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Original Article

RNAi screening with shRNAs against histone methylation-related genes reveals determinants of sorafenib sensitivity in hepatocellular carcinoma cells

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Abstract: Sorafenib is the first drug currently approved to treat advanced hepatocellular carcinoma (HCC). However, very low response rate and acquired drug resistance makes rare patients benefit from sorafenib therapy, therefore it is urgent to find biomarkers for sorafenib sensitivity. Histone modifications, including histone methylation, have been demonstrated to influence the initiation and progression of HCC. It is of great interest to elicit the possibility whether histone methylation plays a role in regulation of sorafenib sensitivity. In present work, a high throughput RNAi screening with 176 shRNA pools against 88 histone methyltransferases (HMTs) and histone demethyltransferases genes was applied to HepG2 cells. Silencing of 3 genes (ASH1L, C17ORF49 and SETD4) was validated to specifically promote HepG2 cells sensitivity to sorafenib. Western blotting results showed that those 3 HMT genes knockdown alone or sorafenib treatments alone both induce AKT/ERK activation. However, combination treatment with sorafenib and silencing of C17ORF49 or SETD4 downregulated AKT phosphorylation and hence induced HCC cells death. Our work may provide potential biomarkers for sorafenib sensitivity and therapeutic combination for sorafenib treatment in HCC patients.

Keywords: Hepatocellular carcinoma, sorafenib, histone methylation, ASH1L, C17ORF49, SETD4, AKT

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common malignancy worldwide [1], with more than 600,000 new cases per year. Approximately 80% of cases arise in Asia and Africa, mainly due to chronic hepatitis B virus (HBV) infection [2]. Patients with unresectable or metastatic disease have a median survival of only a few months [3].

Sorafenib, an oral multikinase inhibitor [4], is the first drug currently approved to treat advanced hepatocellular carcinoma. It has shown in two phase 3 trials involving patients with advanced HCC and Child-Pugh A cirrhosis a statistically significant increase in median overall survival over placebo (SHARP trial [5]: 10.7 months vs 7.9 months; hazard ratio [HR], 0.69; 95% confidence interval [CI], 0.55-0.88; P<0.001. Asia-Pacific trial [6]: 6.5 months vs 4.2 months; HR, 0.68; 95% CI, 0.50-0.93; P=0.014.). However, only 2-3.3% patients in the sorafenib group had a partial response in the two trials. The overwhelming majority of sorafenib patients (54-71%) had stable disease. The tumor response rates to sorafenib were very low. Furthermore, many patients may develop acquired resistance to sorafenib, further narrowing the population who can benefit from sorafenib therapy. Therefore, it is urgent to find biomarkers for sorafenib sensitivity to select suitable patients and to improve the clinical therapy.

Preclinical studies have proposed that activation of PI3K/AKT signaling [7] or epithelial–mesenchymal transition (EMT) [8], overexpression of EGFR and HER3 [9], hypoxia-inducible factor 1 (HIF1α) [10], and nucleophosmin (NPM) [11] all can confer HCC cells resistance to sorafenib. These alterations in HCC cells may

arise from epigenetic and genetic abnormalities. Epigenetic regulations include genomic DNAmethylation, histone modifications, and miRNA regulation, which regulate a variety of important cellular networks. It seems likely that these "epimutations" may occur at a much higher frequency compared to gene mutations and in turn may have important effects on the processes of HCC tumorigenesis and metastasis [12, 13].

