miR-378a enhances the sensitivity of liver cancer to sorafenib by targeting VEGFR, PDGFRβ and c-Raf

HONGXIA FU[1], JICAI ZHANG[1], TONGSHAN PAN[2], SHUYING AI[3], LI TANG[4] and FENGQIN WANG[3]

[1]Department of Clinical Laboratory, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000;
[2]Department of Pediatrics, Danjiangkou First Hospital, Danjiangkou, Hubei 442700; Departments of [3]Oncology and
[4]Neurology, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei 442000, P.R. China

Abstract. Liver cancer is a globally prevalent cancer with poor prognosis. The present study investigated the link between microRNA-378a (miR-378a) expression and the sensitivity of hepatocellular carcinoma (HCC) and hepatoblastoma (HB) cancers to sorafenib therapy. miR-378a expression was determined in liver tissue samples from healthy candidates and patients with liver cancer using the reverse transcription-quantitative polymerase chain reaction. The antitumor effects of miR-378a alone and in combination with sorafenib were investigated in the HB cell line HepG2 and the HCC cell line SMMC-7721 with methyl thiazoyl tetrazolium, colony formation, flow cytometry and Transwell migration assays. The underlying mechanisms were investigated using western blot analysis. miR-378a expression was decreased in tissue samples from patients with liver cancer. HCC and HB cell line proliferation and invasion ability was inhibited by miR-378a. The combination of miR-378a and sorafenib provided the greatest inhibition. Western blot indicated that mitogen activated protein kinase signaling pathway proteins, vascular endothelial growth factor receptor, platelet derived growth factor receptor β, Raf-1 proto-oncogene, serine/threonine kinase and matrix metallopeptidase 2 were regulated by miR-378a alone and to a greater extent when combined with sorafenib. Results suggest that miR-378a can inhibit liver cancer cell growth and enhance the sensitivity of liver cancer cells to sorafenib-based chemotherapies.

Introduction

Liver cancer is a globally prevalent and aggressive cancer with poor prognosis (1). Examples include hepatocellular

Correspondence to: Dr Fengqin Wang, Department of Oncology, Taihe Hospital, Hubei University of Medicine, 32 South Renmin Road, Shiyan, Hubei 442000, P.R. China
E-mail: fqwang042017@hotmail.com

carcinoma (HCC) and hepatoblastoma (HB): HCC is estimated to be the second leading cause of cancer-associated mortality in males and the sixth in females worldwide (2), whereas HB is the most common pediatric liver tumor in the US (3,4). Currently approved therapeutic drugs for liver cancer are ineffective and result in poor patient outcomes (5). Sorafenib is a systemic drug used as a first line therapy for advanced HCC and prolongs the overall survival of patients with HCC from 7.9 to 10.7 months (6). Recent studies have indicated that sorafenib can also induce apoptosis in HB cells and inhibit the progression of HB (7,8). Sorafenib is a small molecule multiple tyrosine kinase inhibitor that can induce apoptosis in cancer cells via the downregulation of vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor β (PDGFRβ), KIT proto-oncogene receptor tyrosine kinase and fms like tyrosine kinase 3 expression levels, as well as through the inhibition of the mitogen-activated protein kinase (MAPK) and Wnt/β-catenin signaling pathways (9,10). However, not all patients with liver cancer benefit from sorafenib therapy and drug resistance is often acquired within 6 month (11).

MicroRNAs (miRNAs) are a class of small, non-coding RNA molecules consisting of 20-22 nucleotides (12). Accumulating evidence has demonstrated that miRNAs have an important role in regulating the growth of various tumors, including those in HCC and HB (13-17). Several studies have reported the important physiological role of miRNA-378a (miR-378a). Chen *et al* (18) reported that miR-378a inhibited prostate cancer via downregulating the MAPK1 signaling pathway. Wei *et al* (19) demonstrated that miR-378a promotes myoblastic differentiation by regulating histone deacetylase 4 in skeletal muscle development and Zhang *et al* (20) indicated that miR-378a activated the pyruvate-phosphoenolpyruvate futile cycle and regulated lipolysis. To the best of the authors' knowledge, the effects of miR-378a alone and in combination with sorafenib in HCC and HB treatment has not yet been studied.

The present study analyzed expression levels of miR-378a in liver tissue samples from healthy donors and patients with liver cancer. The effects of miR-378a alone or in combination with sorafenib therapy on the proliferation and invasion capacities of the HepG2 HB cell line and the SMMC-7721 HCC cell line was also investigated. Results indicated that miR-378a

alone inhibited the proliferation and invasion capacity of both the HB and HCC cell lines. miR-378a also enhanced the sensitivity of HB and HCC cells to sorafenib by targeting VEGFR, PDGFRβ and Raf-1 proto-oncogene, serine/threonine kinase (c-Raf). These results may provide a new avenue for the development of liver cancer therapies.

Materials and methods

Clinical samples. A total of 32 liver cancer and 32 adjacent normal liver tissue samples were obtained from February to October 2016 (Taihe Hospital, Shiyan, China). The mean ± standard deviation (SD) age of candidates was 43.6±7.9 years (range, 36-58 years). A total of 18 male and 14 female samples were obtained. The Ethics Committee of the Shiyan Taihe Hospital Institutional Review Board approved the present study and patients' permission was obtained prior to surgery. Following the surgery, tissue samples were stored at -80°C. Pathological information was obtained following the operation according to the Union of International Cancer Control's TNM classification of Malignant Tumors 2010 (21). Among the patients, 8 cases of stage 3B, 8 cases of stage 3C and 16 cases of stage 4A cancer were identified.

