miR-33a expression sensitizes Lgr5⁺ HCC-CSCs to doxorubicin via ABCA1

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Cancer stem cells (CSCs) are responsible for the unrestrained cell growth and chemo-resistance of malignant tumors. Reports about miR-33a in different type of cancer are limited, and it remains elusive whether there is a link between miR-33a and chemo-resistance of CSCs. Here we report that Lgr5⁺ hepatocellular carcinoma (HCC) cells from primary tissues and cell lines behave similarly to CSCs and are chemo-resistant to doxorubicin. Significantly, reduced miR-33a expression is associated with the chemo-resistance of Lgr5⁺ HCC-CSCs, accompanied by an overexpression of ABCA1 which is identified as target of miR-33a by mainly using miRNA luciferase assay and western-blotting. We demonstrate that down-regulation of miR-33a expression directly contributes to chemo-resistance of Lgr5⁺ HCC-CSCs, and restoring miR-33a expression sensitizes them to doxorubicin via apoptosis by mainly using TUNEL assay, soft agar colony formation assay and xenograft assay. Additionally, reduced miR-33a expression in HCC tissues is associated with chemo-response and poor patient survival, which suggests the therapeutic potential of miR-33a. In conclusion, our work indicates that ectopic miR-33a expression sensitizes Lgr5⁺ HCC-CSCs to doxorubicin via direct targeting ABCA1, which sheds new light on understanding the mechanism of chemo-resistance in HCC-CSCs and contributes to development of potential therapeutics against HCC.

Key words: miR-33a, ABCA1, Lgr5, cancer stem cells, chemo-resistance hepatocellular carcinoma

HCC, which is one of the most intractable cancers worldwide, has persistently increasing rates of both incidence and mortality [1]. Doxorubicin, an anthracycline-based agent, is one of the most widely used anti-HCC drug systematically or locally [2]; especially it is the first-line chemotherapy agent for transarterial chemoembolization (TACE) [3]. However, during long-term doxorubicin chemotherapy, HCC cells may eventually develop acquired chemo-resistance, thereby leading to recurrence and a poor prognosis. There is increasing evidence proving that resistance to HCC therapy is, at least in part, caused by inherent chemo-resistance of a subpopulation of cancer cells [4, 5]. This subpopulation of cancer cells, which is also labeled as CSCs, shares many properties with stem cells [5, 6]. CSCs are responsible for tumor relapse after therapy, are highly tumorigenic, chemotherapy resistant, and able to divide to orchestrate tumor development and progression [7, 8]. Thus, refined investigation on the key molecular mechanisms of chemo-resistance in CSCs will provide novel targets for advancing anticancer therapy.

miRNAs are an abundant class of small non-coding RNAs that inhibit translation or induce mRNA degradation in general by binding to the 3'-UTR of target mRNAs. miRNAs have been identified as tumor promoters or suppressors, regulating the progression of cancers and the state of CSCs [9, 10]. miR-33a is encoded within the intron 16 of SREBP-a key transcriptional modulator of cellular cholesterol homeostasis and is highly conserved in mammals [11]. miR-33a was reported decreased in osteosarcoma cells [12], breast cancer cells [13] and non-small cell lung cancer [14]. However, reports about the expression and biological function of miR-33a in CSCs are rare. Additionally, to date, there are no reports bridging the link between miR-33a and chemo-resistance of HCC-CSCs.

On the other hand, some CSC biomarkers (e.g., CD133, K7, EpCAM) have been identified in HCC, but it is still unclear which biomarker truly represents HCC-CSCs [15, 16]. To date, studies about Lgr5 as the marker for sorting HCC-CSCs are few, especially.

In this study, we aim to evaluate the biological function and the underlying mechanisms of miR-33a in chemo-resistance of doxorubicin in HCC-CSCs. Here, we report that Lgr5⁺ HCC cells from primary HCC tissues and cell lines behave similarly to CSCs and are chemo-resistant to doxorubicin.

Significantly, reduced miR-33a expression is associated with the chemo-resistance of Lgr5^{+} HCC-CSCs, accompanied by an overexpression of ABCA1 which is identified as target of miR-33a. We demonstrate that down-regulation of miR-33a expression directly contributes to chemo-resistance of Lgr5^{+} HCC-CSCs, and restoring miR-33a expression sensitizes them to doxorubicin via apoptosis. Additionally, reduced miR-33a expression in HCC tissues is associated with chemo-response and poor patient survival, suggesting therapeutic potential of miR-33a. In conclusion, our work indicates that ectopic miR-33a expression sensitizes Lgr5^{+} HCC-CSCs to doxorubicin via direct targeting ABCA1.

Patients and methods

Animal and cell culture. Female athymic BALB/c nu/nu mice, 3-4 weeks old, obtained from HFK Bioscience (China), were maintained at the Animal Core Facility at Henan Provincial People's Hospital, under specific pathogen-free (SPF) condition. All studies on mice were conducted in accordance with the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' and were approved by the ethical committee of Henan Provincial People's Hospital. The human HCC cell line PCL8024 and Huh7 were purchased from ATCC (Manassas, USA) and were properly kept in Molecular Medicine Center of Henan Provincial People's Hospital. All the cell lines were authenticated by STR-PCR.

Patients and tumor specimens. Ten patients with primary HCC, who consecutively underwent chemotherapy at Department of Infectious Disease, Henan Provincial People's Hospital, were enrolled into this study from January 2009 to June 2015. Informed consent for the additional core-needle biopsy and experimental use of tumor samples was obtained from all patients, following a protocol approved by the Ethics Committee of Henan Provincial People's Hospital. The tumor response was evaluated by the Response Evaluation Criteria in Solid Tumors (RECIST), which was defined as the following: complete response (CR; disappearance of the disease), partialresponse (PR; reduction of >30%), or progressive disease (PD; enlargement>20%).

Flow cytometry assay. Flow cytometry assay was done on single-cell suspensions obtained by enzymatic digestion of spheres which were derived from primary HCC samples and HCC cell line (PCL8024 and Huh7), using an Epics Altra flow cytometer (Beckman Coulter, USA). The antibodies used for flow cytometry assay were anti-Lrg5-FITC conjugated (Abcam, USA).

Sphere formation and propagation. Single-cell suspensions were obtained from primary HCC samples and HCC cell line (PCL8024 and Huh7). Solid tissues were finely minced by razorblade, washed in DMEM/F12 (Gibico Invitrogen, USA), and then incubated with Accumax 1X (Innovative Cell Technologies, USA) for 30 min at 37 °C. Single-cell suspension was obtained by filtering digested tissue. Single-cells were plated at a density of 10^4 cells in serum-free medium DMEM/F12

(Gibico Invitrogen, USA), supplemented with commercial hormone mix B27 (Gibico Invitrogen, USA), EGF (10 ng/mL; PeproTech, USA), bFGF (10ng/mL; PeproTech, USA), and heparin (2µg/mL; Sigma Aldrich, USA). The medium of all the spheres was replaced with fresh growth factors twice a week until cells started to grow forming floating aggregates. Cultures were expanded by enzymatic digestion of spheres with Accumax 1X (Innovative Cell Technologies, USA), followed by re-plating of both single cells in complete fresh medium. All cells were cultured at 37 °C in a 5% CO_2 humidified incubator.

MTT assay. Lrg5^{+} cells, Lrg5 [-] cells, and differentiated adherent progeny of Lrg5^{+}cells were seeded in 96-well plates at 2000 cells/well, respectively. Cells were then treated with increasing concentrations of doxorubicin from 0.5 to 5 µg/mL for 24 hours. The MTT assay (Sigma Aldrich, USA) was used to determine relative cell growth every 24 h for cell growth curves. 20µl of 5mg/ml MTT was added to the media for 4h incubation at 37°C. Following removal of the culture medium, the remaining crystals were dissolved in 150µl DMSO (Sigma Aldrich, USA). The curve of growth was drawn with the absorbance (A) measured spectrophotometrically in a microplate reader (Bio-Rad, USA) at a wavelength of 490 nm.

