suspended in 250 μl PBS for flow cytometry using the FACSCalibur system.

Electron microscopy. The cells were harvested in PBS for evaluation of apoptosis with electron microscopy at 48 h after the transfection. Firstly, the cells were subsequently fixed in 3.5% glutaraldehyde and 1% OsO₄, then, washed with PBS, dehydrated in ethanol and acetone series, and embedded in Epon 618. Thin sections were cut with a Leica Ultracut UCT microtome, stained with 2% uranyl acetate and lead citrate. Observation followed using JEM-1011 transmission electron microscopy.

Quantitative real-time PCR. To elucidate the mechanism by which Rap1 induces cell growth and inhibits apoptosis in HCC, we examined the expression of NF- κ B p65 and I κ B using quantitative real-time PCR at 48 h after the transfection. Total RNA was isolated from the Rap1 miRNA transfected and control cell lines with TRIzol (Invitrogen; catalog no. 15596-026). Then, the RNA was subjected to real-time PCR analysis with the use of a One Step SYBR PrimeScript RT-PCR kit (Bio-Rad Laboratories) and superscript pre-amplification system (Invitrogen Life Technologies). NF- κ B p65 was amplified using forward primer 5'-GGGAAGGAACG CTGTCAAG-3' and reverse primer 5'-TAGCCTCAGG TACTCCATCA-3'. I κ B was amplified using forward primer 5'-GGATACCTGGAGGATCAGATTA-3' and reverse primer

5'-CCACCTTAGGGAGTAGTAGATCAAT-3'. PCR amplification was performed with 32 cycles of 45 sec denaturing at 94°C, 45 sec annealing at 55°C and 10 min extension at 72°C. A melting curve analysis was conducted to control for the specificity of the amplification products. The expression values were normalized by the β -actin expression. Moreover, the amplified gene fragments were separated in a 2% agarose gel and visualized by ethidium bromide staining and UV light.

Western blot analysis. Western blot analysis was performed as previously described at 24, 48 and 72 h after the transfections. The antibodies included Rap1 monoclonal antibody (Santa Cruz Biotechnology, 60 kDa, mouse), β -actin monoclonal antibody (Abmart; 43 kDa, mouse), goat anti-rabbit and mouse IgG-HRP (Abmart). Cells were collected by trypsin digestion and washed twice with PBS. RIPA lysis buffer (100 μl) (Soledad) was added. Then, protein was extracted, and protein concentrations were determined using BCA protein assay kit (Tiangen) based on the manufacturer's instructions. Protein samples (30 μl) were loaded onto a NuPAGE 8% TA gel (Invitrogen) and run at 80 V. Next, the proteins were transferred to PVDF membranes (Millipore) using an iBlot Gel Transfer Device (Bio-Rad Mini Protein 3) at 200 mA for 1.5 h. The membranes were blocked in 3% BSA blocking buffer for 2 h, exposed to primary antibody overnight at 4°C, and secondary antibody for 2 h at room temperature. After that, the membranes were washed with 1% PBST, and rinsed with Rap1 three times (5 min each time) and with β -actin three times (3 min each time). Excess reagent was removed. The membranes were covered with transparent plastic wraps. Images were acquired using darkroom development techniques.

In vivo studies. The animal protocol was approved by the the Institutional Animal Care and Use Committee of Kunming Medical University. Four-week-old BALB/c nude mice were obtained from Vital River Laboratories (Beijing, China) and maintained in a specific pathogen-free facility. These mice were randomly divided into four groups with 10 mice each group: i) control; ii) 5-FU treated; iii) Rap1 miRNA; and iv) Rap1 miRNA + 5-FU treated group. The mice were implanted subcutaneously into the right flank with Rap1 miRNA stable transfected and control HepG2 cells (1.0 \times 10⁷ cells in 0.2 μl PBS per mouse). When palpable tumors developed (average volume 100 mm³), the mice in 5-FU treated and Rap1 miRNA + 5-FU treated groups were administered intraperitoneally with 5-FU at a dose of 30 mg/kg every 3 days. Those in control and Rap1 miRNA groups were injected with the same volume of 0.9% saline. Tumor size was measured at 3-day intervals with a caliper (calculated volume = shortest diameter² x longest diameter/2). Tumor growth was followed for 25 days from the first injection.