Histone modifications are suggested to orchestrate with DNA methylation to regulate the expression of genes. Among histone modifications, histone methylation is one of the most investigated and is regulated by histone methyltransferases (HMTs) and histone demethyltransferases (HDMTs). SMYD3, encoding a histone methyltransferase involved in the proliferation of cancer cells [14], was expression-enhanced in HCC cell lines and tissues along with the upregulation of trimethylated histone H3 lysine 4 and is associated with the poor prognosis in HCC patients [15]. $p16^{INK4a}$ binds to cyclin-dependent protein kinase 4 (CDK4) and inhibits the ability of CDK4 to interact with cyclin D1. $p16^{INK4a}$ is one of the most altered tumor suppressor gene in human cancer. In HCC, loss of $p16^{INK4a}$ is previously supposed to be mainly caused by aberrant promoter methylation [16], however, recent study demonstrated that $p16^{INK4a}$ silencing by H3K27 trimethylation is an early epigenetic event for regaining tumorigenesis in fusion reprogrammed hepatoma cells [17]. Epigenetic downregulation of the suppressor of cytokine signaling 1 (Socs1) gene is associated with the STAT3 activation and development of hepatocellular carcinoma. The inhibition of the Socs1 expression in HCC was associated with an increase in histone H3 lysine 9, H3 lysine 27, and H4 lysine 20 trimethylation at the Socs1 promoter, but not with DNA promoter methylation [18]. Moreover, sustained JNK1 activation is associated with histone H3 lysines 4 and 9 tri-methylation in human liver cancer [19]. Mixed-lineage leukemia (MLL), an epigenetic regulator, plays critical roles in cell fate, stem cell, and cell cycle decisions. MLL is suggested to interact with ETS2 and mediate the HGF-MET signaling, and may play roles in HCC metastasis [20]. Taken together, histone methylation is involved in HCC initiation, progression, metastasis and prognosis, is there possibility that histone methylation-related genes play roles in sorafenib resistance in HCC cells? It is of great interest to address this question.

In this work, an RNAi screening with shRNA library targeting HMT and HDMT genes was subjected to HepG2 cells. The determinants of sorafenib sensitivity were identified and the underlying mechanisms were investigated by immunoblotting.

Materials and methods

Cell culture

HepG2 (American Type Culture Collection, Rockville, Md.) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 IU/ml) and Streptomycin (100 μg/ml) (Life Technologies) in a humidified atmosphere containing 5% CO_2 at 37°C. Cells in the exponential growth phase were used for all the experiments.

shRNA construction and lentivirus infection

88 genes involving HMTs and HDMTs were subjected to shRNA primer design and eight distinct shRNA fragments for each gene were constructed into lentivirus vector (Invitrogen, BLOCK-iT™ Lentiviral RNAi Expression System, K4944-00), the eight shRNA plasmids for each gene were separated into two groups (A-D and E-H), four plasmids in each group were mixed with equal amount and the mixtures were applied to shRNA lentivirus package, the obtained lentivirus were titered in HEK293T cells according to the manufacturer's protocol. The obtained two shRNA pools for each gene were used for RNAi screening. HepG2 cells were infected with shRNA lentivirus at MOI of 20 in the presence of polybrene (8 μg/ml).

RNAi screening

HepG2 cells (700 cells/well) were plated each well in 96-well plate. 24 h later, cells were treated with 7 different doses of sorafenib (0.100, 0.317, 1.00, 3.17, 10.0, 31.7 and 100 μmol/L) or left untreated. 120 h later, the cell viability was detected by MTS assay. The survival curve of HepG2 cells was plotted by nonlinear regression with the aid of GraphPad Prism 5.0 software.

HepG2 cells (700 cells/well) were seeded in 100 μl of culture medium containing serum per