Quantitative real-time RT-PCR analysis (qPCR). Total RNA was extracted using TRIzol (Invitrogen, USA) and treated with RNase-free DNase (Qiagen, USA). Mature miRNA expression analysis was conducted using a Taq-Man MicroRNA Assays (Applied Biosystems, USA). qPCR was performed using a SYBR Green Reagents (Bio-Rad, USA) on the iQ5 Real-Time PCR Detection System (Bio-Rad, USA), with human U6 as an endogenous control. Primers for mature miR-33a miRNA and U6 snRNA were obtained from Invitrogen. The primer of miR-33a, 5'-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATT-GCACTGGATAGGACTGCAAT-3'; the primer of U6, 5'-CGCTTCACGAGCACATATACTAAAAT-3'.

miRNA luciferase assay. A pMIR-REPORTTM luciferase reporter vector with a miR-33a target sequence cloned into its 3'-UTR (Invitrogen, USA) was used. Luciferase activity was assayed using a luciferase assay kit (Promega, USA).

Lentiviral transduction. Cells dissociated were spin-transduced with 1 ml of NC-si-RNA (Gene Copoeia, USA), *ABCA1*-si-RNA lentiviral knockdowns (Gene Copoeia, USA) packbag or pcDNA3.1-miR-33a (Gene Copoeia, USA) packbag. For both systems, cells were transduced with lentiviral media at a multiplicity of transduction of 35, in the presence of 8 µg/ml polybrene (Sigma-Aldrich, USA) overnight in a 37°C incubator. Stable clones transduced with pcDNA3.1-miR-33a and pcDNA3.1-NC were selected by G418 (800 µg/ml). Stable clones transduced with si-*ABCA1* or si-NC were selected by puromycin (2 µg/ml).

Western blot. Protein extracts were resolved through 8% to 12% SDS-PAGE; transferred to nitrocellulose membranes; and probed with mouse monoclonal antibody against ABCA1 (Santa, USA) or β-actin (Proteintech, USA); probed with per-

oxidase-conjugated secondary antibody (Proteintech, USA). The membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody. Protein expression was detected and quantified using the ODYSSEY Infrared Imaging System (Li-COR Biosciences, USA).

Xenograft assay. Female athymic BALB/c nu/nu mice at 3-4 weeks of age were injected subcutaneously into the right flank with 1×10^5 of Lgr5$^+$HCC-CSCs, and 1×10^5 of Lgr5$^+$HCC-CSCs transduced with pcDNA3.1-miR-33a and pcDNA3.1-NC. The xenografts were monitored until the tumor volumes reached ~100 mm³, each group was randomised into two subgroups that were either left untreated or received intraperitoneal injections of doxorubicin (4 mg/ kg) every 5 days (three cycles). The tumour volume was measured with a calliper using the following formula V(mm³) = 0.52 × length (mm) × width² (mm²).

TUNEL assay. TUNEL staining was performed by the DeadEnd TM Fluorometric TUNEL system according to the manufacturer's instructions (Promega, USA) in xenograph tumor tissues derived from HCC-CSCs, pcDNA3.1-miR-33a-HCC-CSCs and pcDNA3.1-NC-HCC-CSCs following treatment with or without three cycles of doxorubicin. Cells were then observed under a fluorescence microscope (Olympus Optical Co., Germany), and a nucleus with bright green fluorescence staining was recorded as a TUNEL-positive event.

Statistical analysis. SPSS13.0 software was used. Each experiment was performed at least three times. The data were expressed as mean ± SD and one-way ANOVA. An unpaired Student's t-test were used to determine the significant differences of all the results. Significances are ,***, p < 0.001; **,p < 0.01; *, p<0.05.

Results

Lgr5$^+$ HCC-CSCs in human HCC clinical specimens and HCC cell lines exhibit CSC properties and are chemoresistant. We examined the expression of Lgr5 in fresh HCC tissue samples and HCC cell lines (PLC8024 and Huh7) using

flow cytometry. In total, 10 individual patients were analyzed (Table 1). Flow cytometry demonstrated the presence of a rare Lgr5 population ranging from 0.2% to 10.7% in HCC specimens, 7.6% in HCC cell line PLC9024 and 9.8% in HCC cell line Huh7 (Figure 1A, Table 1 and Figure S1). These data identified an expanded pool of Lgr5$^+$ tumor cells in HCC. CSCs are believed to be able to form spheres in serum-free cultivation [17, 18]. Therefore, we cultured 10 cases of primary HCC samples and cell lines (PLC9024 and Huh7) to induce sphere formation (data were not shown). After culturing in the serum-free medium for 2 – 3 weeks, the majority of tumor cells died, but a few tumor cells grew to form spheres. Following the collection of HCC spheres, Lrg5$^+$ cells and Lrg5$^-$ cells were isolated by fluorescence activated cell sorting (FACS). Lrg5$^+$ cells were able to form compact self-renewing spheres, while Lrg5$^-$ cells could not (Figure 1B). Moreover, with increasing concentrations of doxorubicin (DOX) from 0.5 to 5 μg/ml, cell viability in both Lrg5$^-$ cells and differentiated adherent progeny of Lrg5$^+$cells decreased at each concentration compared with that in Lrg5$^+$ cells (Figure 1C). Collectively, these data clearly indicated that Lgr5$^+$ HCC cells were likely to be HCC-CSCs with potent drug resistance to doxorubicin.

miR-33a is reduced in chemo-resistant Lgr5$^+$ HCC-CSCs. Here, we verified that miR-33a expression was significantly reduced in Lgr5$^+$ HCC-CSCs compared with that in Lrg5$^-$ cells by using qRT-PCR (Figure 2A). After differentiation, miR-33a expression dramatically increased (Figure 2A). Transduction of Lgr5$^+$ HCC-CSCs (sphere) with a lentivirus vector carrying miR-33a expression cassette (miR) restored the miR-33a expression to a level comparable with adherent differentiated cells (adh) (Figure 2B). To further investigate the targeting function of miR-33a, we evaluated the percentage of luciferase suppression by transducing a luciferase reporter vector containing a miR-33a target sequence cloned into its 3'-UTR (luc-miR-33a) into Lgr5$^+$ HCC-CSCs and differentiated adherent progeny of Lrg5$^+$ HCC-CSCs. We found that luciferase activity in the differentiated adherent progeny (adh) of Lrg5$^+$ HCC-CSCs was suppressed, whereas Lgr5$^+$ HCC-CSCs transduced with empty vector (ev) and untransduced

Table 1. Expression of Lgr5 in HCC samples

Case	Age	Gender	TNM	Clinical stage	Differenetiation status	Lgr5$^+$ expression	Sphere formation
1	32	Male	T1N0M0	IA	Well	0.2%	Yes
2	55	Female	T2N0M0	IB	Moderately	2.1%	Yes
3	64	Female	T1N1M0	IIA	Moderately	3.2%	No
4	46	Male	T2N1M0	IIA	Poorly	10.7%	Yes
5	57	Female	T2N2M0	IIB	Moderately	5.1%	Yes
6	55	Male	T3N0M0	IIIA	Poorly	4.2%	Yes
7	67	Male	T2N2M0	IIIA	Poorly	0.3%	No
8	61	Male	T2N3M0	IIIB	Poorly	2.1%	Yes
9	38	Male	T3N3M1	IV	Poorly	2.6%	Yes
10	61	Female	T2N3M0	IIIB	Poorly	1.3%	Yes

(un) had almost no change in luciferase activity (PLC8024 left, Huh7 right) (Figure 2C). Transduction of Lrg5+ HCC-CSCs with lenti-miR-33a (miR) restored the suppression of luciferase activity (Figure 2C). Co-transducing the cells with both the reporter vector and the miR-33a ASO weakened the suppression of luciferase activity in the differentiated adherent progeny of Lrg5+ HCC-CSCs (adh) and Lgr5+ HCC-CSCs with ectopic miR-33a expression (miR) (Figure 2C). Thus, these findings indicated that the expression as well as the target function of miR-33a was reduced in Lrg5+ HCC-CSCs.