Cell culture. HepG2 and SMMC-7721 cell lines (American Type Culture Collection, Manassas, VA, US) were cultured in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% fetal bovine serum (FBS; 10438026) (both from Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 IU/ml penicillin and streptomycin (Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37°C.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from tissue samples using TRIzol (Thermo Fisher Scientific, Inc.). Primer was ordered from Thermo Fisher Scientific, Inc. (assay ID: 478349_mir; Thermo Fisher Scientific, Inc.). Reverse transcription was subsequently performed using the TaqMan MicroRNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.) and the thermo-cycling conditions were as follows: 16°C for 30 min, 42°C for 30 min and followed with 85°C for 5 min, then staying at 4°C. miRNA was then examined using the TaqMan MiRNA PCR kit (Applied Biosystems; Thermo Fisher Scientific, Inc.), along with the small nuclear RNA U6 (assay ID: 001973; Thermo Fisher Scientific, Inc.) as an endogenous control, with the following thermo-cycling conditions: 95°C for 20 sec (1 cycle), 95°C for 3 sec and 60°C for 30 sec (40 cycles). miRNA expression was measured using the ΔΔCq method (22). ΔCq was calculated by subtracting the Cq of U6 RNA from the Cq of each miRNA of interest. The ΔΔCq was calculated by subtracting the ΔCq of the control sample from the ΔCq of each sample. Fold change was generated by using the equation $2^{-\Delta\Delta Cq}$.

Luciferase reporter assays. Using TargetScan (http://www.targetscan.org/), putative target sequences of miR-378a were predicted to be in the 3' untranslated region (3'UTR) of VEGFR (AGUCCAGA), PDGFR (GUCCAGA) and c-Raf (AGUCCAG), and synthesized by Shanghai GenePharma Co., Ltd. (Shanghai, China). To evaluate the function of miR-378a, the 3'UTRs of VEGFR, PDGFRβ and c-Raf as well as the miR-378a target sequence (GUCCAG) was cloned into a pMIR-REPORT firefly luciferase microRNA expression reporter vector between *Hind* and *Spe*I sites (Ambion; Thermo Fisher Scientific, Inc.). Details of the PCR thermocycling conditions are as follows: An initial denaturation step at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec, 55°C for 45 sec and 72°C for 30 sec. Vectors were co-transfected with miR-378a mimic into 293 cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), respectively. To test transfection efficiency, luciferase reporter vectors with mutant target sequences (VEGFR-3'UTR-mAUCACUGA, PDGFR-3'UTR-mGAGUGGA and RAF1-3'UTR-mACA GGAG) were transfected in parallel as a negative control. Luciferase activity was measured by Luciferase Assay System (cat. no. E1500; Promega Corporation, Madison, WI, USA) 48 h after transfection.

Transient transfection. miR-378a mimic (3'-ACUGGACUU GGAGUCAGA AGGC-5') and its corresponding negative control (3'-UCAGGAGCGUUGCCUGGCUCGG-5') were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Cells ($5x10^5$/well) were cultured to 60-70% confluence prior to incubation with the miR-378a mimic or miR-378a mimic control at a final concentration of 0.1 μM using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) in a serum-free medium for 48 h.