Immunohistochemistry for Rap1 and caspase-3. At the termination of the experiments, the tumors were removed for histological examination. The tumor samples were fixed in 10% formaldehyde, embedded in paraffin and then cut into 3- μm thick sections. Immunohistochemistry was conducted using streptavidin-peroxidase method with the EnVision Kit (Maixin Bio, Fuzhou, China). The sections were dewaxed

in xylene and rehydrated in a graded series of ethanol. The tissue sections were treated by high temperature and pressure in 10 mmol/l sodium citrate (pH 6.0) for 2 min for antigen retrieval. Next, the primary anti-Rap1 antibody (1:100 dilution; Santa Cruz Biotechnology; 60 kDa) was added to the slides. The slides were incubated at 4°C overnight. After that, the slides were stained with secondary antibody, followed by incubation with diaminobenzidine kit for 10 min, hematoxylin staining, vitrification with dimethylbenzene, and coverslip with mounting medium. The Rap1 and caspase-3 expression were assessed at the center and the invasive front of the tumor in each section. A positive Rap1 expression was defined as nuclear staining at both the cancer central and invasive front. A positive caspase-3 expression was defined as cytoplasmic staining.

Statistical analysis. Data are presented as the means \pm SD. Statistical analysis was performed using the SPSS version 14. The differences were evaluated by t-test. P-values <0.05 were considered to indicate a statistically significant result.

Results

Expression of Rap1 in HCC cell lines. To determine the effect of Rap1 expression on the apoptosis in HCC cells, we first determined the expression of Rap1 in four HCC cell lines and normal liver cells by quantitative RT-PCR. Quantitative RT-PCR revealed that expression of Rap1 was significantly higher in HCC cell lines HuH-7, SMMC-7721, QGY-7703 and HepG2 as compared with normal liver HL-7702 cells. Given the relative high expression of Rap1 in HepG2 cells, this cell line was selected for subsequent experiments to determine whether inhibition of Rap1 expression by miRNA would enhance cells apoptosis.

Effects of Rap1 expression on apoptosis in HepG2 cells. To investigate the anti-apoptotic role of Rap1 in HCC cells, we knocked down the expression of Rap1 in HepG2 using RNA interference (RNAi) techniques with miRNA. Firstly, Rap1 protein levels were evaluated in transfected and control HepG2 cells by western blot analysis to test RNAi efficiency. The level of Rap1 expression in Rap1 miRNA-transfected cells was reduced significantly compared with those in empty plasmid transfected group, and control group at 24, 48 and 72 h after the transfections (Fig. 1). The results suggested that miRNA transfection led to specific suppression of Rap1 protein expression.

The anti-apoptotic effect of Rap1 in HepG2 cells was subsequently confirmed by TUNEL assay and flow cytometric analysis. The flow cytometric analysis revealed that of Rap1 knockdown cells had a significantly higher rate of apoptosis at 48 and 72 h after the transfection when compared with empty plasmid group and control group ($P < 0.05$). At 48 h, $15.8 \pm 0.5\%$ of adherent cells were apoptotic in Rap1 miRNA group, whereas $1.1 \pm 0.2\%$ and $0.3 \pm 0.1\%$ of adherent cells were apoptotic in empty plasmid group and control group, respectively. At 72 h, Rap1 miRNA induced apoptosis in $\sim 33.0 \pm 2.3\%$ of the cells, empty plasmid induced apoptosis in only $8.2 \pm 1.6\%$ of the cells compared with $0.6 \pm 0.1\%$ cell apoptosis in control group (Fig. 2). To gain further

