

# miR-181a induces sorafenib resistance of hepatocellular carcinoma cells through downregulation of RASSF1 expression

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## Key words

Apoptosis, hepatocellular carcinoma, miR-181a, RASSF1, sorafenib

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Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related deaths worldwide.<sup>(1)</sup> Surgical resection and liver transplantation are first-line therapeutic options for early stage HCC patients, and locoregional therapies, including transarterial chemoembolization and radiofrequency ablation, are used to treat patients who are not suitable for surgery.<sup>(2)</sup> Sorafenib is the first systemic drug for patients with advanced HCC.<sup>(3)</sup> Sorafenib inhibits the activity of multi-kinases such as Raf kinase, vascular endothelial growth factor receptor 2 (VEGFR2) and platelet-derived growth factor receptor (PDGFR).<sup>(4)</sup> Although sorafenib could improve the overall survival of HCC patients from 7.9 to 10.7 months, many patients still do not benefit from this therapy and acquired resistance often develops within 6 months.<sup>(3,5)</sup> Therefore, identification of signaling networks that are crucial for the antitumor efficacy of sorafenib will contribute to the rational design of the novel therapeutic approach for HCC.

MicroRNA inhibit translation of target mRNA through binding to their 3' untranslated regions (3'UTR)<sup>(6)</sup> and regulate critical cellular processes such as proliferation and apoptosis in HCC cells.<sup>(7–11)</sup> Abnormal regulation of microRNA may correlate with hepatocarcinogenesis and HCC

Sorafenib, a multi-kinase inhibitor, is the only standard clinical drug for patients with advanced hepatocellular carcinoma (HCC); however, development of sorafenib resistance in HCC often prevents its long-term efficacy. Therefore, novel targets and strategies are urgently needed to improve the antitumor effect of sorafenib. In the present study, we examined the novel mechanisms of sorafenib resistance of HCC cells by investigating the difference in sorafenib sensitivity between two HCC cell lines. Sorafenib induced more apoptosis of HepG2 cells compared to Hep3B cells. Sorafenib exposure to HepG2 cells but not Hep3B cells increased the expression of proapoptotic factor PUMA, and activated PARP and caspase-3. Notably, microRNA-181a (miR-181a) expression levels were lower in HepG2 cells than in Hep3B cells. Exogenous miR-181a expression in HepG2 cells reduced apoptosis, whereas inhibition of miR-181a in Hep3B cells increased apoptosis. In addition, we demonstrated that miR-181a directly targets RASSF1, a MAPK signaling factor, and knockdown of RASSF1 increased sorafenib resistance. Taken together, these results suggest that miR-181a provokes sorafenib resistance through suppression of RASSF1. Our data provide important insight into the novel therapeutic strategy against sorafenib resistance of HCC cells by targeting of miR-181a pathway.

progression.<sup>(12)</sup> For example, microRNA-122 (miR-122) expression is significantly suppressed in HCC and this downregulation is associated with poor prognosis.<sup>(9–11)</sup> In addition, several microRNA, such as miR-222, 1274a and 34a, affect the drug sensitivity of HCC cells.<sup>(13–15)</sup> MiR-181a is highly expressed in cancer stem cells of HCC<sup>(16)</sup> and induces epithelial–mesenchymal transition in ovarian cancer.<sup>(17)</sup> It has also been reported that miR-181a contributes to cancer progression and metastasis in a variety of cancers.<sup>(18,19)</sup> Notably, HCC cell line HepG2 cells express lower levels of miR-181a<sup>(20)</sup> and are more sensitive to sorafenib compared to Hep3B cells.<sup>(21)</sup> These reports suggest that miR-181a may have a role in regulation of sorafenib resistance. In the present study, we found that sorafenib induced more apoptosis in HepG2 cells compared to Hep3B cells. Exogenous miR-181a expression in HepG2 cells reduced apoptosis, whereas inhibition of miR-181a in Hep3B cells increased apoptosis. We also showed that MAPK signaling factor RASSF1 is a target of miR-181a and that knockdown of RASSF1 reduced sorafenib sensitivity. These results suggest that miR-181a plays a critical role in sorafenib resistance through suppression of RASSF1 in HCC cells.

