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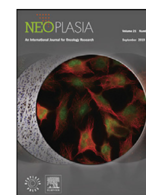

Overcoming ABCB1-mediated multidrug resistance by transcription factor BHLHE40

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A R T I C L E I N F O

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A B S T R A C T

Multidrug resistance (MDR) hinders treatment efficacy in cancer therapy. One typical mechanism contributing to MDR is the overexpression of permeability-glycoprotein (P-gp) encoded by ATP-binding cassette subfamily B member 1 (*ABCB1*). Basic helix-loop-helix family member e40 (BHLHE40) is a well-known transcription factor that has pleiotropic effects including the regulation of cancer-related processes. However, whether BHLHE40 regulates MDR is still unknown. Chromatin immunoprecipitation-seq study revealed BHLHE40 occupancy in the promoter of *ABCB1* gene. Adriamycin (ADM)-resistant human chronic myeloid leukemia cells (K562/A) and human breast cancer cells (MCF-7/A) were established. BHLHE40 expression was downregulated in the ADM-resistant cell lines. Overexpression of BHLHE40 resensitized resistant cells to ADM, promoted cell apoptosis *in vitro* and suppressed tumor growth *in vivo,* whereas BHLHE40 knockdown induced resistance to ADM in parental cells. Moreover, we found that BHLHE40 regulated drug resistance by directly binding to the *ABCB1* promoter (-1605 to -1597) and inactivating its transcription. In consistence, the expression of BHLHE40 was negatively correlated with ABCB1 in various cancer cells, while positively with cancer cell chemosensitivity and better prognosis of patients with breast cancer. The study reveals the role of BHLHE40 as a transcriptional suppressor on the expression of ABCB1, major ABC transporter in chemoresistance. The findings extend the function of BHLHE40 in tumor progression and provides a novel mechanism for the reversal of multidrug resistance.

Introduction

Chemotherapy is one of the major treatment strategies for various cancer types. However, a major impediment hampering the treatment of cancer patients is the emergence of multidrug resistance (MDR), which represents the acquired drug resistance to multiple anti-cancer agents regardless of their structures and action mechanisms [1]. MDR has been explored including changes in drug targets, autophagy, reduced susceptibility to apoptosis and decreased DNA damage and so on [2–4]. Among these mechanisms, the predominant one is the increased expression of ATP-binding cassette (ABC) transporters, such as permeability-glycoprotein (P-gp, encoded by *ABCB1* gene) and multidrug resistance proteins (MRPs, encoded by *ABCC1/ABCC2/ABCC3/ABCC4* genes). The ABC transporters facilitate the outflow transportation of structurally divergent drugs from intracellular compartment to prevent the accumulation of cytotoxic agents in cancer cells [5,6]. P-gp is ubiquitously expressed and its localization on the brain capillary endothelial cells is well-known for the limitation of central nervous system uptake [7]. It participates in chemoresistance of hematological malignancies and solid tumor, including promyelocytic leukemia, adult acute lymphocytic leukemia, bladder cancer and breast cancer, both in cell lines and clinical samples [3,8-11]. Among factors that alter the activity of P-gp, reg-

Abbreviations: ABC, ATP-binding cassette; ADM, adriamycin; BHLHE40, basic helix-loop-helix family member e40; CCK-8, cell counting kit-8; ChIP, chromatin-immunoprecipitation; CML, chronic myeloid leukemia; DEC1, differentiated embryonic chondrocytes-expressed gene 1; EMT, epithelial mesenchymal transition; FBS, fetal bovine serum; IC50, half maximal inhibitory concentration; MDR, multidrug resistance; MRP, multidrug resistance protein; Permeability-glycoprotein, P-gp; Rho123, rhodamine 123; SC, scramble; TCGA, the Cancer Genome Atlas.

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ulated expression contributes the most. However, the underlying mechanisms of the transcriptional regulation of *ABCB1* has not been fully understood.

Basic helix-loop-helix (BHLH) family member e40, also known as differentiated embryonic chondrocytes-expressed gene 1 (DEC1), is a transcription repressor directly binding to the promoters and/or enhancer regions of the target genes [12–14]. Its effects are also ascribed to indirect regulation of gene expression by interacting with RNA polymerase II, other transcription factors or histone modifiers [15]. BHLHE40 is stress-responsive and has a variety of physiological effects including the regulation of neuronal synaptic plasticity, mammalian circadian rhythms, differentiation of chondrocytes and skeletal muscles [16]. Notably, it participates in tumorigenesis and cancer development by affecting cell cycle, cell differentiation, proliferation, apoptosis, and epithelial mesenchymal transition (EMT) of cancer cells [17–20]. Recently, it is also highlighted that BHLHE40 maintains the metabolic homeostasis of tumor infiltrating immune cells and contributes to achieve more effective cancer immunotherapy [21]. As a transcription repressor, whether BHLHE40 suppresses the transcriptional expression of efflux drug transporters through the interaction with the gene promoter is still unknown. It is worthwhile to clearly elaborate the role of BHLHE40 in drug resistance, as it can be a drug target of potential pharmaceutical value for drug design.