Western blot analysis. Cells were lysed for 30 min on ice in radioimmunoprecipitation assay buffer (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with phosphatase inhibitor cocktail (CST Biological Reagents Co., Ltd., Shanghai, China). Protein concentrations were determined using the bicinchoninic acid kit (cat. no. BCA1-1KT; Sigma-Aldrich; Merck KGaA) Proteins (50 μg) were separated on 12% SDS-PAGE prior to transfer onto polyvinylidene difluoride membranes. Membranes were incubated in blocking buffer (I-block, T2015; Thermo Fisher Scientific, Inc.) at room temperature for 2 h then incubated at 4°C overnight with the following primary antibodies: Phospho-(p)-VEGFR-pTyr[1333] (1:1,000, cat. no. SAB4504006; Sigma-Aldrich; Merck KGaA); VEGFR (1:1,000, cat. no. ab46154; Abcam, Cambridge, MA, USA); p-PDGFRβ Tyr[751] (1:1,000, cat. no. 3161; CST Biological Reagents Co., Ltd., Danvers, MA, USA); PDGFRβ (1:1,000, cat. no. Sc-374573; Santa Cruz Biotechnology, Inc., Dallas, TX USA); p-c-Raf, Ser[259] (1:1,000, cat. no. 9421); c-Raf; (1:1,000, cat. no. 9422); p-extracellular signal-related kinase 1/2 (p-ERK1/2) Thr[202/204] (1:1,000, cat. no. 9101); ERK1/2 (1:1,000, cat. no. 9102); and MMP2 (1:1,000; cat. no. 4022) (all from CST Biological Reagents Co., Ltd.) and poly ADP ribose polymerase (PARP, 1:1,000; cat. no. ab32071; Abcam). Membranes were subsequently incubated at room temperature for 2 h with horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig)G (1:2,000, cat. no. 7074) or HRP conjugated anti-mouse IgG (1:2,000, cat. no. 7076) (both from CST Biological Reagents Co., Ltd.) secondary antibodies. β-actin (1:5,000, cat. no. AB8229; Abcam) was used as a loading control. Proteins were visualized using SuperSignal West Pico Plus Chemiluminescent Substrate (cat. no. 34577;
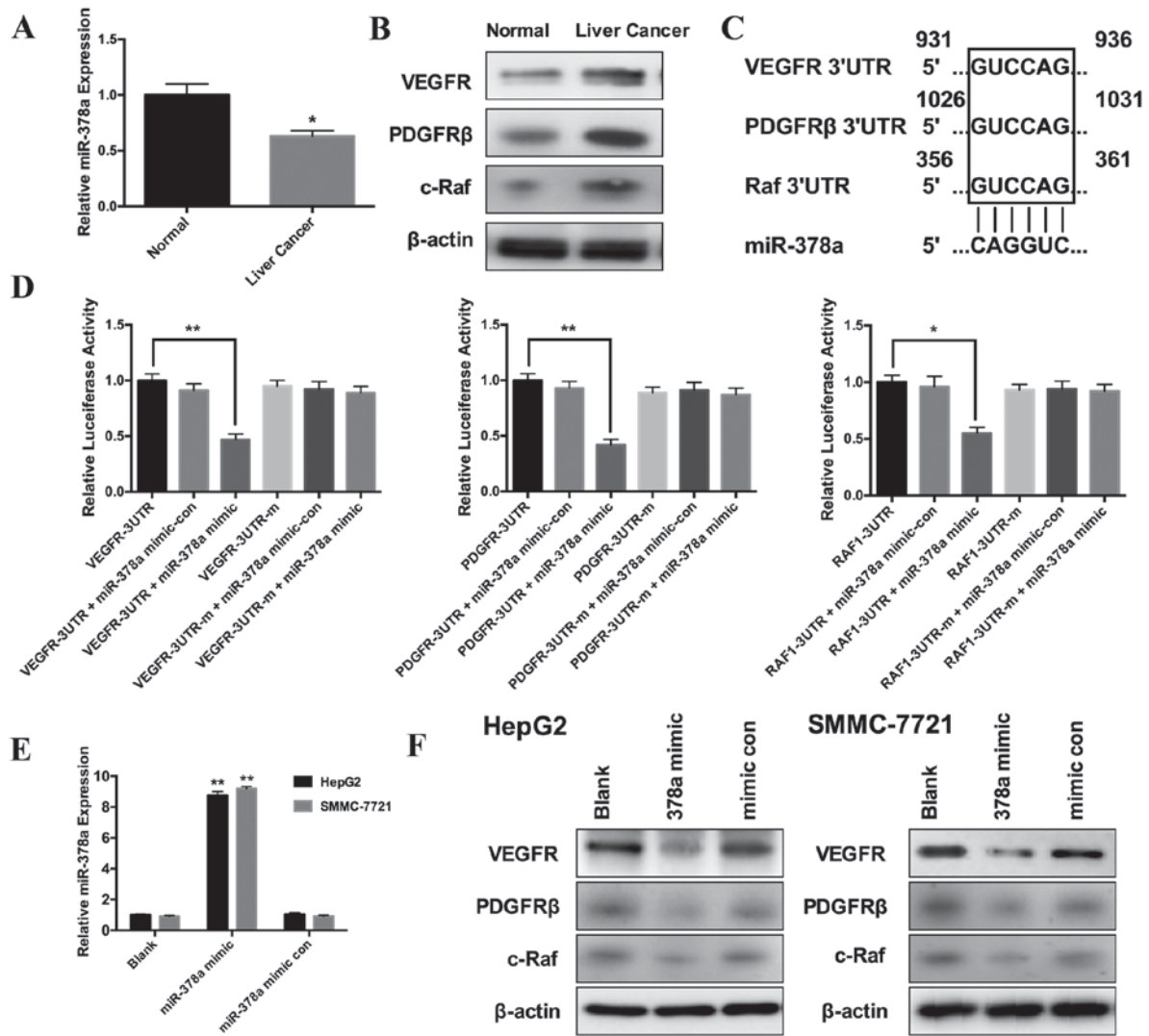
Figure 1. Effects on miR-378a on liver cancer cell *in vitro*. (A) Expression levels of miR-378a quantified by reverse transcription-quantitative polymerase chain reaction. (B) Western blot analysis of VEGFR, PDGFRβ and c-Raf expression in normal and tumor samples. (C) Schematic diagram demonstrating the wild-type or mutated miR-378a binding sites in VEGFR, PDGFRβ and c-Raf 3'UTR fragments. (D) Luciferase reporter assay data of wild-type or mutant 3'UTR of VEGFR, PDGFRβ and c-Raf with miR-378a mimic-con or miR-378a mimic, respectively. $^*P<0.05$, $^{**}P<0.01$. (E) miR-378a expression levels measured in HepG2 and SMMC-7721 cell lines transfected miRNA-378 mimic or mimic-con. $^{**}P<0.01$ vs. blank, n=3/group. (F) The expression level of VEGFR, PDGFRβ and c-Raf in HepG2 and SMMC-7721 cells following transfection with miR-378a mimic. miR, microRNA; VEGFR, vascular endothelial growth factor receptor; PDGFRβ, platelet derived growth factor receptor β; c-Raf, Raf-1 proto-oncogene serine/threonine kinase; 3'UTR, 3' untranslated region; 3'UTRm, mutated 3'UTR; mimic-con, miR mimic control.

Thermo Fisher Scientific, Inc.). ImageJ software version 1.50i (National Institutes of Health, Bethesda, MD, USA) was used for density analysis.

Cell viability assay. Untransfected and miR-378a mimic transfected HepG2 and SMMC-7721 cells (48 h after transfection) were seeded into a 96-well plate (1,500 cells/well). Cells were treated with sorafenib (S7397, Selleck Chemicals, Houston, TX, USA; 0.25, 0.5, 1, 2, 4, 8 and 16 μM) for 72 h prior to incubation with 1 mg/ml of methyl thiazoyl tetrazolium (MTT; Sigma-Aldrich; Merck KGaA) for 4 h at 37°C in a CO_2 incubator. Mitochondrial reduction of MTT in DMSO to formazan was tested by culture at 37°C for 15 min. The amount of formazan absorbed was measured at 570 nm absorbance, with 450 nm used as the reference wavelength. IC_{50} values were calculated and expressed as a mean ± SD of three independent experiments.

Colony formation assay. The cells were seeded at a density of 200 cells/ml into 6-well culture plates for 24 h, then washed with PBS and cultured with DMEM (Invitrogen; Thermo Fisher Scientific, Inc.) in the presence or absence of sorafenib (EC_{10}=1.8±0.2 μM, concentration set as 0.25, 0.5, 1, 2, 4, 8 and 16 μM). Colonies were allowed to grow for 14 days. Cells were subsequently fixed in 70% ethanol for 15 min at room temperature and stained with 0.2% crystal violet at room temperature for 20 min. Positive colony formations (>50 cells/colony) were counted. EC_{50} was calculated as the concentration of sorafenib eliciting a half-maximal response.

Flow cytometry assay. Following the treatment with sorafenib (0.25, 0.5, 1, 2, 4, 8 and 16 μM) for 72 h, cells were fixed with 70% cold ethanol for 30 min at 4°C, stained with 200 μl propidium iodide (50 μg/ml) at room temperature for 15 min

and analyzed by flow cytometry. The data were analyzed using Cellquest Pro version 5.1 software (BD Biosciences, San Jose, CA, US). The experiments were repeated at least three times.