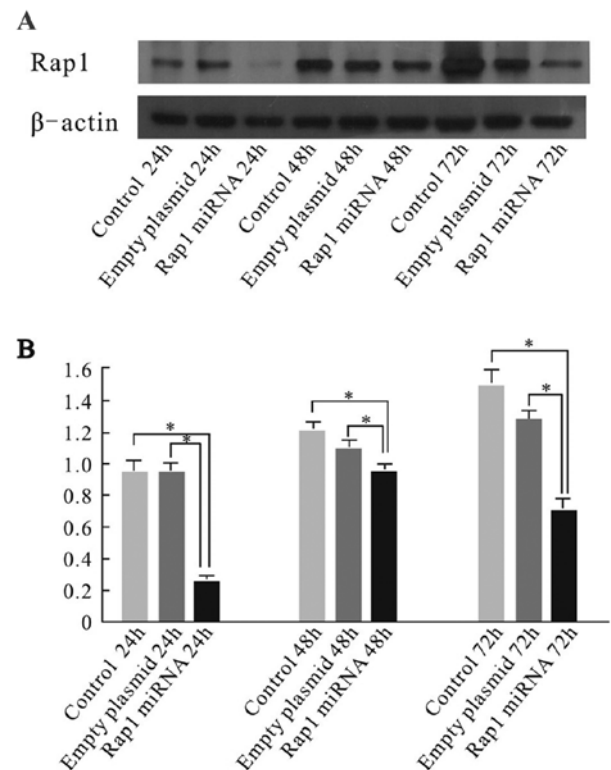


Figure 1. Western blot analysis of Rap1 expression in Rap1 miRNA transfected and control HepG2 cells, β -actin was used as a control (* $P < 0.05$).

information about the morphological changes, the cells from the three groups were evaluated of apoptosis with electron microscopy at 48 h after the transfection. Electron microscopy revealed that some shrinking cells with a ring of condensed chromatin at the interior surface of the nuclear envelope in Rap1 miRNA group (Fig. 3), which was consistent with the result of flow cytometric analysis. Take together, these results suggested Rap1 miRNA increased apoptosis in HepG2 cells.

Next, we investigated whether Rap1 miRNA would enhance chemosensitivity to 5-FU in HepG2 cells. HepG2 cells were treated with 0, 0.1, 0.2, 0.3, 0.4, 0.5 or 0.6 μ M 5-FU for 24 at 48 h after the transfection. The cell viability was evaluated using the MTT assay. MTT assays revealed the Rap1 miRNA-transfected cells showed increased sensitivity to 5-FU as compared with the empty plasmid-transfected, and the control cells (Fig. 4).

Suppression of Rap1 increases cell apoptosis through NF- κ B signaling in HepG2 cells. We next investigated the mechanism responsible for the anti-apoptotic effect of Rap1 in HepG2 cells. We examined the expression of NF- κ B p65 and I κ B at mRNA level at 48 h after the transfection. Quantitative real-time RT-PCR analysis revealed that the NF- κ B p65 mRNA was downregulated significantly, whereas the I κ B mRNA was greatly upregulated in Rap1 miRNA-transfected cells compared with those in control cells ($P < 0.05$) (Figs. 5 and 6), suggesting that NF- κ B was suppressed in Rap1 miRNA-transfected HepG2 cells. These results indicate that Rap1 plays an anti-apoptotic role via the NF- κ B/I κ B pathway.