## Materials and Methods

**Cell culture.** HepG2 and Hep3B cells were cultured in DMEM (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (MBL, Nagoya, Japan).

**Cell viability assay.** HepG2 and Hep3B cells were seeded onto a 96-well plate at a density of  $1 \times 10^4$  and  $0.5 \times 10^4$  cells/well, respectively. After 72 h, viable cells were quantified using the Cell Counting Kit-8 (Dojin Chemical, Kumamoto, Japan) according to the manufacturer's protocol. Absorbance was measured at 450 nm with a reference wavelength of 600 nm using a microplate reader for 96-well plates (Tecan, Zurich, Switzerland).

**Transfection.** HepG2 cells and Hep3B cells were transfected with 100 nM pre-miR-181a or anti-miR-181a (Life Technologies, Carlsbad, CA, USA) using Lipofectamine 2000 (Life Technologies) according to the manufacturer's protocol. Cells were transfected with 20  $\mu$ M siRASSF1 and negative control siRNA using Lipofectamine 2000.

**Quantitative-RT PCR of miRNA.** Total RNA was isolated from the cells with TRIzol Reagent (Life Technologies). Expression of miR-181a was detected using TaqMan MicroRNA Assays (Applied Biosystems, Foster City, CA, USA), the MicroRNA RT Kit (Applied Biosystems) and the Taqman Universal PCR Master Mix II with UNG (Applied Biosystems) and the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's protocol. RNU6B was used as an internal control.

**Quantitative-RT PCR of mRNA.** Total RNA was isolated from the cells with TRIzol reagent. Expression of mRNA was detected using SuperScript II Reverse Transcriptase

(Invitrogen, Carlsbad, CA, USA) and FastStart SYBR Green Master (Roche Diagnostics, Mannheim, Germany) by the LightCycler 2.0 (Roche Diagnostics).

**Hoechst staining.** Cells were treated with Hoechst 33342 for 30 min and photographed with a microscope (IX-71) (OLYMPUS, Tokyo, Japan) and analyzed using an image analysis system (inForm 2.0) (PerkinElmer, Norwalk, CT, USA).

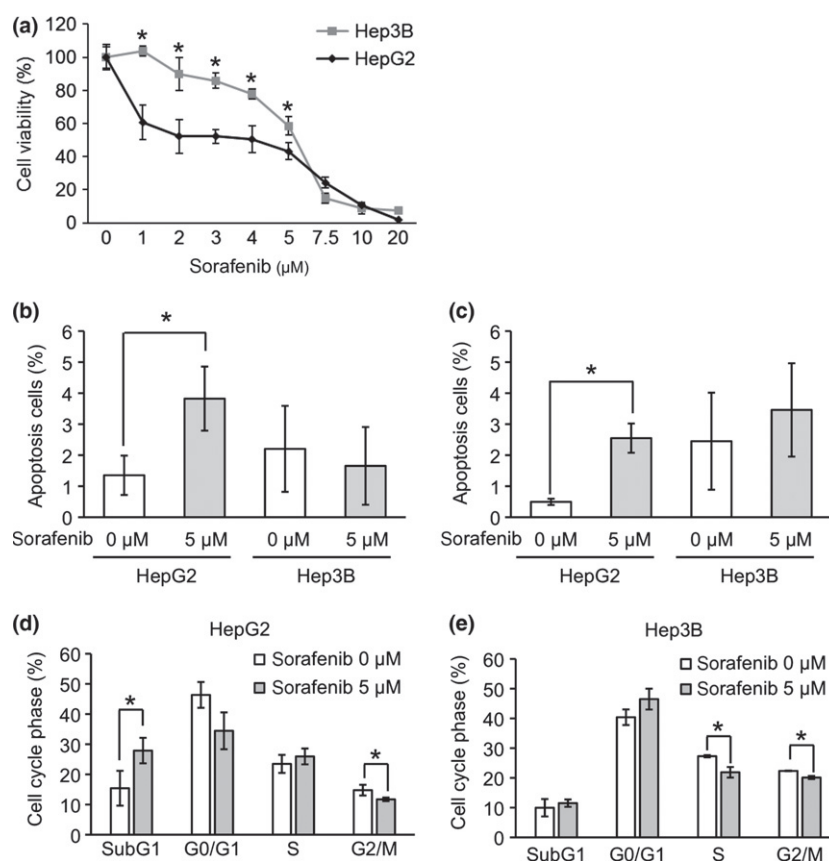
**Caspase 3/7 activity analysis.** Apoptosis was quantified using the Caspase-Glo 3/7 Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol.