ABCA1 is a direct target gene of miR-33a. In an effort to determine the potential downstream mRNA targets regulated by miR-33a, we integrated mRNA expression profiling findings with in prediction algorithms of PicTar and TargetScan, we found that the seed sequence of miR-33a had a perfect match with the 3'-UTR of the ABCA1 mRNA. To examine the targeting function of miR-33a to the 3'-UTRs of ABCA1 mRNA, we used luciferase reporter constructs containing the miR-33a recognition site or a mutated sequence at the site from the 3'-UTR of ABCA1 mRNA. Transfection of 293T cells with a miR-33a
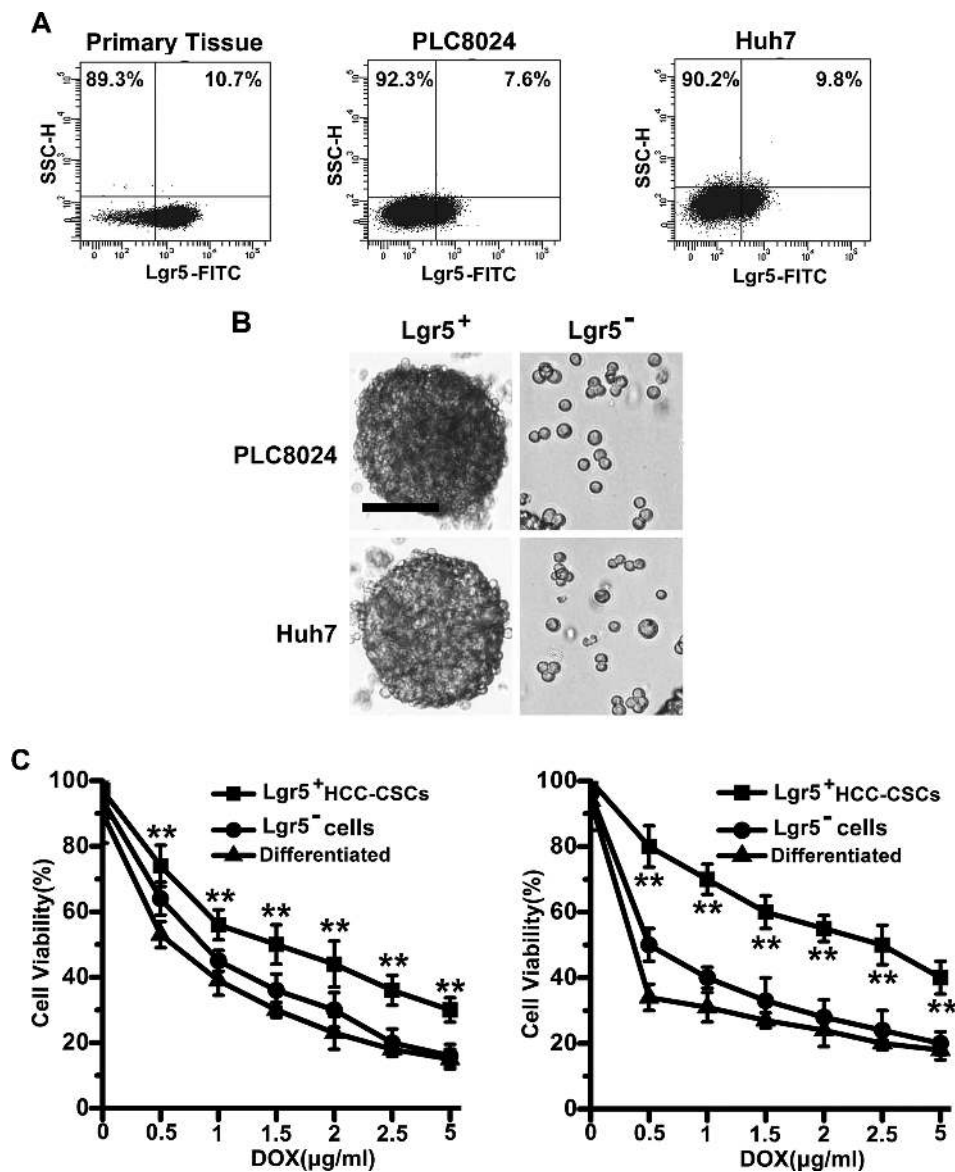

Figure 1. Lgr5+ HCC cells displayed stem cell-like features and were resistant to chemotherapy drug doxorubicin. (A) The expression of Lgr5 in fresh HCC tissue samples and HCC cell lines (PLC8024 and Huh7) was examined by using flow cytometry. **(B)** Phase-contrast images of the ability of tumor spheres formation by seeding with Lgr5+ cells and Lgr5 – cells in serum-free medium. Scale bar, 100 μm. **(C)** With the presence of doxorubicin (DOX), cell viability of Lgr5+ cells, Lgr5 – cells, and differentiated adherent progeny of Lgr5+ cells were determined by MTT. Note: Columns, mean of three individual experiments; SD,**, P<0.01.

mimic suppressed the luciferase activity of the reporter vectors containing the 3'-UTR of wild-type ABCA1 (Figure 3A). The statistical comparisons of luciferase activity were made among each group of wild-type ABCA13'-UTR, including untransduced group (un), transduced with empty vector (nc) and with

miR-33a inhibitor. We found that tansducing Lgr5+ HCC-CSCs (sphere) with miR-33a inhibitor resulted in increased luciferase activity of the reporter vector containing the 3'-UTR of wild-type ABCA1 in comparison with that in Lgr5+ HCC-CSCs transduced with empty vector (nc) and untransduced Lgr5+
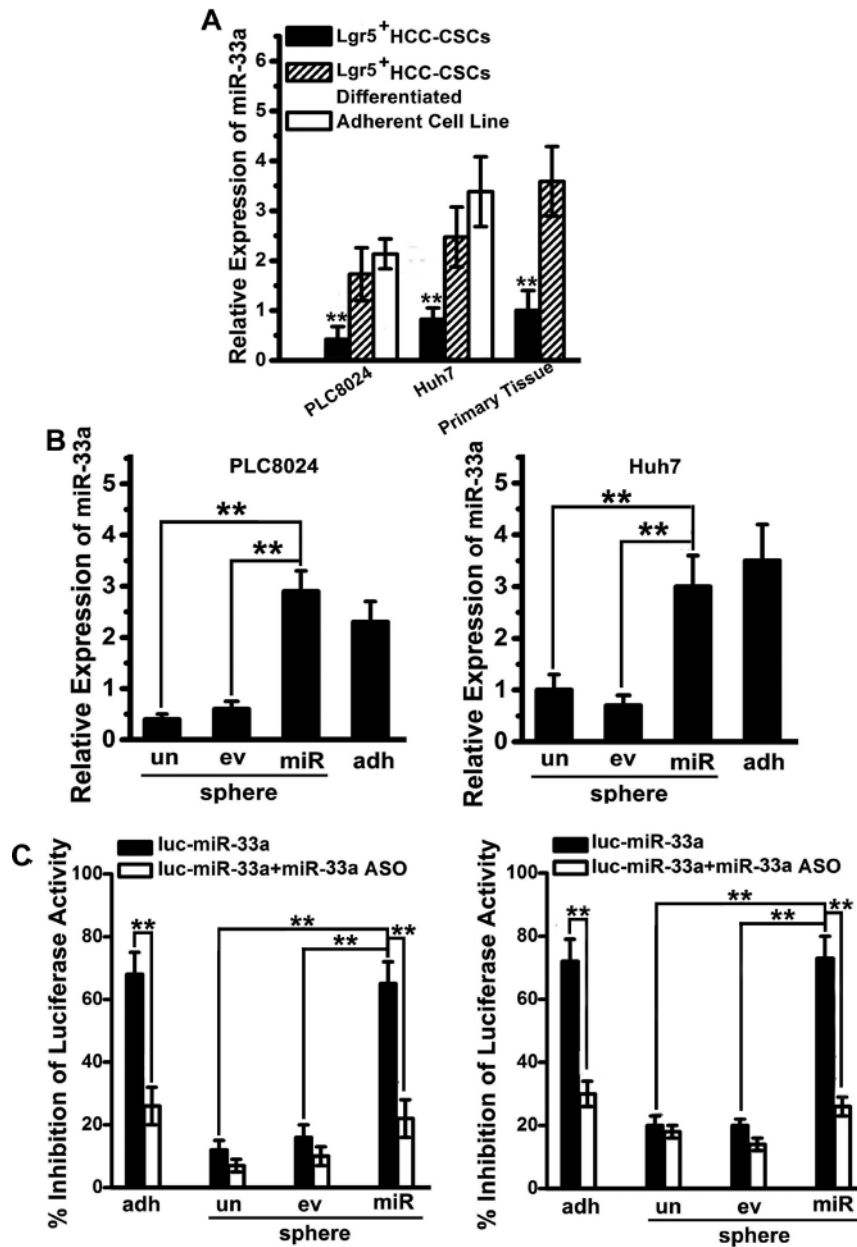
Figure 2. miR-33a was reduced in Lgr5+ HCC-CSCs. (A) Relative expression of miR-33a in Lgr5+ HCC-CSCs, HCC adherent cell line (PLC8024 and Huh7) cells, and differentiated adherent progeny of Lgr5+ HCC-CSCs were examined by qPCR. Note: Columns, mean of three individual experiments; SD,**, P < 0.01 vs. untransduced cells. **(B)** Lgr5+ HCC-CSCs transduced with a lenti-miR-33a (miR), showed increased miR-33a expression compared with that in untransduced group (un), Lgr5+ HCC-CSCs upon transduction with lentivirus vector alone (ev) and adherent PLC8024/ Huh7 cells (adh). Note: Columns, mean of three individual experiments; SD,**, P < 0.01 vs. uninfected cells. **(C)** Luciferase activity was decreased following transduction of a reporter vector carrying a miR-33a targeting sequence at its 3'-UTR (PLC8024 left, Huh7 right). Note: Columns, mean of three individual experiments; SD,**, P < 0.01 vs. Lgr5+ HCC-CSCs. Transduction with a lentivirus vector carrying miR-33a antagomiR reduced endogenous or exogenous miR-33a activity. Note: Columns, mean of three individual experiments; SD,**, P < 0.01 vs. untransduced cells (un). 'ev' means transduction with lentivirus vector alone; 'adh' means adherent PLC8024/ Huh7 cells; 'miR' means Lgr5+ HCC-CSCs transduced with a lenti-miR-33a.