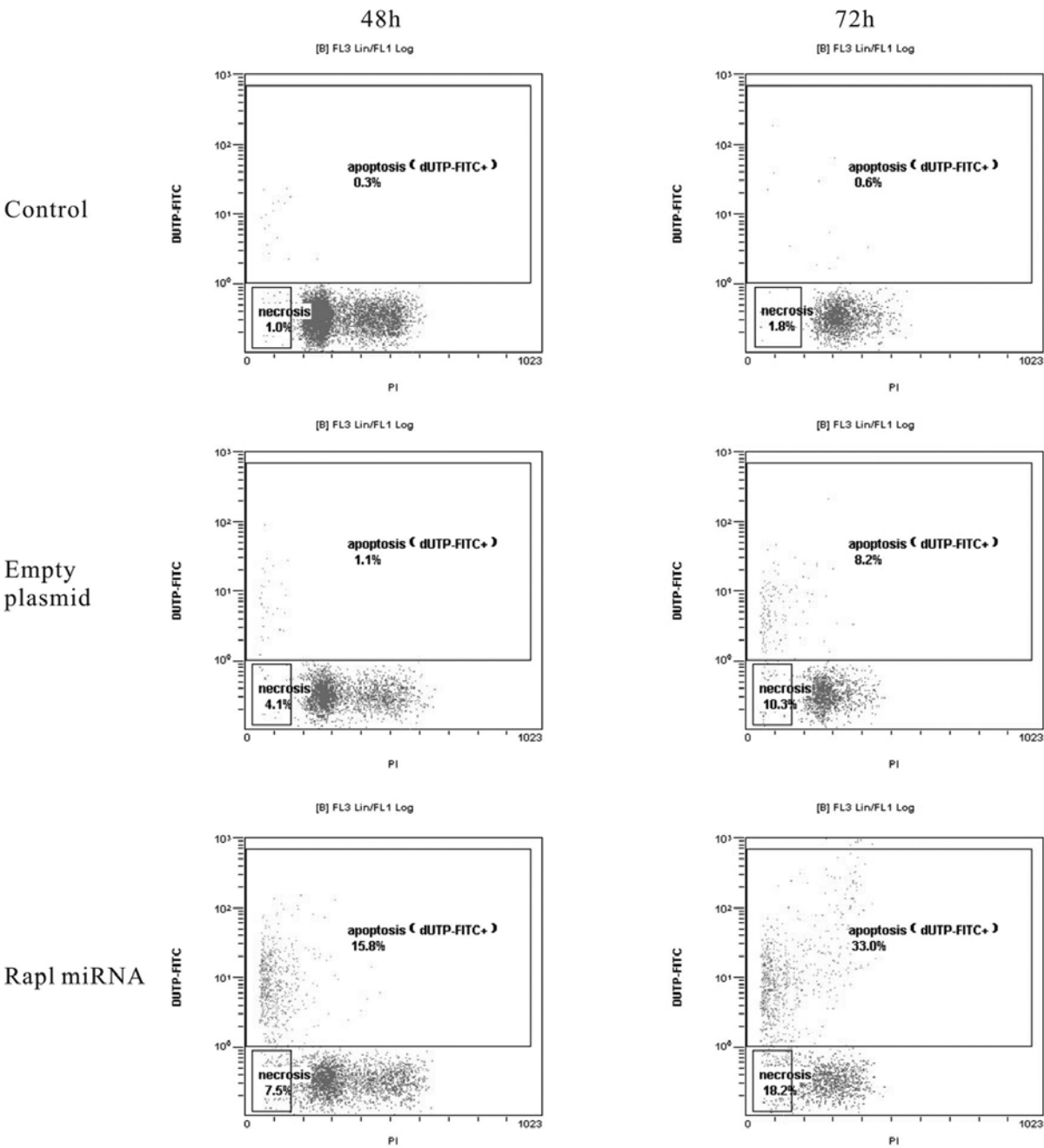


Figure 2. Flow cytometric analyses of the percentages of apoptotic cells at 48 and 72 h after the transfection.

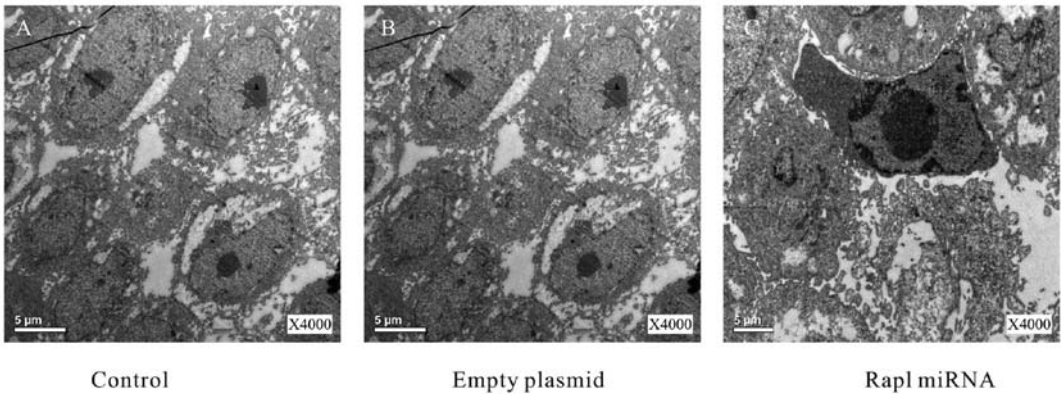


Figure 3. Electron microscopy images (original magnification, x4000) of HepG2 cells. Cell shrinkage and chromatin condensation were noted in Rap1 miRNA group at 48 h after the transfection.

