Combination of Matrine and Sorafenib Decreases the Aggressive Phenotypes of Hepatocellular Carcinoma Cells

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Key Words

Apoptosis · Combination therapy · Growth suppression ·
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Abstract

Background: The aim of this study was to determine the effects of matrine (a natural alkaloid) on sorafenib-induced cytotoxicity against hepatocellular carcinoma (HCC) cells, and to explore the molecular mechanisms involved. **Methods:** HepG2 and Hep3B cells were treated with matrine alone or in combination with sorafenib, and cell viability and apoptosis were assessed. The involvement of micro (mi)RNA-21 in the action of matrine was examined. **Results:** Matrine significantly augmented the antiproliferative activity of sorafenib in a dose-dependent manner. Matrine significantly increased apoptosis, coupled with enhanced cleavage of caspase-3 and poly (ADP-ribose) polymerase. miRNA-21-overexpressing HCC cells showed a marked decrease in matrine-induced growth suppression and the expression of phosphatase and tensin homolog (PTEN). The suppressive effect of combining matrine and sorafenib was significantly reduced by miRNA-21 overexpression or PTEN inhibition. **Conclusion:** Matrine in combination with sorafenib leads to increased cytotoxic effects against HCC cells, at least partially, via the suppression of miRNA-21 and the subsequent induction of PTEN.

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Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths worldwide [1]. The poor prognosis of HCC largely results from the asymptomatic nature of the early stage of the disease [2]. For advanced HCC, no effective therapies are currently available. Sorafenib (previously known as BAY 43-9006) is the first oral multi-kinase inhibitor and shows cytotoxic effects against a variety of cancers [3]. Sorafenib has been approved by the US Food and Drug Administration for the treatment of advanced HCC. Two randomized, placebo-controlled, phase III trials have demonstrated that sorafenib offers survival benefits to patients with advanced HCC; however, the improvement in overall survival is limited, with only 2–3 months of prolongation [4, 5]. At present, many attempts have been made to develop a sorafenib-based combination with other anticancer reagents to improve therapeutic efficacy [6].

Matrine is one of the major alkaloid components of *Sophora flavescens* Ait, a traditional Chinese medicine, and possesses a variety of pharmacological effects, including anticancer effects [7]. It has been documented that matrine induces growth inhibition and apoptosis in V600EBRAF-harboring melanoma cells via the activation of phosphatase and tensin homolog (PTEN), a well-known tumor suppressor [8]. Zhang et al. [9] reported that matrine promotes apoptosis in human acute myeloid

leukemia cells via the activation of caspase-3. Caspase-3 activation and subsequent cleavage of poly (ADP-ribose) polymerase (PARP) play a critical role in mediating apoptosis [10, 11]. Matrine also exerts anticancer activity in HCC cells. It has been reported that matrine inhibits cell proliferation in human HCC HepG2 cells [12]. Zhang et al. [13] confirmed that matrine causes growth suppression and promotes apoptotic and autophagic death in HCC cells. When used with conventional chemotherapeutic agents, matrine was found to provide synergistic inhibitory effects on tumor growth in an animal model of gastric cancer [14]. However, its effect on the efficacy of sorafenib in HCC is still not known.

In breast cancer, matrine has been found to inhibit cell growth via the modulation of the micro (mi)RNA-21/PTEN/Akt pathway [15]. miRNAs are a large class of small noncoding RNAs involved in the posttranscriptional regulation of target genes [16, 17]. Compelling evidence indicates that miRNA-21 plays an oncogenic role in HCC by promoting cell proliferation, migration and invasion, which are causally linked to targeting PTEN [18].

In this study, we explored the combined effects of sorafenib and matrine on HCC cell proliferation and apoptosis and checked whether the anticancer property of matrine in HCC was mediated by modulation of the miRNA-21/PTEN axis.

Materials and Methods

Reagents and Antibodies

Dulbecco's modified Eagle medium, fetal bovine serum, penicillin, streptomycin and Lipofectamine 2000 transfection reagent were purchased from Invitrogen (Carlsbad, Calif., USA), miRNA-21 mimic, negative mimic and mirVana miRNA isolation kit from Ambion (Foster City, Calif., USA), PTEN small interfering RNA (siRNA) and control siRNA from Cell Signaling Technology (Beverly, Mass., USA), Taqman miRNA assays from Applied Biosystems (Foster City, Calif., USA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), phenylmethylsulfonyl fluoride, aprotinin, leupeptin and sodium orthovanadate from Sigma-Aldrich (St. Louis, Mo., USA), an annexin V apoptosis kit from Becton Dickinson Biosciences (San Diego, Calif., USA) and enhanced chemiluminescence reagents from Amersham Biosciences (Piscataway, N.J., USA). Rabbit anti-cleaved caspase-3, anti-cleaved PARP, anti-PTEN and anti- β -actin antibodies were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA).

