Regorafenib diminishes the expression and secretion of angiogenesis and metastasis associated proteins and inhibits cell invasion via NF-κB inactivation in SK-Hep1 cells

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Abstract. The aim of the present study was to investigate the effects of regorafenib on the nuclear factor κ-light-chain-enhancer of activated B cells (NF)-κB-modulated expression of angiogenesis- and metastasis-associated proteins and cell invasion in human hepatocellular carcinoma SK-Hep1 cells. The SK-Hep1 cells were treated with different concentrations of NF-κB inhibitor 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4,6-diamine (QNZ) or regorafenib for 24 or 48 h. The effects of QNZ and regorafenib on cell viability, NF-κB activation, expression and secretion levels of angiogenesis- and metastasis-associated proteins and cell invasion were evaluated with MTT assays, western blotting, ELISA, gelatin zymography and cell invasion assays. The results demonstrated that QNZ and regorafenib significantly reduced the expression and secretion levels of the angiogenesis- and metastasis-associated proteins vascular endothelial growth factor, tumor necrosis factor-α, interleukin (IL)-1β, IL-6, matrix metalloproteinase (MMP)-2 and MMP-9, NF-κB activation and cell invasion. In conclusion, the inhibition of NF-κB activation induces anti-angiogenic and antimetastatic effects in SK-Hep1 cells. Regorafenib reduces the level of expression and secretion of angiogenesis- and metastasis-associated proteins and cell invasion through the suppression of NF-κB activation in SK-Hep1 cells.

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Introduction

Human hepatocellular carcinoma (HCC) has the highest mortality rate worldwide (1). Angiogenesis, the development of new blood vessels, is a prerequisite for tumor growth and metastasis in HCC (2). Tumor angiogenesis is modulated by angiogenic factors including vascular endothelial growth factor (VEGF), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6. The expression of VEGF, a major mediator of angiogenesis, is upregulated by the stimulation of inflammatory cytokines, such as TNF-α, IL-1β and IL-6. The overexpression of angiogenic factors is exhibited in and associated with poor prognoses in HCC (2-6).

Metastasis is the major cause of mortality in patients with cancer (7). Matrix metalloproteinases-2 (MMP-2) and MMP-9, which represent 72 kD and 92 kD type IV collagenases, respectively, degrade the basement membrane of normal cells and result in tumor invasion and subsequent metastasis. The detection of high expression levels of MMP-2 and MMP-9 in HCC has been suggested as a biomarker for poor prognosis (8,9).

Nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), which is assembled via the dimerization of five subunits: RELA p65; RELB; NF-κB 1 p50; NF-κB 2 p52; and c-Rel, serves an important role in immunity, inflammation and tumorigenesis (10). The NF-κB p50/p65 heterodimer is associated with tumorigenesis and is generally sequestered in the cytoplasm by an inhibitor of κBα, IκBα. Activators of NF-κB induce the phosphorylation and degradation of IκBα through IκB kinase and 26S proteasome, respectively, resulting in the NF-κB nuclear translocation and expression of NF-κB target oncogenes (11). A number of angiogenesis- and metastasis-associated proteins are encoded by NF-κB target oncogenes in HCC cells (12). The inhibition of NF-κB activation induces anti-angiogenic and antimetastatic effects via the suppression of the NF-κB signal cascade (13). NF-κB was revealed to be more highly expressed in the nucleoli of HCC cells compared with normal hepatic tissue, which was associated with poor prognoses (14). However, a number of

therapeutic agents, and radiation, induce apoptosis signal and trigger NF-κB activity in HCC *in vitro* and *in vivo* (13,15,16). Therefore, the development of novel inhibitors of NF-κB signaling may be useful for preventing angiogenesis and metastasis in patients with HCC.