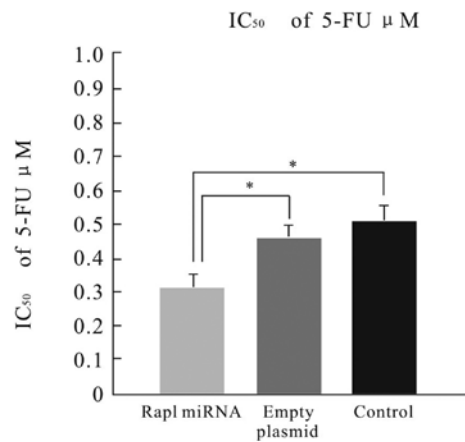


Figure 4. Growth inhibition by 5-FU in HepG2 cells. HepG2 cells were plated on 96-well plates and treated with 0, 0.1, 0.2, 0.3, 0.4, 0.5 or 0.6 μ M 5-FU for 24 h. Cell survival was determined using MTT assay. Each value represents the mean \pm SD from triplicate measurements. (* P <0.05).

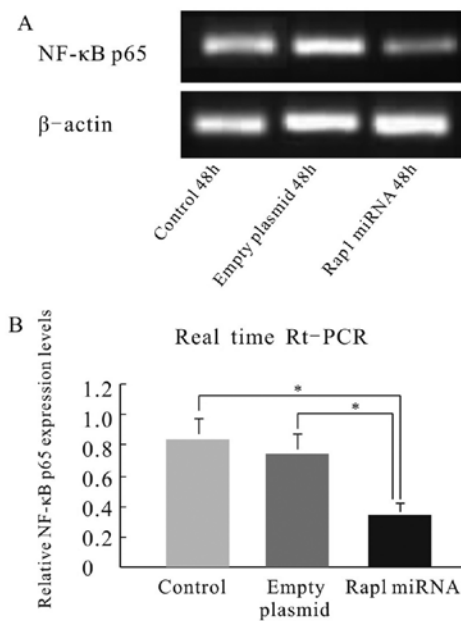


Figure 5. At 48 h after the transfection, the expression of NF- κ B p65 mRNA was determined with quantitative real-time RT-PCR analysis. The NF- κ B p65 mRNA was downregulated significantly when compared with that in control cells (* P <0.05).

Rap1 miRNA reduces HepG2 tumor growth in vivo. To determine the efficacy of Rap1 miRNA alone or combined with 5-FU *in vivo*, we used a BALB/c murine xenograft tumor model with Rap1-deleted and control HepG2 cells. The size of the tumors was measured in three dimensions twice a week for 3 weeks, and the tumor volume was calculated. Mean tumor volume in Rap1-miRNA group was 851.2 ± 32.6 mm³ compared to the average tumor volume of 1494.7 ± 126.2 mm³ in control group at the termination of the experiment (P <0.05). Rap1 miRNA combined with 5-FU treatment led to a reduction of tumor growth even further as compared with 5-FU alone (624.9 ± 45.3 mm³ vs. 1424.0 ± 108.6 mm³; P <0.05) (Fig. 7). The results suggested that Rap1 miRNA inhibited liver cancer progression and enhanced sensitivity of HepG2 cells to 5-FU *in vivo*.

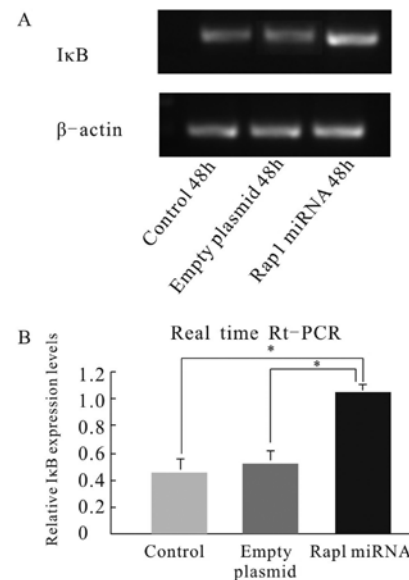


Figure 6. At 48 h after the transfection, the expression of IκB was determined with quantitative real-time RT-PCR analysis. The IκB mRNA was up-regulated significantly when compared with that in control cells (* P <0.05).