Cell Culture

Human HCC cells (HepG2 and Hep3B) were purchased from the American Type Culture Collection (Manassas, Va., USA). They were maintained in Dulbecco's modified Eagle medium

supplement with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C in a 5% CO₂ humidified incubator.

Cell Treatment and Transfection

HepG2 and Hep3B cells at a density of 4×10^5 cells/well in 6-well plates or 3×10^3 cells/well in 96-well plates were exposed to sorafenib at 2.5 μ M [19] for 48 h in the presence or absence of different concentrations (0.4–1.6 g/l) of matrine. After treatment, cells were tested for proliferation and apoptosis. For transient overexpression of miRNA-21, cells were transfected with 50 nM miRNA-21 mimic or negative mimic using Lipofectamine 2000 transfection reagent. For knockdown of PTEN expression, cells were transiently transfected with PTEN or control siRNA at a concentration of 100 nM, using Lipofectamine 2000 Transfection reagent. After incubation for 24 h, the transfected cells were treated with matrine (0.4 g/l) alone or along with sorafenib. The cells were collected 48 h later and tested for proliferation.

miRNA-21 Expression Analysis

Total RNA was isolated from cells using the mirVana miRNA isolation kit, according to the manufacturer's instructions. The expression level of mature miRNA-21 was determined using Taqman miRNA assays. Briefly, cDNA was synthesized with an miRNA-specific stem-loop primer, and quantitative PCR was performed using specific TaqMan microRNA assay primers. The relative miRNA-21 amount normalized to the U6 small nuclear RNA level was calculated using the comparative cycle threshold ($\Delta\Delta C_t$) method [20]. Each assay was carried out in triplicate.

Cell Proliferation Assay

Cell proliferation was determined using the MTT method. Briefly, after drug treatment for 48 h, the MTT solution (5 mg/ml) was added to the cell cultures and incubated at 37°C for 4 h. After the removal of MTT, dimethyl sulfoxide solution was added to dissolve formazan crystals. Absorbance was measured at a wavelength of 570 nm. Triplicate wells were tested for each treatment.

Apoptosis Analysis by Annexin-V/Propidium Iodide Staining

After drug treatment, cells were harvested by trypsinization and resuspended in phosphate-buffered saline. Cell apoptosis was determined using the annexin V apoptosis kit. The cells were stained with fluorescein isothiocyanate-conjugated annexin V and propidium iodide solution (20 μ g/ml) for 15 min in the dark. Apoptotic cells (annexin V-positive) were analyzed by flow cytometry (Becton Dickinson Biosciences). Each assay was performed in triplicate.

Western Blot Analysis

Cells were rinsed with ice-cold phosphate-buffered saline and lysed in lysis buffer (10 mM Tris/HCl pH 6.8, 10% glycerol, 2% sodium dodecyl sulfate, 1% Triton X-100, 1% Nonidet P-40) containing 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin and 1 mM sodium orthovanadate. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, Mass., USA). Membranes were incubated in blocking solution, probed with various antibodies, and then visualized by means of horseradish peroxidase-conjugated secondary

Fig. 1. Effects of sorafenib and matrine alone or in combination on HCC cell proliferation. HepG2 (**a**) and Hep3B cells (**b**) were exposed to sorafenib (2.5 μ M) or matrine (0.4 g/l) alone or the combination of sorafenib with matrine at 0.4, 0.8 and 1.6 g/l. After 48-hour incu-

bation, cell proliferation was determined using the MTT assay. The proliferation in untreated cells (control) was arbitrarily assigned 100%. * $p < 0.05$ vs. control; # $p < 0.05$ vs. sorafenib or matrine alone.

Fig. 2. Effects of sorafenib and matrine alone or in combination on apoptosis of HCC cells. HepG2 and Hep3B cells were exposed to sorafenib (2.5 μ M) and matrine (0.4 g/l) alone or in combination. After 48-hour incubation, cell apoptosis was assessed. **a** The cells were stained with annexin V and propidium iodide and the percentage of apoptotic cells was determined using flow cytometry.

* $p < 0.05$ vs. control; # $p < 0.05$ vs. sorafenib or matrine alone. **b** Cells were tested for apoptotic markers by Western blot analysis. Representative blots of 3 independent experiments with similar results are shown. β -Actin was used as the loading control. c-casp-3 = Cleaved caspase-3; c-PARP = cleaved PARP.

antibodies and enhanced chemiluminescence reagents. Densitometry was performed using Quantity One software (Bio-Rad, Hercules, Calif., USA).

Statistical Analysis

Data are presented as means $\pm$ standard deviation. The difference among the means of multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by the Tukey test. A p value of <0.05 was considered statistically significant.