Regorafenib, or Stivarga®, a novel oral multiple kinase inhibitor, is a member of the group of biaryl urea compounds and is similar to sorafenib in chemical structure. The addition of fluorine to the center of the phenyl group means that regorafenib may exhibit greater range of activity against oncogenic receptor tyrosine kinases and intracellular signaling kinases compared with sorafenib (17). Regorafenib has been approved for the treatment of metastatic colorectal cancer and advanced gastrointestinal stromal tumors. A randomized double-blinded phase III study of regorafenib in patients with HCC who have progressed after sorafenib treatment is ongoing (18). In our previous studies, sorafenib was revealed to be an inhibitor of NF-κB signaling and reduced NF-κB-modulated expression of proteins including MMP-9 and VEGF in HCC *in vitro* and *in vivo* (13,19). However, whether regorafenib, a sorafenib derivative, induces anti-angiogenic and antimetastatic effects through the suppression of NF-κB activation in HCC cells remains unknown. The aim of the present study was to investigate the effects of regorafenib on NF-κB-modulated expression of angiogenesis- and metastasis-associated proteins and cell invasion in HCC SK-Hep1 cells by using western blotting, ELISA, gelatin zymography and Matrigel invasion assays. The effects of NF-κB inactivation on the expression of angiogenesis- and metastasis-associated proteins and cell invasion in SK-Hep1 cells were also evaluated.

Materials and methods

Chemicals. Regorafenib was provided by Bayer Corporation (Whippany, NJ, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin (PS) were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). TNF-α, IL-1β and IL-6 ELISA kits and NF-κB inhibitor QNZ were purchased from eBioscience, Inc. (San Diego, CA, USA) and Apexbio Technology LLC (Houston, TX, USA), respectively. Matrigel and Transwell (8-μm pore size) were obtained from Selleck Chemicals (Houston, TX, USA) and Corning Life Sciences (Tewksbury, MA, USA), respectively. Primary antibodies for β-actin and TNF-α were obtained from Thermo Fischer Scientific, Inc. (Waltham, MA, USA). Primary antibodies for NF-κB p65 and IL-1β were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies for MMP-9 and VEGF were purchased from EMD Millipore (Billerica, MA, USA). Primary antibodies for MMP-2, IL-6, and NF-κB p65 were purchased from OriGene Technologies, Inc., (Rockville, MD, USA), Abbiotec LLC (San Diego, CA, USA), and Santa Cruz Biotechnology (Dallas, TX, USA). Secondary antibodies were bought from Jackson ImmunoResearch Laboratoires, Inc. (West Grove, PA, USA).

Cell culture. Hepatocellular carcinoma SK-Hep1 cells were used in the present study. The SK-Hep1 cells were provided by Professor Jing-Gung Chung from the Department of Biological Science and Technology, China Medical University, Taichung, Taiwan. The cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained at 37˚C and 5% CO_2 in a humidified incubator (1).

MTT assay. The SK-Hep1 cells were seeded into 96-well plates with $2x10^4$ cells/well and incubated at room temperature overnight, then treated with regorafenib (0, 5, 10, 15, 20 and 25 μM) in 0.1% DMSO or QNZ (0, 0.05, 0.1, 0.2 and 0.4 μM) in 0.1% dimethyl sulphoxide for 12, 24, and 48 h. The effects of regorafenib and QNZ on cell viability were analyzed by MTT assay as described previously (16).

Western blotting. A total of $2x10^6$ SK-Hep1 cells were incubated at room temperature in a 10-cm diameter dish overnight, then treated with 20 μM regorafenib or 0.1 μM QNZ for 12 or 24 h. The total cellular proteins in all treatment groups were extracted using 1 mM phenylmethanesulfonyl fluoride, 0.5% NP-40, 120 mM NaCl, and 50 mM Tris-HCl pH 8.0 lysis buffer. The protein expressions of VEGF, MMP-2, MMP-9, IL-1β, IL-6, TNF-α, pNF-κB, and NF-κB were evaluated with western blotting assays as described by Wang *et al* (20). The protein expression was quantified by densitometry using ImageJ software version 1.50 (National Institutes of Health, Bethesda, MD, USA) in order to determine the density of regions of interest. β-actin was used as the internal control.

ELISA. The SK-Hep1 cells were seeded into 12-well plates with $1x10^5$ cells/well and incubated overnight at room temperature, then treated with 20 μM regorafenib or 0.1 μM QNZ for 12 h. ELISA kits were used to measure the levels of IL-1β, IL-6 and TNF-α secreted by the cells in media. The procedure followed the protocols provided by manufacturer.

Gelatin zymography assay. The SK-Hep1 cells were cultured in 6-well plates with $1x10^6$ cells/well and incubated overnight at room temperature, then treated with 20 μM regorafenib or 0.1 μM QNZ for 12 h. The secretion of active MMP-2 and MMP-9 from the cells in medium was analyzed with gelatin zymography assays as described previously (19). ImageJ software version 1.50 was used to quantify the bands of MMP-2 and MMP-9 activity.

