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Augmenter of liver regeneration potentiates doxorubicin anticancer efficacy by reducing the expression of ABCB1 and ABCG2 in hepatocellular carcinoma

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Hepatocellular carcinoma (HCC) is highly chemoresistant and therefore challenges both physicians and patients. Augmenter of liver regeneration (ALR), previously also known as 'hepatic stimulator substance', is reported to inhibit the epithelial–mesenchymal transition (EMT) in HCC, one of the frequent events that occur in cancer metastasis, suggesting that ALR is involved in HCC. In this study, we report for the first time that the transfection of *ALR* enhances the antitumor effect of chemotherapy with doxorubicin, a typical anticancer drug, on HCC *in vitro* and *in vivo*. The efflux of doxorubicin from *ALR*-transfected HCC cells is efficiently suppressed. This implies the intracellular retention of doxorubicin in tumor cells, which is at least partly attributable to the effective inhibition of ABCB1 and ABCG2 transporter expression in *ALR*-expressing cells. The downregulation of *ALR* expression by short hairpin RNA diminishes the antitumor effect of ALR. We further demonstrate that ALR inhibits the AKT/Snail signaling pathway, resulting in the downregulation of ABCB1 and ABCG2 expression. In conclusion, our results suggest that ALR is a potential chemotherapeutic agent against HCC.
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Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer-related death worldwide.[1] Notably, the prevalence of HCC is increasing. Whereas hepatitis-C-related cirrhosis is the dominant risk factor for patients with HCC in North America, there is a higher incidence of hepatitis B in Africa and Asia.[2] Despite the recent improvement in the early diagnosis of HCC with imaging techniques, its treatment is still a challenge to both physicians and patients. Depending on the stage, there are several treatment modalities for HCC, including surgery, radiotherapy and chemotherapy.[3] For patients with HCC, the effectiveness of chemotherapy is strongly associated with the residual liver function and the physical condition of the patient. A challenging issue for HCC chemotherapy is that HCC is highly chemoresistant, displaying multidrug resistance (MDR) to functionally and structurally different anticancer drugs.[4,5]

The ATP-binding cassette (ABC) transporters comprise of one of the largest and oldest families of membrane proteins. Using energy from the hydrolysis of ATP, ABC transporters translocate several types of compounds, including some chemotherapeutic drugs, across the cellular and intracellular membranes.[6] One of the major mechanisms underlying the failure of chemotherapy attributable to MDR is believed to be related to the ABC-transporter-mediated exclusion of anticancer drugs.[7] To date, at least 49 ABC-transporter superfamily members have been identified.[6] Among them, ATP-binding cassette transporter subfamily B member 1 (ABCB1), ATP-binding cassette transporter subfamily C member 1 (ABCC1), ATP-binding cassette transporters subfamily C member 10 (ABCC10), and ATP-binding cassette transporter subfamily G member 2 (ABCG2) have been shown to be the major mediators of the efflux of anticancer drugs from cancer cells, and have been designated MDR-ABC transporters.[8] ABCB1 and ABCG2 are strongly involved in the acquisition of drug resistance in HCC and the downregulated expression of these two MDR-ABC transporters could allow the accumulation of anticancer drugs within HCC cells, improving the drugs' efficacy.[9–11]

Augmenter of liver regeneration (ALR), also known as hepatic stimulator substance or growth factor ERV1, was initially reported as a mitogenic factor that promotes liver regeneration after partial hepatectomy in rats.[12] It was later demonstrated that ALR used alone did not stimulate the growth of primary cultured hepatocytes, but instead, enhanced DNA replication in hepatocytes *in vitro*, if added

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in combination with other mitogens, such as epidermal growth factor.[13] In 1996, the *ALR* complementary DNA (cDNA) sequence was first reported by Giorda et al. and the human *ALR* gene was identified and mapped to chromosome 16.[14] In a subsequent investigation, we found that the *ALR* gene is driven by a TATA-less promoter that contains several regulatory elements, such as activator protein 1 (AP1)/AP4 *cis*-acting elements [15] and hepatic nuclear factor 4α (HNF4α) recognition sites.[16] These genetic features imply that ALR is involved in the regulation of liver regeneration.

The products of *ALR* consist of two splicing forms, a small molecule of 15 kD and a larger molecule of 23 kD. Whereas the small form is secreted as an extracellular cytokine that promotes hepatic growth,[17] the larger form localizes in the mitochondrial intermembrane space and acts as a flavin-adenine-dinucleotide-linked sulfhydryl oxidase (SOX), with a redox-active CXXC motif.[18,19] The 23-kD ALR is reported to function in the maturation of Fe–S proteins, preventing the generation of excess reactive oxygen species.[18,19] It has been demonstrated that ALR protects hepatocytes from various injuries,[20–24] including by CCl_4, D-galactosamine, H_2O_2, ethanol, and cadmium. The protection of hepatocytes by ALR is also associated with the suppression of the mitochondrial permeability transition and the inhibition of mitochondrion-mediated apoptosis.[22] Our study also suggested that increased ALR expression in hepatocytes forestalls cell apoptosis by inhibiting endoplasmic reticulum stress [25] and reducing lipotoxicity to the mitochondria by the preservation of carnitine palmitoyl-transferase1 (CPT1) activity.[26]

Experimental and clinical studies have shown that ALR is closely associated with several liver diseases. Enhanced *ALR* messenger RNA (mRNA) and protein expression are found in the livers of patients with cirrhosis, cholangiocellular carcinoma, and HCC.[27] It has also been reported that *ALR* expression correlates inversely with the HCC tumor grade and tumor metastasis.[28] Although HCC metastasis has long been associated with increased drug resistance, little information is yet available to explain the relationship between ALR and HCC in terms of chemoresistance.

In the present study, we found that *ALR*-transfected HCC cells were sensitive to doxorubicin, a typical anticancer drug. The inhibition of tumor growth by *ALR* transfection also correlated with the regulation of AKT/Snail signaling pathway, which resulted in the reduced expression of ABCB1 and ABCG2 and the intracellular retention of doxorubicin. All the antitumor effects of ALR were clearly diminished when its expression was downregulated with short hairpin RNA (shRNA). This study implies that ALR warrants further study as a potential molecular target for the chemotherapeutic treatment against HCC.

MATERIALS AND METHODS

Reagents

Dulbecco's modified Eagle's medium and fetal bovine serum were purchased from Hyclone (Pittsburgh, PA, USA). Doxorubicin hydrochloride and verapamil were purchased from Sigma–Aldrich (St Louis, MO, USA). FuGene HD, the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay, and the Caspase-Glo 3/7 Assay were purchased from Promega (Madison, WI, USA). The Annexin V–FITC/PI Apoptosis Kit was purchased from BD (Franklin Lake, NJ, USA). The radioimmunoprecipitation assay lysis buffer was purchased from Applygen (Beijing, China). The antibody directed against glyceraldehyde 3-phosphate dehydrogenase was purchased from KangCheng (Shanghai, China). The antibody against ALR (11293-1-AP) was purchased from Proteintech (Rosemont, IL, USA). The antibodies against cleaved poly-(ADP-ribose) polymerase (cleaved PARP; 9541) and ABCG2 (4477) were purchased from Cell Signaling Technology (Beverly, MA, USA).The antibody against ABCB1 (ab170904), Snail (ab180714), total AKT (ab8805), phosphoT308-AKT (ab38449), and phosphoS473-AKT (ab81283) were purchased from Abcam (Cambridge, UK). The RNeasy Mini Kit and QuantiNova SYBR Green RT–PCR Kit were purchased from Qiagen (Dusseldorf, Germany). The cDNA Synthesis Kit was purchased from TaKaRa (Dalian, China).