Results

Matrine Augments the Antiproliferative Effect of Sorafenib in HCC Cells

Compared to untreated control, sorafenib (2.5 μ M) or matrine (0.4 g/l) alone caused a 10–30% reduction in the proliferation of HepG2 (fig. 1a) and Hep3B (fig. 1b) cells

after 48-hour incubation. The combination with matrine significantly ($p < 0.05$) augmented the antiproliferative activity of sorafenib in HCC cells, compared to sorafenib alone. In the presence of matrine (0.4 g/l), sorafenib treatment inhibited the proliferation of HepG2 and Hep3B cells by 52 and 57%, respectively ($p < 0.05$ vs. untreated control). If not stated otherwise, the concentration of matrine used in the following experiments was 0.4 g/l.

Combining Sorafenib with Matrine Enhances Apoptotic Death of HCC Cells

Exposure to sorafenib or matrine alone led to modest apoptosis in HepG2 cells (7.8 ± 1.2 and $11.5 \pm 3.7\%$, respectively) relative to control ($2.9 \pm 0.6\%$; fig. 2a). Notably, the combination of sorafenib with matrine promoted significant apoptosis in HepG2 cells, with an apoptotic rate of $26.5 \pm 4.8\%$. Similar proapoptotic effects of combined

Fig. 3. Effects of matrine and sorafenib alone or in combination on miRNA-21 and PTEN expression. Cells were treated with sorafenib (2.5 μ M) and matrine (0.4 g/l) alone or in combination for 48 h and tested for gene expression. **a** Real-time PCR analysis was done to measure miRNA-21 expression changes. The miRNA-21 expression level in untreated cells (control) was arbitrarily assigned 100%. Data are representative of 3 independent experiments. **b** Western blot analysis of PTEN protein levels. Top panels show representative blots of 3 independent experiments. Bottom panels demonstrate densitometric analysis of PTEN protein expression. Data are expressed as fold change of control. * $p < 0.05$ vs. control.

sorafenib and matrine treatment were observed in Hep3B cells (fig. 2a). Western blot analysis demonstrated that treatment with combined sorafenib and matrine resulted in a marked enhancement in the cleavage of caspase-3 and PARP, compared to treatment with each alone (fig. 2b).

Exposure to Matrine but Not Sorafenib Inhibits miRNA-21 and Increases PTEN in HCC Cells

Real-time PCR analysis revealed that exposure to matrine significantly ($p < 0.05$) decreased the expression of miRNA-21 in HepG2 and Hep3B cells, compared to control cells (fig. 3a). The miRNA-21 expression was similarly reduced in HCC cells with combined sorafenib and

matrine. However, sorafenib treatment had no significant effect on miRNA-21 expression in HCC cells. Western blot analysis revealed that matrine significantly induced the PTEN protein expression in HCC cells, but such induction was not enhanced by combined matrine and sorafenib (fig. 3b).

miRNA-21 Overexpression Antagonizes the Anticancer Activity of Matrine Alone or in Combination with Sorafenib by Targeting PTEN

MTT assay revealed that pretransfection with miRNA-21 mimic almost completely abrogated matrine (0.4 g/l)-induced growth suppression in HepG2 and Hep3B cells (fig. 4a). miRNA-21 overexpression significantly ($p < 0.05$) blocked the induction of PTEN expression by matrine alone (fig. 4b). Similar to overexpression of miRNA-21, the repression of PTEN expression with siRNA significantly ($p < 0.05$) reduced the growth suppression by matrine in HCC cells (fig. 4c). Western blot analysis confirmed the effective reduction of PTEN protein in HCC cells by the delivery of PTEN siRNA but not control siRNA (fig. 4d). The inhibition of HCC cell proliferation by combining matrine and sorafenib was significantly counteracted by pretransfection with miRNA-21 mimic or PTEN siRNA (fig. 4a, c).

Discussion

Sorafenib administration has become a new standard therapy for patients with advanced HCC [5]. To improve its therapeutic efficacy, many sorafenib-based combined modalities have been tested [21–23]. However, their overall outcomes are unsatisfactory. There is an urgent need for the development of novel therapeutic approaches to advanced HCC. The combination of sorafenib with bioactive natural compounds may lead to increased therapeutic efficacy. For instance, Wan et al. [24] reported that combined treatment with sorafenib and tetrandrine induces synergistic apoptosis in cancer cells via the activation of reactive oxygen species/Akt signaling. Cohen et al. [25] demonstrated that a combination of sorafenib with withaferin A, a natural withanolide, significantly enhances apoptotic death in both papillary and anaplastic thyroid cancer cells. Our data showed that the combination of sorafenib and matrine caused a significant increase in growth suppression and apoptosis induction, relative to each agent alone. At the molecular level, combined treatment with sorafenib and matrine resulted in a marked elevation in the cleavage of