Cell invasion assay. Matrigel-coated Transwell chambers were used to measure cell invasion. The 8-μm pore sized Transwell chambers were inserted into 96-well plates and coated with 50 ml Matrigel, then incubated in 5% CO_2 at 37˚C for 1 h. The SK-hep1 cells in serum-free DMEM were treated with 20 μM regorafenib or 0.1 μM QNZ and $2x10^5$ cells/well were added in the apical chamber of the Transwell, followed by the addition of DMEM supplemented with 10% FBS to the basolateral chamber and subsequent incubation for 12 h. Sterile cotton swabs were used to remove the Matrigel in the Transwell chamber. The invasive cells in the bottom of the Transwell were fixed with a mixture of 3:1 methanol and acetic acid and stained with 0.5% crystal violet for 15 min and then were counted under a light microscope at magnification, x100 (21).

Statistical analysis. The significance of difference between treatment group and control was verified by Student's t-test.

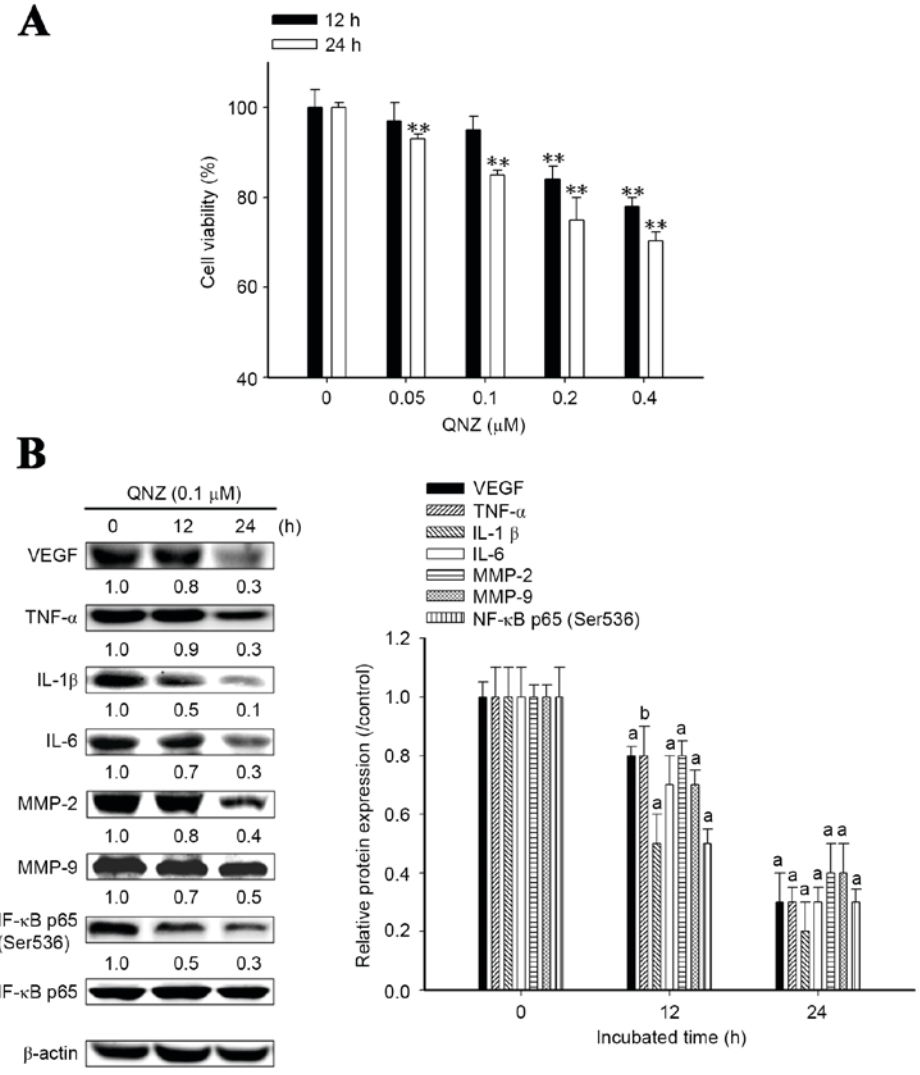

Figure 1. Effects of NF-κB inhibitor QNZ on cell viability and NF-κB-modulated expression of angiogenesis- and metastasis-associated proteins in SK-Hep1 cells. Cells were treated with 0, 0.05, 0.1, 0.2 and 0.4 μM of QNZ in 0.1% DMSO for various time intervals. (A) Change of cell viability was evaluated with MTT assay. **P<0.01 vs. control. (B) Changes in protein expression levels including VEGF, TNF-α, IL-1β, IL-6, MMP-2, MMP-9, active form of NF-κB p65 [NF-κB p65 (Ser 536)] and total NF-κB p65 (NF-κB p65) were demonstrated with western blotting. ^{a}P<0.01 and ^{b}P<0.05 vs. control. VEGF, vascular endothelial growth factor; TNF-α, tumor necrosis factor α; IL, interleukin; MMP, matrix metalloproteinase; NF-κB p65, nuclear factor-κB tumor protein 65; DMSO, dimethyl sulfoxide; QNZ, 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine.

