British Journal of Cancer

ARTICLE

Molecular Diagnostics

Low miR-10b-3p associated with sorafenib resistance in hepatocellular carcinoma

Yu-Yun Shao[1,2,3,9], Pai-Sheng Chen[4,9], Liang-In Lin[5], Bin-Shyun Lee[1], Andrew Ling[6], Ann-Lii Cheng[1,2,3,7], Chiun Hsu[1,2,3] and Da-Liang Ou[1,8]✉

BACKGROUND: Sorafenib is one of the standard first-line therapies for advanced hepatocellular carcinoma (HCC). Unfortunately, there are currently no appropriate biomarkers to predict the clinical efficacy of sorafenib in HCC patients. MicroRNAs (miRNAs) have been studied for their biological functions and clinical applications in human cancers.
METHODS: In this study, we found that miR-10b-3p expression was suppressed in sorafenib-resistant HCC cell lines through miRNA microarray analysis.
RESULTS: Sorafenib-induced apoptosis in HCC cells was significantly enhanced by miR-10b-3p overexpression and partially abrogated by miR-10b-3p depletion. Among 45 patients who received sorafenib for advanced HCC, those with high miR-10b-3p levels, compared to those with low levels, exhibited significantly longer overall survival (OS) (median, 13.9 vs. 3.5 months, $p = 0.021$), suggesting that high serum miR-10b-3p level in patients treated with sorafenib for advanced HCC serves as a biomarker for predicting sorafenib efficacy. Furthermore, we confirmed that cyclin E1, a known promoter of sorafenib resistance reported by our previous study, is the downstream target for miR-10b-3p in HCC cells.
CONCLUSIONS: This study not only identified the molecular target for miR-10b-3p, but also provided evidence that circulating miR-10b-3p may be used as a biomarker for predicting sorafenib sensitivity in patients with HCC.

British Journal of Cancer (2022) 126:1806–1814; https://doi.org/10.1038/s41416-022-01759-w

BACKGROUND

MicroRNAs (miRNAs) are non-coding RNAs that play important roles in gene regulation, often suppressing gene expression either by blocking mRNA translation or enhancing mRNA degradation. In hepatocellular carcinoma (HCC), several miRNAs have been demonstrated to play important roles in regulating genes important to cell cycle progression, migration, metastatic potential, and angiogenesis [1–3]. In this manner, different miRNAs can act as pro-oncogenic drivers or tumor suppressors depending on which genes they regulate.

Following tissue injury, miRNAs may be released passively into circulation and may also be actively secreted into circulation via exosomes [4, 5]. Secreted miRNAs may play important roles in regulating gene expression and function in the target cells [6, 7]. Cancer cells may even secrete anti-oncogenic miRNAs into the serum as a tumorigenic mechanism [8]. Given the relative ease of collecting patient blood samples and measuring a miRNA panel, circulating miRNAs thus have the potential to serve as useful biomarkers for predicting treatment sensitivity and cancer prognosis [9, 10].

Circulating miRNAs have therefore been extensively explored as biomarkers for early diagnosis in many cancers, including HCC [8, 11–15]. Certain miRNAs or patterns (i.e., 'signature' of miRNAs) have been associated with advanced tumor stage and poor survival [16–19], leading to the development of a plasma microRNA panel in diagnosing early-stage HCC [20]. Nevertheless, there remain challenges in using these miRNA panels, including the large heterogeneity in the plasma miRNA levels of HCC patients [8].

Sorafenib, a multikinase inhibitor, is one of the standard first-line therapies for advanced HCC [21]. Given the relatively modest survival benefit and large variations in patient response to sorafenib, finding biomarkers that predict patient response to sorafenib treatment has important clinical implications [22]. Several studies have explored the correlation between levels of different miRNAs in patients with advanced HCC and the efficacy of sorafenib treatment [23–31]. Candidate miRNAs have been identified by comparing the expression of miRNAs between sorafenib-sensitive and sorafenib-resistant HCC cells in vitro [23–26, 28–31] or by comparing the

[1]Graduate Institute of Oncology, College of Medicine, National Taiwan University, Taipei City 10055, Taiwan. [2]Department of Oncology, National Taiwan University Hospital, Taipei City 10048, Taiwan. [3]National Taiwan University Cancer Center, Taipei City 10672, Taiwan. [4]Department of Medical Laboratory Science and Biotechnology, National Cheng Kung University, Tainan City 70101, Taiwan. [5]Graduate Institute of Clinical Laboratory Sciences and Medical Biotechnology, College of Medicine, National Taiwan University, Taipei City 10048, Taiwan. [6]Columbia University Vagelos College of Physicians and Surgeons, New York, NY 10032, USA. [7]Department of Internal Medicine, National Taiwan University Hospital, Taipei City 10048, Taiwan. [8]YongLin Institute of Health, National Taiwan University, Taipei City 10672, Taiwan. [12]These authors contributed equally: Yu-Yun Shao, Pai-Sheng Chen. ✉email: dlou@ntu.edu.tw

Received: 10 August 2021 Revised: 29 January 2022 Accepted: 11 February 2022
Published online: 2 March 2022

serum miRNAs levels between patients with or without clinical benefit from sorafenib treatment [24, 25, 27, 29]. These studies have suggested potential correlations between the expression of specific circulating miRNAs, such as miR-122 and miR-34a, and response to molecular targeted therapy for HCC patients.

In this study, we investigated the roles of miR-10b-3p (hsa-miR-10b-3p) in regulating HCC cell response to sorafenib therapy. miR-10b (the complementary miRNA of miR-10b-3p) has already been extensively studied as a driver of epithelial-mesenchymal transitions and cancer metastasis in many cancers [18, 32–36], including HCC cell lines. Compared to miR-10b, miR-10b-3p has been less well studied. At the molecular level, miR-10b-3p was demonstrated to target CMTM5 and appeared to promote tumor growth and cell proliferation in HCC cell lines [19]. The biological role of miR-10b-3p specifically relating to sorafenib treatment and sensitivity, however, remains unclear.

We used in vitro and in vivo models of HCC simulating the acquired resistance to sorafenib in HCC patients to explore the mechanisms by which miR-10b-3p modulates resistance of HCC cells to sorafenib. These models were also used to explore the potential clinical use of miR-10b-3p as a prognostic marker and a predictive biomarker in response to sorafenib treatment.

METHODS

Cell lines and reagents. The HCC cell lines, HepG2, PLC5, Tong and Hep3B, were obtained from the American Type Culture Collection (ATCC), and the Huh-7 cell line was from the Health Science Research Resources Bank. Sorafenib (Bayer-Schering Pharma, West Haven, CT) was dissolved in DMSO for in vitro experiments, and the final concentration of DMSO was kept below 0.1%. The sorafenib-resistant cell lines, Huh-7R and HepG2R, were generated by continuous treatment of Huh-7 and HepG2 cells with sorafenib up to 10 μM and maintained as previously described [37, 38]. The antibodies used for western blotting were cyclin E1 (BD Bioscience, San Diego, CA, USA), phospho-cyclin E1, phospho-Rb, Mcl-1, E2F1, caspase 3, PARP-1, GAPDH (Cell Signaling Technology, Danvers, MA, USA), Rb, CDK2, and Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell viability and apoptosis assays. Cell viability was assessed using an MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) assay as previously described [37]. The IC_{50} values after drug treatment were calculated using CompuSyn software (ComboSyn, Paramus, NJ). The fraction of apoptotic cells after drug treatment was assessed by sub-G1 fraction analysis using flow cytometry [37].