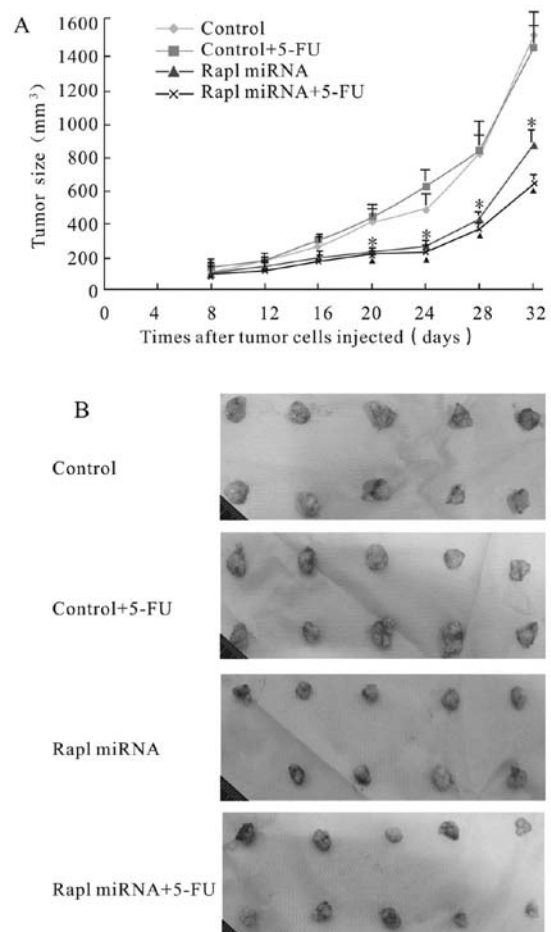


Figure 7. Effects of Rap1 miRNA and 5-FU on HepG2 tumor growth in nude mice. (A) Cohorts of 10 nude mice received subcutaneous injection with 1×10^7 Rap1 miRNA transfected or control HepG2 cells. On day 8 after the injection of tumor cells, the mice received intraperitoneal injection of 5-FU or the same volume of 0.9% saline twice a week, and tumor size was measured from day 8 to 32. * P <0.05 compared to the control; * P <0.05 compared to the control + 5-FU. (B) The tumors were removed from the mice in each group for immunostaining analysis at day 32.

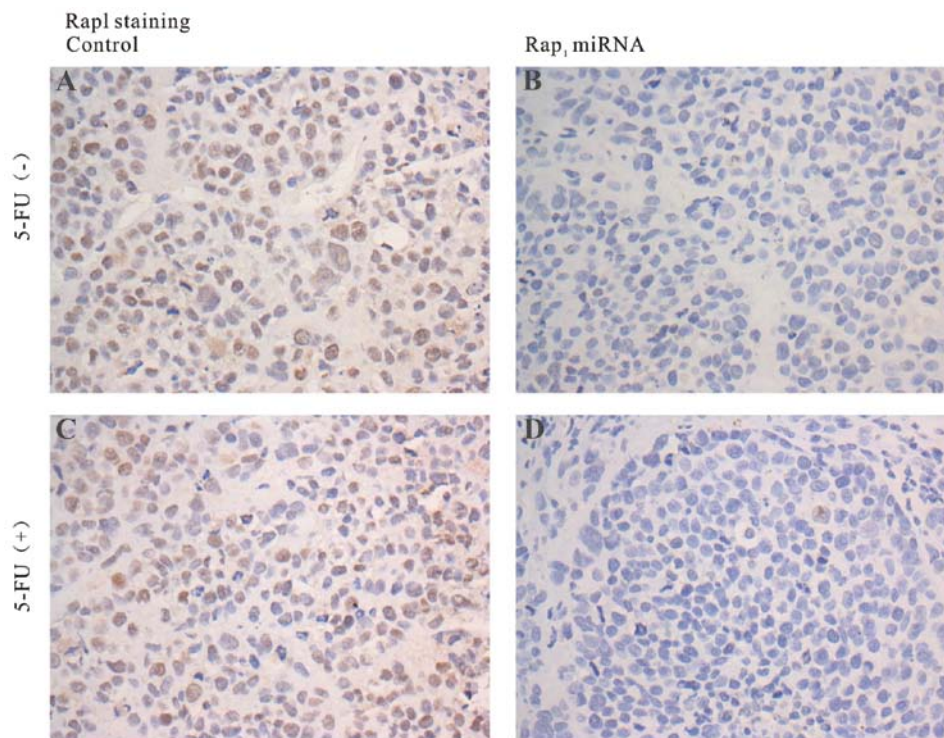


Figure 8. An immunohistochemical analysis of Rap1 in HepG2 tumors *in vivo* (original magnification, x400). Rap1 was undetectable in Rap1 knockdown tumors, whereas nuclear staining of Rap1 was observed in control tumors.