HCC-CSCs (un) (Figure 3B). Besides, we showed that ABCA1 protein was significantly elevated in Lgr5^{+} HCC-CSCs (sphere) compared with that in the differentiated adherent progeny of Lrg5^{+} HCC-CSCs (adh) (Figure 3C). To further examine whether miR-33a affects ABCA1 expression, we transduced Lgr5^{+} HCC-CSCs with lenti-miR-33a. We found that restoring the expression of miR-33a reduced ABCA1 expression in

Lgr5[+] HCC-CSCs (Figure 3D). Meanwhile, knock-down of ABCA1 with lenti-si-ABCA1 reduced ABCA1 expression in Lgr5^{+} HCC-CSCs (Figure 3D). Therefore, our data implied that miR-33a regulated ABCA1 expression by directly targeting their 3'-UTRs and the reduction of miR-33a might result in the increase of ABCA1 protein by relieving translational repression at the 3'-UTRs of ABCA1.
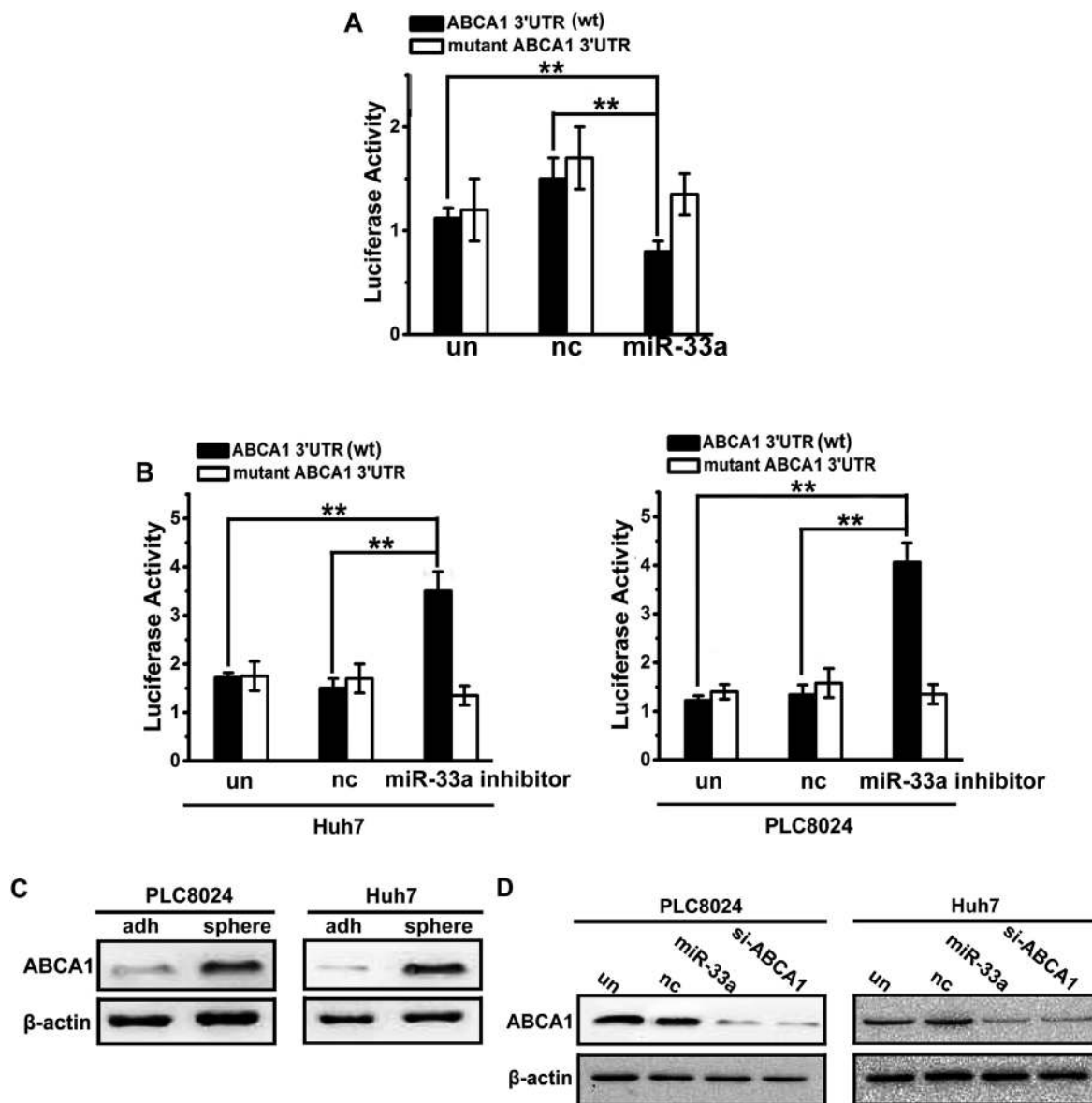

Figure 3. ABCA1 is the target of miR-33a. (A) 293T cells were transfected with 100 nmol/L miR-33a mimics or scrambled oligonucleotide as a negative control (nc). Expression of luciferase with the putative miR-33a target site in wild-type (wt) or mutated 3'-UTR from ABCA1 was measured in a luminometer. Note: Columns, mean of three individual experiments; SD,*, P < 0.05 vs. untransduced cells. (B) Adherent PLC8024 and Huh7 cells were transfected with 100 nmol/L miR-33a inhibitor or scrambled oligonucleotide as a negative control (nc). Expression of luciferase with the putative miR-33a target site in wild-type (wt) or mutated 3'UTR from ABCA1 was measured in a luminometer. Note: Columns, mean of three individual experiments; SD,**, P < 0.01 vs. untransfected cells (un). (C) Western-blotting with anti-ABCA1 antibodies showed that ABCA1 protein was elevated in Lgr5^{+} HCC-CSCs (sphere) compared with that in their differentiated cells (adh). (D) Western-blotting showed that ABCA1 protein decreased with the ectopic expression of miR-33a in Lgr5^{+} HCC-CSCs. Transduction of lenti-si-ABCA1 in Lgr5^{+} HCC-CSCs, compared with that in untransduced (un) or empty vector cells (nc), led to a reduction in the expression of ABCA1.