Screening for candidate miRNAs. miRNAs were extracted with mirVana miRNA Isolation kit (Ambion, Austin, TX, for cell lysate samples) or mirVana PARIS kit (for serum and culture media samples) according to the manufacturer's protocol. The miRNA quality was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies). Candidate miRNAs were selected by comparing the miRNA profiles between the Huh-7 (sorafenib-sensitive) and Huh-7R (sorafenib-resistant) cells using a TaqMan® human microRNA array v2.0 on an Applied Biosystems 7900HT Real-Time PCR System (Applied Biosystems, Foster City, CA) and quantified using the TaqMan miRNA Assay Kit (Applied Biosystems), The mammalian U6 (MammU6) gene was used to determine the relative miRNA expression levels, and several endogenous controls, including RNU44, RNU48, miR-16 and miR-24, were used to validate the MammU6 control. The relative miRNA amount of the target gene/MammU6 gene was calculated using the ΔCt (threshold cycle) method as follows: relative expression = 2-ΔCt, where ΔCt = Ct (target gene) - Ct (MammU6). miR-10b-3b was selected for further exploration.

Identification of genes targeted by miR-10b-3p in HCC cells. Microarray analysis with the Agilent SurePrint G3 Human GE 8x60K Microarrays System (Agilent Technologies) was used to identify genes targeted by miR-10b-3p in HCC cells after sorafenib treatment. Huh-7 cells overexpressing miR-10b-3p were treated with sorafenib or DMSO control for 24 h. RNA extraction, cDNA synthesis, and cDNA quantification proceeded as described above.

Modulation of pre-miR and anti-miR of miR-10b-3p expression. To measure the effects of miR-10b-3p on cellular signaling activities, cells were transfected with pre-miR or anti-miR of miR-10b-3p (Ambion, Austin, TX) using the siPORT NeoFx siRNA transfection reagent (Ambion, Austin, TX) and then treated with sorafenib at the indicated concentrations and times. The transfected cells were subjected subsequent flow cytometry. Protein and RNA extracted from the cells were subjected to Western blotting and qRT-PCR analysis, as previously described [37].

In vivo miRNA-10b-3p expression in HCC xenografts. The protocol for the in vivo studies was approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University. All the animal studies were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. The orthotopic HCC model was established by injecting 1×10^6 Huh-7 or Huh-7R cells into the sub-capsular area of the left liver lobe of male SCID mice at 6–7 weeks of age, as previously described [39], to analyze miR-10b-3p expression by in situ hybridization (miRCURY LNA microRNA ISH optimization kit, EXIQON). In brief, formalin-fixed, paraffin-embedded tumor sections were deparaffinized, rehydrated, fixed in 4% paraformaldehyde, and pre-hybridized in hybridization buffer containing 20 nmol/L of DIG-labeled Locked Nucleic Acid (LNA™) probe (Exiqon, Denmark) at 50 °C for 2 hours. Slides were hybridized overnight with the diluted probe in a humidified chamber at 50 °C. The slides were then blocked with 2% sheep serum, 2 mg/mL BSA in PBST), and incubated with anti-DIG-AP Fab fragments (1:1000, Roche Diagnostics) at 4 °C overnight. Signals were detected by incubating sections in NBT-BCIP solution (Roche Diagnostics) and imaged by TissueFAXS (TissueGnostics, Vienna, Austria) with a 20× lens. The staining intensity was categorized as high, moderate, or low.

Depletion of miR-10b-3p expression in HCC xenograft

The protocol for the xenograft experiments was approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University, as described above. The 2×10^6 Huh-7/miR-10b-3p-suppressing cells (Anti-10b-3p) or Huh-7/ miR-scrambled-suppressing cells (Anti-NC) were injected subcutaneously into the flank of male BALB/c athymic (nu+/nu+) mice (six weeks old). Tumor size was measured with calipers every seven days after tumor formation. Tumor volume was calculated by using the following formula: volume (mm^3) = $(\text{width})^2 \times \text{length} \times 0.5$. When tumor volume reached ~200 mm^3, the mice were randomized and began to receive sorafenib (10 mg/kg/day) by gavage. After four weeks of administration, the mice were sacrificed and removed, photographed, and weighted, as previously described [37].

Luciferase Reporter Assay. The 587 bp sequence of the CCNE1 3′-untranslated region (UTR) containing either the predicted miR-10b-3p binding site was synthesized and cloned into the pmirGLO dual-luciferase miRNA target expression vector (Promega) by Omics Biotechnology Co., Ltd. The luciferase reporter assay was performed as previously described [37].

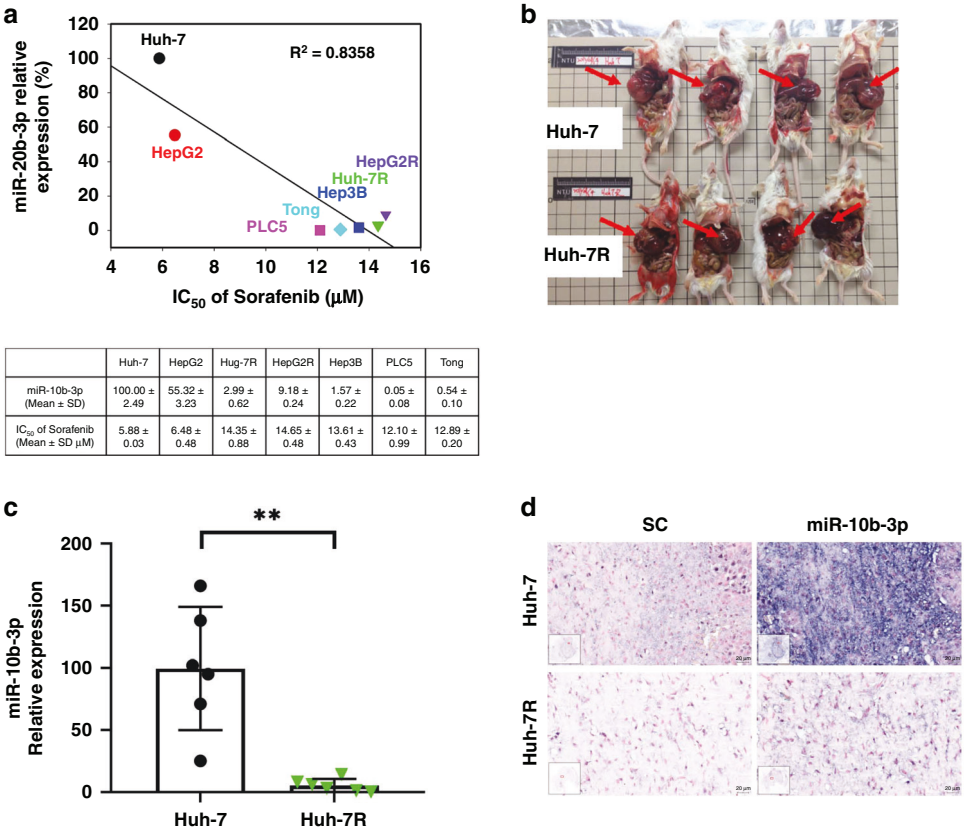
Patient samples. We enrolled patients who received sorafenib as the first-line therapy for advanced HCC at NTUH. All patients were histologically or clinically diagnosed to have HCC. Clinical diagnosis followed the guidelines of the American Association for the Study of Liver Diseases [40]. It was only allowed in patients with cirrhosis or chronic hepatitis B or C when typical imaging patterns were found in dynamic imaging. Sera from 6 healthy volunteers were used as control. For patients with HCC, serum samples were collected before treatment initiation and 4 weeks after the start of sorafenib treatment. Total RNA was isolated, using the mirVana PARIS kit (Ambion) described above. The patient's demographic features, objective tumor response, treatment duration, date of disease progression, and survival data were retrieved from the medical records (Supplemental Table S1). This study was approved by the Institute Research Ethical Committee of NTUH.

Statistical analysis. Comparisons were analyzed using the Student's *t* test, chi-square test, and one-way ANOVA as appropriate. Significance was defined as $p < 0.05$. Kaplan–Meier analysis was performed to estimate survival, and the log-rank test was used to compare the survival between patients with high and low serum miR-10b-3p levels univariately. In the multivariate analysis, a Cox proportional hazards model was used to adjust for other potential OS predictors including gender, age, macrovascular invasion, hepatitis virus infection, extrahepatic involvement, serum alpha-fetoprotein level, the ALBI grade, the CLIP score, performance status, and prior HCC treatment. A stepwise variable selection procedure was used to determine the best fit model with the significance levels for entry and for stay set at ≥0.15.