Cell Culture and Plasmid Transfection

The wild-type hepatoma cell line HepG2 and the human liver cancer cell lines BEL-7402, Hep3B, and Huh7 were maintained in our laboratory. The stably *ALR*-transfected HepG2 cells (*ALR*-tx), *ALR*-knockdown HepG2 cells (*ALR*-shRNA), and vector-transfected HepG2 cells (Vector-tx) were established in our laboratory, as described previously.[26] All the cells were grown as adherent monolayers in drug-free culture medium for >2 weeks before assay. All the cell lines were incubated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a humidified incubator at 37 °C under 5% CO_2.

Western Blotting

Cells were washed with phosphate-buffered saline (PBS) and lysed with radioimmunoprecipitation assay buffer. The cell debris and insoluble proteins were removed by centrifugation at 12 500 *g* at 4 °C for 10 min. Samples containing 50 μg of protein were separated with 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE). The expression levels of ALR, cleaved PARP, ABCB1, ABCG2, Snail, p-AKT T308, and p-AKT S473 were determined with a western blotting analysis using the appropriate antibodies. The relative amount of protein in each band was normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase or total AKT with the Image J software (National Institutes of Health, Bethesda, MD, USA).

Cytotoxicity Tested with MTS Assay

A 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) colorimetric assay was used to detect the cytotoxicity of the anticancer drug to cells. Cells were seeded evenly at a density

of 5×10^3 per well in a 96-well microtiter plate and cultured overnight. The *ALR*-tx and *ALR*-shRNA HepG2 cells were incubated for 48 h in medium containing 1% fetal bovine serum and doxorubicin at a final concentration of 0.0, 0.5, 1.0, 2.0, 4.0, 8.0, or 16.0 μg/ml. The Mock (untransfected) and Vector-tx HepG2 cells were used as the controls. MTS solution (20 μl) was then added to each well, and the microtiter plates were incubated for a further 1 h. The absorbance was determined at 490 nm with a Microplate Spectrophotometer (BioTek, Winooski, VT, USA). The cell inhibition rates and the half maximal inhibitory concentrations (IC_{50}) were calculated.

Colony-Forming Assay

A total of 1×10^4 cells were plated on ϕ100 mm dishes in complete culture medium or in complete culture medium containing 1.88 μg/ml doxorubicin and incubated for 48 h at 37 °C, after which the cells were cultured in complete culture medium for 14 days. The cells were then stained with hematoxylin. The colonies were quantified with the Image-J-software.

Caspase 3/7 Activity

Cells were seeded evenly at a density of 5×10^3 per well in a 96-well microtiter plate and cultured overnight. They were then treated with or without 1.88 μg/ml doxorubicin (IC_{50} for Vector-tx cells) for 24 or 48 h. Caspase activity was then detected with the Caspase-Glo 3/7 Assay Kit, according to the manufacturer's protocol. Luminescence was recorded with the Glomax 96 Microplate-Luminometer (Promega).

Apoptosis Analysis

The cells were plated in six-well microtiter plates, cultured overnight, and then treated with or without 1.88 μg/ml doxorubicin (IC_{50} for Vector-tx cells) for 24 h or 48 h. Cell apoptosis was then analyzed with the Annexin V–FITC/PI Apoptosis Kit, according to the manufacturer's protocol, with flow cytometry (LSRFortessa SORP; BD, Franklin Lake, NJ, USA).

Tumor Xenograft Assay and Treatment

Six-week-old NOD/SCID mice, purchased from the Academy of Military Sciences (Beijing, China), were used for these experiments. All the animals received humane care during the study and the relevant experimental protocols were approved by the Ethics Committee of Capital Medical University (Beijing, China). The animals were housed at a controlled room temperature (22–25 °C), and autoclaved food and water were available *ad libitum*. *ALR*-tx or *ALR*-shRNA cells were suspended at a density of 1×10^7 per 200 μl in PBS and injected subcutaneously under the left armpit of each mouse. Wild-type HepG2 cells were injected under the right armpit of each mouse as the control. The injection sites were palpated every day to evaluate tumor growth. The tumors were measured every 3 days with calipers and the volumes were calculated as $ab^2/2$ (a and b represent the length and width of the tumor, respectively). Tumors that failed to reach a volume of 30 mm^3 by the start of treatment were not used in this study. The mice were randomized into four groups ($n = 4$ in each group) and treated with one of the following regimens: (a) Mock/*ALR*-tx+doxorubicin (1.8 mg/kg, intraperitoneal [*ip*], once every 3 days [q3d] × 7), (b) Mock/*ALR*-tx+normal saline (NS; *ip*, q3d × 7), (c) Mock/*ALR*-shRNA +doxorubicin (1.8 mg/kg, *ip*, q3d × 7) and (d) Mock/*ALR*-shRNA+NS (*ip*, q3d × 7). At the end of the experimental period, the mice were killed, and the excised tumors were weighed and fixed in 40% formaldehyde. Fresh tumor samples were analyzed with transmission electron microscopy and Western blotting.

Hematoxylin and Eosin Staining

After the mice were killed, the tissues from the xenograft tumors were dissected. Sections (5 μm thick) were cut from the formaldehyde-fixed paraffin-embedded tissues for histological examination. The tissue sections were conventionally stained with HE and examined with light microscopy (Leica Microsystems, Wetzlar, Germany).

Transmission Electron Microscopy

The freshly dissected tumor tissues from the mice were fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.4) at 4 °C for 2 h, washed in PBS, and postfixed with 1% osmium tetraoxide in PBS. The cells were then dehydrated stepwise in a graded series of isoamyl acetate and with a hexamethyldisilazane flow process. The tissue samples were then embedded in epoxy resin, and ultrathin sections were stained with uranyl acetate/lead citrate and viewed with a transmission electron microscope (Hitachi, Tokyo, Japan).

Intracellular Retention of Doxorubicin

Cells were seeded evenly at a density of 5×10^3 per well in a 96-well microtiter plate and cultured overnight. On the following day, the cells were treated with increasing doses of doxorubicin (HepG2 cell line: 2.5, 5.0, and 10.0 μM; Bel-7402 cell line: 1.25, 2.5, and 5.0 μM) for 2 h, and then washed with PBS. The intracellular retention of doxorubicin was analyzed with a high-content analyzer (ArrayScan XTI; Thermo Scientific, Rockford, IL, USA). Cells were also seeded on sterile coated cover slips and allowed to grow overnight. On the following day, the cells were incubated with or without 3 μM verapamil for 1 h. They were then treated with doxorubicin (2.5 μM for HepG2 cell line and 1.25 μM for Bel-7402 cell line) for 2 h, washed with PBS, and examined with a laser scanning confocal microscope (Leica Microsystems).

RNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from the cells with the RNeasy Mini Kit (Qiagen), and 2 μg of total RNA was reverse-transcribed into cDNA with the cDNA Synthesis Kit (TaKaRa), according

to the manufacturer's protocol. The transcript levels of *ABCB1*, *ABCC1*, *ABCC10*, and *ABCG2* were quantified with qPCR, and the amplicon expression in each sample was normalized to the expression of 18S rRNA. The primers used to amplify *ABCB1* were forward 5′-GGGATGGTCAGTGTTG ATGGA-3′ and reverse 5′-GCTATCGTGGTGGCAAACAATA-3′; *ABCC1*: forward 5′-TAAAAGAGGATGCCCAGGTG-3′ and reverse 5′-ACCCTGTGATCCACCAGAAG-3′;*ABCC10*: forward 5′-CTCCCACTGGATCTCTCAGC-3′ and reverse 5′-TC GCATACACGGTGAGGTAG-3′; and *ABCG2*: forward 5′-AC GATATGGATTTACGGCTTT-3′ and reverse 5′-TCGATGC CCTGCTTTACCAA-3′. Calculations were based on the comparative cycle threshold Ct method ($2^{-\Delta\Delta Ct}$).

Statistical Analysis

The results are expressed as means ± s.d. Significance was determined with Student's *t* test using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). *P* values < 0.05 were considered statistically significant. All experiments were repeated at least twice.