Cell invasion assay. Matrigel (356235; BD Biosciences, San Jose, CA, USA) was pre-coated on the upper compartment of Millicell cell culture inserts containing 8.0-μm pores (PSET010R5; EMD Millipore, Temecula, CA, USA). DMEM (~600 μl) with 10% FBS was added to the lower compartment and 200 μl serum free culture medium with 0.1% BSA containing 2×10^5 HepG2 cells were seeded to the upper compartment. After culture with sorafenib (0.25, 0.5, 1, 2, 4, 8 and 16 μM) for 24 h at 37°C in a humidified 5% CO_2 environment. Cells that had migrated to the lower compartment were fixed with 70% ethanol for 15 min at room temperature and stained with 0.2% crystal violet at room temperature for 20 min and counted under the phase contrast microscope (Olympus CX41; Olympus Corporation, Tokyo, Japan), magnification, x100.

Statistical analysis. Statistical analysis was completed with SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) and presented as the mean ± standard deviation for repeated measures. All experiments were repeated at least three times. A one-way analysis of variance test was used for comparison of differences between groups. Tukey's multiple comparison was used as the post hoc test. $P \leq 0.05$ was considered to indicate a statistically significant difference.

Results

Sorafenib can suppress tumor growth through the inhibition of multiple tyrosine kinases, including VEGFR, PDGFRβ and c-Raf. These three genes were input into TargetScan to identify seed matched miRNA predictions. miR-378a was predicted to target all three genes. Thus, miR-378a expression levels were investigated in liver tissue samples from healthy donors and patients with liver cancer. RT-qPCR indicated that the expression level of miR-378a in liver cancer samples was significantly reduced compared with normal liver samples (Fig. 1A). Western blot analysis indicated increased expression of VEGFR, PDGFRβ and c-Raf in liver cancer samples (Fig. 1B). These data suggest that miR-378a has effects similar to sorafenib and therefore may have the potential to treat liver cancer.

The ability of miR-378a to target VEGFR, PDGFRβ and c-RAF mRNA was subsequently confirmed through the construction of luciferase expression vectors harboring wild-type and mutant 3'UTR target sequences of the genes: VEGFR-3UTR, VEGFR-3UTR-m; PDGFRβ-3UTR, PDGFRβ-3UTR-m; Raf-3UTR and Raf-3UTR-m (Fig. 1C). The assay revealed that the wild-type target sequences had significantly lower luciferase activity than their respective mutants in 293 cells in the presence of the miR-378a mimic. By contrast, the miR-378a mimic did not reduce luciferase activity when miR-378a seed sequences at 3'UTR of these three gene were mutated. (Fig. 1D). These data indicated that miR-378a may target VEGFR, PDGFRβ and c-Raf mRNA in 293 cells.

Table I. Anti-proliferative activity of miR1-378a and sorafenib on HepG2 and SMMC-7721 cells.

	IC_{50}	
Treatment	HepG2	SMMC-7721
Sorafenib	5.5±0.5 μM	4.7±0.4 μM
miR-378a mimic	-	-
miR-378a mimic control	-	-
Sorafenib + miR-378a mimic	3.2±0.4 μM[a]	2.8±0.3 μM[a]
Sorafenib + miR-378a mimic control	4.8±0.6 μM	4.9±0.5 μM

[a]P<0.05 vs. sorafenib alone. Untransfected and miR-378a mimic transfected HepG2 and SMMC-7721 cells were treated with sorafenib. After 72 h of treatment, IC_{50} values were calculated. miRNA-378a, microRNA-378a; mimic-con, mimic control.

Table II. miR-378a alone or combined with sorafenib affected the invasion capacity of HepG2 cells.

Groups	Cell count	Inhibition rate (%)
Non-treatment	8,884±214	-
Sorafenib	2,168±99[b]	75.6
miR-378a mimic	5,740±203[a]	35.4
miR-378a mimic control	8,657±375	2.6
Sorafenib + miR-378a mimic	1,245±88[b]	86.0
Sorafenib + miR-378a mimic control	2,215±102[b]	75.1

[a]P<0.05; [b]P<0.01. Untransfected and miR-378a transfected HepG2 cells were treated with sorafenib for 24 h. Results were reported as mean migrated cells/5 field ± standard deviation of three independent experiments. The inhibition rate was calculated according to the formula: Inhibition rate (%) = [Cell count (non-treatment) - cell count (treatment)]/cell count (non-treatment) x 100. miRNA-378a, microRNA-378a; mimic-con, mimic control.

The miR-378a mimic or the miR-378a mimic control were transfected into HepG2 and SMMC-7721 cells. The expression of miR-378a was significantly upregulated in miR-378a mimic transfected HepG2 and SMMC-7721 cells, compared with untransfected and miR-378a mimic negative control transfected cells (Fig. 1E). Western blotting also confirmed that VEGFR, PDGFRβ and c-Raf expression levels were significantly lower in miR-378a mimic transfected HepG2 and SMMC-7721 cells compared with cells transfected with the mimic control (Fig. 1F).