**Cell cycle analysis.**  $1 \times 10^6$  cells were fixed with 70% EtOH overnight at 4°C. Cells were treated with RNase for 30 min at room temperature, stained with PI for 60 min and analyzed using FACSaria (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

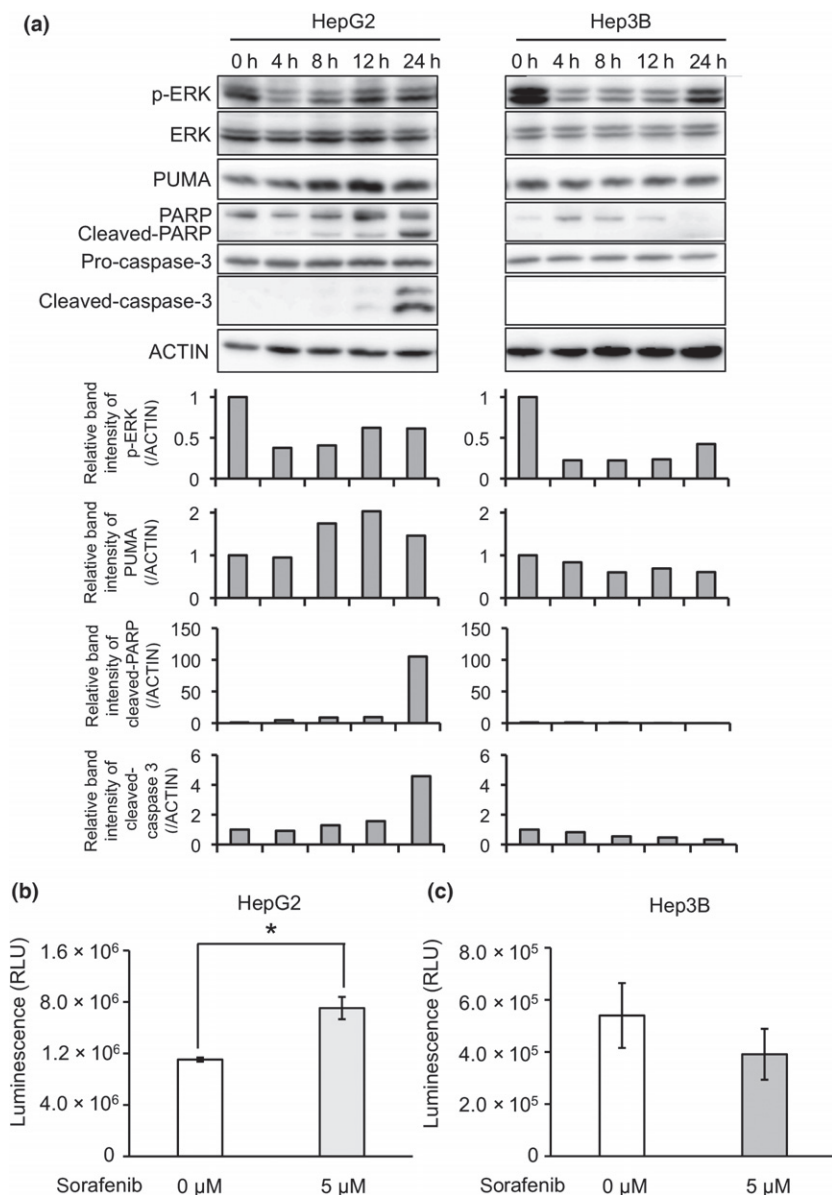
**Western blot analysis.** The primary antibodies used in this study were polyclonal antibodies against human  $\beta$ -ACTIN, RASSF1 (Santa Cruz, CA, USA), ERK, phospho-ERK (Thr202/Tyr204), caspase-3 and PARP (Cell Signaling Technology, Beverly, MA, USA). HRP-labeled secondary antibodies were used for the detection of primary antibodies and the bands were detected by Image-Quant LAS4000 (GE Healthcare UK, Little Chalfont, UK).