RESULTS

ALR Sensitizes Hepatoma Cells to the Antitumor Effects of Doxorubicin

To clarify the link between ALR and chemoresistance in hepatoma cells, we transfected HepG2 cells with the *ALR* gene, and the expression of ALR in the four cell lines (Mock, Vector-tx, *ALR*-tx, and *ALR*-shRNA cells) was confirmed with western blotting. As shown in Figure 1a, ALR expression was 1.4-fold higher in the *ALR*-tx cells and significantly reduced (by 90%) in the *ALR*-shRNA cells compared with that in the vector-transfected cells.

The cells were then treated with increasing doses (0, 0.5, 1, 2, 4, 8, or 16 μg/ml) of doxorubicin for 48 h. Cell survival was assessed with MTS reagent and the cell growth inhibition rate and IC_{50} values were calculated (Figure 1b). The difference in the growth inhibition rates of the *ALR*-tx and *ALR*-shRNA cells was obvious at low doses (0.5–2 μg/ml) and reached a plateau at doses > 8 μg/ml. The control vector-transfected cells displayed an IC_{50} of 1.88 μg/ml, whereas the *ALR*-tx cells had a lower IC_{50} of 1.23 μg/ml. (ca. 34.6% lower, $P < 0.05$). Surprisingly, the IC_{50} of the *ALR*-shRNA cells increased to 2.87 μg/ml, $>$2-fold higher than that of the *ALR*-tx cells ($P < 0.01$). A colony-forming assay also showed that the chemoresistance of the HepG2 cells was significantly reduced in the *ALR*-tx cells, whereas the ALR-shRNA cells had an increased capacity to form colonies (Figure 1c).

We then tested whether the ALR-associated antitumor effect was specific to HepG2 cells. To do so, other HCC cell lines, including Bel-7402, Hep3B, and Huh7 cells, were used. The *ALR* plasmid was delivered into these cells and the ALR expression levels were detected 24 h after transfection (Figure 1d). The ALR-expressing cells appeared more sensitive to doxorubicin than the control cells when evaluated with an MTS assay (Figure 1e).

Taken together, these results suggest that the transfection of HCC cells with *ALR* enhanced the antitumor efficacy of doxorubicin against these cells.

ALR Enhances Doxorubicin-Induced Apoptosis *In Vitro*

The activation of the caspase signaling pathway and the consequent apoptosis are believed to be the cardinal mechanism of many antitumor drugs, including doxorubicin. To test whether the ALR-related antitumor effect of doxorubicin is associated with caspase activation, caspase 3 activity, and cleaved PARP were analyzed. As shown in Figure 2a, after doxorubicin treatment for 24 h, caspase 3 activity was markedly increased in the *ALR*-tx cells, whereas there were negligible changes in the other cell lines. At 48 h after treatment, all the cells showed higher caspase 3 activity, but this increase was remarkably inhibited in the *ALR*-shRNA cells. Consistent with this, treatment with doxorubicin also resulted in the cleavage of PARP protein, a marker of cellular apoptosis, in the *ALR*-tx cells at 24 h, whereas this increase was lower in the *ALR*-shRNA cells at 48 h than in the other cell lines (Figure 2b). These results suggest that the *ALR*-transfection sensitizes the chemotherapy of doxorubicin which occurs at the earlier exposure time, while, the inhibition of ALR expression reverses this effect.

Doxorubicin-induced apoptosis was also analyzed with Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) flow cytometry, and the calculation of the apoptotic rates is shown (Figure 2c). After treatment with doxorubicin for 24 h, the apoptotic rate in the Mock cells and vector-transfected cells clearly increased by 20.3 and 11.5%, respectively. However, the *ALR*-transfected cells displayed a significantly greater increase in the apoptotic rate (38.3%). Likewise, when the cells were treated with doxorubicin for 48 h, the apoptotic rates in the Mock cells and vector-transfected cells were 32.7 and 28.9%, respectively, whereas that in the *ALR*-transfected cells was 39.4%.

These results suggest that the transfection of HCC cells with *ALR* increased the caspase-activation-induced apoptosis induced by doxorubicin.

ALR Increases the Antitumor Effect of Doxorubicin *In Vivo*

To confirm that ALR increases the antitumor effect of doxorubicin *in vivo*, we created xenograft mouse models with *ALR*-tx or *ALR*-shRNA cells. Equal amounts of *ALR*-tx or *ALR*-shRNA cells were injected under the left armpits of NOD/SCID mice, and untransfected HepG2 cells were injected under the right armpit of each mouse as the control. The administration of 1.8 mg/kg doxorubicin (*ip* $3q \times 7$) retarded the growth of the *ALR*-tx xenografts. However, *ALR*-shRNA transfection reduced this effect over a period of 21 days (Figure 3a). After treatment with doxorubicin, the tumor inhibition rates were 54.12, 82.37, and 9.94% for the Mock, *ALR*-tx, and *ALR*-shRNA mice, respectively (Figure 3b). The tumor growth rate was significantly lower