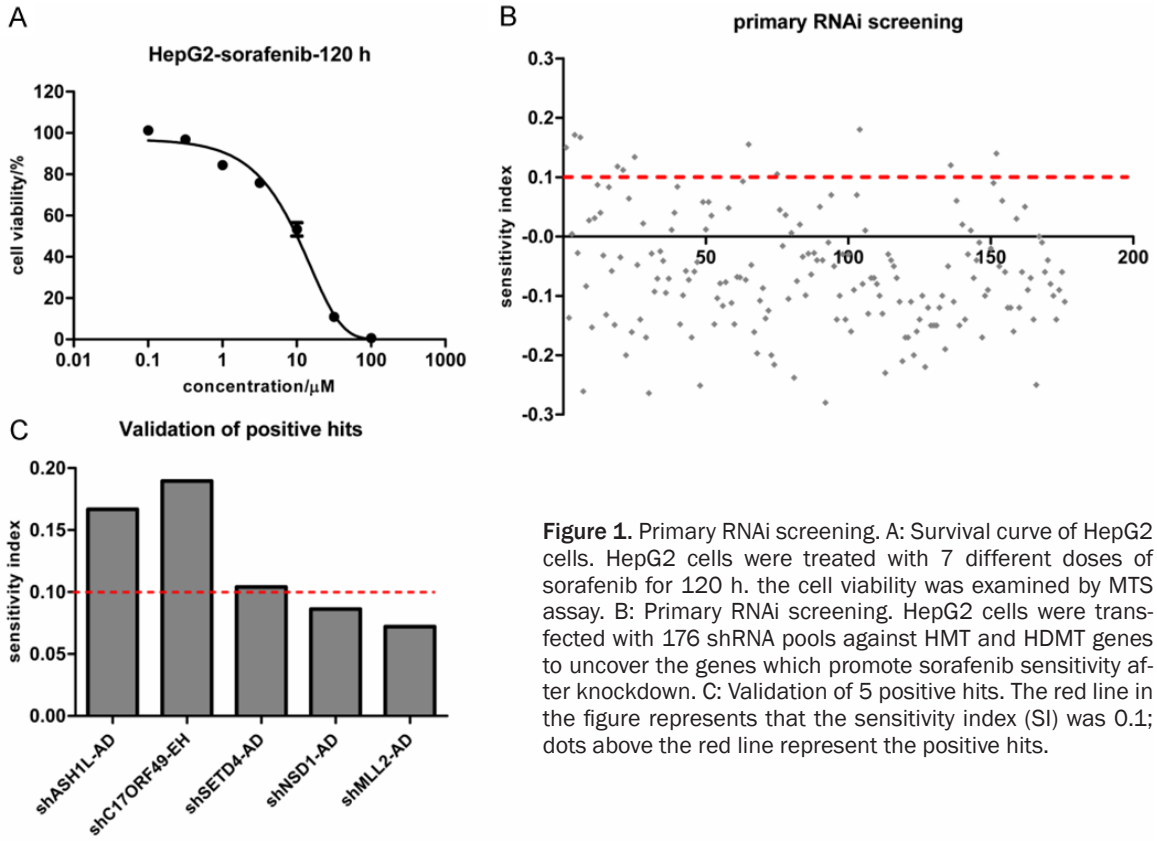

Figure 1. Primary RNAi screening. A: Survival curve of HepG2 cells. HepG2 cells were treated with 7 different doses of sorafenib for 120 h. the cell viability was examined by MTS assay. B: Primary RNAi screening. HepG2 cells were transfected with 176 shRNA pools against HMT and HDMT genes to uncover the genes which promote sorafenib sensitivity after knockdown. C: Validation of 5 positive hits. The red line in the figure represents that the sensitivity index (SI) was 0.1; dots above the red line represent the positive hits.

Table 1. 11 positive hits in the primary RNAi screening

Gene	SI	*P* value	Gene	SI	*P* value
ASH1L	0.1501	0.0013	MLL5	0.1119	0.0017
ASH2L	0.1710	0.0246	NSD1	0.1341	0.0069
C17ORF49	0.1668	0.0006	PRDM7	0.1396	0.0010
JMJD5	0.1834	0.0133	SETD4	0.1547	0.0093
KDM5C	0.1162	0.0005	SMYD2	0.1054	0.0012
MLL2	0.1184	0.0031			

well in a 96-well plate. 24 h later, the cells were treated with control shRNA (shCtrl) or shRNA library for HMTs and HDMTs. 72 h later, the cells were treated with 5 μmol/L Sorafenib or left untreated for 120 h. Then 20 μl of MTS (CellTiter 96 AQueous One Solution Reagent; Promega) was added to each well for 2 h at 37°C. After incubation, the absorbance was read at a wavelength of 490 nm according to the manufacturer's protocol. Every treatment was triplicate in the same experiment.