Data were presented as the mean ± standard error. P<0.05 was considered to indicate a statistically significant difference. All experiments were performed three times.

Results

NF-κB inhibitor reduces cell viability and expression of angiogenesis and metastasis associated proteins in SK-Hep1 cells. To confirm the effects of NF-κB inactivation on the expression of angiogenesis- and metastasis-associated proteins, the SK-Hep1 cells were treated with a range of concentrations of QNZ for various time intervals. The effects of QNZ on cell viability, expression of angiogenesis- and metastasis-associated proteins, and NF-κB p65 in the SK-Hep1 cells were evaluated with an MTT assay and western blotting. Fig. 1A illustrates that the cell viability significantly reduced as the QNZ concentration and incubation time increased compared with the control. Fig. 1B indicates that the levels of expression of angiogenesis- and metastasis-associated proteins, along with NF-κB p65, were significantly inhibited by QNZ in a time-dependent manner.

Regorafenib induces cytotoxicity and inhibits expression of NF-κB-modulated angiogenesis- and metastasis-associated proteins in SK-Hep1 cells. The present study suggested that regorafenib affects the levels of expression of NF-κB-modulated angiogenesis- and metastasis-associated proteins by treating SK-Hep1 cells with different concentration of regorafenib for various time intervals. The effects of regorafenib on the cell viability and expression levels of the angiogenesis- and metastasis-associated proteins and NF-κB p65 in SK-Hep1 cells were evaluated with MTT assay and western blotting. Fig. 2A indicates that cell viability was significantly decreased by regorafenib in a dose- and time-dependent manner. Regorafenib significantly reduced expression of angiogenesis- and metastasis-associated proteins along with NF-κB p65 in a