The inhibitory capacity of sorafenib on cell proliferation in untransfected or miR-378a mimic transfected liver cancer cells was evaluated by MTT assay (Table I). After 72 h, sorafenib displayed anti-proliferative activity in untransfected HepG2 and SMMC-7721 cells with IC_{50} values of 5.5±0.5 and 4.7±0.4 μM, respectively. Liver cancer cells transfected with miR-378a mimic had significantly lower IC_{50} values for sorafenib, at 3.2±0.4 μM in HepG2 and 2.8±0.3 μM in SMMC-7721 cells.
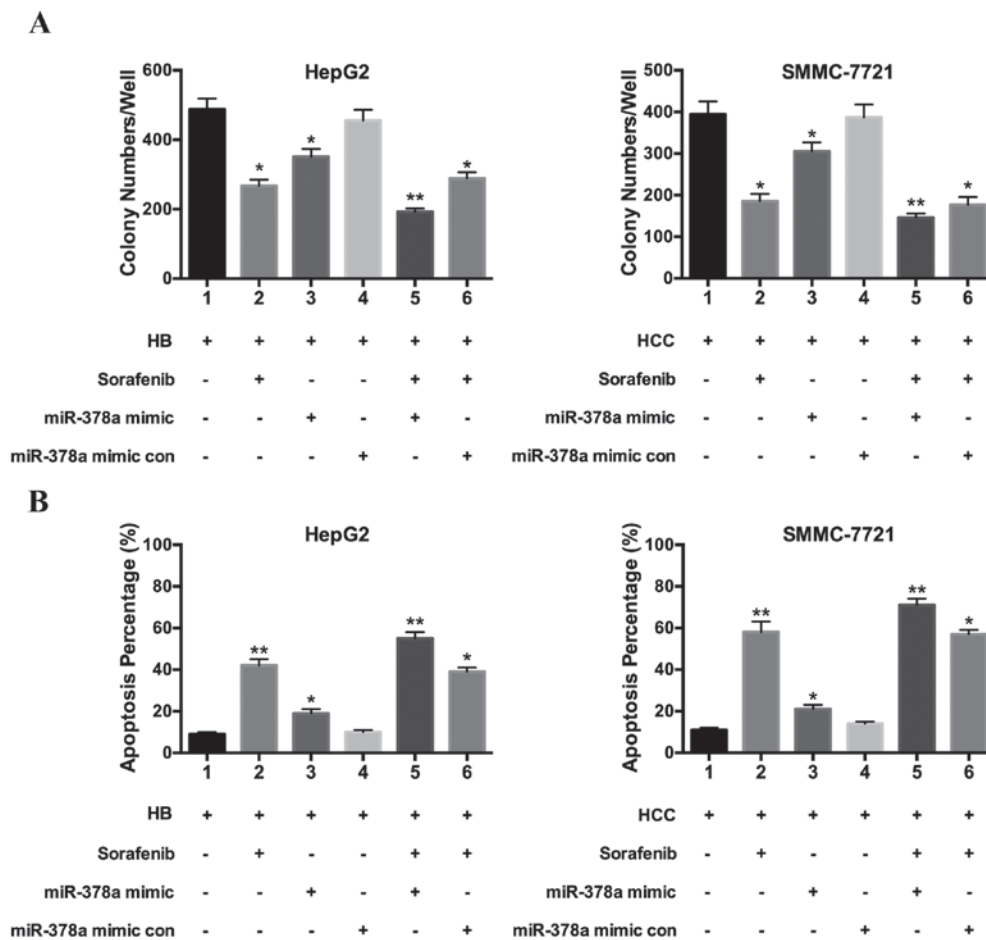
Figure 2. miR-378a alone, sorafenib alone and their combination inhibited proliferation and induced apoptosis in HepG2 and SMMC-7721 cells. (A) Colony formation was inhibited in HepG2 and SMMC-7721 cell lines by sorafenib, miR378a and their combination. *P<0.05, **P<0.01 vs. untreated group. (B) Apoptosis of HepG2 and SMMC-7721 cell lines was induced by sorafenib, miR-378a and their combination. *P<0.05, **P<0.01 vs. untreated group. HB, hepatoblastoma; HCC, hepatocellular carcinoma; miR-378a, microRNA-378a.

The antitumor activity of sorafenib in liver cancer cells was further confirmed in colony formation experiments. The calculated EC_{50} values of sorafenib in untransfected HepG2 and SMMC-7721 cell lines were 2.1±0.2 and 1.8±0.2 μM, respectively. EC_{50} values of sorafenib in miR-378a mimic transfected HepG2 and SMMC-7721 were significantly lower, at 1.6±0.2 and 1.3±0.2 μM, respectively (Fig. 2A). Changes in cell cycle distribution and apoptosis were subsequently analyzed in both untransfected and miR-378a mimic transfected HepG2 and SMMC-7721 cells with a flow cytometry assay. Treatment with sorafenib for 72 h increased the proportion of cells in the G0-G1 phase and decreased the proportion of cells in the S and G2/M phase (data not shown). These results suggest that the inhibition of proliferation by sorafenib in HepG2 and SMMC-7721 cells is in part due to a G1 phase arrest. Furthermore, a larger proportion of cells were dead cells in the miR-378a mimic transfected group compared with untransfected cells. Sorafenib increased the proportion of dead cells in the miR-378a transfected group from 50±5 to 61±6%, demonstrating a possible synergistic inhibitory effect of miR-378a and sorafenib on liver cancer cell growth (Fig. 2B).

Invasion capacity is an important characteristic of malignant cells. Therefore, the ability of miR-378a to reduce HepG2 cell invasion activity was investigated using Transwell assays. Compared with controls, miR-378a mimic transfection reduced the invasive potential of HepG2 cells by 35.4%, and sorafenib treatment in miR-378a mimic transfected HepG2 cells reduced the invasive potential by 86.0% (Fig. 3; Table II). This inhibition rate was significantly higher than that of sorafenib treated untransfected HepG2 cells (75.6% reduction). These data demonstrate that sorafenib or miR-378a alone affect the invasive potential of HepG2 cells in vitro and miR-378a enhances the sensitivity of HepG2 cells to sorafenib.