RESULTS

miR-10b-3p levels may correlate with response of HCC cell lines to sorafenib

The miRNA expression profiles of sorafenib-sensitive (Huh-7) and sorafenib-resistant (Huh-7R and Hep3B) HCC cell lines were compared, and 29 miRNAs with a greater than 3-fold difference



	Huh-7	HepG2	Hug-7R	HepG2R	Hep3B	PLC5	Tong
miR-10b-3p (Mean ± SD)	100.00 ± 2.49	55.32 ± 3.23	2.99 ± 0.62	9.18 ± 0.24	1.57 ± 0.22	0.05 ± 0.08	0.54 ± 0.10
IC_{50} of Sorafenib (Mean ± SD μM)	5.88 ± 0.03	6.48 ± 0.48	14.35 ± 0.88	14.65 ± 0.48	13.61 ± 0.43	12.10 ± 0.99	12.89 ± 0.20

Fig. 1 miR-10b-3p as a predictive biomarker of response to sorafenib treatment in HCC cell lines in vitro and in vivo. A Quantitative RT-PCR of miR-10b-3p miRNA expression in HCC cell lines. IC_{50} of HCC cell lines after sorafenib treatment. HCC cells were treated with sorafenib at the indicated concentrations in 96-well plates for 72 h, and cell viability was assessed by MTT assay. Points, mean ($n = 3$); bars, SD of three independent experiments. **B** The orthotopic HCC model was established by injecting 1×10^6 Huh-7 or Huh-7R cells into the sub-capsular area of the left liver lobe of male SCID mice at the age of 6–7 weeks. **C** After implantation for 30 days, mouse sera were collected in BD serum tubes for quantitative RT-PCR analysis of serum miR-10b-3p ($n = 6$). **D** After implantation for 30 days, tumor tissues of mice were collected, formalin-fixed, and paraffin-embedded for analysis of tumor miR-10b-3p. Huh-7 or Huh-7R tumor tissues of mice were stained with the miR-10b-3p probe (miR-10b-3p) or a scramble-control probe (SC) by in situ hybridization (miRCURY LNA microRNA ISH optimization kit). Positive signals are shown in dark blue; nuclei are counterstained in red. Scale bar: 20 μm.

in expression levels between sorafenib-sensitive and sorafenib-resistant cell lines were selected (Supplementary Table S2). From this pool, 9 candidate miRNAs with a greater than 4-fold difference were selected for confirmation by quantitative RT-PCR, and miR-10b-3p was one of the most significantly downregulated miRNAs found in both sorafenib-resistant cell lines (Supplementary Table S3). The IC_{50} of sorafenib, determined by MTT assay, was highly correlated with the miR-10b-3p levels of individual HCC cell lines, with significantly lower miR-10b-3p levels found in sorafenib-resistant cell lines (Fig. 1A). The orthotopic liver cancer models (Fig. 1B) demonstrated that mice bearing sorafenib-resistant tumors (Huh-7R) had significantly lower levels of miR-10b-3p in serum (Fig. 1C) and in the tumors (Fig. 1D).

The changes in miR-10b-3p levels in HCC cells and in the culture medium after sorafenib treatment were then examined. The HCC cells were treated for 24 h, when no evident morphological changes of the cells were observed, and the cells and the culture medium were collected for miR-10b-3p quantification. The levels of miR-10b-3p increased significantly both in the cells (Fig. 2A) and in the culture medium (Fig. 2B) when the cells were treated at greater than the IC_{50} of sorafenib (10 μM for sorafenib-sensitive and 20 μM for sorafenib-resistant HCC cells, respectively). These findings suggested that secreted miR-10b-3p may serve as a biomarker for HCC patients who received sorafenib treatment, and miR-10b-3p may play a functional role in sorafenib-induced cell death.

Serum miR-10b-3p levels may help predict the clinical outcome of HCC patients who received sorafenib treatment

Since tissue biopsies are unavailable from advanced HCC patients, we collected their blood samples for analysis of circulating miR-10b-3p. Serum samples from a cohort of patients who received sorafenib treatment for advanced HCC were tested for the potential of circulating miR-10b-3p as a sorafenib therapeutic biomarker. A total of 45 patients were enrolled (Supplementary Table S1). Compared with healthy volunteers ($n = 6$), HCC patients tended to have higher miR-10b-3p levels, calculated using qRT-PCR analysis and the ΔCt (threshold cycle) method (Fig. 3A). The HCC patients were divided into high- ($n = 27$) and low- miR-10b-3p ($n = 18$) groups, with the cut-off value set at the third quartile (0.1015) of healthy controls (Fig. 3A). The baseline characteristics of the high- vs. low- miR-10b-3p patients were compared in Supplementary Table S1.

Patients with high miR-10b-3p levels had significantly longer overall survival (OS) (median, 13.9 vs. 3.5 months, $p = 0.021$; Fig. 3B) but not progression-free survival (Fig. 3C). In multivariate analysis, high miR-10b-3p level remained as an independent predictor of longer OS (hazard ratio 0.276, $p = 0.003$; Table 1). For patients with available serum samples obtained after sorafenib treatment (24 patients in the high-miR-10b-3p group and 15 in the low-miR-10b-3p group), we further evaluated whether the increase in miR-10b-3p levels was associated with therapeutic efficacy. The results indicated that only in the low-miR-10b-3p

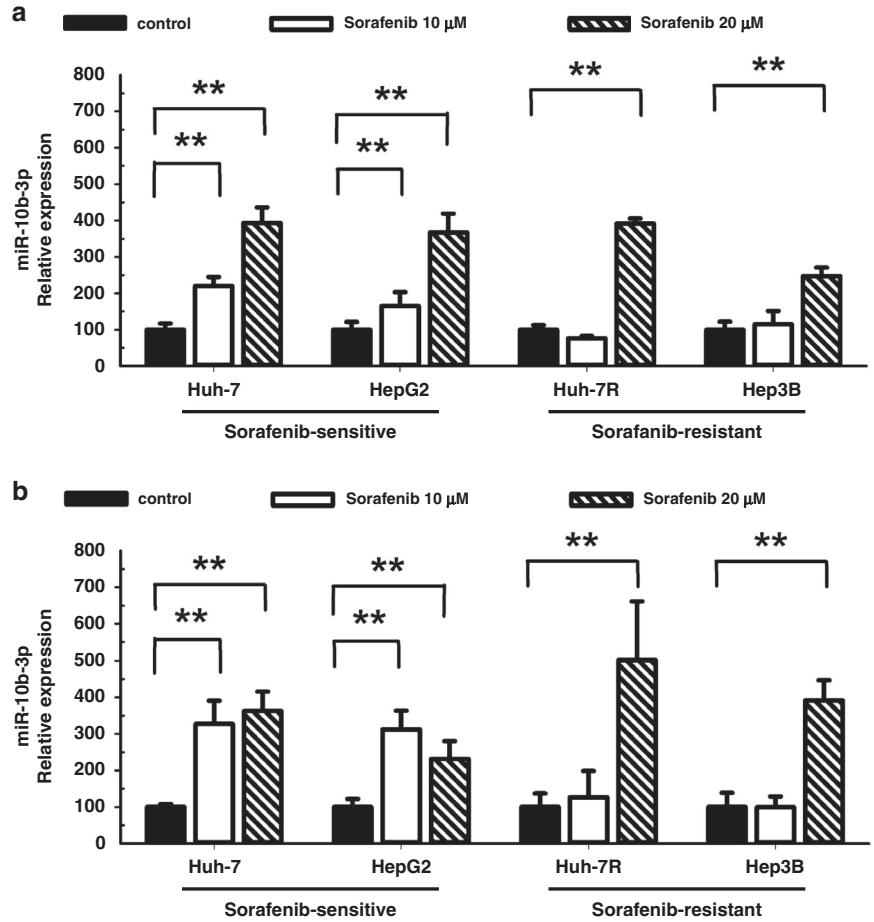


Fig. 2 Expression of miR-10b-3p was induced by sorafenib in HCC cells. A miR-10b-3p induction after sorafenib treatment. HCC cells were treated for 24 h. Quantitative RT-PCR analysis of miR-10b-3p levels in cell lysates. **B** Induced secretion of miR-10b-3p after sorafenib treatment. HCC cells were treated for 24 h before collection of conditioned medium for analysis by quantitative RT-PCR. Each value is the mean ± SD. $^{**}p < 0.01$ compared with control.

group, an increase in serum miR-10b-3p levels was associated with better overall survival (OS) (Fig. 3D, E).