Four distinct shRNA species targeting each positive pool were used to revalidate hits from the primary screening. A significance threshold of $p<0.05$ (Student's t test) was used for each individual shRNA. Validation of RNAi gene silencing was measured by quantitative PCR as described below.

Data analysis: derivation of drug sensitivity

The derivation of sensitivity index was as described before [21]. In order to establish the influence of shRNA-induced knockdown of gene expression on sorafenib sensitivity, the individual effects of sorafenib and shRNAs on viability were taken into account. The viability effect of shRNA without sorafenib compared to shCtrl was designated Rc/Cc. The effect of the sorafenib on viability of shCtrl-transfected cells was designated Cd/Cc. This enabled a calculation of the Expected combined effect of shRNA and sorafenib on cell viability: Rc/Cc*Cd/Cc. The Observed combined effects of sorafenib and shRNA on cell viability compared to untreated shCtrl-transfected cells was designated Rd/Cc. Therefore, an index of antagonism or sensitivity (SI) for each shRNA was calculated by subtracting the Observed combined effect of sorafenib and shRNA (Rd/Cc) from the Expected total viability effect: SI = (Rc/Cc*Cd/Cc) - (Rd/Cc). In order for

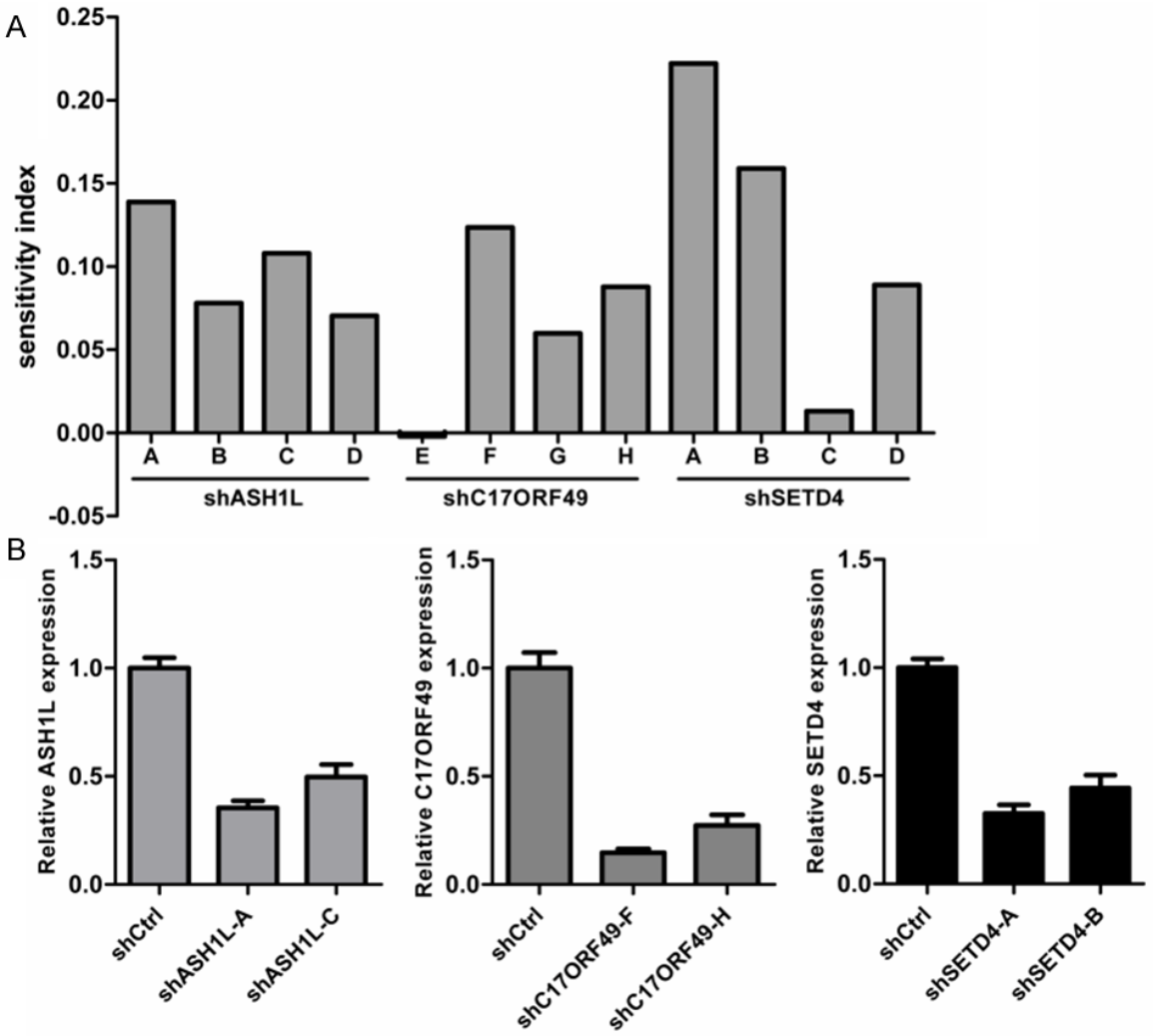