**DNA constructs.** For functional analysis of miR-181a, segments of the 3'-UTR RASSF1 mRNA sequences were synthesized. The products were then subcloned into the HindIII-XbaI site of a pGL3-Promoter Vector (Promega).

**Luciferase reporter assay.** The cells were co-transfected with firefly luciferase plasmid (Promega), renilla luciferase plasmid and microRNA with Lipofectamine 2000, and luciferase assays were performed with a dual-luciferase reporter assay system (Promega) at 48 h after transfection, in accordance with the



**Fig. 1.** Sorafenib induced more apoptosis and lower cell viability of HepG2 cells compared to Hep3B cells. (a) HepG2 and Hep3B cells were cultured with sorafenib (0–20  $\mu$ M) for 72 h and cell viability was analyzed by WST assay. (b, c) HepG2 and Hep3B cells were cultured with 0 or 5  $\mu$ M of sorafenib for 24 h (b) or 48 h (c) and the number of apoptotic cells was quantified by Hoechst staining and presented as a percentage of total cell number. Seven fields were randomly chosen, photographed from each group, and statistically analyzed. \* $P < 0.05$ , compared between 0 and 5  $\mu$ M treatment of sorafenib. (d, e) Cell-cycle analysis was performed by flow cytometry using HepG2 cells (d) or Hep3B cells (e) treated with 0 or 5  $\mu$ M of sorafenib for 48 h. \* $P < 0.05$ , compared between 0 and 5  $\mu$ M treatment of sorafenib.



**Fig. 2.** Apoptosis related-molecules were increased/activated in sorafenib-treated HepG2 cells. (a) Western blot analysis of phospho-ERK, ERK, PUMA, PARP, caspase-3 and ACTIN using HepG2 or Hep3B cells treated with 5 μM sorafenib for 0–24 h. Quantification of western blot bands using ImageJ software (national institutes of health, Bethesda, MD), which are normalized by ACTIN, were displayed as bar graphs. (b) HepG2 and Hep3B cells were treated with sorafenib (0 or 5 μM) for 48 h and activities of caspase-3/7 were measured. \**P* < 0.05, compared between 0 and 5 μM treatment of sorafenib.

manufacturer's instructions. Luminescent signals were quantified with a luminometer (MiniLumat LB9506; Berthold GmbH&Co. KG, Wildbad, Germany), and each value for firefly luciferase activity was normalized by renilla luciferase activity.