The functional roles of miR-10b-3p in sorafenib efficacy for HCC cells

The effects of miR-10b-3p over-expression or depletion on sorafenib-induced apoptosis in HCC cells were evaluated by transient transfection of miR-10b-3p mimics (Fig. 4A) or antagomirs (Fig. 4B). Overexpression of miR-10b-3p significantly enhanced sorafenib-induced apoptosis (Fig. 4A), whereas depletion of miR-10b-3p suppressed sorafenib-induced apoptosis (Fig. 4B). This finding was further confirmed for apoptosis-related proteins by western blotting, which showed cleavage of PARP-1 and caspase 3 (Supplementary Fig. S1). Overexpression of miR-10b-3p significantly enhanced sorafenib-induced cleavage of PARP-1 and caspase 3 (Supplementary Fig. S1A), whereas depletion of miR-10b-3p suppressed sorafenib-induced cleavage of PARP-1 and caspase 3 (Supplementary Fig. S1B). These phenomena appeared more prominent in the sorafenib-sensitive Huh-7 and HepG2 cells (Fig. 4 and Supplementary Fig. S1). The effects of mimics and antagomirs on cell viability, as determined by MTT assay, showed consistent trends (Supplementary Fig. S2). A subcutaneous liver cancer model was used to analyze the effect of miR-10b-3p on sorafenib resistance in vivo. We demonstrated that mice showing depletion of miR-10b-3p (Anti-10b-3p) in sorafenib-sensitive tumors (Huh-7) had significantly suppressed sorafenib-induced anti-tumor efficacy (Fig. 4C).

The potential downstream targets of miR-10b-3p were explored using gene expression microarray data of Huh-7 cells. Eighteen genes were found to be downregulated in Huh-7 cells treated with miR-10b-3p over-expression, sorafenib, or both (Fig. 5A). Among these genes, cyclin E1 (CCNE1) was selected for further analysis because CCNE1 has been found a critical regulator of sorafenib resistance [37]. A reporter system of wild type and mutant 3′-UTRs of CCNE1, which contained a potential binding site for miR-10b-3p, was established to verify whether CCNE1 is the direct target of miR-10b-3p in Huh-7 and HepG2 cells, which had relatively high endogenous miR-10b-3p (Fig. 5B). The construct with mutant miR-10b-3p binding site at the CCNE1 3′-UTR demonstrates luciferase activity, while the construct with wild-type CCNE1 3′-UTR failed to do so, suggesting that endogenous miR-10b-3p directly recognized and suppressed CCNE1 expression (Fig. 5C). To clarify the regulatory mechanism of miR-10b-3p overexpression or depletion, sorafenib-induced cyclin E1 and downstream in HCC cells were evaluated by transient transfection of miR-10b-3p mimics (Fig. 5D) or antagomirs (Supplementary Fig. S3). Overexpression of miR-10b-3p downregulated endogenous CCNE1 mRNA, cyclin E1, and Rb protein levels, but not CDK2 and E2F1 expression in both sorafenib-sensitive and sorafenib-resistant HCC cells (Fig. 5D), whereas depletion of miR-10b-3p slightly upregulated cyclin E1 and Rb protein expression (Supplementary Fig. S3), consistent with the apoptosis-enhancing effects of miR-10b-3p overexpression (Fig. 4A). Previous study, we demonstrated that cyclin

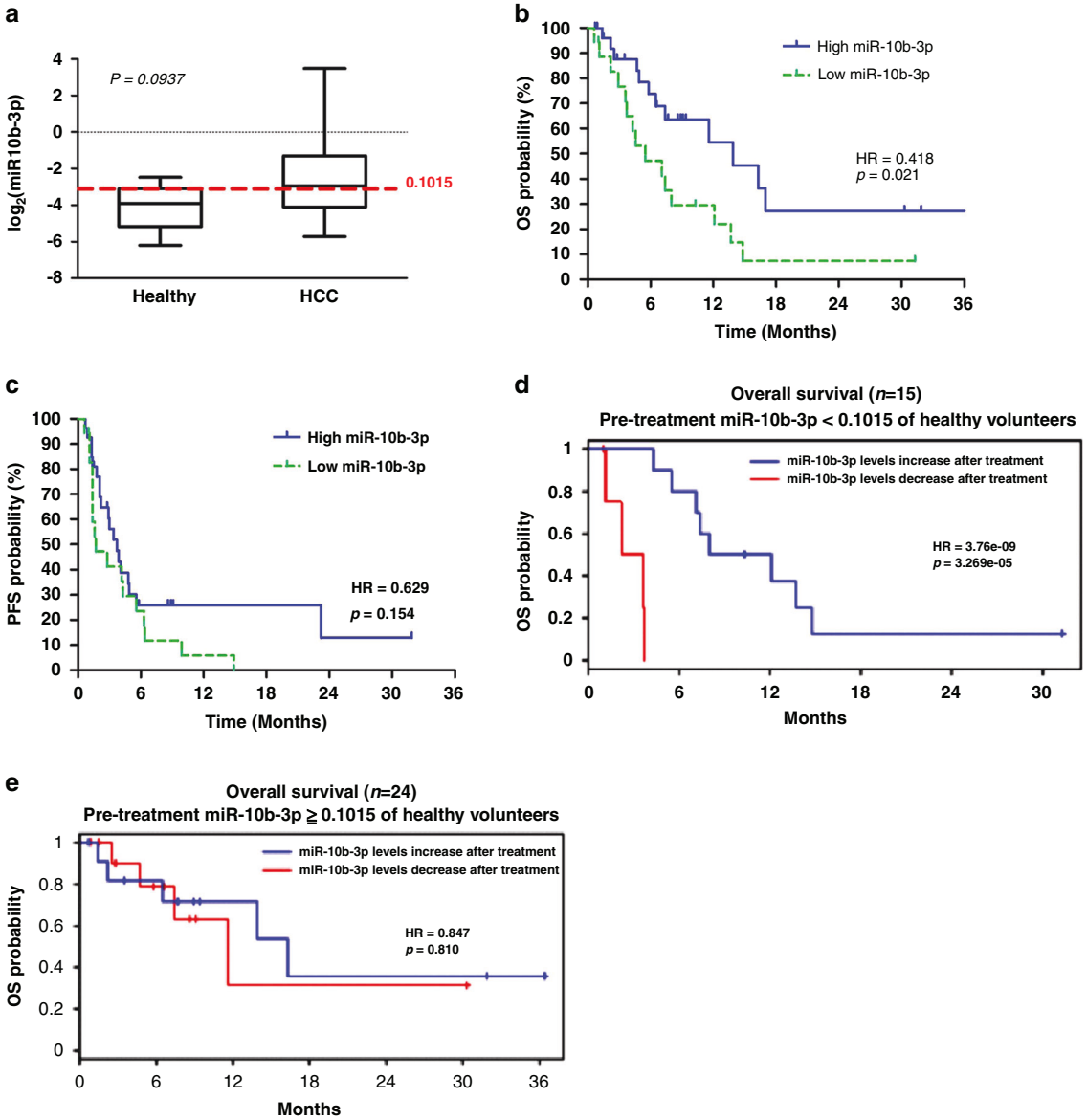


Fig. 3 Expression of miR-10b-3p was associated with better prognosis of HCC patients. A Comparison of serum levels of miR-10b-3p in healthy volunteer controls ($n = 6$) and advanced HCC patients before sorafenib treatment ($n = 45$) by Q-RT-PCR analysis. The expression levels were represented by the relative amount of target gene (miR-10b-3p) vs. control gene (MammU6). $2^{-\Delta CT}$, where $\Delta CT = CT$ (target gene) $-$ CT (control gene). The cut-off value was set at the third quartile (0.1015) of healthy controls, and then the patients were divided into two groups. **B** The cut-off value was set at third quartile (0.1015) of healthy controls; then the patients were divided into two groups. High miR-10b-3p (≥0.1015) predicts good patient survival before accepting sorafenib treatment. Kaplan–Meier survival curves of overall survival and (**C**) progression-free survival for HCC patients whose serum expressed high vs. low levels of miR-10b-3p *p*, log-rank test. **D** Low miR-10b-3p (˂0.1015) patients were divided into two groups. Patients whose miR-10b-3p levels increased after sorafenib treatment had better survival. **E** High miR-10b-3p (≥0.1015) patients were divided into two groups. There was no difference in survival between patients whose miR-10b-3p levels increased vs. patients whose levels decreased after sorafenib treatment.