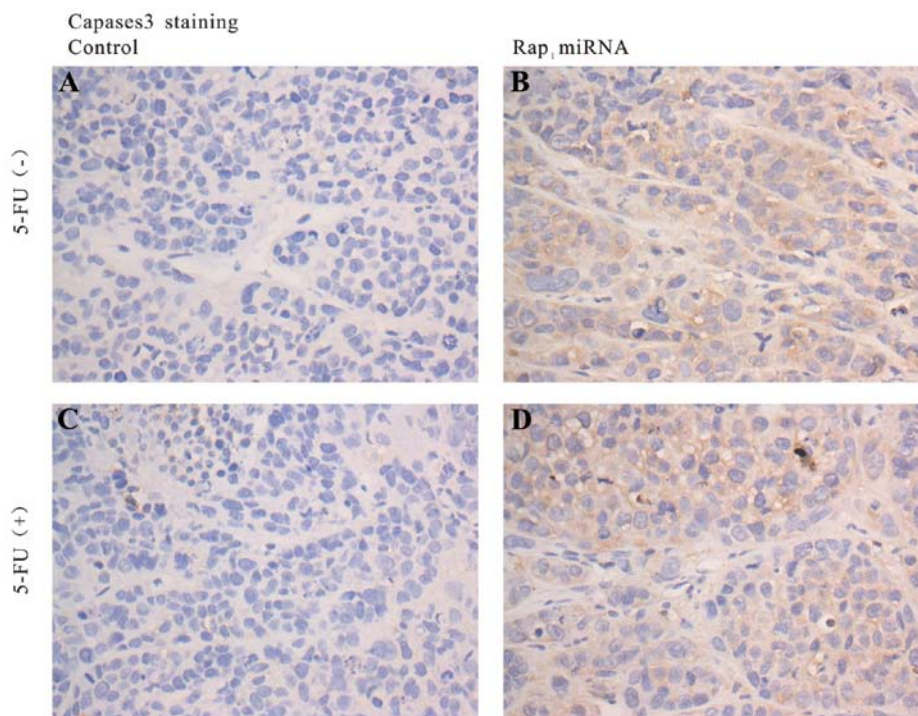


Figure 9. An immunohistochemical analysis of caspase-3 in HepG2 tumors *in vivo* (original magnification, x400). Cytoplasm staining of caspase-3 was detected in Rap1 knockdown tumors, whereas very little or no staining was detectable in control tumors.

We also investigated the expression of Rap1 and caspase-3 expression using immunohistochemical staining in harvested tumors. Immunohistochemical staining analyses revealed that Rap1 expression was significantly inhibited in Rap1 miRNA tumors, whereas Rap1 overexpression was observed in control

tumors (Fig. 8). Caspase-3 expression was positive in Rap1 miRNA treated tumors, and negative in the controls (Fig. 9). The results demonstrated that knockdown of Rap1 dramatically enhanced caspase-mediated apoptosis in the mouse model of HCC.

Discussion

Previous studies have demonstrated that arterial infusion chemotherapy improves the outcome of patients with advanced HCC (10,11). 5-FU regimen is widely used in arterial infusion chemotherapy for patients with advanced HCC (10). However, HCC is highly resistant to chemotherapy in some patients. Defect in apoptosis is a principal mechanism of drug resistance in HCC (5). Several studies have revealed that telomerase activity was associated with increased apoptotic and chemotherapeutic resistance in HCC (12-14). The expression of telomeric repeat-binding factor (TRF)1, TRF2 and TRF1 interacting protein 2 (TIN2) was found to correlate with the progression of hepatocarcinogenesis, and markedly upregulated from dysplastic nodules to HCC (15,16). As a member of telomere-binding proteins, Rap1 plays a role in maintaining telomere homeostasis and genomic integrity (17). In a recent study, Rap1 signaling was identified as a crucial factor in TNF α -induced apoptosis in breast cancers (9). However, the role of Rap1 on HCC progression and its underlying molecular mechanisms remain largely unknown. Therefore, in the present study, we examined the effect of knocking down Rap1 expression on apoptosis and 5-FU chemosensitivity in HCC cells, and analyzed its mechanisms.