Ectopic miR-33a expression sensitizes Lgr5+ HCC-CSCs to doxorubicin. To investigate whether the down-regulation of miR-33a expression in Lgr5+ HCC-CSCs contributes to doxorubicin resistance, a lentiviral-based approach was used to restore miR-33a expression with lenti-miR-33a, or to knock down ABCA1 expression with lenti-si-ABCA1. Without doxorubicin, the cell viability of Lgr5+ HCC-CSCs (un) was not significantly changed upon transduction with lenti-miR-33a, lenti-si-ABCA1, or empty lentivirus (nc) (Figure 4A), suggesting that the dysregulation of miR-33a and ABCA1 were not required for Lgr5+ HCC-CSCs survival. With increasing concentration of doxorubicin, the percentage of viable cells in lenti-miR-33a-HCC-CSCs and lenti-si-ABCA1-HCC-CSCs decreased more rapidly than that in si-NC-HCC-CSCs (Figure 4A). There was no statistical difference between cell viability in si-NC-HCC-CSCs and untransduced group. At each concentration, doxorubicin induced the highest growth inhibition

in Lgr5+ HCC-CSCs with the transduction of lenti-si-ABCA1 compared with lenti-miR-33a-HCC-CSCs, si-NC-HCC-CSCs and untransduced group (Figure 4A). Next, we employed a soft agar colony formation assay to further evaluate the growth of lenti-miR-33a-HCC-CSCs with treatment of doxorubicin. With the presence of doxorubicin at 2μg/ml, colony formation in lenti-miR-33a-HCC-CSCs and lenti-si-ABCA1-HCC-CSCs reduced compared with that in si-NC-HCC-CSCs and untransduced group (Figure 4B).

Furthermore, we built xenograft tumor model using si-NC-HCC-CSCs, lenti-miR-33a-HCC-CSCs, or untransduced HCC-CSCs, respectively. We found that xenograft tumors in lenti-miR-33a-HCC-CSCs group were slightly smaller (Figure 5A), although no significant difference was observed. Upon intratumor injection with doxorubicin, the size of xenograft tumors in lenti-miR-33a-HCC-CSCs group was significantly reduced in comparison with that in si-NC-
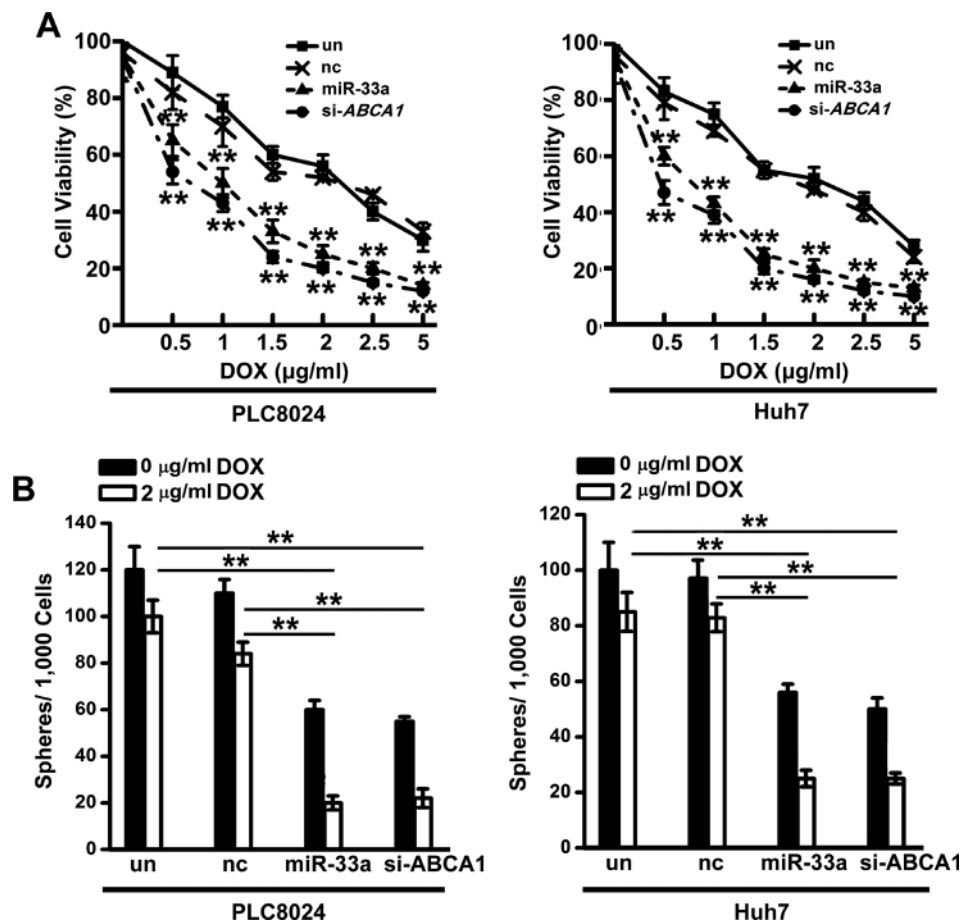

Figure 4. Cell viability of Lgr5+ HCC-CSCs is reduced upon transduction with lenti-miR-33a or lenti-si-ABCA1 under chemotherapy. (A) With increasing concentrations of doxorubicin from 0.5 to 5 μg/mL, the proportion of viable cells upon transduction with lenti-miR-33a or lenti-si-ABCA1, but not with lentivirus vector alone (nc), dropped more rapidly than in untransduced cells (un). Note: Columns, mean of three individual experiments; SD,**, P<0.01. **(B)** Soft agar colony formation assay showed that in the presence of adriamycin at 2μg/mL, colony formation in soft agar, counted 20 days after incubation, was reduced in Lgr5+ HCC-CSCs with lenti-miR-33a or lenti-si-ABCA1 transduction compared with the untransduced cells (un) or those transduced with an empty lentivirus vector (nc). Note: Columns, mean of three individual experiments; SD,**, P<0.01.

HCC-CSCs and untransduced HCC-CSCs group (Figure 5A). Besides, we found that the expression of miR-33a increased significantly in lenti-miR-33a-HCC-CSCs group compared with that in si-NC-HCC-CSCs, or untransduced HCC-CSCs (Figure S2, Supplementary Information). While, the expression of ABCA1 was reduced in lenti-miR-33a-HCC-CSCs group (Figure S2, Supplementary Information).

These data further implied that miR-33a expression contributing to chemo-resistance of Lgr5+ HCC-CSCs in vivo might be via ABCA1.