E1 suppression by sorafenib in HCC cells correlated with therapeutic efficacy of sorafenib [37]. These results suggest that miR-10b-3p may be an upstream regulator of CCNE1 expression and may involve in sorafenib resistance in HCC cells (Fig. 5E).

DISCUSSION

In this study, we demonstrated that miR-10b-3p was one of the most significantly downregulated miRNAs found in sorafenib-resistant cell lines in vitro and in vivo. Interestingly, sorafenib treatment dose-dependently enhanced the endogenous expression and secretion of miR-10b-3p in all tested HCC cell lines. In our cohort of sorafenib-treated patients, high serum miR-10b-3p levels correlated with significantly longer OS, suggesting that serum miR-10b-3p may serve as a predictive biomarker for sorafenib treatment response in advanced HCC patients. Because Q3 of the healthy controls was close to the HCC patients' median value, patients with low or high mir-10b-3p did not to differ drastically in number. Furthermore, we found that miR-10b-3p-regulated Cyclin E1 expression is involved in controlling sorafenib resistance in HCC cells. The overall survival results for our patient cohort differ from those of other studies on miR-10b-3p in which high serum miR-10b-3p levels were associated with worse survival in HCC patients cohorts [19]. However, these previous studies included all HCC

Table 1. Multivariate analyses of overall survival.

Covariate	Coefficient	Hazard Ratio	95% C.I.	*p* value
Overall survival				
High miR-10b-3p	−1.2871	0.276	0.120–0.635	0.0025
Extrahepatic involvement	1.1963	3.308	1.328–8.238	0.0102
CLIP ≥ 3	1.3444	3.836	1.441–10.210	0.0071
ECOG = 0 (vs. 1 or 2)	−1.1102	0.329	0.142–0.765	0.0097

Cox's proportional hazards model: $n = 45$, adjusted generalized $R^2 = 0.402$.

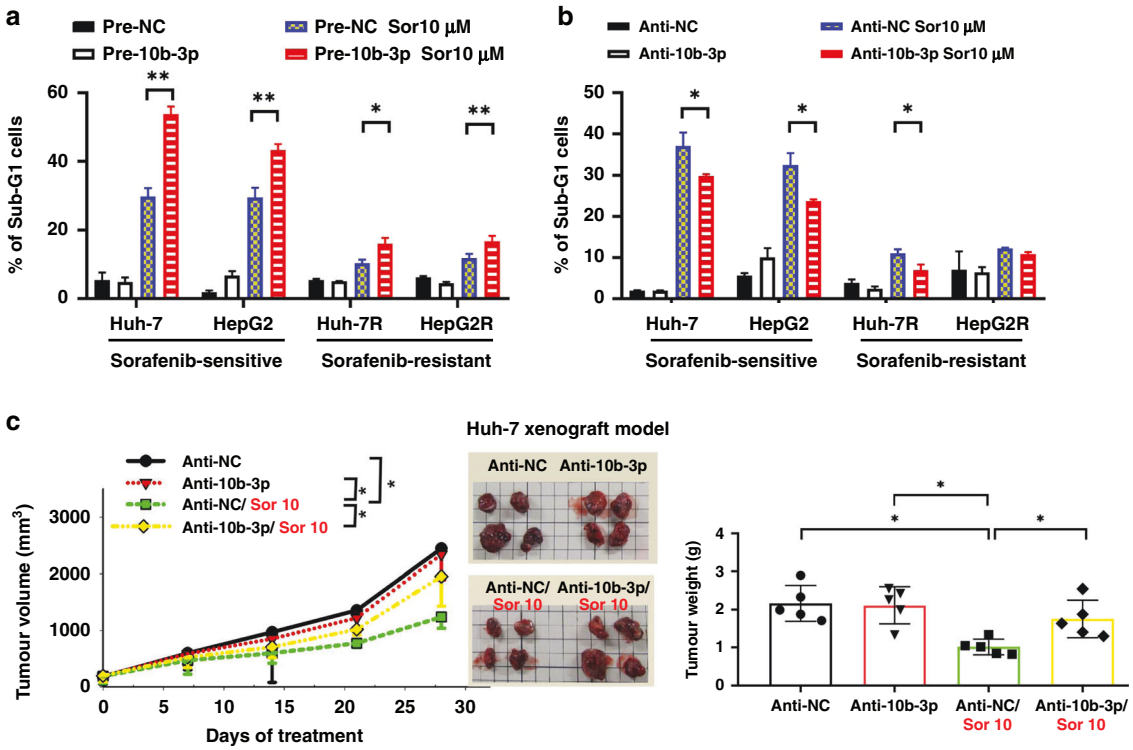
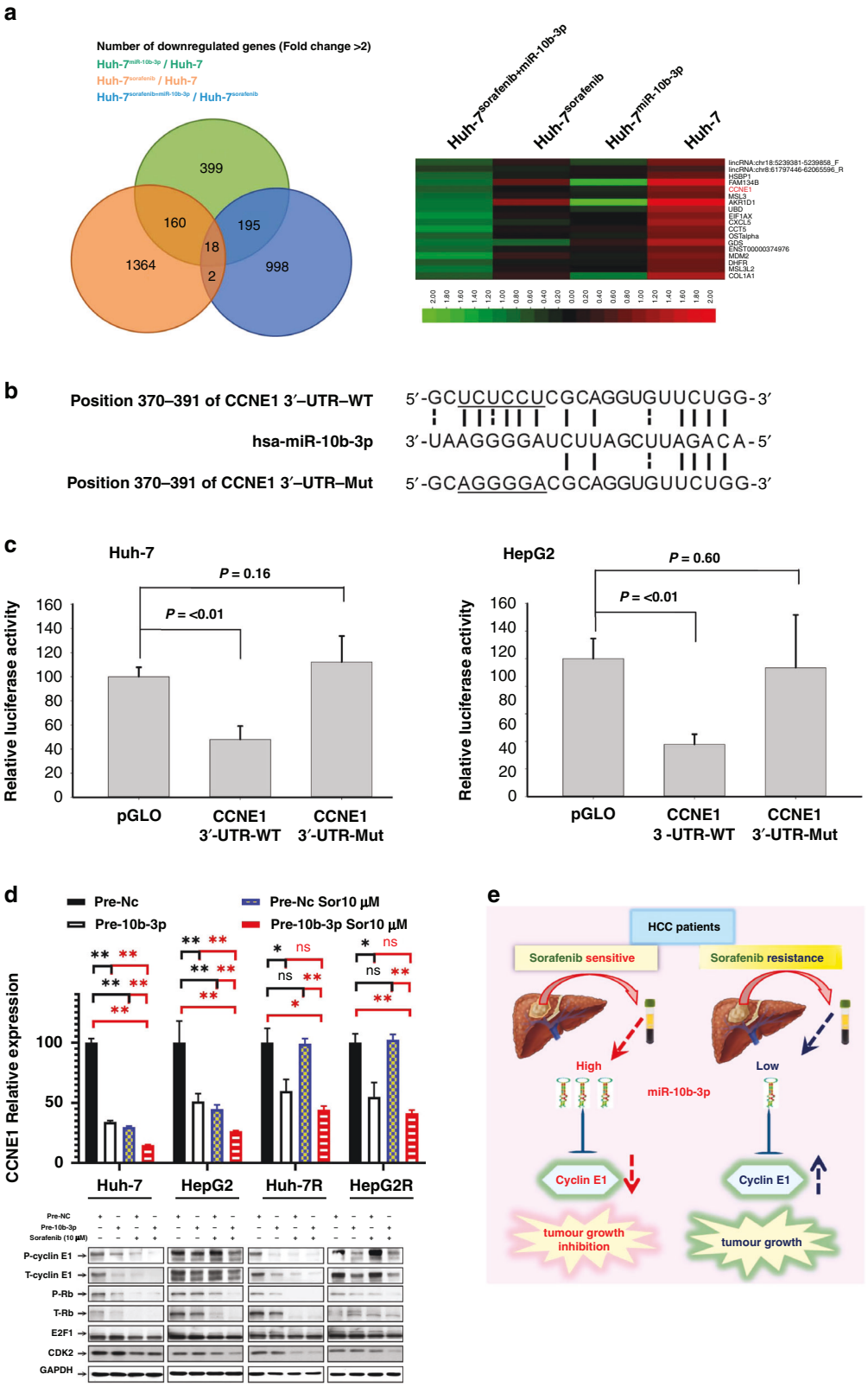