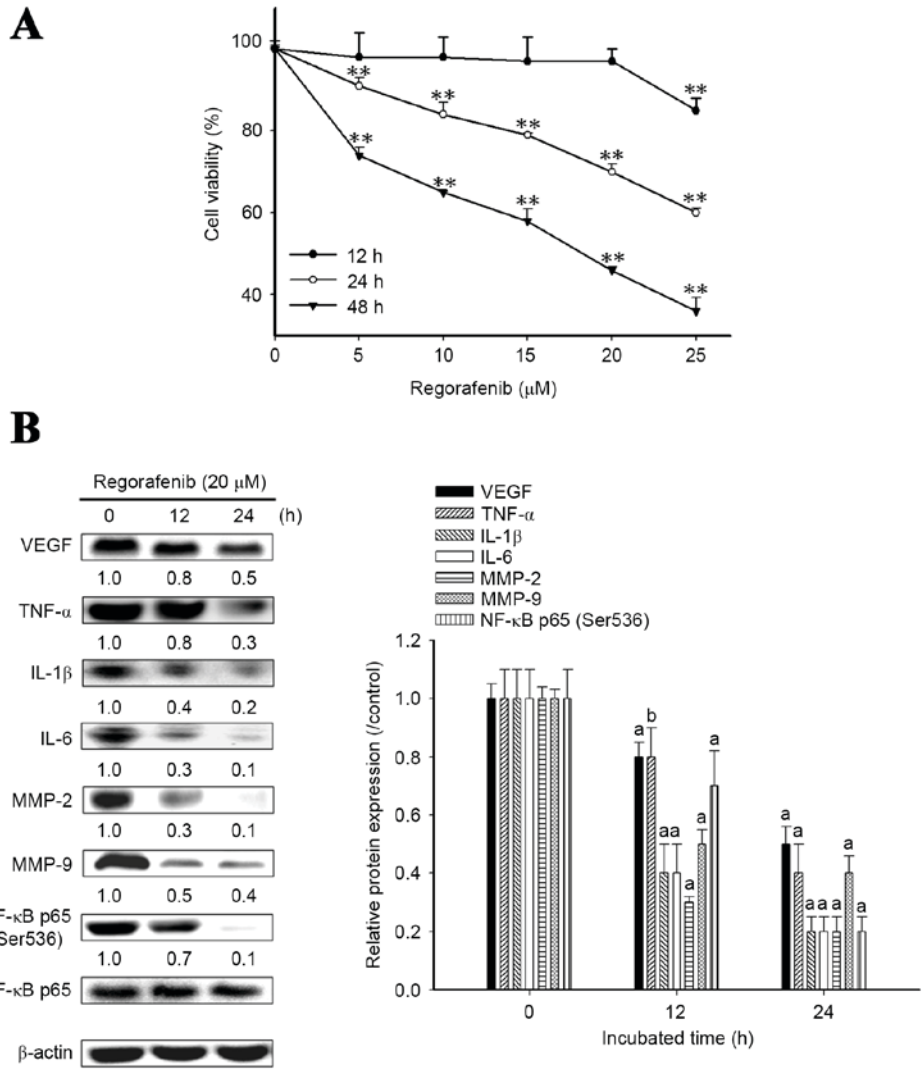

Figure 2. Effects of regorafenib on cell viability and NF-κB-modulated expression of angiogenesis- and metastasis-associated proteins in SK-Hep1 cells. Cells were treated with 0, 5, 10, 15, 20 and 25 μM regorafenib in 0.1% DMSO for various time intervals. (A) Change of cell viability was evaluated with MTT assay. **P<0.01 vs. control. (B) Change of protein expression level including VEGF, TNF-α, IL-1β, IL-6, MMP-2, MMP-9 and active form of NF-κB p65 [NF-κB p65 (Ser 536)] and total NF-κB p65 (NF-κB p65) were demonstrated with western blotting. ^{a}P<0.01 and ^{b}P<0.05 vs. control. VEGF, vascular endothelial growth factor; TNF-α, tumour necrosis factor α; IL, interleukin; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; NF-κB p65, NF-κB tumor protein 65; DMSO, dimethyl sulfoxide.

time dependent manner by 20-80% compared with the control (Fig. 2B).

Regorafenib and QNZ reduce secretion of angiogenesis associated cytokines in SK-Hep1 cells. ELISA was used to evaluate the effects of regorafenib and QNZ on the secretion of angiogenesis-associated cytokines TNF-α, IL-1β and IL-6 in the SK-Hep1 cells. TNF-α, IL-1β, and IL-6 secretion were significantly inhibited by 5-55% compared with the control, as demonstrated in Fig. 3A-C with unchanged cell viability, as illustrated in Figs. 1A and 2A, when the cells were treated with 0.1 μM QNZ or 20 μM regorafenib for 12 h.

Regorafenib and QNZ inhibit secretion of metastasis-associated proteins and cell invasion in SK-Hep1 cells. The effect of regorafenib and QNZ on the secretion of active MMP-2 and MMP-9 and cell invasion in the SK-Hep1 cells was investigated using gelatin zymography and cell invasion assays. Regorafenib and QNZ reduced the protein levels of MMP-2 and MMP-9, as demonstrated in Figs. 1B and 2B, and inhibited the secretion of active MMP-2 and MMP-9, as illustrated in Fig. 4A. It was also revealed that regorafenib and QNZ significantly decreased the number of cells that invaded the Matrigel by 50-70% compared with the control, as demonstrated in Fig. 4B.

Discussion

Regorafenib, a novel sorafenib derivative, has been approved by the Food and Drug Administration (FDA) for the treatment of metastatic colorectal cancer and advanced gastrointestinal stromal tumors (18). Our previous studies indicated that sorafenib, as an NF-κB signal inhibitor, inhibits the vorinostat- and 12-O-tetradecanoylphorbol-13-acetate-induced expression of angiogenesis- and metastasis-associated proteins through the inhibition of NF-κB activation in HCC cells *in vitro*