Raf kinases are key regulators of the MAPK cascade, and upregulated signaling in this pathway has an important role in various types of cancer. The expression levels of MAPK pathway proteins in untransfected and miR-378a mimic transfected HepG2 cells were analyzed using western blotting in order to investigate the mechanism underlying the inhibition of tumor growth by sorafenib (Fig. 4). Results indicated that sorafenib reduced the level of three tyrosine kinase targets: p-PDGFRβ, p-VEGFR and p-c-Raf, in both untransfected and miR-378a mimic transfected HepG2 cells. MAPK signaling pathway proteins activity were significantly downregulated by sorafenib in HepG2 cells (P<0.05). Invasion regulatory protein matrix metallopeptidase 2 (MMP2) was downregulated by sorafenib in HepG2 cells. Concurrent with these results, the pro-apoptotic protein PARP was significantly increased in sorafenib treated
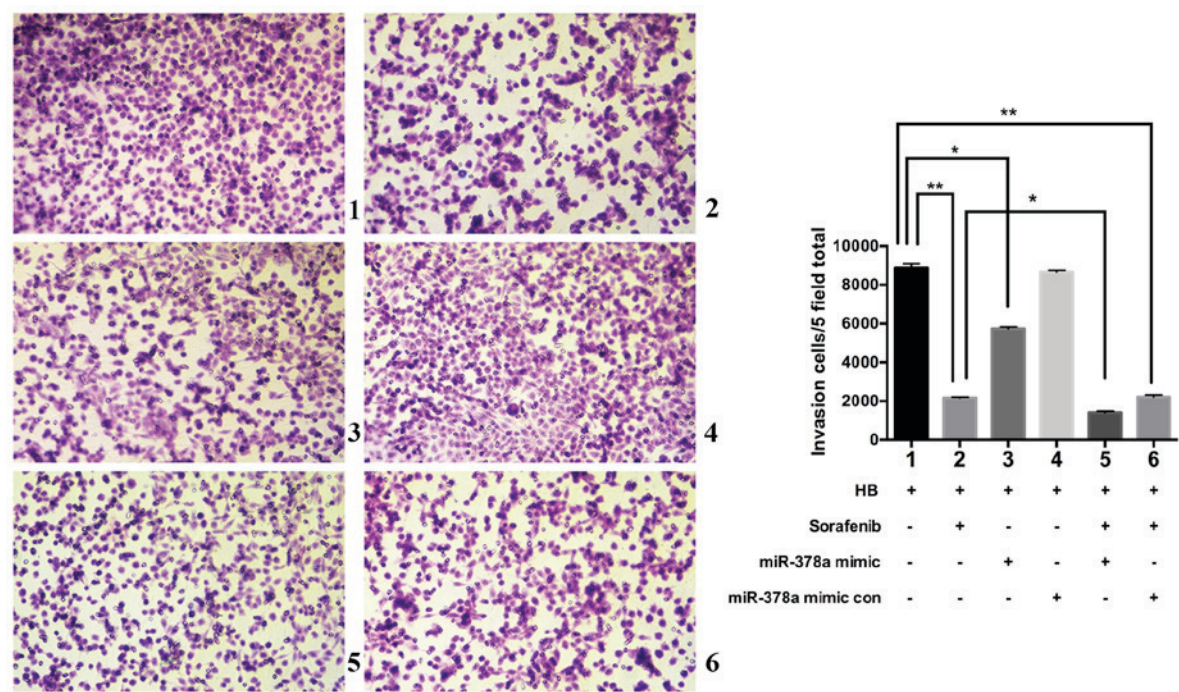
Figure 3. Sorafenib affected the invasion capacity of untransfected and miR-378a transfected HepG2 cells. Cells were treated with sorafenib for 24 h. The HepG2 non-treatment group was used as a control. The data are presented as the mean ± standard deviation. *P<0.05, **P<0.005. HB, hepatoblastoma; miR-378a, microRNA-378a.
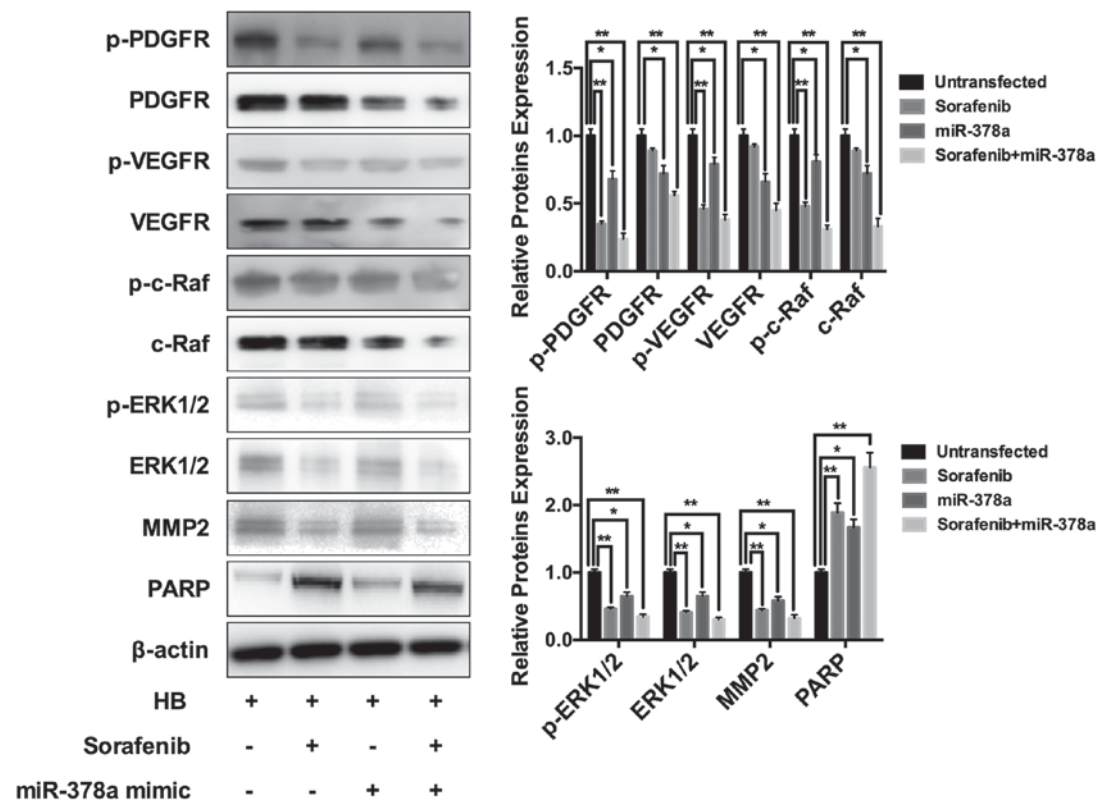

Figure 4. Western blot analysis of PDGFRβ, VEGFR, c-Raf, mitogen-activated protein kinase signaling, MMP2 and PARP expression and their semi-quantitative data in untransfected and miR-378a transfected HepG2 cell line. *P<0.05, **P<0.01. p, phosphorylated; PDGFR, platelet derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor; c-Raf, raf-1 proto-oncogene serine/threonine kinase; ERK, extracellular signal-regulated kinase; MMP2, matrix metallopeptidase 2; PARP, poly-(ADP-ribose) polymerase; HB, hepatoblastoma; miR-378a, microRNA-378a.