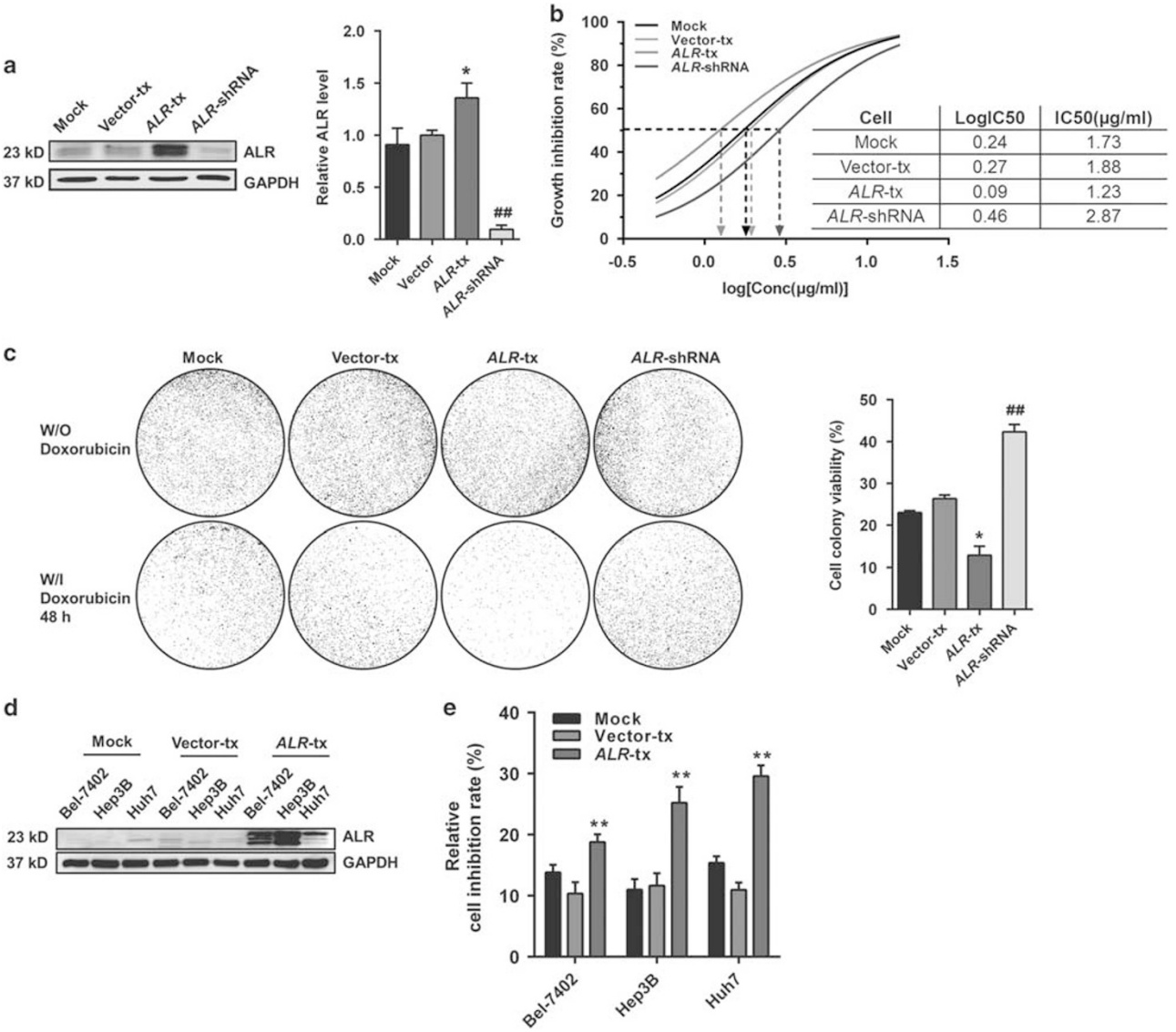

Figure 1 ALR influences the anticancer effect of doxorubicin in hepatoma cells. (**a**–**c**) HepG2 cells were stably transfected with *ALR* or *ALR* was knocked down with shRNA; untransfected (Mock) and stably vector-transfected cells were used as the controls. (**a**) Western blotting analysis of ALR expression in the cells (Mock, Vector-tx, *ALR*-tx, *ALR*-shRNA). (**b**) Cells were treated with increasing doses (0, 0.5, 1, 2, 4, 8, or 16 μg/ml) of doxorubicin for 48 h; the survival rates were assessed with MTS reagent, and the cell growth inhibition rates and IC_{50} values were calculated. (**c**) A total of 1×10^4 cells were plated and treated with or without 1.88 μg/ml doxorubicin (IC_{50} of Vector-tx) for 48 h, then the growth medium was changed, after 14 days, the plates were stained with hematoxylin and the cell colony viabilities were calculated. (**d**) Bel-7402, Hep3B, and Huh7 cells were transfected with pcDNA3.0 alone or the pcDNA3.0/ALR plasmid, using FuGene HD Transfection Reagent. Twenty-four hours after transfection, ALR expression was analyzed with western blotting. Cells in were treated with or without 1.0 μg/ml of doxorubicin for 48 h, and cytotoxicity was detected with an MTS colorimetric assay (**e**). Graph shows the means ± s.d. of triplicate experiments. $^*P<0.05$, $^{**}P<0.01$, when *ALR*-tx cells were compared with Vector-tx cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$, when *ALR*-shRNA cells were compared with Vector-tx cells.

in the *ALR*-tx mice than in the *ALR*-shRNA mice during the 21 days of doxorubicin treatment (Figure 3c and d).

The histological changes in the tumor tissues were also examined. Compared with the control group, focal necrosis was clearly observed in the Mock group, whereas marked necrosis was seen in the *ALR*-tx group. On the contrary, the knockdown of *ALR* expression (*ALR*-shRNA) reduced the doxorubicin-induced necrosis in the tumor tissues (Figure 3e).

Ultrastructural observations supported the results of HE staining. As shown in Figure 3f, in the Mock group, after treatment with doxorubicin for 21 days, typical apoptotic nuclei were observed, with the chromatin condensed into several large masses close to the nuclear envelope, whereas in the *ALR*-tx samples, the nuclear membrane was markedly disrupted and karyolysis was clearly apparent (indicated with yellow arrows).

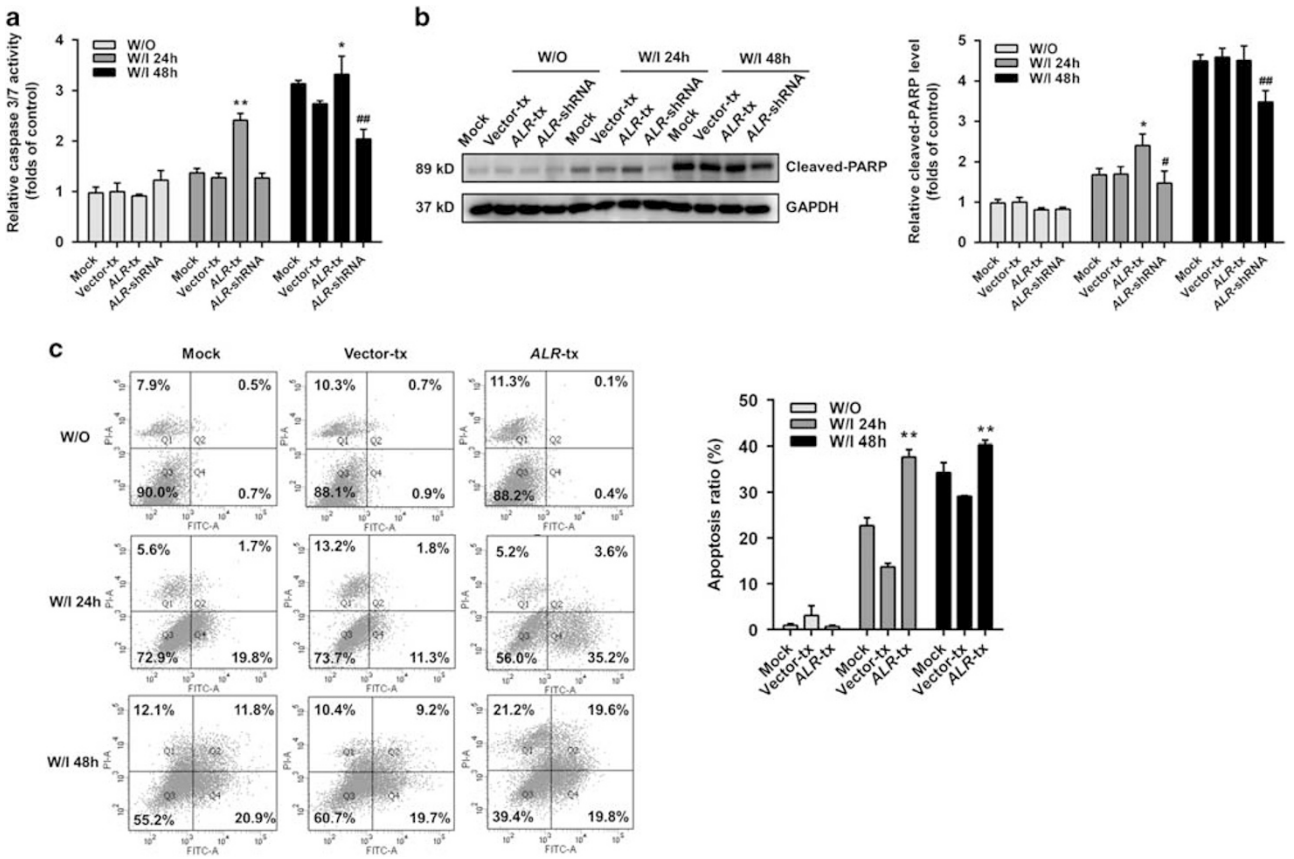

Figure 2 ALR increases doxorubicin-induced apoptosis *in vitro*. Cells were treated with or without 1.88 μg/ml doxorubicin for 24 h or 48 h. Caspase activity was detected with the Caspase-Glo 3/7 Assay Kit (**a**), cleaved PARP was analyzed with western blotting (**b**), and apoptosis was analyzed with flow cytometry (**c**). Graph shows the means ± s.d. of twice experiments. $^{*}P<0.05$, $^{**}P<0.01$, when *ALR*-tx cells were compared with Vector-tx cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$, when *ALR*-shRNA cells were compared with Vector-tx cells.

These results demonstrate that treatment with ALR increased the antitumor effects of doxorubicin in a xenograft model.