Figure 2. ASH1L, C17ORF49 and SETD4 silencing specifically promotes sorafenib sensitivity. A: Four shRNA fragments composed of the positive pools were revalidated for their sorafenib-sensitizing effects on HepG2 cells. There were at least two shRNA fragments for each gene that markedly promote HepG2 cell sensitivity to sorafenib following treatment. B: Efficacy of gene silencing was examined by qPCR.

the SI to usefully predict antagonism or sensitivity, an additional criteria that Rd/Cd<0.95 (for sensitizing shRNAs) was employed for hit selection. Those shRNAs with SI>0.10 were designated positive hits.

Quantitative PCR

HepG2 cells were infected with lentiviral shRNA under condition above mentioned. After 24 h, the culture medium was refreshed. RNA was extracted 72 h later and cDNA was synthesized using PrimeScript RT reagent kit with gDNA Eraser (Takara, RR074A) for RT-PCR with oligo-dT. Real-time qPCR was performed on CFX-96 (Bio-lab), with endogenous control Actb. Gene expression was calculated relative to expression of Actb endogenous control and adjusted relative to expression in shCtrl-infected cells. The primers for qPCR validation were as follows: Actb: forward (F): 5'-GCATCCCCCAAAGTTCACAA-3', reverse (R): 5'-GGACTTCCTGTAACAACGCATCT-3'; ASH1L: F: 5'-TGCAACGCCATCTACTCTTCT-3', R: 5'-AGCTGTGCCAACTTTTCTGTT-3'; C17ORF49: F: 5'-GAAACAGAAGGCTGATGTGACACT-3', R: 5'-CCTTCAATATCCACCACGTCACT-3'; SETD4: F: 5'-TCACGTTGGAGAAAGCATGAAG-3', R: 5'-TCCAGGAACAGCCGTTGATT-3'.

Protein isolation and western blotting

Cell pellets were resuspended in 1×SDS loading buffer (1 mmol·L^{-1} Na_3VO_4, 10 mmol·L^{-1} NaF, 1 mmol·L^{-1} PMSF) containing protease inhibi-