Fig. 4 Expression of miR-10b-3p sensitizes HCC cells in response to sorafenib treatment. HCC cell lines were transfected with pre-miR of miR-10b-3p (pre-10b-3p) or a negative-control (pre-NC) and treated with the indicated sorafenib concentration for 72 h. **A** Effects of miR-10b-3p overexpression on sorafenib-induced apoptosis assessed by Sub-G1 analysis. HCC cell lines were transfected with anti-miR directed against miR-10b-3p (anti-10b-3p) or a negative-control (anti-NC) and treated with indicated sorafenib concentration for 72 h. **B** Effects of miR-10b-3p depletion on sorafenib-induced apoptosis assessed by Sub-G1 analysis. Each value is the mean ± SD of three independent experiments. *$p < 0.05$; **$p < 0.01$, compared with pre-NC or anti-NC (sorafenib 10 μM). **C** Depletion of miR-10b-3p (Anti-10b-3p) in sorafenib-sensitive tumors (Huh-7) suppressed sorafenib-induced anti-tumor efficacy in a subcutaneous liver cancer model. The growth curve and tumor weight of xenograft tumors in nude mice; $n = 5$ in each group; Data are presented as the mean ± SD; *$p < 0.05$.

patients, whereas the patients in our cohort received sorafenib treatment after initial measurement of their serum miR-10b-3p levels. This difference may suggest that the serum miR-10b-3p level has different implications for patient survival depending on sorafenib treatment status. The mechanisms underlying the differences in survival characteristics between these patient cohorts should be further studied.

The biological functions of miR-10b-3p have not been as thoroughly characterized as another mature miRNA product generated from the same precursor, miR-10b-5p. The biological roles of miR-10b-5p as an oncogenic miRNA promoting cancer metastasis through HOXD10 and KLF4 [41] and proliferation, migration, and invasion in HCC cells [42] have been well documented. A previous study found that in breast cancer, miRNA-10b-3p suppresses proliferation and reduces in vivo tumor growth by targeting BUB1, PLK1 and CCNA2 [43]. Our study has identified a novel tumor-suppressive mechanism in which miR-10b-3p induction suppresses Cyclin E1 expression, in turn prompting sorafenib-mediated cell death (Fig. 5D).

miR-10b-3p was reported to be associated with worse survival in HCC patients [19]. However, suggested a pro-oncogenic role of miR-10b-3p through the inhibition of CMTM5 in HCC cell lines without sorafenib treatment [19]. The difference in our results and the results of the study by Guan et al. may suggest that sorafenib treatment potentially affects the CMTM5 and Cyclin E1 pathways in a manner such that miR-10b-3p adopts a greater tumor suppressor role following sorafenib treatment. For example, it may be that sorafenib inhibits factors in the CMTM5 pathway, which allows miR-10b-3p to act primarily as a tumor suppressor through its interactions with the Cyclin E1 pathway in sorafenib-treated HCC. In light of previous studies, our current findings may also suggest that the balance between oncogenic miR-10b-5p and the tumor-suppressive function of miR-10b-3p may be critical in modulating HCC progression and regulating cancer metastasis in sorafenib-treated HCC.



In addition to intracellular functions, numerous miRNAs have been identified to mediate intercellular communication. These secreted miRNAs in circulation could be informative biomarkers, with potential in therapeutic applications [7]. Circulating miRNAs in serum have also been linked to different pathological processes of the liver, including inflammation, injury, fibrosis, and cancer. For example, serum miR-122, one of the best-studied miRNAs as well as the most abundantly-expressed miRNA in the liver, has been found to be elevated in mice with liver injury and inflammation [22, 44]. The potential tumorigenic mechanism of secreting tumor

Fig. 5 miR-10b-3p was a possible mediator of sorafenib efficacy through down-regulation of cyclin E1 in HCC cell lines. A The target genes in the miR-10b-3p-expressing Huh-7 cells (Huh-7$^{miR\text{-}10b\text{-}3p}$), sorafenib-treated Huh-7 cells (Huh-7^{sorafenib}), and sorafenib-treated miR-10b-3p-expressing Huh-7 cells (Huh-7$^{sorafenib+miR\text{-}10b\text{-}3p}$) following microarray data analysis (left panel). Eighteen RNA targets selected by microarray to analyze gene expression in Huh-7 cells transfected with miR-10b-3p or treated with sorafenib (right panel). **B** The putative binding site of miR-10b-3p on the 3′-UTR of the human CCNE1 gene determined by computational predictions. CCNE1 3′-UTR-WT sequence and the CCNE1 3′-UTR-Mut in which the underlined sequence was mutagenized to abolish miR-10b-3p binding. **C** The constructs of the cyclin E1 3′-UTR (+370 to +391 bp) were transfected into Huh-7 and HepG2 cells, and the relative luciferase activity was shown. **D** Cyclin E1 mRNA expression in HCC cell lines was measured using qRT-PCR from RNA extracted from cells transfected with and overexpressing miR-10b-3p (Pre-10b-3p) or a negative control (Pre-NC) treated with sorafenib (10 μM) for 24 h. HPRT was used as an internal control (top panel). Cyclin E1 and downstream protein (E2F1, Rb, and CDK2) expression was detected by western blotting of total protein from cells transfected with and overexpressing miR-10b-3p (Pre-10b-3p) and the negative control (Pre-NC) treated with sorafenib (10 μM) for 24 h. GAPDH was used as an internal loading control (bottom panel). Each value is the mean ± SD of independent experiments. *$p < 0.05$; **$p < 0.01$. **E** Schematic representation of the miR-10b-3p sensitizes HCC cells in response to sorafenib treatment through the downregulated Cyclin E1 expression.

suppressor miRNAs out of the cell [8] also merits further study with miR-10b-3p and any other potential miRNA biomarkers as their molecular mechanisms become more well understood.

The biological role of circulating miRNAs has demonstrated their use as predictive, diagnostic, and prognostic biomarkers as well as targets for therapy in HCC and in many other cancers [8, 34, 45–48]. The recent development of a miRNA classifier is valuable for detecting preclinical hepatocellular carcinoma, providing patients with a chance of curative resection and longer survival [12]. Similar hope arises from a panel of seven miRNAs that can identify small-size, early-stage, and alpha-fetoprotein-negative hepatocellular carcinoma in patients at risk [49]. In this study, we not only reveal a functional mechanism of miR-10b-3p mediated cell death in the regulation of sorafenib sensitivity, but also found that secreted miR-10b-3p, which is induced by sorafenib treatment, maybe an independent prognostic biomarker for HCC patients. Our study had some limitations. We used healthy volunteers as the control group, although patients with chronic liver disease may be a better choice. However, the control group was only used to determine the optimal cutoff point for the miR-10b-3p level in patients with HCC. In addition, the sample size was relatively small and the study lacked a histological diagnosis. However, such limitations may not substantially influence our outcomes. Our results should be further validated by a prospective study involving multicenter clinical trials.