Fig. 4. The miRNA-21/PTEN axis mediates the effects of matrine on the proliferation of HCC cells. Cells were pretransfected with miRNA-21 mimic or negative control oligonucleotides (Neg-oligo), followed by exposure to matrine alone or in combination with sorafenib for 48 h. **a** Cell proliferation was determined using the MTT assay. The proliferation in untreated cells (control) was arbitrarily assigned 100%. * $p < 0.05$ vs. control; # $p < 0.05$ vs. matrine alone; & $p < 0.05$ vs. combined matrine and sorafenib. **b** Western blot analysis of PTEN protein levels. Top panels show representative blots of 3 independent experiments. Bottom panels demonstrate densitometric analysis of PTEN protein expression. Data are

expressed as fold change of control. * $p < 0.05$ vs. control; # $p < 0.05$ vs. matrine alone. **c** Cells were pretransfected with PTEN siRNA (si-PTEN) or control siRNA and treated with matrine alone or in combination with sorafenib for 48 h. Cell proliferation was examined using the MTT assay. * $p < 0.05$ vs. control; # $p < 0.05$ vs. matrine alone; & $p < 0.05$ vs. combined matrine and sorafenib. **d** Western blot analysis of PTEN protein levels in HCC cells transfected with si-PTEN or control siRNA. Top panels show representative blots of 3 independent experiments. Bottom panels show densitometric results. * $p < 0.05$ vs. control (untreated cells).

caspase-3 and PARP, two indicators of caspase-mediated apoptosis. To the best of our knowledge, this is the first report about the synergistic anticancer activity of sorafenib and matrine.

Matrine shows cytotoxic effects against various cancers, including melanoma [8], acute myeloid leukemia [9] and HCC [12]. Zhang et al. [13] reported that matrine is capable of inducing apoptosis and autophagy in HCC cells. Our data confirmed the proapoptotic activity of matrine in HCC cells, which was associated with the promotion of caspase-3 and PARP cleavage. Induction of caspase-3-mediated apoptosis by matrine has also been described in several other types of cancer cells such as gastric cancer cells [26]. Reduced expression of oncogenes rep-

resents an important mechanism for suppressing tumor growth. miRNA-21 has been found to act as an oncogene in HCC by accelerating tumor cell proliferation, migration and invasion [18]. Notably, our data revealed that matrine treatment resulted in a significant reduction in miRNA-21 expression in HCC cells. Ectopic expression of miRNA-21 antagonized the reduction in the proliferation of matrine-treated HCC cells. Likewise, matrine-induced growth suppression was impaired by pretransfection of PTEN siRNA. PTEN has been identified as a direct target of miRNA-21 [18]. Consistently, our data demonstrated that miRNA-21 overexpression repressed the PTEN expression in matrine-treated HCC cells. PTEN is a potent tumor suppressor and its upregulation by phar-

macological or genetic approaches leads to apoptotic death in tumor cells [27, 28]. Taken together, our data suggest that matrine upregulates the expression of PTEN via the inhibition of miRNA-21 expression, which in turn contributes to apoptotic death in HCC cells. The modulation of the miRNA-21/PTEN axis by matrine has also been reported in breast cancer cells [15].

Several miRNAs have been found to be involved in the sensitivity of HCC cells to sorafenib [29, 30]. For instance, Bai et al. [29] demonstrated that miRNA-122-expressing HCC cells are more susceptible to sorafenib than those lacking miRNA-122 expression. Similarly, the expression of let-7 miRNAs potentiates sorafenib-induced apoptosis in HCC cells via targeting the antiapoptotic protein, Bcl-xL [30]. The delivery of miRNA-193b [31] and miRNA-193a [32] also sensitizes HCC cells to sorafenib cytotoxicity. We showed that in contrast to matrine, sorafenib failed to alter the expression of miRNA-21 in HCC cells. Most interestingly, the delivery of either miRNA-21 mimic or PTEN siRNA significantly blocked the growth inhibition induced by combined treatment with matrine

and sorafenib. These results indicate that matrine-mediated sensitization to sorafenib is at least partially attributable to alteration of the miRNA-21/PTEN signaling. miRNA-21 has been shown to confer resistance to interferon- α and 5-fluorouracil in HCC cells [33]. Our findings further highlight the role of miRNA-21 in regulating the susceptibility of HCC cells to systemic therapeutic agents.

In conclusion, our data show that the anticancer activity of matrine alone or in combination with sorafenib in HCC is mediated, at least partially, by suppression of miRNA-21 and subsequent upregulation of PTEN. Matrine represents a promising natural compound for use in the enhancement of the therapeutic efficacy of sorafenib in HCC.

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