ALR Increases the Intracellular Retention of Doxorubicin

As discussed above, ALR promoted the antitumor effects of doxorubicin against HCC both *in vitro* and *in vivo*. It is well known that the retention of doxorubicin inside cells is essential for its cellular cytotoxicity. Therefore, we investigated whether ALR enhances the retention of doxorubicin inside cells, thus increasing its cytotoxicity for HCC cells. The intracellular content of doxorubicin was determined with a high-content analyzer and confocal microscopy, based on its own fluorescence. As shown in Figure 4a, after treatment with different doses of doxorubicin (2.5, 5, or 10 μM) for 2 h, the HepG2 cells displayed different intracellular drug retention rates, which was highest in the *ALR*-tx cells but greatly reduced in the *ALR*-shRNA cells, ie, less doxorubicin accumulated in the *ALR*-knockdown cells than in the *ALR*-tx cells. We also observed the intracellular retention of doxorubicin (2.5 μM; Figure 4b) with fluorescence microscopy. The amount of doxorubicin, represented by the red fluorescent signal, clearly increased in the intracellular compartment and nuclei of the *ALR*-tx cells, but this increase appeared smaller when *ALR* was knocked down. Verapamil, a classic MDR efflux pump inhibitor, was used as a positive control. The doxorubicin accumulation in Bel-7402 cells transfected with the *ALR*-expressing plasmid or *ALR* shRNA (Figure 4c and d) showed identical patterns to that in HepG2 cells.

These results suggest that ALR increases the retention of doxorubicin within the nuclei of cells, which allows the cells to be readily killed by chemotherapy.

ALR Inhibits ABCB1 and ABCG2 Expression *In Vitro* and *In Vivo*

The resistance of tumor cells to chemotherapy is mainly attributed to the presence of MDR, and ABC transporters that mediate the efflux of chemotherapeutic agents are believed to be the most important factors in MDR. The inhibition of ABC transporter activity enhances the efficacy of anticancer drugs in HCC cells and improves their cytotoxicity. Therefore, we investigated the MDR-ABC transporter responsible for the *ALR*-related antitumor effect. The expression of the *ABCB1*, *ABCC1*, *ABCC10*, and *ABCG2* genes in the *ALR*-tx

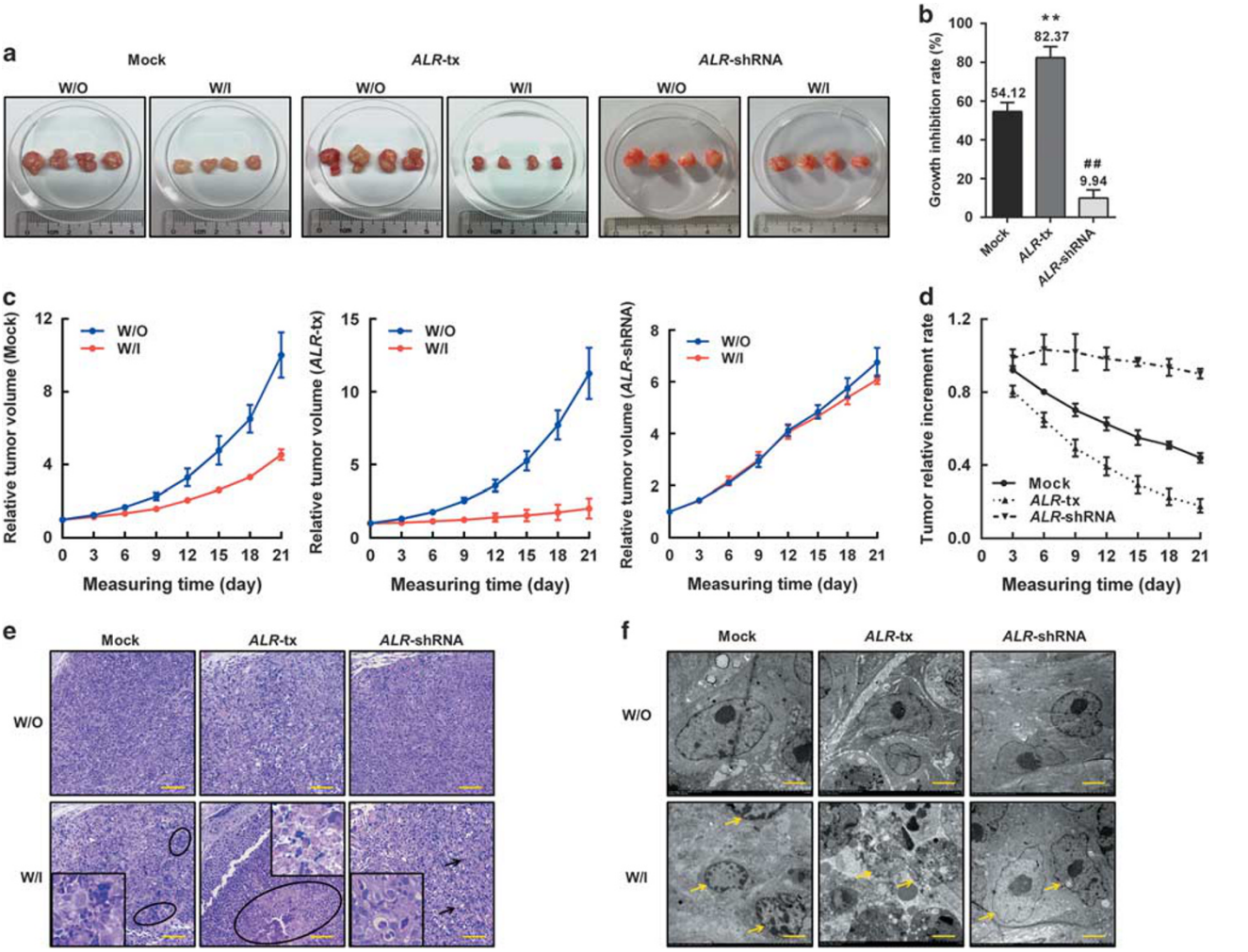

Figure 3 ALR enhances the anticancer effects of doxorubicin in xenograft tumors. The *in vivo* effect of ALR was examined in 6-week-old NOD/SCID mice using stably transfected HepG2 cells, treated with or without doxorubicin (1.8 mg/kg, *ip*, q3d × 7). The tumors (**a**) were excised after treatment, and the tumor inhibition rates (**b**), relative tumor volumes (**c**), and relative tumor rates of increase (**d**) were calculated. Graph shows the means ± s.d. of four experiments. $^{**}P<0.01$, when *ALR*-tx tumors were compared with Mock tumors; $^{\#\#}P<0.01$, when *ALR*-shRNA tumors were compared with Mock tumors. The microstructures of the tumor tissues from each group were observed after HE staining (**e**) and the ultrastructure was observed with transmission electron microscopy (**f**).

HCC cells was quantified with qPCR. As shown in Figure 5a, among the four genes tested, only the expression of *ABCB1* and *ABCG2* changed significantly in the *ALR*-tx and shRNA knockdown hepatocytes. The protein levels of ABCB1 and ABCG2 were then checked with western blotting. We showed that the transfection of cells with *ALR* reduced the expression of ABCB1 and ABCG2, whereas *ALR* knockdown increased the ABCG2 levels (Figure 5b). The expression of ALR, ABCB1, and ABCG2 in the xenografts was identical to that seen in HepG2 cells (Figure 5c).

ALR Transfection Improves the Intracellular Retention of Doxorubicin

ALR reduced ABCB1 and ABCG2 expression and increased the intracellular retention of doxorubicin, consequently promoting the antitumor effects of doxorubicin on HCC *in vitro* and *in vivo*. To confirm this phenomenon, a rescue experiment, in which *ALR*-shRNA cells were transfected with an *ALR*-containing plasmid, was performed to confirm that *ALR* transfection actually inhibits the expression of ABCB1 and ABCG2. As shown in Figure 6a, after *ALR* rescue, the expression of ABCB1 and ABCG2 was significantly suppressed (by 39.7 and 33.6%, respectively) compared with that in the *ALR*-shRNA cells. Simultaneously, the intracellular level of doxorubicin increased >2-fold after *ALR* rescue (Figure 6b and c). These data further verify that ALR expression is closely associated with the functions of ABCB1 and ABCG2, which may regulate the efflux of doxorubicin.