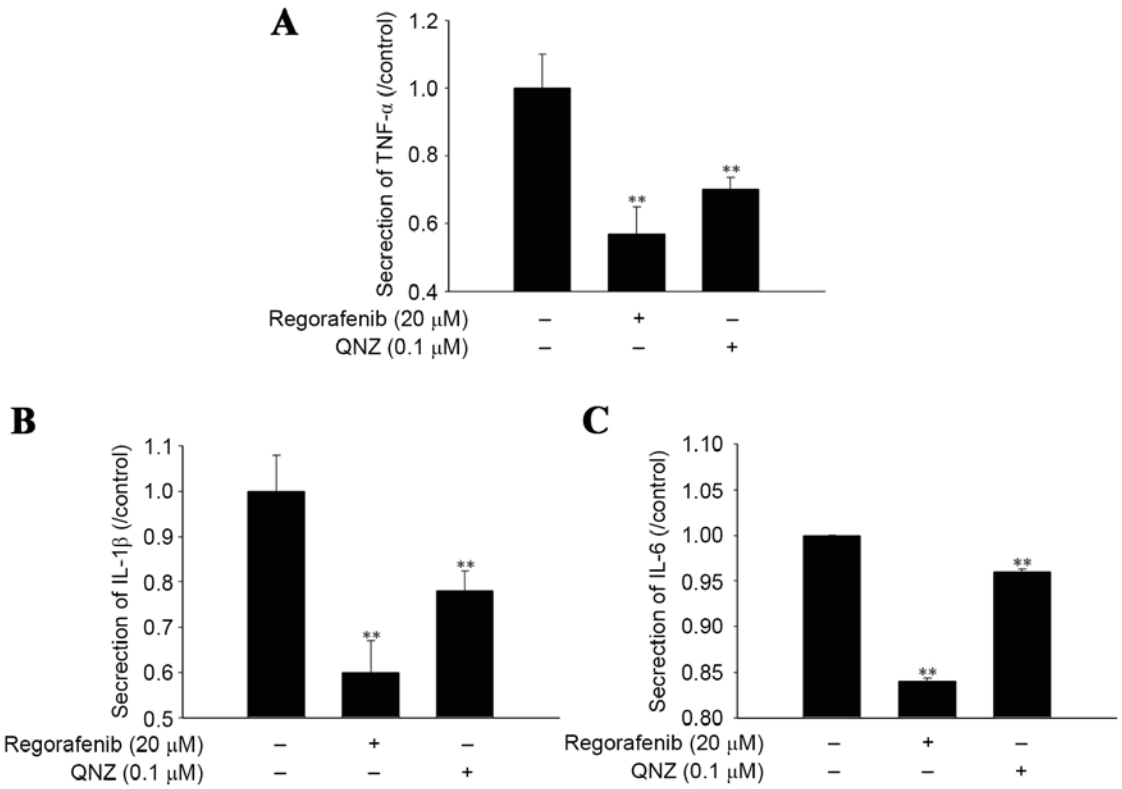

Figure 3. Effects of regorafenib and QNZ on secretion of inflammatory cytokines TNF-α, IL-1β and IL-6 in SK-Hep1 cells. Cells were treated with 20 μM regorafenib or 0.1 μM QNZ for 12 h. The secretion of cytokines was measured using ELISA. (A) Secretion of TNF-α. (B) Secretion of IL-1β. (C) Secretion of IL-6. **P<0.01 vs. control. TNF-α, tumor necrosis factor α; IL, interleukin; QNZ, 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine.

and *in vivo* (13,19). However, whether regorafenib triggers anti-angiogenic and antimetastatic effects via the inhibition of NF-κB activation in HCC cells remains unknown. Therefore, in the present study, the effects of regorafenib on the levels of expression of NF-κB-modulated angiogenesis- and metastasis-associated proteins and cell invasion in HCC cells were investigated *in vitro*. It was revealed that QNZ, a specific NF-κB inhibitor, reduces the cell viability and expression levels of angiogenesis- and metastasis-associated proteins VEGF, TNF-α, IL-1β, IL-6, MMP-2, MMP-9 and NF-κB p65. Additionally, it was identified that regorafenib induces cytotoxicity and inhibits the expression of angiogenesis- and metastasis-associated proteins VEGF, TNF-α, IL-1β, IL-6, MMP-2, MMP-9 and NF-κB p65. Regorafenib and QNZ significantly decrease the levels of cytokines TNF-α, IL-1β and IL-6 secreted by the cells in media. Finally, regorafenib and QNZ also significantly decrease the secretion of active MMP-2 and MMP-9 and the number of invading cells.