In addition, to detect whether doxorubicin-mediated cell death is generated by apoptosis, TUNEL apoptosis index was examined in every tumor group. Intratumor injection with doxorubicin in xenograft tumors derived from HCC-CSCs
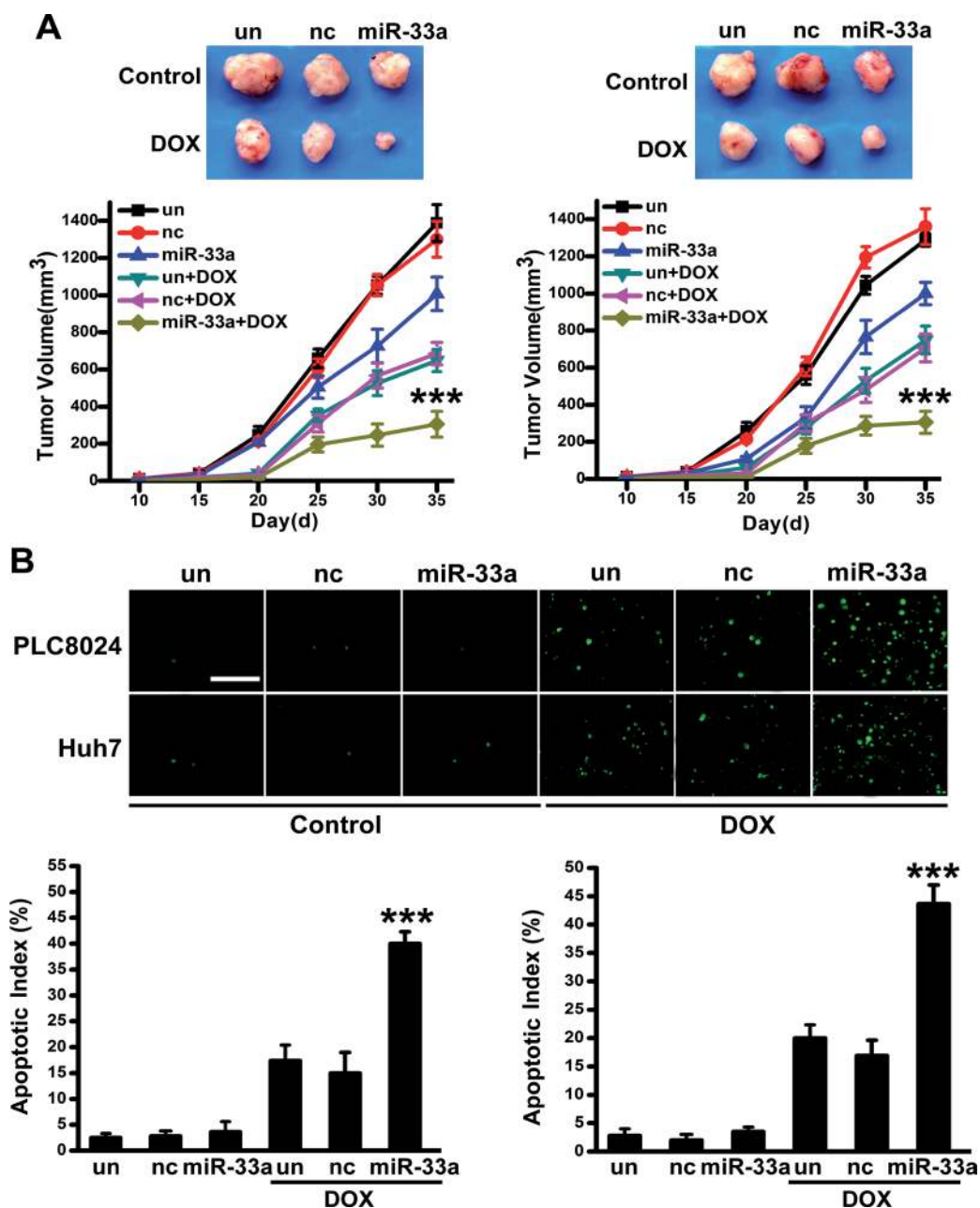

Figure 5. Ectopic expression of miR-33a sensitizes Lgr5+ HCC-CSCs to doxorubicin *in vitro* and *in vivo*. (A) The potential of tumor initiation of Lgr5+ HCC-CSCs (un), lenti-miR-33a, or lenti-NC-HCC-CSCs fractions by subcutaneous injection, and representative tumor volumes were measured following treatment with or without three cycles of doxorubicin. Note: Columns, mean of three individual experiments; SD,***, P<0.001. **(B)** Apoptosis in xenograft tumors derived from Lgr5+ HCC-CSCs (un), lenti-miR-33a, or lenti-NC-HCC-CSCs fractions following treatment with or without three cycles of doxorubicin were detected by TUNEL assay. Scale bar, 100 μm. Note: Columns, mean of three individual experiments; SD,***, P<0.001.

resulted in an increased TUNEL index compared with intra-tumor injection with glucose (control) in xenograft tumors derived from HCC-CSCs (Figure 5B). Significantly, with the treatment of doxorubicin, xenograft tumors in lenti-miR-33a-HCC-CSCs group showed an obviously higher TUNEL index than those in si-NC-HCC-CSCs group and untransduced HCC-CSCs group (Figure 5B). Taken together, our data demonstrated that down-regulation of miR-33a expression directly contributed to their chemo-resistance, and restoring miR-33a expression could sensitize HCC-CSCs to doxorubicin via apoptosis.

Down-regulation of miR-33a in HCC tissues correlates chemotherapy resistance and poor patient survival. To investigate whether miR-33a expression is related to chemo-resistance in HCC patient cohort, 10 HCC patients who exhibited CR (chemosensitive), PR (chemosensitive), or PD (chemoresistant) according to RECIST criteria were enrolled. We found that miR-33a expression in CR HCC tissues was the highest among the different chemo-responsive HCC tissues (Figure 6A). It implied a link between miR-33a expression and chemo-response in HCC patients. Additionally, CR HCC tissues showed the lowest expression of ABCA1, while PD HCC tissues showed the highest one (Figure S3, Supplementary Information). It suggested that ectopic miR-33a expression might be related to chemo-resistance in HCC patient cohort via targeting ABCA1. Moreover, survival curve showed that patients with high miR-33a expression (≥2.5 fold) survived obviously longer than patients with low miR-33a expression (<2.5 fold) (Figure 6B). These data suggested a potential pre-dicted value of miR-33a in chemo-resistance.

Discussion

HCC-CSCs are suggested responsible for the chemo-resist-ance of HCC, but the mechanisms underlying chemo-resistance of HCC to conventional therapies remain unclear [19, 20]. Stud-ies have suggested that eradication of HCC-CSCs is required to achieve a complete remission and to prevent disease recur-rence [18, 29]. Thus, investigation of major regulators and the underlying mechanisms which contribute to chemo-resistance of HCC-CSCs need to be focused on.

Lgr5[+] is a unique biomarker expressed in stem cells in epi-thelia of the intestine and colon [21, 22]. However, it remains unclear whether the Lgr5^{+} neoplastic counterparts contribute to the progression of HCC. Here, we found that 90% of HCC tissues were positive for anti-Lgr5 staining in HCC tissues. The ability of sphere formation and chemo-resistance to doxorubicin in Lgr5^{+} HCC cells was higher than that in both Lrg5^{−} cells and differentiated adherent progeny of Lrg5^{+}cells. We preliminary revealed that Lgr5^{+} HCC cells were a subset of previously unrecognized tumor cells that displayed the proper-ties of HCC-CSCs. Given that Lgr5^{+} tumor cells are crucial for the development and progression of colorectal cancer [23-25], we reasonably deduce that Lgr5^{+} HCC-CSCs may be important for the pathogenesis and drug resistance of HCC. However,
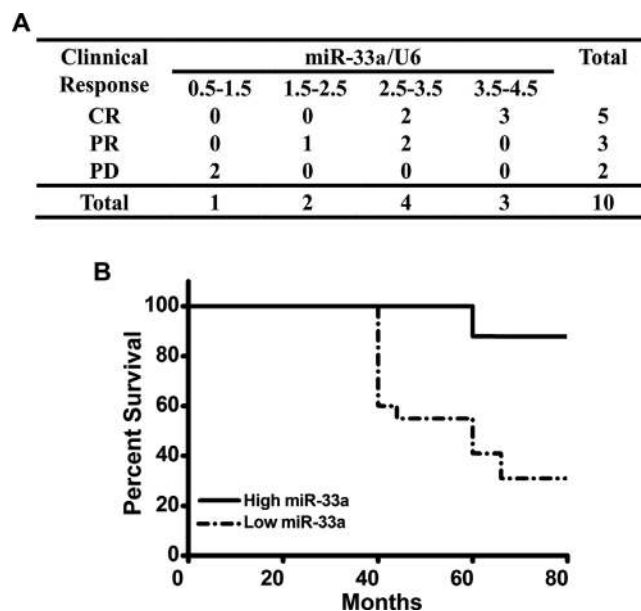

Figure 6. miR-33a reduction in HCC tissues correlates chemotherapy re-sistance and poor patient survival. (A) The correlation of clinical response and miR-33a expression in 10 HCC patients (CR: disappearance of the disease; PR: reduction of >30%; PD: enlargement>20%) were built. **(B)** Kaplan–Meier survival curves for HCC patients plotted against miR-33a expression showed a statistical difference in survival between patients with high – and low – miR-33a.

the exact function, mechanisms, and the prognosis value of Lgr5 in HCC need further study.

miR-33a was reported decreased in some types of cancer [12, 13]. However, reports about the expression and biological function of miR-33a in HCC-CSCs are rare. We report here, for the first time, that the reduction of miR-33a is an independ-ent and significant factor affecting the chemo-resistance of HCC-CSCs. We verified that miR-33a was reduced in Lgr5^{+} HCC-CSCs compared with that in Lrg5^{−} cells and differentiated adherent progeny of Lrg5^{+}cells. We, then, demonstrated that the expression as well as the target function of miR-33a was reduced in Lrg5^{+} HCC-CSCs by a series of luciferase assays and western-blotting. Asad Jan [26], et, al. found that ABCA1 is the target gene of miR-33 in mouse brain cells. ABCA1 expression in HCC-CSCs is associated with chemo-resistance and reduced survival times of patients with HCC [27]. By using luciferase reporter constructs, our data implied that miR-33a regu-lated ABCA1 expression by directly targeting their 3'-UTRs. Western-blotting with anti–ABCA1 antibodies further showed that the reduction of miR-33a might result in the increase of ABCA1 protein by relieving translational repression at the 3'-UTRs of ABCA1. Furthermore, our data demonstrated that down-regulation of miR-33a expression directly contributed to their chemo-resistance to doxorubicin by *in vitro* and *in vivo* functional assays.