Rap1 mRNA expression was initially assessed in 4 HCC cell lines using real-time PCR analysis. Rap1 expression was detected in all the cell lines. Given the relative high expression of Rap1 in HepG2 cells, the cells were selected for Rap1 knockdown assay. Western blot analyses revealed that the expression of Rap1 was significantly suppressed in Rap1 knockdown HepG2 cells compared with those in the empty plasmid transfected, and control cells at 24, 48 and 72 h after transfection. *In vivo*, immunohistochemical analyses revealed that Rap1 expression in resected tumors was lower in the Rap1-miRNA group than that in control group, which was consistent with the *in vitro* results. The results suggested miRNA interference was effective in knocking down the expression of Rap1 *in vitro* and *in vivo*.

Subsequently, the effects of Rap1 miRNA on apoptosis and 5-FU chemosensitivity were investigated in HepG2 cells. The flow cytometric analysis revealed that the cells in Rap1 knockdown group had a significantly higher rate of apoptosis at 48 and 72 h after the transfection compared with those in empty plasmid group and control group. The pro-apoptotic effect of Rap1 miRNA was further confirmed by electron microscopy. *In vivo*, caspase-3 expression in resected tumors was higher in the Rap1-miRNA group than in control group. The results demonstrated that knockdown of Rap1 was effective in inducing apoptosis in HepG2 cells.

Rap1 knockdown also resulted in a significant decrease in cell proliferation rate and an increase in 5-FU sensitivity in HepG2 cells. MTT assay showed that Rap1 miRNA cells showed increased sensitivity to 5-FU ($IC_{50}=0.31 \mu M$) as compared with empty plasmid-transfected cells, or control cells. At the termination of the experiment *in vivo*, the mice in Rap1 miRNA transfected group had a significant reduction in tumor growth as compared with the control group. Rap1 miRNA combined with 5-FU treatment led to a reduction of tumor growth even further as compared with 5-FU alone. These results are in agreement with those reported by Teo *et al* (9)

who found that knockdown of Rap1 by siRNAs significantly sensitized MCF7 cells to TNF α -induced and adriamycin-induced apoptosis. Collectively, the findings suggest that increased Rap1 expression plays an anti-apoptotic role in HCC cells, inhibition of Rap1 with miRNA enhanced apoptosis and 5-FU sensitivity both *in vitro* and *in vivo* xenografts.

We found that knockdown of Rap1 resulted in increased NF- κ B p65 and decreased I κ B mRNA levels in HepG2 cell line. Similarly, Teo *et al* found that there was a positive correlation between high levels of cytoplasmic Rap1 and high levels of nuclear NF- κ B in breast cancer (9). NF- κ B is a transcription factor with five subunits: RelA (p65), RelB, c-Rel, p50/NF- κ B1, and p52/NF- κ B2. The heterodimeric complex of p50/p65 (RelA) is predominantly detected in the cells. NF- κ B dimers are complexed with the inhibitory protein I κ B and maintained in an inactive state in most cell types (18). Previous studies have demonstrated that 5-FU induces apoptosis, and activate the NF- κ B pathway in colon carcinoma and HCC models (19,20). Activated NF- κ B pathway is known to play a vital role in apoptosis resistance in various malignancies including HCC through regulation of its downstream target genes (18,21). Inhibiting NF- κ B can increase 5-FU sensitivity in HCC (20). Some studies demonstrated NF- κ B induced the expression of inhibitor of apoptosis protein-1 (c-IAP1) and c-IAP2. NF- κ B also upregulates the expression of Bcl-2 family (such as Bcl-2 and Bcl-xL), which plays a pivotal role in the inhibition of apoptosis (8,21). It is likely that downstream gene targets of NF- κ B are involved in Rap1-related apoptosis mechanism in HCC cells. Rap1 promotes cell survival through an NF- κ B-dependent pathway in HCC.

In summary, the present study revealed that the combined Rap1 inhibition and chemotherapy inhibited tumor development and induced higher apoptosis rates than chemotherapy alone and control group in HepG2 cells. Our data suggest that inhibition of Rap1 is a promising target of therapeutic approaches to render HCC cells more sensitive to apoptosis and chemotherapy. Targeting of Rap1 via RNA interference is a potential therapeutic strategy.

Acknowledgements

The present study was supported by grants from the Applied Basic Research Programs of Science and Technology Commission Foundation of Yunnan Province (no. 2011FB064 and no. 2012FB065). The authors thank Dr Eduardo Briceño for reviewing the manuscript.

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