ALR Inhibits ABCB1 and ABCG2 Expression Partly Through the Inhibition of AKT/Snail Signaling Pathway

An earlier study has shown that the transfection of *ALR* could inhibit the expression of Snail in HCC cells.[28] The activation of the AKT/Snail signaling pathway is known to contribute to

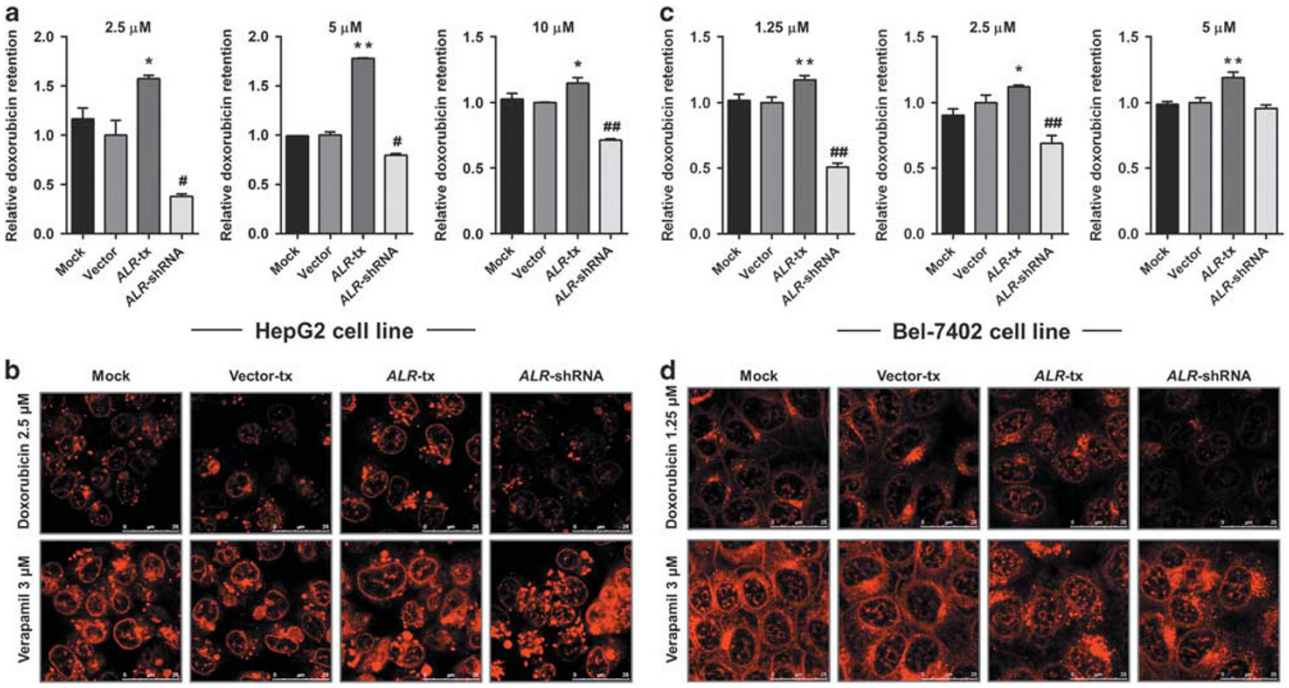

Figure 4 ALR increases the retention of doxorubicin fluorescence in hepatoma cells. HepG2 cells were treated with 2.5, 5, or 10 μM doxorubicin for 2 h, and then the intracellular retention of doxorubicin was examined with a high-content analysis (**a**); the same cells were pretreated or not with 3 μM verapamil for 1 h, and then treated with 2.5 μM doxorubicin for 2 h, the intracellular retention of doxorubicin was examined with laser scanning confocal microscopy based on its own fluorescence (**b**). Bel-7402 cells were treated with 1.25, 2.5, or 5 μM doxorubicin for 2 h, and then the intracellular retention of doxorubicin was examined with a high-content analysis (**c**); the same cells were pretreated or not with 3 μM verapamil for 1 h, and then treated with 1.25 μM doxorubicin for 2 h, the intracellular retention of doxorubicin was examined with laser scanning confocal microscopy based on its own fluorescence (**d**). Graph shows the means ± SD of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$, when *ALR*-tx cells were compared with Vector-tx cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$, when *ALR*-shRNA cells were compared with Vector-tx cells.

drug resistance by increasing the expression of MDR-ABC transporters, after which the overexpression of Snail increases the promoter activity of ABC transporters.[29,30] Given the critical role of the AKT/Snail signaling pathway in drug resistance, we were interested in exploring whether ALR-enhanced antitumor effect of doxorubicin is related to the AKT/Snail/MDR-ABC signaling pathway. A western blotting analysis showed that the transfection of HepG2 cells with *ALR* inhibited the phosphorylation of AKT at Ser473 and Thr308, and that the level of Snail was reduced. The phosphorylation of AKT and the expression of Snail were increased in cells treated with *ALR* shRNA (Figure 7). These data imply that the reduced expression of ABCB1 and ABCG2 regulated by ALR might, at least in part, be associated with the AKT/Snail pathway, resulting in elevated doxorubicin accumulation and alleviating tumor drug resistance.

DISCUSSION

ALR was originally described as a hepatic factor that promotes liver regeneration. Recently, enhanced ALR expression has been detected in the livers of patients with cirrhosis and cholangiocellular or HCC. Gandhi et al. reported that the liver-specific deletion of ALR exacerbated alcohol-induced liver injury, and accelerated the development of steatohepatitis, liver fibrosis, and even HCC.[31,32] They demonstrated that ~60% of $ALR^{-/-}$ mice developed HCC within 12 months of birth. These hepatic lesions are probably attributable to severe mitochondrial impairment, although the essential mechanism remained unclear. An analysis of 53 tissues from HCC patients showed that ALR expression correlated inversely with tumor grade and tumor angioinvasion.[28] Dayoub et al. demonstrated that the overexpression of ALR in HCC cells attenuated the epithelial–mesenchymal transition (EMT) *in vitro* and *in vivo*.[28] The role of ALR in the development and metastasis of HCC prompted us to investigate the potential functions of ALR in HCC treatment. In this study, we report for the first time that ALR participates in the anticancer effect by increasing the accumulation of doxorubicin in HCC cells. This enhanced intracellular accumulation of doxorubicin seems to be related to the inhibition of the AKT/Snail signaling pathway, resulting in the downregulation of ABCB1 and ABCG2 and a consequent increase in the lethal effect of anticancer drugs on HCC cells. The therapeutic potential of ALR for HCC is an innovative concept, and its expression could be used as an index in selecting the appropriate therapeutic strategy for HCC.