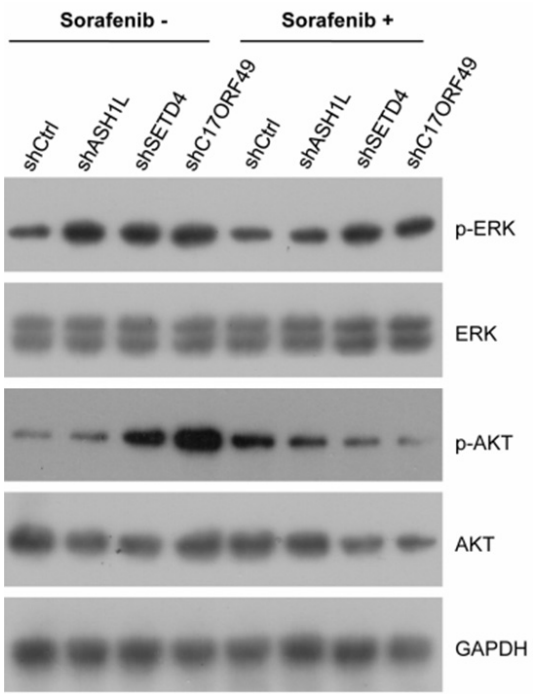

Figure 3. Western blotting assay in HepG2 cells. ASH1L, C17ORF49 and SETD4 were silencing in HepG2 cells and treated with 5 μmol/L sorafenib or left untreated. 72 h later, cell lysates were subjected to SDS-PAGE and immunoblotted to 0.40 μm of PVDF membrane. And then, GAPDH, ERK, p-ERK, AKT and p-AKT were examined by antibodies.

tors. Lysates (20 μg each lane) were applied to SDS-PAGE. Immunoblotting of Abs specific for GAPDH (Abmart, 080922), AKT (Santa Cruz, sc-8312), p-AKT (Santa Cruz, SC-7985-R), ERK (Abclonal, A0228) and p-ERK (Cell signaling, #9106S, pT202/204) were detected using HRP-conjugated anti-mouse (Promega) or anti-rabbit (Promega) and visualized by chemiluminescence detection system (Millipore, WBKLS0500).

Results

HMTs and HDMTs are involved in resistance to sorafenib

To access the roles of HMT and HDMT genes on sorafenib sensitivity in HCC cells, an RNAi screening was designed to be performed in HepG2 cells. As the first step of screening, the concentration of sorafenib used to treat cells was determined by MTS assay (**Figure 1A**). Since we are looking for genes that enhance the sensitivity to sorafenib following knockdown by shRNA, the cytotoxic effect of sorafenib on HepG2 cells should not be too acute to observe the synergetic effect of RNAi and sorafenib. So, the concentration of sorafenib was selected to be 5 μmol/L, the cell viability was inhibited by 20% or so at this concentration.

HepG2 cells were transfected with 176 shRNA pools (totally 88 genes, two shRNA pools for each gene) targeting HMT and HDMT genes. Cells were transfected, followed 72 h later with sorafenib treatment for a further 120 h, before cell viability was measured using MTS assay. To identify proteins whose knockdown promoted a drug-sensitizing phenotype, we analyzed data obtained after sorafenib treatment, utilizing the sensitivity index (SI) equation. **Figure 1B** shows all shRNAs that alter sorafenib action in HepG2 cells. 11 positive hits (SI>0.10, $p<0.05$) identified in primary screening were list in **Table 1**. And then, 5 positive pools (shASH1L-AD, C17ORF49-EH, shSETD4-AD, shNSD1-AD, shMLL2-AD) were validated with the same protocol and 3 out of these 5 pools were identified in this validation (**Figure 1C**).

ASH1L, C17ORF49 and SETD4 specifically promotes sorafenib sensitivity following knockdown

To validate the specificity of the effects observed and rule out the possibility of off-target effects in RNAi screening, the most potent growth affecting hits were re-assayed using each of the four different shRNA species that comprise the SMART pools. For ASH1L, C17ORF49 and SETD4, there are at least two shRNA fragments that markedly promote HepG2 cell sensitivity to sorafenib following RNAi (**Figure 2A**), indicating that sorafenib-sensitizing phenotype by gene silencing is unlikely to be caused by off-targets effect. Moreover, the gene silencing efficacy of the two shRNA fragments caused the most outstanding sorafenib-sensitizing phenotype for these three genes were examined by quantitative PCR (**Figure 2B**). The results showed that the two shRNAs targeting the 3 genes that caused the most significant sorafenib-sensitizing effects were also shown to cause the most significant gene silencing. Taken together, ASH1L, C17ORF49 and SETD4 silencing specifically promotes sorafenib sensitivity.