HepG2 cells. It was demonstrated that miR-378a also regulated the expression level of PARP and MMP2. Furthermore, the expression level of MAPK signaling proteins was significantly lower in miR-378a mimic transfected cells compared with

untransfected HepG2 cells. These data suggest that sorafenib or miR-378a alone can regulate MAPK signaling and the expression of its subsequent downstream proteins and miR-378a can enhance the sensitivity of HepG2 cells to sorafenib.

Discussion

Sorafenib is the mainstay therapy recommended for the treatment of liver cancer, despite its low clinical response rate (6,11). miRNAs have a critical role in the biological behavior of liver cancer (23). Various studies have indicated the effectiveness of miRNAs against HCC and HB is due to their ability to inhibit cell growth, invasion ability, angiogenesis and induce tumor cell apoptosis (24-27). miR-378a has been reported to regulate multiple physiological activities, including myoblast differentiation, adipogenesis, and tumor growth (28), such as those found in colorectal cancer and rhabdomyosarcoma (19,29-31). A recent study reported that miR-378a enhances the sensitivity of MCF-7 breast cancer cells to tamoxifen by targeting golgi transport 1A protein (32). However, to the best of our knowledge, the effect of miR-378a in combination with sorafenib treatment in liver cancer cells has not yet been studied.

The present study revealed that miR-378a levels were decreased in liver cancer tissues. Upregulated miR-378a may inhibit proliferation and induce apoptosis of HepG2 and SMMC-7721 cells. To further examine the mechanisms underlying these effects, VEGFR, PDGFRβ and c-Raf were selected as candidate miR-378a target genes by *in silico* algorithms. These three genes are also important targets of sorafenib. Experiments revealed that mRNA levels of these three candidate genes were downregulated in 293 cells transfected with miR-378a mimic, which was consistent with the computational analysis. The luciferase reporter assay suggested that miR-378a regulates the expression of VEGFR, PDGFRβ and c-Raf by directly targeting the 3'UTR of these genes. VEGFR, PDGFRβ and c-Raf regulate various cellular processes, including proliferation, apoptosis, the stress response and mitotic checkpoints in cancer cells (33-36). In addition, these target genes mediate the activation of the MAPK signaling pathway. Inhibition of the MAPK/ERK1/2 pathway has been demonstrated to be beneficial in numerous cancer studies (33,37,38). The present study indicated miR-378a or sorafenib alone can inhibit MAPK/ERK1/2 signaling and enhance the expression of the pro-apoptotic protein PARP. A synergistic effect was observed in tumor cells transfected with miR-378a and subsequently treated with sorafenib. miR-378a also enhanced the G1 phase arrest and anti-invasion effects of sorafenib on liver cancer cells (data not shown).

In conclusion, the inhibitory effect of sorafenib on tumor cell proliferation and invasion activities was enhanced by combined treatment with miR-378a. These findings could potentially be applied to develop alternative approaches to liver cancer therapy.

References

1. El-Serag HB and Rudolph KL: Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. Gastroenterology 132: 2557-2576, 2007.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global Cancer Statistics, 2012. Ca Cancer J Clin 65: 87-108, 2015.
3. McLaughlin CC, Baptiste MS, Schymura MJ, Nasca PC and Zdeb MS: Maternal and infant birth characteristics and hepatoblastoma. Am J Epidemiol 163: 818-828, 2006.
4. Trobaugh-Lotrario AD, Meyers RL, O'Neill AF and Feusner JH. Unresectable hepatoblastoma: Current perspectives. Hepatic Med 9: 1-6, 2017.
5. Livraghi T, Mäkisalo H and Line PD: Treatment options in hepatocellular carcinoma today. Scand J Surg 100: 22-29, 2011.
6. Cheng AL, Kang YK, Chen Z, Tsao CJ, Qin S, Kim JS, Luo R, Feng J, Ye S, Yang TS, et al: Efficacy and safety of sorafenib in patients in the Asia-Pacific region with advanced hepatocellular carcinoma: A phase III randomised, double-blind, placebo-controlled trial. Lancet Oncol 10: 25-34, 2009.
7. Rodríguez-Hernández A, Navarro-Villarán E, González R, Pereira S, Soriano-De Castro LB, Sarrias-Giménez A, Barrera-Pulido L, Álamo-Martínez JM, Serrablo-Requejo A, Blanco-Fernández G, et al: Regulation of cell death receptor S-nitrosylation and apoptotic signaling by sorafenib in hepatoblastoma cells. Redox Biology 6: 174-182, 2015.
8. Shanmugam N, Valamparampil JJ, Scott JX, Vij M, Narasimhan G, Reddy MS and Rela M: Complete remission of refractory hepatoblastoma after liver transplantation in a child with sorafenib monotherapy: A new hope? Pediatric Blood Cancer: Jul 17, 2017 (Epub ahead of print).
9. Dazert E. Colombi M, Boldanova T, Moes S, Adametz D, Quagliata L, Roth V, Terracciano L, Heim MH, Jenoe P and Hall MN: Quantitative proteomics and phosphoproteomics on serial tumor biopsies from a sorafenib-treated HCC patient. Proc Natl Acad Sci USA 113: 1381-1386, 2016.
10. Galuppo R, Maynard E, Shah M, Daily MF, Chen C, Spear BT and Gedaly R: Synergistic inhibition of HCC and liver cancer stem cell proliferation by targeting RAS/RAF/MAPK and WNT/β-catenin pathways. Anticancer Res 34: 1709-1713, 2014.
11. Llovet JM, Ricci S, Mazzaferro V, Hilgard P, Gane E, Blanc JF, de Oliveira AC, Santoro A, Raoul JL, Forner A, et al: Sorafenib in advanced hepatocellular carcinoma. N Engl J Med 359: 378-390, 2008.
12. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
13. Song X, Wang Z, Jin Y, Wang Y and Duan W: Loss of miR-532-5p in vitro promotes cell proliferation and metastasis by influencing CXCL2 expression in HCC. Am J Transl Res 7: 2254-2261, 2015.
14. Liu Y, Ding Y, Huang J, Wang S, Ni W, Guan J, Li Q, Zhang Y, Ding Y, Chen B and Chen L: MiR-141 suppresses the migration and invasion of HCC cells by targeting Tiam1. PLoS One 9: e88393, 2014.
15. Gao B, Gao K, Li L, Huang Z and Lin L: miR-184 functions as an oncogenic regulator in hepatocellular carcinoma (HCC). Biomed Pharmacother 68: 143-148, 2014.
16. He J, Guo X, Sun L, Wang N and Bao J: Regulatory network analysis of genes and microRNAs in human hepatoblastoma. Oncol Lett 12: 4099-4106, 2016.
17. Cairo S, Wang Y, de Reyniès A, Duroure K, Dahan J, Redon MJ, Fabre M, McClelland M, Wang XW, Croce CM and Buendia MA: Stem cell-like micro-RNA signature driven by Myc in aggressive liver cancer. Proc Natl Acad Sci USA 107: 20471-20476, 2010.
18. Chen QG, Zhou W, Han T, Du SQ, Li ZH, Zhang Z, Shan GY and Kong CZ: miR-378 suppresses prostate cancer cell growth through downregulation of MAPK1 in vitro and in vivo. Tumor Biol 37: 2095-2103, 2016.
19. Wei X, Li H, Zhang B, Li C, Dong D, Lan X, Huang Y, Bai Y, Lin F, Zhao X and Chen H: miR-378a-3p promotes differentiation and inhibits proliferation of myoblasts by targeting HDAC4 in skeletal muscle development. RNA Biol 13: 1300-1309, 2016.
20. Zhang Y, Li C, Li H, Song Y, Zhao Y, Zhai L, Wang H, Zhong R, Tang H and Zhu D: miR-378 activates the pyruvate-pep futile cycle and enhances lipolysis to ameliorate obesity in mice. EBioMedicine 5: 93-104, 2016.
21. International Union Against Cancer (UICC): TNM classification of malignant tumors. Sobin LH, Gospodarowicz MK and Wittekind Ch (eds). 7th editon. Wiley-Blackwell, Oxford, 2010.
22. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