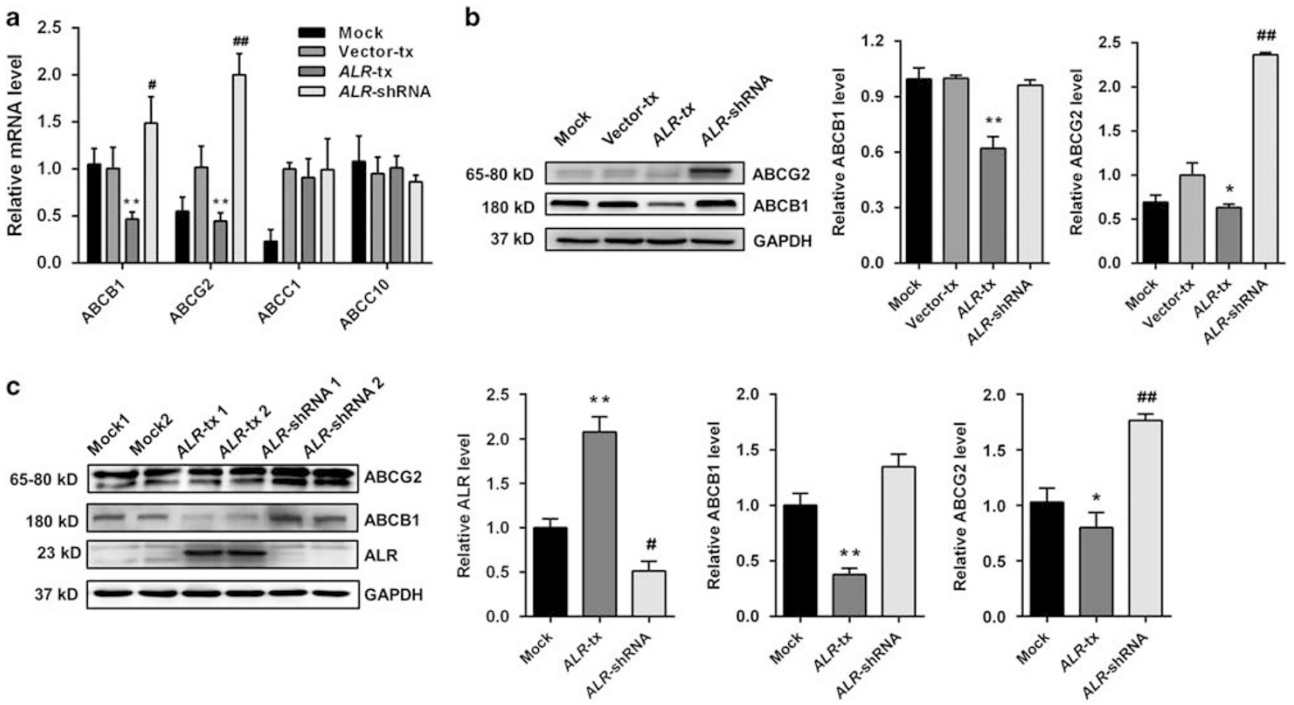

Figure 5 ALR reduces *ABCB1* and *ABCG2* expression *in vitro* and *in vivo*. (**a**) Total mRNA was extracted from human HCC HepG2 cells, Mock, Vector-tx, *ALR*-tx, and *ALR*-shRNA cells, and the expression of the *ABCB1*, *ABCC1*, *ABCC10*, and *ABCG2* genes was quantified with qPCR. (**b**) Expression of the ABCB1- and ABCG2 proteins in the four cell lines was analyzed with western blotting. (**c**) Changes in ALR, ABCB1, and ABCG2 expression in the xenografts were also determined with western blotting. Graph shows the means ± s.d. of triplicate experiments. $^{*}P<0.05$, $^{**}P<0.01$, when *ALR*-tx cells were compared with control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, when *ALR*-shRNA cells were compared with control.

The MDR status of cancer cells allows them to resist the cytotoxicity of structurally and mechanistically unrelated chemotherapeutic drugs, and is based on several mechanisms, including efflux transporters, the regulation of apoptosis and autophagy, DNA repair, and epigenetic regulation.[33] In humans, the activation of MDR is a cardinal factor in the failure of cancer chemotherapy. Today, the majority of patients with advanced-stage HCC experience no effect when chemotherapy is administered, causing an overall poor prognosis for HCC patients.[34] Therefore, the development of an effective strategy to inhibit or inactivate MDR has become an urgent issue for those with chemotherapy-resistant tumors.

In this study, we have demonstrated for the first time a link between ALR expression and the resistance of HCC to chemotherapeutic drugs. We tested cell sensitivity to doxorubicin in the presence or absence of ALR, and showed that the IC_{50} of doxorubicin for HCC cells was more than double after *ALR* knockdown than when *ALR* was expressed. *ALR* transfection promoted the DNA-damage-induced apoptosis and necrosis caused by doxorubicin both *in vitro* and *in vivo*.

To investigate the mechanism by which ALR sensitizes HCC cells, we investigated the intracellular retention of doxorubicin. Consistent with previous reports, doxorubicin mainly accumulated in the cell nuclei to exert its antitumor effect. However, less doxorubicin accumulated in the *ALR*-knockdown cells, which may explain the higher IC_{50} for doxorubicin during treatment. Likewise, ALR overexpression significantly increased the intracellular retention of doxorubicin, providing clues to the mechanism of ALR action.

ABC transporters, particularly ABCB1 and ABCG2, play important roles in the efflux of xenobiotic compounds.[35] The overexpression of ABCB1 and ABCG2 has been shown to enhance MDR in some kinds of cancers, including breast, colon, ovary, stomach, and liver cancers.[36,37] ABCB1, also known as P-glycoprotein, was the first ABC transporter identified.[38] Physiologically, ABCB1 is an apical membrane transporter that protects the placenta, liver, intestine, and blood–brain barrier cells against xenobiotics and cellular toxins. A wide variety of ABCB1 substrates have been identified, including paclitaxel, vincristine, and doxorubicin.[8] ABCG2, also called 'breast cancer resistance protein' (BCRP), was originally cloned from breast tumor cell lines and placental tissue in 1998, and was quickly recognized as a major factor in MDR.[39] The overexpression of ABCG2 confers significant resistance to many chemotherapeutic substrates, including mitoxantrone, methotrexate, and doxorubicin.[40] The expression of ABCB1 and ABCG2 was investigated here to determine the mechanism by which ALR reduces doxorubicin efflux. The overexpression of ALR increased the sensitivity of HCC cells to doxorubicin, which