A number of molecular mechanisms about chemo-resistance have been proposed, including the upregulation

of multi-drug transporters from the ABC superfamily, active DNA damage repair mechanisms, and resistance to apoptosis [28]. Our data comfirmed that restoring miR-33a expression could sensitize HCC-CSCs to doxorubicin via apoptosis. Other pathways which played important roles in chemo-resistance of cancer cells may also be involved. Additionally, we observed that miR-33a expression was related to chemo-response and poor survival in HCC patient cohort. These data suggested a potential predicted value of miR-33a in chemo-resistance.

In conclusion, we report that Lgr5⁺HCC cells from primary tissues and cell lines behave similarly to CSCs and are chemo-resistant to doxorubicin. Significantly, reduced miR-33a expression is associated with the chemo-resistance of Lgr5⁺ HCC-CSCs, accompanied by an overexpression of ABCA1 which is identified as target of miR-33a. Additionally, reduced miR-33a expression in HCC tissues is associated with chemo-response and poor patient survival, suggesting the therapeutic potential of miR-33a. Here, we demonstrate that ectopic miR-33a expression sensitizes Lgr5⁺ HCC-CSCs to doxorubicin via direct targeting ABCA1, which sheds new light on understanding the mechanism of chemo-resistance in HCC-CSCs and contributes to development of potential therapeutics against HCC.

Supplementary information is available in the online version of the paper.

References

[1]   FORNER A., LLOVET JM, BRUIX J. Hepatocellular carcinoma. Lancet 2012; 379: 1245–1255. https:/doi.org/10.1016/S0140-6736(11)61347-0

[2]   TACAR O, SRIAMORNSAK P, DASS CR. Doxorubicin: an update on anticancer molecular action, toxicity and novel drug delivery systems. J Pharm Pharmacol 2013; 65: 157–170. https://doi.org/10.1111/j.2042-7158.2012.01567.x

[3]   FORNER A, GILABERT M, BRUIX J, RAOUL JL. Treatment of intermediate-stage hepatocellular carcinoma. Nat Rev Clin Oncol 2014; 11:. 525–535. https://doi.org/10.1038/nrclinonc.2014.122

[4]   WION D, BERGER F. Cancer stem cells. N Engl J Med 2006; 355: 2703; author reply 2703. https://doi.org/10.1056/NEJMc062882

[5]   MAGEE JA, PISKOUNOVA E, MORRISON SJ. Cancer stem cells: impact, heterogeneity, and uncertainty. Cancer Cell 2012; 21: 283–296. https://doi.org/10.1016/j.ccr.2012.03.003

[6]   GE G, ZHOU C, REN Y, TANG X, WANG K et al. Enhanced SLC34A2 in breast cancer stem cell-like cells induces chemotherapeutic resistance to doxorubicin via SLC34A2-Bmi1-ABCC5 signaling. Tumour Biol 2016; 37: 5049–5062. https://doi.org/10.1007/s13277-015-4226-0

[7]   YAMASHITA T, WANG XW. Cancer stem cells in the development of liver cancer. J Clin Invest 2013; 123: 1911–1918. https://doi.org/10.1172/JCI66024

[8]   SU J, WU S, TANG W, QIAN H, ZHOU H et al. Reduced SLC27A2 induces cisplatin resistance in lung cancer stem cells by negatively regulating Bmi1-ABCG2 signaling. Mol Carcinog 2015. doi: 10.1002/mc.22430 https://doi.org/10.1002/mc.22430

[9]   DeSANO JT, XU L. MicroRNA regulation of cancer stem cells and therapeutic implications. AAPS J 2009; 11: 682–692. https://doi.org/10.1208/s12248-009-9147-7

[10] ESQUELA-KERSCHER A, SLACK FJ. Oncomirs – microRNAs with a role in cancer. Nat Rev Cancer 2006; 6: 259–269. https://doi.org/10.1038/nrc1840

[11] O'CONNOR MD, WEDERELL E, ROBERTSON G, DELANEY A, MOROZOVA O et al. Retinoblastoma-binding proteins 4 and 9 are important for human pluripotent stem cell maintenance. Exp Hematol 2011; 39: 866–879 e1.

[12] ZHANG J, WANG D, XIONG J, CHEN L, HUANG J. MicroRNA-33a-5p suppresses growth of osteosarcoma cells and is downregulated in human osteosarcoma. Oncol Lett 2015; 10: 2135–2141. https://doi.org/10.3892/ol.2015.3503

[13] ZHANG C, ZHANG Y, DING W, LIN Y, HUANG Z et al. MiR-33a suppresses breast cancer cell proliferation and metastasis by targeting ADAM9 and ROS1. Protein Cell, 2015. 6(12): p. 881–889. https://doi.org/10.1007/s13238-015-0223-8

[14] YANG L, ZHANG Y, DING W, LIN Y, HUANG Z et al. MircoRNA-33a inhibits epithelial-to-mesenchymal transition and metastasis and could be a prognostic marker in non-small cell lung cancer. Sci Rep 2015; 5: 13677. https://doi.org/10.1038/srep13677

[15] JASZBERENYI M, RICK FG, POPOVICS P, BLOCK NL, ZARANDI M et al. Potentiation of cytotoxic chemotherapy by growth hormone-releasing hormone agonists. Proc Natl Acad Sci U S A 2014; 111: 781–786. https://doi.org/10.1073/pnas.1322622111

[16] LAI CH, PARK KS, LEE DH, ALBEROBELLO AT, RAFFELD M et al. HSP-90 inhibitor ganetespib is synergistic with doxorubicin in small cell lung cancer. Oncogene 2014; 33: 4867–4876. https://doi.org/10.1038/onc.2013.439

[17] JEONG CH, CHO YY, KIM MO, KIM SH, CHO EJ et al. Phosphorylation of Sox2 cooperates in reprogramming to pluripotent stem cells. Stem Cells 2010; 28: 2141–2150. https://doi.org/10.1002/stem.540

[18] SU J, WU S, WU H, LI L, GUO T. CD44 is functionally crucial for driving lung cancer stem cells metastasis through Wnt/beta-catenin-FoxM1-Twist signaling. Mol Carcinog 2015. doi: 10.1002/mc.22443 https://doi.org/10.1002/mc.22443

[19] BECK B, BLANPAIN C. Unravelling cancer stem cell potential. Nat Rev Cancer 2013; 13: 727–738. https://doi.org/10.1038/nrc3597

[20] ROSKAMS T. Liver stem cells and their implication in hepatocellular and cholangiocarcinoma. Oncogene 2006; 25: 3818–3822. https://doi.org/10.1038/sj.onc.1209558

[21] SULPICE L, RAYAR M, DESILLE M, TURLIN B, FAUTREL A et al. Molecular profiling of stroma identifies osteopontin as an independent predictor of poor prognosis in intrahepatic cholangiocarcinoma. Hepatology 2013; 58: 1992–2000. https://doi.org/10.1002/hep.26577

[22] GIANNELLI G, FRANSVEA E, BERGAMINI C, MARI-NOSCI F, ANTONACI S. Laminin-5 chains are expressed differentially in metastatic and nonmetastatic hepatocellular carcinoma. Clin Cancer Res 2003; 9: 3684–3691.