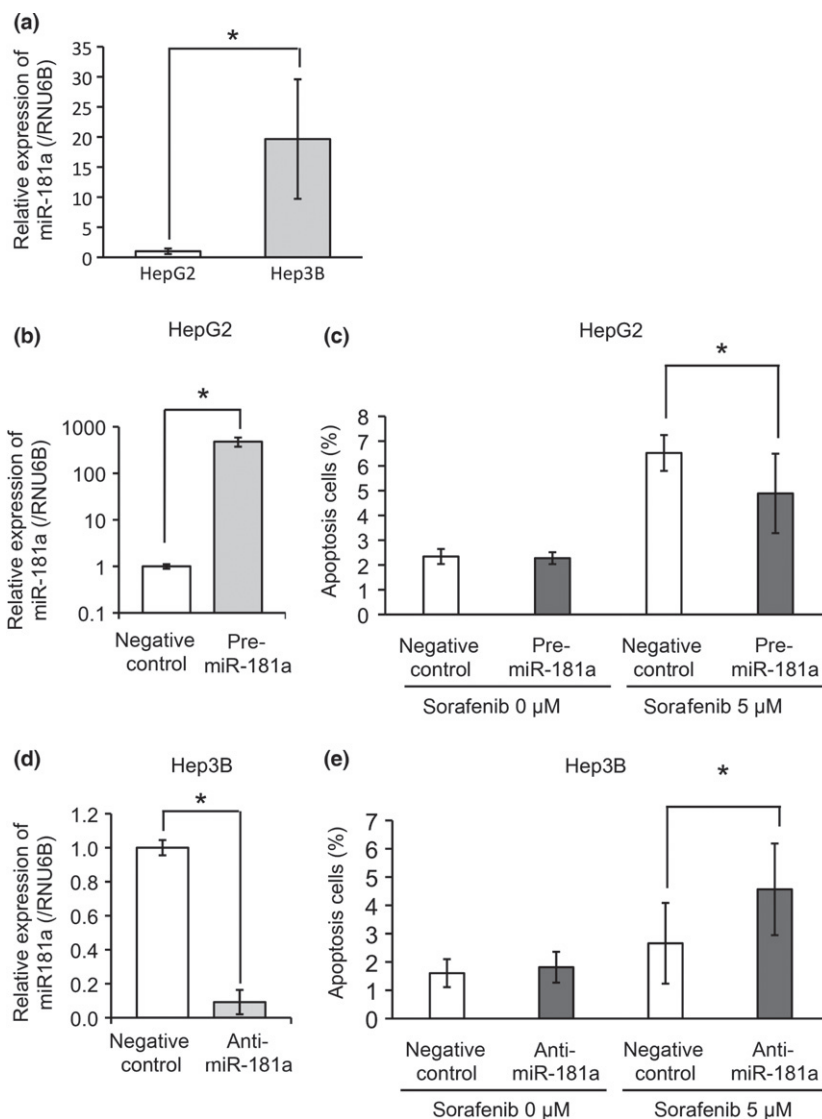
**Statistical analysis.** All the values were expressed as mean ± SD. The differences between the two groups were analyzed using an unpaired two-tailed Student's *t*-test. A *P*-value <0.05 was considered to be significant.

## Results

**Sorafenib induced more apoptosis and lower cell viability of HepG2 cells compared to Hep3B cells.** To examine the sorafenib sensitivity of HepG2 cells and Hep3B cells, cell viability assay was performed. HepG2 and Hep3B cells were cultured with sorafenib (0–20 μM) for 72 h and assayed by Cell Counting Kit-8. HepG2 cells showed higher sensitivity to sorafenib than Hep3B cells, which is consistent with a previous report (Fig. 1a).<sup>(21)</sup> Next, we investigated whether apoptosis is

induced by sorafenib treatment. Cells were stained with Hoechst and apoptotic cells were analyzed. Sorafenib significantly increased the rate of apoptosis of HepG2 cells, but did not increase the rate of apoptosis of Hep3B cells (Fig. 1b,c). Consistently, cell cycle analysis by FACS revealed that sorafenib-treated HepG2 cells clearly increased sub-G1 population, while Hep3B cells did not. These results indicate that sorafenib induced higher apoptotic rate and lower cell viability of HepG2 cells compared to Hep3B cells (Fig. 1d,e).

**Apoptosis related-molecules were increased/activated in sorafenib-treated HepG2 cells.** Next, we examined expression levels of apoptosis regulators with sorafenib treatment by western blot analysis. Quantification of western blot bands, which were normalized by ACTIN, is shown in Figure 2a. Sorafenib inhibits activity of RAF kinase, which reduces phosphorylation of ERK (p-ERK). We confirmed that p-ERK was reduced by sorafenib treatment (Fig. 2a). Quantified data combined with western blot clearly showed that sorafenib treatment induced apoptosis markers, PUMA, cleaved-PARP and