Pikarsky *et al* (22) suggested that NF-κB activation is essential for promoting inflammation-associated HCC. NF-κB signaling has been identified as a biomarker for cancer that is indicative of a self-sufficiency in proliferative growth signals, an insensitivity to growth inhibitory signals, an ability to evade apoptosis, an unlimited replicative potential and the induction of angiogenesis and metastasis by modulating the levels of expression of effector proteins (6,23,24). The inhibition of NF-κB signaling reduces the expression of angiogenesis- and metastasis-associated proteins and results in the suppression of tumor progression (13,23,24). Our previous studies revealed that the transfection of inhibitor of nuclear factor κBβ mutant vector, a super repressor of NF-κB activation, decreased the protein expression of MMP-9 and VEGF in HCC and oral cancer cells (19,25). Huang *et al* (26) demonstrated that the inhibition of NF-κB activation is associated with the suppression of angiogenesis, invasion, and metastasis in prostate cancer cells. In the present study, QNZ, a specific inhibitor of NF-κB activation, was used to investigate the effects of NF-κB inactivation on the levels of angiogenesis- and metastasis associated-proteins and cell invasion in SK-Hep1 cells. It was demonstrated that QNZ significantly decreases the expression levels and secretion of angiogenesis- and metastasis-associated proteins and cell invasion in the SK-Hep1 cells. Baud and Karin (23) suggested that NF-κB signaling may be a potential molecular target for cancer therapy and the development of the NF-κB pathway inhibitors may offer an effective treatment approach for certain types of cancer.

Constitutive NF-κB activation was demonstrated in HCC and used as a biomarker for poor prognosis. Jin *et al* (14) used immunohistochemistry (IHC) to evaluate the expression and location of NF-κB proteins in HCC and surrounding normal hepatic tissues. It was identified that NF-κB nuclear translocation in HCC was significantly increased compared with adjacent normal liver tissue. In addition, NF-κB nuclear translocation was significantly higher in patients with HCC exhibiting adverse clinical features including advanced staging, portal vein tumor thrombus, and the involvement of regional lymph nodes compared with other patients with early-stage disease. Chemotherapeutic agents and radiation are able to trigger the NF-κB-modulated expression of angiogenesis- and metastasis-associated proteins in