It must be noted that the validity of reference miRNAs has been found to be unstable, especially with the abundance of reports identifying individual miRNAs in primarily small studies [50, 51]. Validation of these smaller miRNA studies using larger datasets that are combinations of independent datasets merits further research [51]. Nevertheless, our findings demonstrate the biological significance and underscore new clinical applications of miR-10b-3p as a potential prognostic marker, a predictive marker for monitoring sorafenib response, and a potential druggable target.

DATA AVAILABILITY

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

REFERENCES

1. Zhang PF, Wei CY, Huang XY, Peng R, Yang X, Lu JC, et al. Circular RNA circTRIM33-12 acts as the sponge of MicroRNA-191 to suppress hepatocellular carcinoma progression. Mol Cancer. 2019;18:105.
2. Liu X, Chen D, Chen H, Wang W, Liu Y, Wang Y, et al. YB1 regulates miR-205/200b-ZEB1 axis by inhibiting microRNA maturation in hepatocellular carcinoma. Cancer Commun. 2021. https://doi.org/10.1002/cac2.12164
3. Komoll RM, Hu Q, Olarewaju O, von Dohlen L, Yuan Q, Xie Y, et al. MicroRNA-342-3p is a potent tumour suppressor in hepatocellular carcinoma. J Hepatol. 2021;74:122–34.
4. Chen Y, Buyel JJ, Hanssen MJ, Siegel F, Pan R, Naumann J, et al. Exosomal microRNA miR-92a concentration in serum reflects human brown fat activity. Nat Commun. 2016;7:11420.
5. Matsumura T, Sugimachi K, Iinuma H, Takahashi Y, Kurashige J, Sawada G, et al. Exosomal microRNA in serum is a novel biomarker of recurrence in human colorectal cancer. Br J Cancer. 2015;113:275–81.
6. Xu L, Beckebaum S, Iacob S, Wu G, Kaiser GM, Radtke A, et al. MicroRNA-101 inhibits human hepatocellular carcinoma progression through EZH2 down-regulation and increased cytostatic drug sensitivity. J Hepatol. 2014;60:590–8.
7. Chen X, Liang H, Zhang J, Zen K, Zhang CY. Secreted microRNAs: a new form of intercellular communication. Trends Cell Biol. 2012;22:125–32.
8. Bai X, Liu Z, Shao X, Wang D, Dong E, Wang Y, et al. The heterogeneity of plasma miRNA profiles in hepatocellular carcinoma patients and the exploration of diagnostic circulating miRNAs for hepatocellular carcinoma. PLoS One. 2019;14:e0211581.
9. Brase JC, Wuttig D, Kuner R, Sultmann H. Serum microRNAs as non-invasive biomarkers for cancer. Mol Cancer. 2010;9:306.
10. Shi Y, Zhang DD, Liu JB, Yang XL, Xin R, Jia CY, et al. Comprehensive analysis to identify DLEU2L/TAOK1 axis as a prognostic biomarker in hepatocellular carcinoma. Mol Ther Nucleic Acids. 2021;23:702–18.
11. Hung CH, Hu TH, Lu SN, Kuo FY, Chen CH, Wang JH, et al. Circulating microRNAs as biomarkers for diagnosis of early hepatocellular carcinoma associated with hepatitis B virus. Int J Cancer. 2016;138:714–20.
12. Lin XJ, Chong Y, Guo ZW, Xie C, Yang XJ, Zhang Q, et al. A serum microRNA classifier for early detection of hepatocellular carcinoma: a multicentre, retrospective, longitudinal biomarker identification study with a nested case-control study. Lancet Oncol. 2015;16:804–15.
13. Parpart S, Roessler S, Dong F, Rao V, Takai A, Ji J, et al. Modulation of miR-29 expression by alpha-fetoprotein is linked to the hepatocellular carcinoma epigenome. Hepatology. 2014;60:872–83.
14. Zanutto S, Ciniselli CM, Belfiore A, Lecchi M, Masci E, Delconte G, et al. Plasma miRNA-based signatures in CRC screening programs. Int J Cancer. 2020;146:1164–73.
15. Lu X, Lu J, Wang S, Zhang Y, Ding Y, Shen X, et al. Circulating serum exosomal miR-92a-3p as a novel biomarker for early diagnosis of gastric cancer. Future Oncol. 2021;17:907–19.
16. Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, et al. Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J Clin Oncol. 2010;28:1721–6.
17. Kleivi Sahlberg K, Bottai G, Naume B, Burwinkel B, Calin GA, Borresen-Dale AL, et al. A serum microRNA signature predicts tumor relapse and survival in triple-negative breast cancer patients. Clin Cancer Res. 2015;21:1207–14.
18. Sheedy P, Medarova Z. The fundamental role of miR-10b in metastatic cancer. Am J cancer Res. 2018;8:1674–88.
19. Guan L, Ji D, Liang N, Li S, Sun B. Up-regulation of miR-10b-3p promotes the progression of hepatocellular carcinoma cells via targeting CMTM5. J Cell Mol Med. 2018;22:3434–41.
20. Zhou J, Yu L, Gao X, Hu J, Wang J, Dai Z, et al. Plasma microRNA panel to diagnose hepatitis B virus-related hepatocellular carcinoma. J Clin Oncol. 2011;29:4781–8.
21. Llovet JM, Kelley RK, Villanueva A, Singal AG, Pikarsky E, Roayaie S, et al. Hepatocellular carcinoma. Nat Rev Dis Prim. 2021;7:6.
22. Kanthaje S, Makol A, Chakraborti A. Sorafenib response in hepatocellular carcinoma: MicroRNAs as tuning forks. Hepatol Res. 2018;48:5–14.
23. Azumi J, Tsubota T, Sakabe T, Shiota G. miR-181a induces sorafenib resistance of hepatocellular carcinoma cells through downregulation of RASSF1 expression. Cancer Sci. 2016;107:1256–62.
24. Fornari F, Pollutri D, Patrizi C, La Bella T, Marinelli S, Casadei Gardini A, et al. In hepatocellular carcinoma miR-221 modulates sorafenib resistance through inhibition of caspase-3-mediated apoptosis. Clin Cancer Res. 2017;23:3953–65.