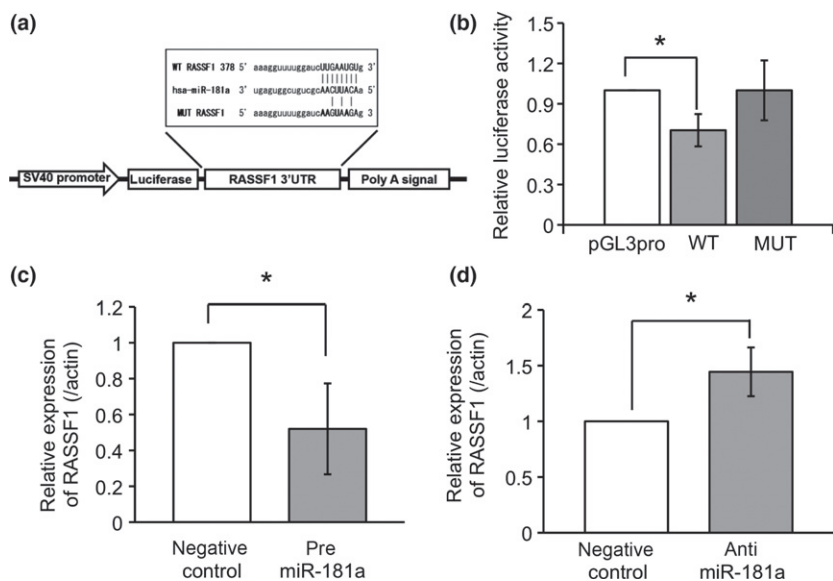
**Fig. 3.** miR-181a plays a critical role in sorafenib resistance. (a) The expression levels of miR-181a of HepG2 and Hep3B cells were measured by quantitative-RT-PCR (qRT-PCR). (b) Pre-miR-181a or negative control miR was transfected into HepG2 cells and miR-181a expression levels were analyzed by qRT-PCR. (c) Pre-miR-181a or negative control miR was transfected into HepG2 cells and cells were treated with sorafenib for 48 h, then apoptosis cells were measured by Hoechst 33342 staining. (d) Anti-miR-181a or negative control miR was transfected into Hep3B cells and miR-181a expression levels were analyzed by qRT-PCR. (e) Anti-miR-181a or negative control miR was transfected into Hep3B cells and cells were treated with sorafenib for 48 h, then apoptosis cells were measured by Hoechst 33342 staining. \* $P < 0.05$ , compared between two groups.

cleaved-caspase-3 in HepG2 but not in Hep3B cells (Fig. 2a). We also measured caspase-3/7 activity. Consistent with western blot analysis, caspase-3/7 activity was significantly increased in HepG2 cells but not in Hep3B cells with sorafenib treatment (Fig. 2b,c). These results indicate that sorafenib induced apoptosis of HepG2 cells through increase/activation of proapoptotic factors.

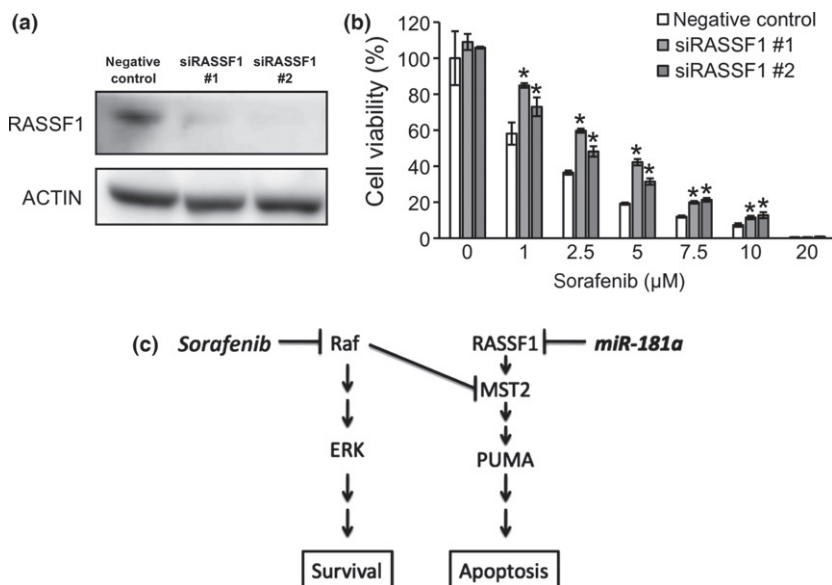
**miR-181a plays a critical role in sorafenib resistance.** It has been reported that miR-181a is involved in regulation of proliferation and drug resistance of cancer cells. Thus, we next examined the expression levels of miR-181a in HepG2 cells and Hep3B cells by quantitative-RT PCR (qRT-PCR). Notably, HepG2 cells expressed lower levels of miR-181a compared to Hep3B cells (Fig. 3a). To test whether miR-181a expression levels affect sorafenib sensitivity, pre-miR-181a was transfected into HepG2 cells (Fig. 3b), and the rate of apoptosis was examined by Hoechst staining. As shown in Figure 3c, sorafenib-induced apoptosis was reduced by ectopic expression of miR-181a in HepG2 cells. Conversely, when we inhibited miR-181a by anti-miR-181a treatment in Hep3B cells (Fig. 3d), sorafenib-induced apoptosis was increased (Fig. 3e). These results suggest that miR-181a plays a critical role in sorafenib resistance.