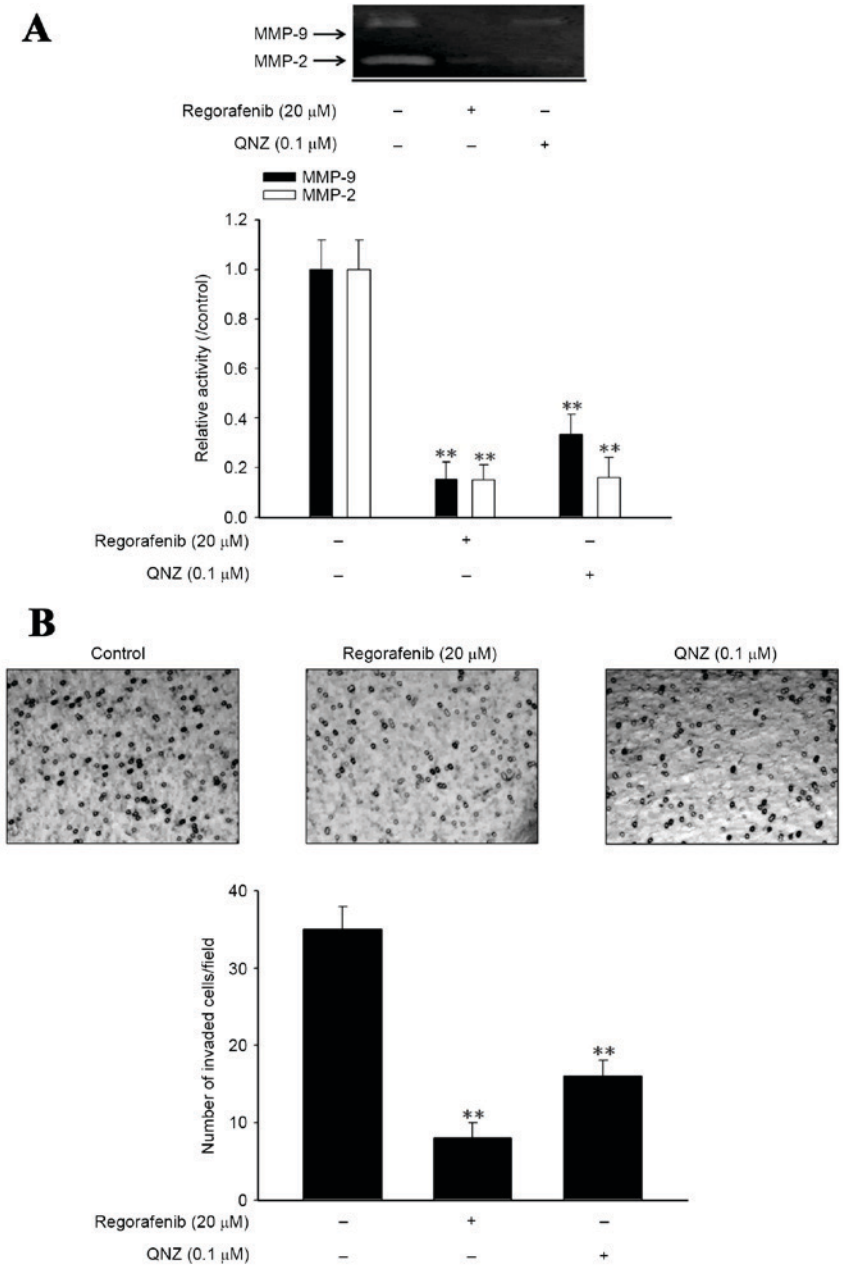

Figure 4. Effects of regorafenib and QNZ on the secretion of active MMP-2 and MMP-9, and cell invasion. Cells were treated with 20 μM regorafenib or 0.1 μM QNZ for 12 h. The secretion of active MMP-2 and MMP-9 and cell invasion were evaluated with gelatin zymography and cell invasion assays. (A) Change in active MMP-2 and MMP-9 secretion. (B) Change in invaded cell numbers, magnification, x100. **P<0.01 vs. control. MMP, matrix metalloproteinase; QNZ, 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine.

HCC cells via the activation of NF-κB signaling (15,16). Sorafenib, a multiple kinase inhibitor, has been approved by the FDA for the treatment of advanced HCC (13). Previous studies have demonstrated that sorafenib inhibits endogenous NF-κB activity and reverses the anticancer drug and carcinogen-induced NF-κB activity with the inhibition of MMP-9 and VEGF protein expression in HCC *in vitro* and *in vivo* (13,19). Carr *et al* (27) suggested that regorafenib, or fluoro-sorafenib, inhibits tumor growth via the induction of caspase-dependent apoptosis in HCC cells. However, the effect of regorafenib on NF-κB-modulated angiogenesis and metastasis in HCC cells remains unknown.

The main purpose of the present study was to investigate if regorafenib affects the NF-κB-modulated expression of angiogenesis- and metastasis-associated proteins and cell invasion in SK-Hep1 cells. The role of NF-κB inactivation on the anti-angiogenic and antimetastatic effects was investigated using QNZ. Based on the data from previous studies, it was suggested that the blockage of NF-κB activation triggers anti-angiogenic and antimetastatic effects in HCC cells. Then, regorafenib was used and revealed similar findings to the experiments with QNZ. Regorafenib significantly decreased the level of NF-κB activation and the levels of expression of angiogenesis- and metastasis-associated proteins. The secretion of inflammatory cytokines TNF-α, L-1β and IL-6 were significantly decreased by regorafenib treatment. Regorafenib suppressed the secretion of active MMP-2 and MMP-9 and the number of cells invaded in SK-Hep1 cells. The effects of

regorafenib on NF-κB activation in HCC may be different in other types of cancer cells. Chen *et al* (28) revealed that regorafenib inhibits colorectal tumor growth through the activation of the NF-κB-modulated protein expression of p53 upregulated modulator of apoptosis. Regorafenib is hypothesized to induce anti-angiogenic and antimetastatic effects through the suppression of NF-κB activation in SK-Hep 1 cells *in vitro*.

In conclusion, the present study demonstrated that regorafenib reduced the expression and secretion of the angiogenesis- and metastasis-associated proteins VEGF, TNF-α, IL-1β, IL-6, MMP-2 and MMP-9, and cell invasion through the inhibition of NF-κB activation. Therefore, regorafenib may exhibit a potential clinical application as an NF-κB signal inhibitor to limit angiogenesis and metastasis in HCC.

Acknowledgements

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