[23] NAGAMATSU K, KOMORI M, KURODA S, TANAKA K. Dynamic light scattering studies on hydrodynamic properties of fibrinogen-fibronectin complex. J Biomol Struct Dyn 1992; 9: 807–820. https:/doi.org/10.1080/07391102.1992.10507957

[24] LAPLANTE M, SABATINI DM. mTOR signaling in growth control and disease. Cell 2012; 149: 274–293. https:/doi.org/10.1016/j.cell.2012.03.017

[25] CHIBA T, KITA K, ZHENG YW, YOKOSUKA O, SAISHO H et al. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties.

Hepatology 2006; 44: 240–251. https:/doi.org/10.1002/hep.21227

[26] JAN A, KARASINSKA JM, KANG MH, DEHAAN W, RUDDLE P et al. Direct intracerebral delivery of a miR-33 antisense oligonucleotide into mouse brain increases brain ABCA1 expression. [Corrected]. Neurosci Lett 2015; 598: 66–72. https:/doi.org/10.1016/j.neulet.2015.05.007

[27] TAM KH, YANG ZF, LAU CK, LAM CT, PANG RW et al. Inhibition of mTOR enhances chemosensitivity in hepatocellular carcinoma. Cancer Lett 2009; 273: 201–209. https:/doi.org/10.1016/j.canlet.2008.08.018

[28] ACKER T, PLATE KH. A role for hypoxia and hypoxia-inducible transcription factors in tumor physiology. J Mol Med (Berl) 2002; 80: 562–575. https:/doi.org/10.1007/s00109-002-0355-1

Figure S1 The expression of Lgr5 in fresh HCC tissue samples and HCC cell lines (PLC8024 and Huh7) was examined by using flow cytometry. Paralleled comparison was stained IgG-FITC.
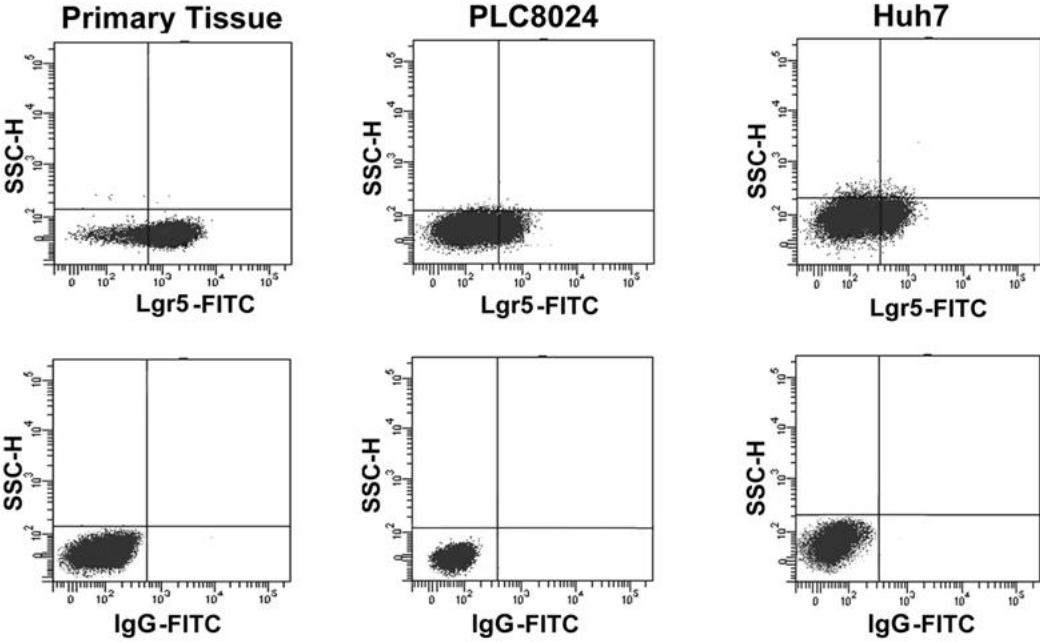
Figure S2 Relative expression of miR-33a and ABCA1 in xenograft tumors derived from Lgr5^{+} HCC-CSCs (un), lenti-miR-33a (miR-33a), or lenti-NC-HCC-CSCs (nc) fractions, following treatment with or without three cycles of doxorubicin, were examined by qPCR. Note: Columns, mean of three individual experiments; SD,**, P < 0.01.
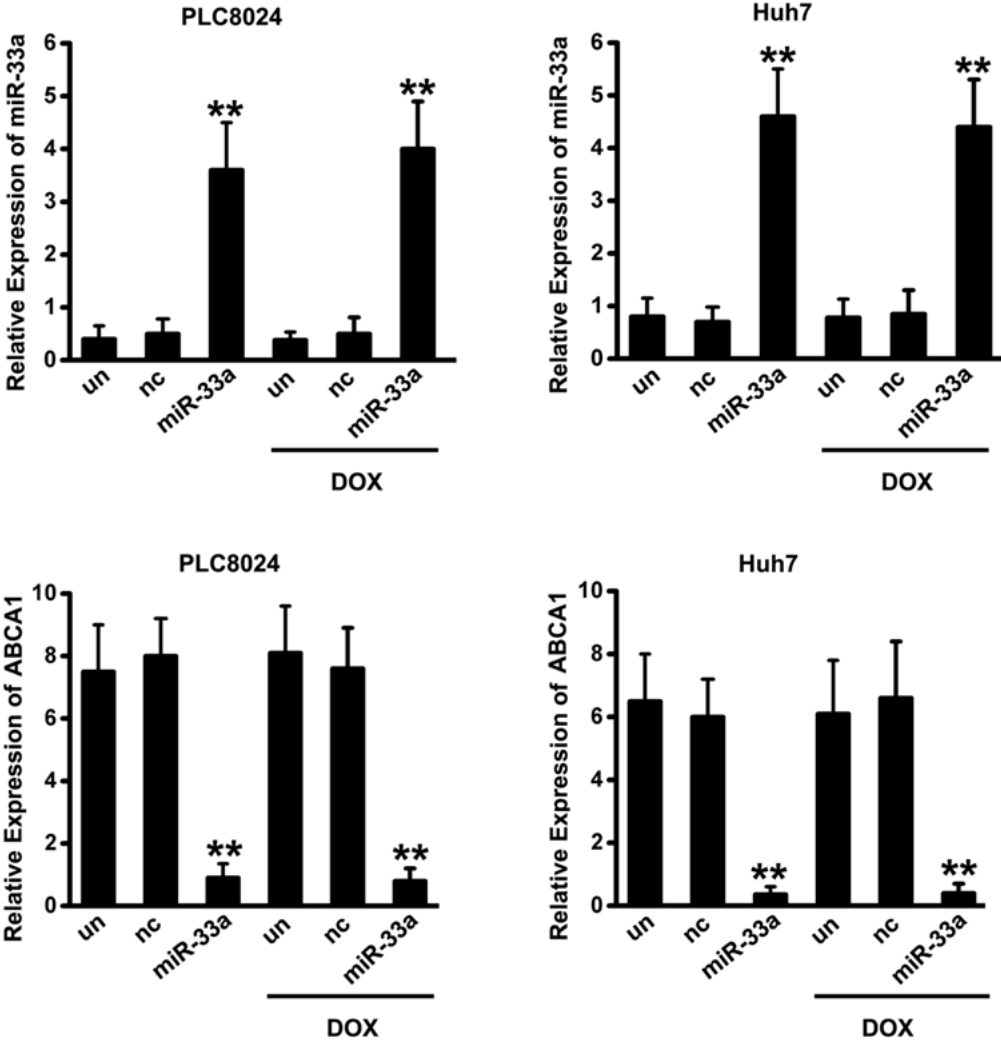
Figure S3 Relative expression of ABCA1 in different group of clinical response in 10 HCC patients (CR: disappearance of the disease; PR: reduction of >30%; PD: enlargement>20%) were examined by qPCR. Note: Columns, mean of three individual experiments.