**miR-181a downregulates RASSF1 expression.** To elucidate the target of miR-181a, we performed a database analysis using miRanda. We searched the candidates in MAPK pathway and apoptosis-related genes and found RASSF1 as a target gene of miR-181a (Fig. 4a). Then, we performed a luciferase assay using reporter plasmid containing WT or mutant sequence of 3' UTR region of RASSF1 (Fig. 4a). Ectopic expression of WT miR-181a reduced luciferase activity compared to control, whereas that of mutant miR-181a did not (Fig. 4b). In addition, pre-miR-181a suppressed RASSF1 expression in HepG2 cells (Fig. 4c), whereas anti-miR181a in Hep3B cells increased it (Fig. 4d). These results indicate that RASSF1 is a target of miR-181a.

**Knockdown of RASSF1 reduced sorafenib sensitivity.** The finding that RASSF1 is a target of miR-181a suggests that RASSF1 may regulate sensitivity to sorafenib. To test this possibility, we knocked down RASSF1 using two kinds of siRNA against RASSF1 (Fig. 5a) in HepG2 cells, and cell viability assay with sorafenib treatment was performed. Knockdown of RASSF1 significantly increased cell viability against sorafenib (Fig. 5b), suggesting that RASSF1 plays a critical role in sorafenib sensitivity.



**Fig. 4.** miR-181a downregulates RASSF1 expression. (a) Schematic representation of the predicted and mutated miR-181 binding sites in the RASSF1 3'UTR. (b) HepG2 cells were transfected with pGL3-3'UTR RASSF1 (wild type or mutated) or pGL3-promoter vector (control) with pRL-TK vector, and luciferase assay was performed. \* $P < 0.05$ , compared between WT or MUT and pGLpro control. (c) Relative expression of RASSF1 normalized by ACTIN was analyzed by quantitative-RT-PCR (qRT-PCR) using HepG2 cells transfected with pre-miR-181a or negative control miR. (d) Relative expression of RASSF1 normalized by ACTIN was analyzed by qRT-PCR using Hep3B cells transfected with anti-miR-181a or negative control. \* $P < 0.05$ , compared between two groups.



**Fig. 5.** Knockdown of RASSF1 reduced sorafenib sensitivity. (a) RASSF1 was knocked down by siRNA and western blot analysis of RASSF1 was performed. ACTIN was used as an internal control. (b) Cells were treated with sorafenib (0–20  $\mu$ M) and two kinds of siRNA against RASSF1 (#1 and #2) or negative control siRNA, and cell viability assay was performed. \* $P < 0.05$ , compared between siRASSF1 and negative control. (c) Summarized models of miR-181a-mediated sorafenib resistance in hepatocellular carcinoma cells.