23. Khare S, Zhang Q and Ibdah JA: Epigenetics of hepatocellular carcinoma: Role of microRNA. World J Gastroenterol 19, 5439-5445, 2013.
24. Nakao K, Miyaaki H and Ichikawa T: Antitumor function of microRNA-122 against hepatocellular carcinoma. J Gastroenterol 49: 589-593, 2014.
25. Zhu H, Wang G, Zhou X, Song X, Gao H, Ma C, Chang H, Li H, Liu FF, Lu J and Ma J: miR-1299 suppresses cell proliferation of hepatocellular carcinoma (HCC) by targeting CDK6. Biomed Pharmacother 83: 792-797, 2016.
26. Huang CY, Huang XP, Zhu JY, Chen ZG, Li XJ, Zhang XH, Huang S, He JB, Lian F, Zhao YN and Wu GB: miR-128-3p suppresses hepatocellular carcinoma proliferation by regulating PIK3R1 and is correlated with the prognosis of HCC patients. Oncol Rep 33: 2889-2898, 2015.
27. Chang L, Li K and Guo T: miR-26a-5p suppresses tumor metastasis by regulating EMT and is associated with prognosis in HCC. Clin Transl Oncol 19: 695-703, 2016.
28. Krist B, Florczyk U, Pietraszek-Gremplewicz K, Józkowicz A and Dulak J: The role of miR-378a in metabolism, angiogenesis, and muscle biology. Int J Endocrinol 2015: 281756, 2015.
29. Huang N, Wang J, Xie W, Lyu Q, Wu J, He J, Qiu W, Xu N and Zhang Y: miR-378a-3p enhances adipogenesis by targeting mitogen-activated protein kinase 1. Biochem Biophys Res Commun 457: 37-42, 2015.
30. Li H, Dai S, Zhen T, Shi H, Zhang F, Yang Y, Kang L, Liang Y and Han A: Clinical and biological significance of miR-378a-3p and miR-378a-5p in colorectal cancer. Eur J Cancer 50: 1207-1221, 2014.
31. Megiorni F, Cialfi S, McDowell HP, Felsani A, Camero S, Guffanti A, Pizer B, Clerico A, De Grazia A, Pizzuti A, *et al*: Deep Sequencing the microRNA profile in rhabdomyosarcoma reveals down-regulation of miR-378 family members. BMC Cancer 14: 880, 2014.
32. Ikeda K, Horie-Inoue K, Ueno T, Suzuki T, Sato W, Shigekawa T, Osaki A, Saeki T, Berezikov E, Mano H and Inoue S: miR-378a-3p modulates tamoxifen sensitivity in breast cancer MCF-7 cells through targeting GOLT1A. Sci Rep 5: 13170, 2015.
33. Li C, Li Y, Lv H, Li S, Tang K, Zhou W, Yu S and Chen X: The novel anti-neuroblastoma agent PF403, inhibits proliferation and invasion in vitro and in brain xenografts. Int J Oncol 47: 179-187, 2015.
34. Gao J, Liu X, Yang F, Liu T, Yan Q and Yang X: By inhibiting Ras/Raf/ERK and MMP-9, knockdown of EpCAM inhibits breast cancer cell growth and metastasis. Oncotarget 6: 27187-27198, 2015.
35. Moreno L, Popov S, Jury A, Al Sarraj S, Jones C and Zacharoulis S: Role of platelet derived growth factor receptor (PDGFR) over-expression and angiogenesis in ependymoma. J Neurooncol 111: 169-176, 2013.
36. Ghosh S, Sullivan CA, Zerkowski MP, Molinaro AM, Rimm DL, Camp RL and Chung GG: High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. Hum Pathol 39: 1835-1843, 2008.
37. Burotto M, Chiou VL, Lee JM and Kohn EC: The MAPK pathway across different malignancies: A new perspective. Cancer 120: 3446-3456, 2014.
38. Song X, Wei Z and Shaikh ZA: Requirement of ERα and basal activities of EGFR and Src kinase in Cd-induced activation of MAPK/ERK pathway in human breast cancer MCF-7 cells. Toxicol Appl Pharmacol 287: 26-34, 2015.