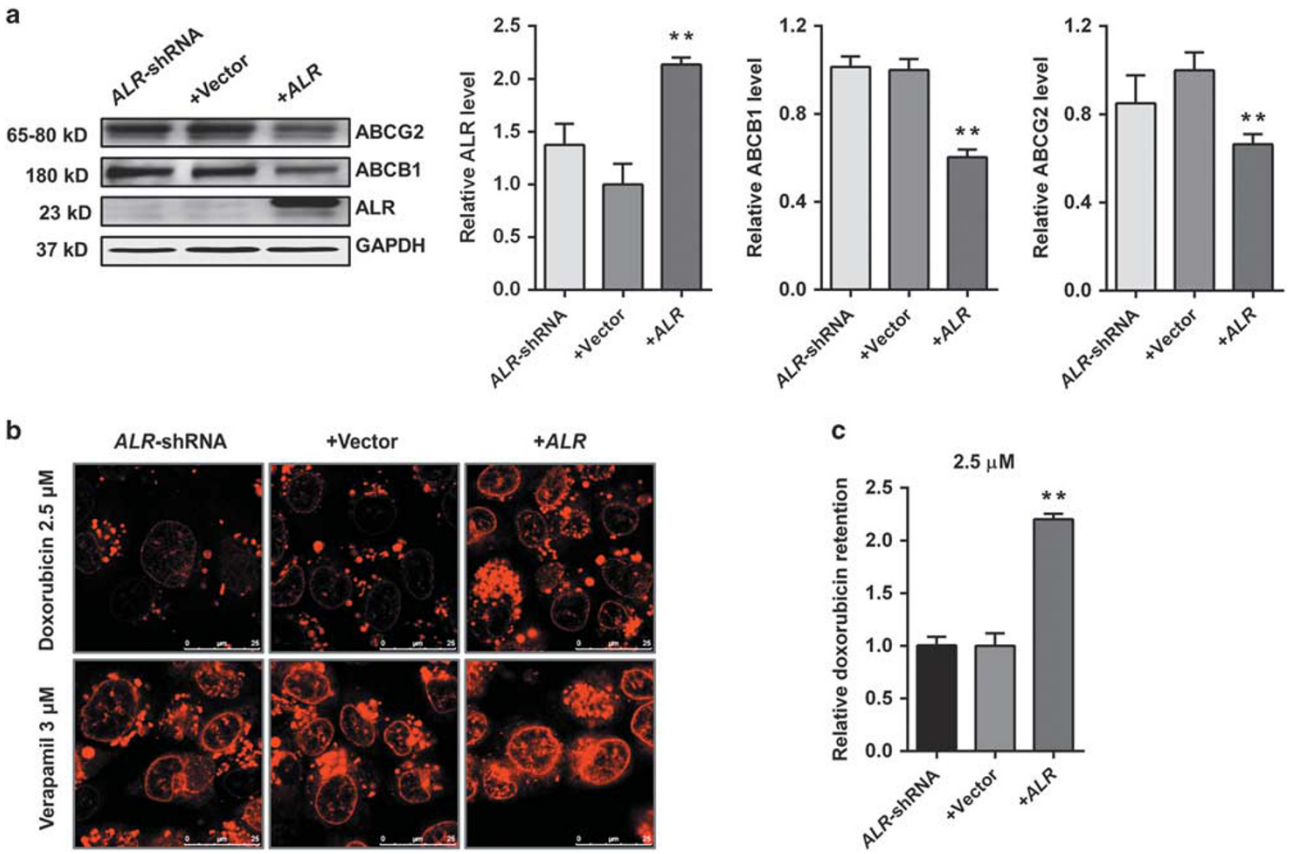

Figure 6 *ALR* transfection rescues the effect of doxorubicin in *ALR*-knockdown cells. *ALR*-shRNA cells were transfected with the *ALR* plasmid or the vector only (pcDNA 3.0) using FuGene HD Transfection Reagent. Twenty-four hours after transfection, (**a**) the expression of ALR, ABCB1, and ABCG2 was analyzed with western blotting. Cells were pretreated or not with 3 μM verapamil, and then treated with 2.5 μM doxorubicin for 2 h. The intracellular retention of doxorubicin was examined with laser scanning confocal microscopy (**b**) and with a high-content analysis (**c**). Graph shows the means ± s.d. of triplicate experiments. $^{**}P<0.01$, when *+ALR* cells were compared with +Vector cells.

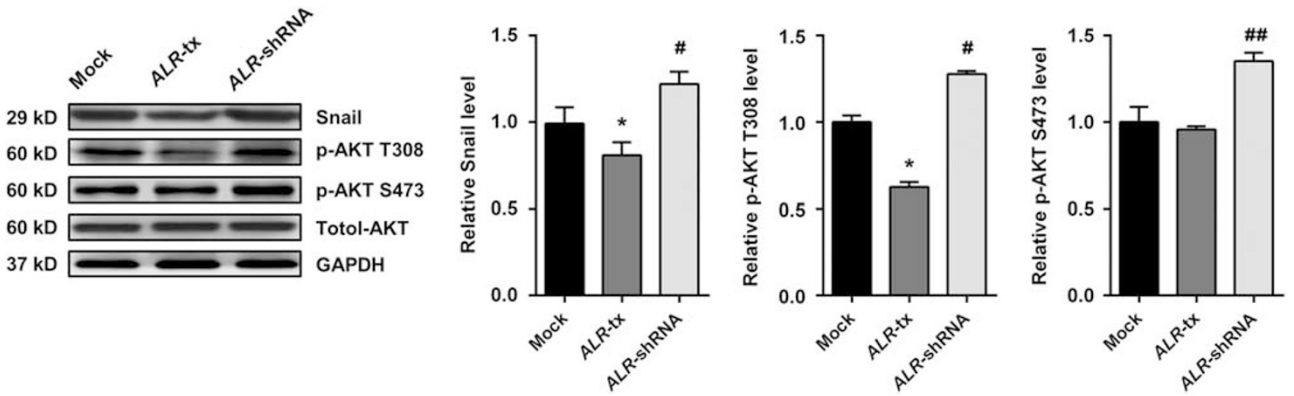

Figure 7 ALR regulates the AKT/Snail signaling pathway. Expression of the Snail, p-AKT T308, p-AKT S473 proteins in Mock, *ALR*-tx, *ALR*-shRNA cell lines was analyzed with western blotting. Graph shows the means ± SD of triplicate experiments. $^{*}P<0.05$ when *ALR*-tx cells were compared with Mock cells; $^{\#}P<0.05$, $^{\#\#}P<0.01$, when *ALR*-shRNA cells were compared with Mock cells.

was accompanied by the downregulation of ABCB1 and ABCG2. Unfortunately, in this experiment, it is noted that the suppression of the of ABCB1/ABCG2 expression by ALR transfection is not so heavy, which we predict might be attributed to a fact that regulation of these transporter expression may be very complicated. ALR, supposed to be one of these potential regulators, could not play a cardinal or key role for the regulation. In this case, alternation in MDR expression caused by ALR transfection might be limited. It has also been suggested that ABCB1 and ABCG2, which are

plasma-membrane-bound transporters, become dysfunctional when translocated to the cytoplasm.[11] Whether ALR alters the translocation of ABCB1 and ABCG2 and, if so, the mechanisms involved, remain to be determined. On the basis of our results, we hypothesize that ALR sensitizes HCC cells to doxorubicin at least partly through the inhibition of ABCB1 and ABCG2 expression.

Cancer metastasis has long been associated with increased drug resistance. Previous studies by the Dayoub group and our laboratory have shown that the transfection of *ALR* inhibits the metastasis of HCC by downregulating the expression of Snail, a widely known transcription factor that mediates the EMT.[28] It is noteworthy that the activation of the AKT/Snail signaling pathway contributes to drug resistance in many types of cancers.[29,41] Recently, Nieh et al. proposed that head and neck squamous cell carcinoma cells express high levels of ABCB1 and ABCG2 in response to increasing Snail levels.[42] Similarly, Saxena et al. demonstrated that several ABC transporters contain binding sites for EMT regulators, such as Twist and Snail, and that these factors modulate the promoter activities of ABC transporter genes.[30] On the basis of the aforementioned findings, we investigated whether ALR enhanced the antitumor effect of doxorubicin through the inhibition of the AKT/Snail/MDR-ABC signaling pathway. We demonstrated that the altered expression of ALR markedly modulated the activity of the AKT/Snail signaling pathway in human HCC cells. Therefore, we assume that ALR reduces the phosphorylation of AKT and the expression of Snail, and consequently inhibits the promoter activity of the ABC transporters, leading to the downregulation of ABCB1 and ABCG2. However, the pathway that ALR regulates MDR expression remains largely unexplored although AKT/Snail route might be one potential. Our next attempt will be more closely dealing with this focus.

As it was discovered that the overexpression of ABC transporters in cancer cells mediates the cells' resistance to anticancer drugs, there has been an ongoing effort to develop inhibitors of these transporters to increase the concentrations of anticancer drugs within cells. In the last 10 years, many modulators of ABC transporters have been identified. Among these, some compounds inhibit hepatic and intestinal cytochrome P450 enzymes, reducing the metabolism and clearance of the drugs, thereby causing systemic toxicity.[43] ALR is clearly an important intracellular survival factor for hepatocytes, allowing them to circumvent this toxic effect. The most commercially successful chemotherapeutic antitumor drugs mainly target ABCB1, and few compounds target other ABC transporters. Because HCC cells always develop MDR, which is attributed to multiple ABC transporters, a strategy that targets a single molecule might predictably fail.[44] On the basis of our results, the ALR-related antitumor effect is not exclusively attributable to the inhibition of ABCB1, and ABCG2 might also play a role in the mobilization of chemotherapeutic agents against HCC.

In conclusion, inducing the overexpression of ALR in cancerous hepatocytes improves their sensitivity to antitumor drugs by increasing the retention of intracellular drugs, at least partly through the modulation of the AKT/Snail/MDR-ABC (ABCB1 and ABCG2) signaling pathway. In the future, ALR may be used to improve the overall prognosis of HCC when it is combined with common chemotherapeutic drugs.

ACKNOWLEDGMENTS

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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