## Discussion

In the present study, we showed that sorafenib induced more apoptosis and reduced viability of HepG2 cells compared to Hep3B cells. Sorafenib treatment in HepG2 cells but not in Hep3B cells increased expression of apoptosis factor PUMA, and activated PARP and caspase-3. We found that miR-181a expression levels were lower in HepG2 cells than in Hep3B cells, and exogenous miR-181a expression in HepG2 cells reduced apoptosis, whereas inhibition of miR-181a in Hpe3B cells increased apoptosis. In addition, we demonstrated that RASSF1 is a target of miR-181a, and knockdown of RASSF1 increased sorafenib resistance. Together, these results suggest that miR-181a provokes sorafenib resistance through repression of RASSF1 expression. Our data provide important insight into a novel therapeutic strategy against sorafenib resistant HCC cells by targeting of miR-181a pathway. A critical future challenge is to explore the relationship between RASSF1 expression level and treatment response for sorafenib-based chemotherapy in HCC patients.

It has been reported that miR-181a has oncogenic functions in various cancers.<sup>(16–19)</sup> Consistent with these reports, we here demonstrate that miR-181a induces sorafenib resistance of HCC cells. We showed that miR-181a directly represses expression of RASSF1 in HCC cells, which is consistent with a recent report that miR-181a and miR-181b are highly expressed in acute promyelocytic leukemia and downregulate RASSF1 expression.<sup>(22)</sup> In addition, as well as miR181-a and miR-181b, miR-181c, which has been shown to promote pancreatic cancer cell chemoresistance,<sup>(23)</sup> are aberrantly expressed in various cancers, including osteosarcoma and lung adenocarcinoma.<sup>(18,24)</sup> These findings raise the possibility that miR-181b and miR-181c have a similar function to miR-181a in HCC cells. In addition, Ji *et al.*<sup>(16)</sup> indicate that all members of miR-181s are associated with HCC cells with features of hepatic cancer stem cells, suggesting that HepG2 cells, which have mature features of HCC cells, express lower levels of miR-181s. Indeed, HepG2 cells expressed lower levels of miR-181a in our experiment. Therefore, we have additionally

determined expression levels of miR-181b and miR-181c in HepG2 and Hep3B HCC cells by qPCR analyses (Fig. S1). qPCR results showed that miR-181b was significantly upregulated in Hep3B cells compared to HepG2 cells similar to miR-181a, whereas miR-181c levels were comparable. Taken together, these results suggest that, in addition to miR-181a, miR-181b may also provoke sorafenib resistance in HCC cells.

RASSF1 is a tumor suppressor gene and elicits apoptosis through induction of PUMA (Fig. 5c).<sup>(25)</sup> In contrast, Raf kinase is known to enhance cell survival through activation of ERK (Fig. 5c).<sup>(26)</sup> Raf also binds to MST2, which is a downstream regulator of RASSF1, and inhibits RASSF1 signal pathway (Fig. 5c).<sup>(27)</sup> Therefore, inhibition of Raf by sorafenib and low expression of miR-181a may enhance RASSF1-MST2-PUMA-mediated apoptotic signaling (Fig. 5c). In addition to Raf, sorafenib simultaneously inhibits growth factor receptors including VEGFR2 and PDGFR in tumor-associated endothelial cells, thus inhibiting endothelial cell growth and neoangiogenesis. Here we show that miR-181a suppresses apoptotic signaling factor RASSF1, which elicits sorafenib resistance of HCC cells. Based on our results, miR-181a may also impact on apoptotic signaling in endothelial cells. If this is the case, suppression of miR-181a could also enhance

sorafenib-induced apoptosis provoked by inhibition of VEGFR and PDGFR of endothelial cells.

Recently, several miRNA delivery systems to the liver have been developed. For example, miR-221 is frequently overexpressed in HCC and systemic delivery of a cholesterol-tagged anti-miR-221 to orthotopic HCC tumors appeared to reduce tumor cell growth and promote survival.<sup>(28)</sup> In addition, adeno-associated virus, lentivirus and nano-sized lipid particles are also useful methods for delivering miRNA to liver cancers.<sup>(29–32)</sup> Therefore, based on our data, development of the anti-miR-181a delivery system with low side effects may also be a promising therapeutic approach against HCC.

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### Disclosure Statement

The authors have no conflict of interest to declare.

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### Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Fig. S1.** miR-181b and miR-181c expression levels in HepG2 and Hep3B cells.