25. Gramantieri L, Pollutri D, Gagliardi M, Giovannini C, Quarta S, Ferracin M, et al. MiR-30e-3p influences tumor phenotype through MDM2/TP53 axis and predicts sorafenib resistance in hepatocellular carcinoma. Cancer Res. 2020;80:1720–34.
26. He C, Dong X, Zhai B, Jiang X, Dong D, Li B, et al. MiR-21 mediates sorafenib resistance of hepatocellular carcinoma cells by inhibiting autophagy via the PTEN/Akt pathway. Oncotarget. 2015;6:28867–81.
27. Nishida N, Arizumi T, Hagiwara S, Ida H, Sakurai T, Kudo M. MicroRNAs for the prediction of early response to sorafenib treatment in human hepatocellular carcinoma. Liver Cancer. 2017;6:113–25.
28. Tan W, Lin Z, Chen X, Li W, Zhu S, Wei Y, et al. miR-126-3p contributes to sorafenib resistance in hepatocellular carcinoma via downregulating SPRED1. Ann Transl Med. 2021;9:38.
29. Weng H, Zeng L, Cao L, Chen T, Li Y, Xu Y, et al. circFOXM1 contributes to sorafenib resistance of hepatocellular carcinoma cells by regulating MECP2 via miR-1324. Mol Ther Nucleic Acids. 2021;23:811–20.
30. Xu Y, Huang J, Ma L, Shan J, Shen J, Yang Z, et al. MicroRNA-122 confers sorafenib resistance to hepatocellular carcinoma cells by targeting IGF-1R to regulate RAS/RAF/ERK signaling pathways. Cancer Lett. 2016;371:171–81.
31. Zhao W, Ma B, Tian Z, Han H, Tang J, Dong B, et al. Inhibiting CBX4 efficiently protects hepatocellular carcinoma cells against sorafenib resistance. Br J Cancer. 2021;124:1237–48.
32. Ouyang H, Gore J, Deitz S, Korc M. microRNA-10b enhances pancreatic cancer cell invasion by suppressing TIP30 expression and promoting EGF and TGF-beta actions. Oncogene. 2014;33:4664–74.
33. Zhang P, Hong H, Sun X, Jiang H, Ma S, Zhao S, et al. MicroRNA-10b regulates epithelial-mesenchymal transition by modulating KLF4/Notch1/E-cadherin in cisplatin-resistant nasopharyngeal carcinoma cells. Am J Cancer Res. 2016;6:141–56.
34. Monroig-Bosque PdC, Shah MY, Fu X, Fuentes-Mattei E, Ling H, Ivan C, et al. OncomiR-10b hijacks the small molecule inhibitor linifanib in human cancers. Sci Rep. 2018;8:13106.
35. Khalighfard S, Alizadeh AM, Irani S, Omranipour R. Plasma miR-21, miR-155, miR-10b, and Let-7a as the potential biomarkers for the monitoring of breast cancer patients. Sci Rep. 2018;8:17981.
36. Zhu Q, Gong L, Wang J, Tu Q, Yao L, Zhang J-R, et al. miR-10b exerts oncogenic activity in human hepatocellular carcinoma cells by targeting expression of CUB and sushi multiple domains 1 (CSMD1). BMC Cancer. 2016;16:806–806.
37. Hsu C, Lin LI, Cheng YC, Feng ZR, Shao YY, Cheng AL, et al. Cyclin E1 inhibition can overcome sorafenib resistance in hepatocellular carcinoma cells through Mcl-1 suppression. Clin Cancer Res. 2016;22:2555–64.
38. Lai HH, Li CW, Hong CC, Sun HY, Chiu CF, Ou DL, et al. TARBP2-mediated destabilization of Nanog overcomes sorafenib resistance in hepatocellular carcinoma. Mol Oncol. 2019;13:928–45.
39. Ou DL, Shen YC, Yu SL, Chen KF, Yeh PY, Fan HH, et al. Induction of DNA damage-inducible gene GADD45beta contributes to sorafenib-induced apoptosis in hepatocellular carcinoma cells. Cancer Res. 2010;70:9309–18.
40. Marrero JA, Kulik LM, Sirlin CB, Zhu AX, Finn RS, Abecassis MM, et al. Diagnosis, staging, and management of hepatocellular carcinoma: 2018 practice guidance by the American Association for the Study of Liver Diseases. Hepatology. 2018;68:723–50.
41. Ma L. Role of miR-10b in breast cancer metastasis. Breast Cancer Res. 2010;12:210.
42. Li QJ, Zhou L, Yang F, Wang GX, Zheng H, Wang DS, et al. MicroRNA-10b promotes migration and invasion through CADM1 in human hepatocellular carcinoma cells. Tumour Biol. 2012;33:1455–65.
43. Biagioni F, Bossel Ben-Moshe N, Fontemaggi G, Canu V, Mori F, Antoniani B, et al. miR-10b*, a master inhibitor of the cell cycle, is down-regulated in human breast tumours. EMBO Mol Med. 2012;4:1214–29.
44. Calkins KL, Thamotharan S, Ghosh S, Dai Y, Devaskar SU. MicroRNA 122 reflects liver injury in children with intestinal failure-associated liver disease treated with intravenous fish oil. J Nutr. 2020;150:1144–50.
45. Yang N, Ekanem NR, Sakyi CA, Ray SD. Hepatocellular carcinoma and microRNA: new perspectives on therapeutics and diagnostics. Adv Drug Deliv Rev. 2015;81:62–74.
46. Vasuri F, Visani M, Acquaviva G, Brand T, Fiorentino M, Pession A, et al. Role of microRNAs in the main molecular pathways of hepatocellular carcinoma. World J Gastroenterol. 2018;24:2647–60.
47. Marisi G, Cucchetti A, Ulivi P, Canale M, Cabibbo G, Solaini L, et al. Ten years of sorafenib in hepatocellular carcinoma: Are there any predictive and/or prognostic markers? World J Gastroenterol. 2018;24:4152–63.
48. Lu JW, Ho YJ, Yang YJ, Liao HA, Ciou SC, Lin LI, et al. Zebrafish as a disease model for studying human hepatocellular carcinoma. World J Gastroenterol. 2015;21:12042–58.
49. Li LM, Hu ZB, Zhou ZX, Chen X, Liu FY, Zhang JF, et al. Serum microRNA profiles serve as novel biomarkers for HBV infection and diagnosis of HBV-positive hepatocarcinoma. Cancer Res. 2010;70:9798–807.
50. Xiang M, Zeng Y, Yang R, Xu H, Chen Z, Zhong J, et al. U6 is not a suitable endogenous control for the quantification of circulating microRNAs. Biochem Biophys Res Commun. 2014;454:210–4.
51. Nagy Á, Lánczky A, Menyhárt O, Győrffy B. Validation of miRNA prognostic power in hepatocellular carcinoma using expression data of independent datasets. Sci Rep. 2018;8:9227.

ACKNOWLEDGEMENTS

This work was financially supported by the Cancer Biology Research Group, Center of Precision Medicine, National Taiwan University, Taipei, Taiwan. The authors thank the National Center for High-performance Computing for computer time and facilities as well as the second Core Lab, Department of Medical Research, National Taiwan University Hospital for providing laboratory facilities. D-LO was supported by National Taiwan University YongLin Institute of Health Scholar.

AUTHOR CONTRIBUTIONS

Y-YS and P-SC contributed equally to this work. Study concept and design: D-LO, CH; Methodology and technical support: Y-YS, P-SC, B-SL, and D-LO; Analysis and interpretation of data: Y-YS, P-SC, LL, AL, CH, and D-LO; Writing, review, and/or revision of the manuscript: Y-YS, P-SC, and D-LO; Study supervision: A-LC and CH.

FUNDING

This study was supported by the following research grants: NTU-109L901403, NTU-110L901404 (from Ministry of Education, Taiwan), MOST 106-2314-B-002-229-MY3, MOST 107-3017-F-002-002, MOST 107-2314-B-002-210-MY3, MOST 108-2314-B-002-075-MY3, MOST 108-3017-F-002-004, MOST 109-2634-F-002-043, 109-2314-B-002-229 -MY3, MOST 110-2634-F-002-044 (from Ministry of Science and Technology, Taiwan), YongLin Chair Grant S-01, (from National Taiwan University), UN108-010, UN109-051 (from National Taiwan University Hospital).

COMPETING INTERESTS

A-LC is a consultant for and a member of the speaker's bureau of Bayer-Schering Pharma. A-LC is a consultant of Novartis, Merck Serono, Eisai, Merck Sharp & Dohme (MSD) Corp., ONXEO, Bayer HealthCare Pharmaceuticals Inc., Bristol-Myers Squibb (BMS) Company, and Ono Pharmaceutical Co., Ltd. A-LC is an Associate Editor of Liver Cancer. CH received research grants from BMS/ONO, Roche, and Ipsen and received honorarium from the following pharmaceutical companies: AstraZeneca, Bayer, BMS/ONO, Eisai, Eli Lilly, Ipsen, Merck Serono, MSD, Novartis, Roche, TTY Biopharm.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol for the in vivo studies was approved by the Institutional Animal Care and Use Committee of the College of Medicine, National Taiwan University (No. 20130360). All the animal studies were performed according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. We enrolled patients who received sorafenib as the first-line therapy for advanced HCC at NTUH. This study was approved by the Institute Research Ethical Committee of NTUH (No. 201401040RIND).

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41416-022-01759-w.

Correspondence and requests for materials should be addressed to Da-Liang Ou.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.