Western blotting showed that SETD4 and C17ORF49 silencing and sorafenib treatment synergistically inactivate AKT signaling

To uncover the underlying mechanisms by which silencing of ASH1L, SETD4 and C17ORF49 promotes sorafenib sensitivity, immunoblotting assay were performed for ERK and AKT signaling (**Figure 3**), well-known signaling essential for cancer cells proliferation and survival. The results showed that: 1) In HepG2 cells treated with control shRNA (shCtrl), sorafenib treatment made that ERK signaling was not altered significantly whereas AKT signaling was obviously upregulated; 2) In the absence of sorafenib treatment, ERK and AKT signaling were all upregulated following ASH1L, C17ORF49 and SETD4 knockdown; 3) Although ERK signaling was modestly downregulated after gene silencing and sorafenib treatment compared to that following gene silencing alone, there was no significantly downregulation in ERK signaling, compared to negative control treated with shCtrl and sorafenib. While the AKT signaling was obviously synergistically inactivated by sorafenib treatment and gene silencing, especially SETD4 and C17ORF49 silencing.

Discussion

Sorafenib is the first drug currently approved to treat advanced hepatocellular carcinoma (HCC). However, very low response rate and acquired drug resistance makes rare patients benefit from sorafenib therapy, therefore it is urgent to find biomarkers for sorafenib sensitivity. Histone modifications, including histone methylation, have been demonstrated to influence the initiation and progression of HCC. It is of great interest to elicit the possibility whether histone methylation plays a role in regulation of sorafenib sensitivity.

In present work, a high throughput RNAi screening with 176 shRNA pools against 88 HMT and HDMT genes was applied to HepG2 cells. Silencing of 3 genes (ASH1L, C17ORF49 and SETD4) was validated to specifically promote HepG2 cells sensitivity to sorafenib.

ASH1L is a trithorax group histone methyltransferase that is involved in gene activation or suppression. ASH1L catalyzes the dimethylation and trimethylation of Lys36 of histone H3 (H3K36) and hence activates the transcription of downstream molecules [22]. Xia et al reported that Ash1l-mediated H3K4 methylation at the Tnfaip3 promoter suppresses interleukin-6 production and inflammatory autoimmune diseases by inducing the ubiquitin-editing enzyme A20 [23]. ASH1L along with other epigenetic regulators has been found to mutate or amplify in lung cancer cell lines [24]. Furthermore, ASH1L stimulates migration of lung cancer cells through Cdk5/p35 pathway [25]. C17ORF49 is a component of chromatin complexes such as the MLL1/MLL and NURF complexes, its function remains largely unknown. SETD4 is found to be expressed in oocytes [26] and early embryos [27], however, little is known about its function.

Three underlying mechanisms have been found to support sorafenib therapy [28]. First, sorafenib blocks HCC cell proliferation by inhibiting BRaf and Raf1/c-Raf serine/threonine kinase phosphorylation in the mitogen-activated protein kinase pathway. Second, sorafenib induces apoptosis by reducing eIF4E phosphorylation and downregulating Mcl-1 levels in tumor cells. Third, sorafenib prevents tumor-associated angiogenesis by inactivating vascular endothelial growth factor receptors (VEGFR-2 and -3) and the platelet-derived growth factor receptor-b. In addition, expression of enhancer of zeste homolog 2 (EZH2), a mammalian histone methyltransferase that contributes to the epigenetic silencing of target genes that regulate cancer cell growth and survival, has been associated with sorafenib resistance of HCC cells [29]. ShRNA-mediated EZH2 knockdown or EZH2 inhibition with 3-deazaneplanocin A treatment promoted sorafenib-induced hepatoma cell growth arrest and apoptosis. In our work, silencing of 3 HMT genes (ASH1L, C17ORF49 and SETD4) synergizes with sorafenib to induce HepG2 cells death. The mechanisms were investigated by immunoblotting assay.

Western blotting results showed that those 3 HMT genes knockdown alone or sorafenib treatment alone both induce AKT/ERK activation. However, combination treatment with sorafenib and silencing of C17ORF49 or SETD4 downregulated AKT phosphorylation and hence induced HCC cells death. The underlying mechanisms warrant further investigation.

Collectively, our work may provide potential biomarkers for sorafenib sensitivity and therapeutic combination for sorafenib treatment in HCC patients.

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Disclosure of conflict of interest

None.

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