Although some drugs have been developed to target P-gp, they fail in the phase of clinic trial due to poor inhibition efficacy or unbearable toxicity, including verapamil, quinidine, amiodarone and cyclosporine A [5]. Therefore, further elucidation on the regulatory mechanisms of P-gp is crucial for the reversal of multidrug resistance. In the present study, we search NCBI GEO datasets and dig into the occupancy of BHLHE40 in the promoter of the multidrug resistance-associated ABC efflux transporters in the informative ChIP-seq genome binding profile (GSE125729, GSM935616, GSM935566 and GSE106000). On this basis, experiments are designed to testify the hypothesis that BHLHE40 attenuates ABCB1-mediated adriamycin (ADM) resistance and further explore the underlying molecular mechanisms.

Methods and materials

Cell lines and culture conditions

Human chronic myeloid leukemia (CML) cell line K562 cells and human breast cancer cell line MCF-7 cells were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China) and characterized by STR assays. ADM induced drug-resistant K562/A cells and ADM-resistant MCF-7/A cells were established by the prolonged treatment in the presence of ADM. Drug sensitivity was evaluated after the establishment of the cell lines. K562 and K562/A cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), in a humidified environment with 5% CO_2 at 37°C. MCF-7 and MCF-7/A cells were cultured in DMEM medium (Gibco) with the same conditions. To maintain drug resistance, ADM (10 μM) was supplied to K562/A and MCF-7/A until at least 2 weeks before all the experiments.

Bioinformatics analysis

ChIP-seq datasets of GM12878 (GSM935430), K562 (GSM935616), and HepG2 (GSM935616) cells which were pulled down by the BHLHE40 specific antibody were selected for analysis. Firstly, according to the fold change (FC>7 or <-7) and p-value (p-value<0.0001) provided by narrow peak, the sequences with significant changes were screened. To link the peak coordinates with their closest genes, peaks were annotated to genes within (+700bp, -2000bp) around the transcription start site using ChIPseeker, a R package [22]. Then, after removing the antisense strand, long non-coding RNA, microRNA, ribosomal RNA and repeated genes, a cluster of 671 genes was obtained with significance in the ChIP-seq datasets of all three cell lines. The above genes were then subjected to Gene Ontology analysis.

Real-time RT-PCR

Total RNA was extracted by using TRIZOL reagent (Invitrogen) according to the manufacturer's instruction. The first strand cDNA was synthesized adopting oligo (dT) and M-MLV reverse transcriptase (Vazyme, Nanjing, China). Quantitative PCR was performed with SYBR green PCR kit (Vazyme, Nanjing, China) on the Applied Biosystems StepOne Real-Time PCR Systems. All signals from each target gene were conducted in triplicate and normalized to corresponding GAPDH. The relative mRNA expression was calculated according to the $-2^{-\Delta\Delta Ct}$ and expressed as the mean ± SD. The Primer sequences used in the study were shown in Supplementary Table 1.

Western blot analysis

Total proteins from cells were extracted using RIPA buffer (Abcam) and protein concentrations were measured with BCA protein assay. Equal amounts of protein were fractionated through 10% SDS-PAGE gels and electroblotted to PVDF membranes. The membranes were blocked with 10% non-fat milk and then incubated overnight at 4°C with respective primary antibodies. After repeated washing, horseradish peroxidase-conjugated secondary antibody was used for 1 h at room temperature. Protein bands were visualized with an enhanced chemiluminescence system (ThermoFisher). The levels of protein expression were quantified by densitometric analysis with Image J software (NIH), and GAPDH as a control. Antibodies used in the present study were as follows: BHLHE40 (1:3000, Abcam), P-gp (1:5000, Abcam), GAPDH (1:3000, Bioworld, USA). The goat anti-rabbit IgG conjugated with horseradish peroxidase was from Pierce Chemical (1:5000, Pierce, USA).

Plasmid construction and cell transfection

Overexpression and shRNA vectors for BHLHE40 were constructed and transfected as previously described [23]. ABCB1 promoter sequence (-5052 to +120 bp) were cloned to pGL3 reporter vector and named as pABCB1-Luc. The binding sites in ABCB1 promoter were mutated using a Mut Express II Fast Mutagenesis Kit V2 (Vazyme, Nanjing, China) according to the instructions, and the mutations were confirmed by direct DNA sequencing. The stable cell lines expressing BHLHE40 or shRNA were generated by transfection of lentivirus (purchased from Obio Technology, Shanghai, China).

Drug resistance assay

In vitro drug cytotoxicity was measured by Cell Counting Kit-8 (CCK-8, Beyotime, China) assays. Cells incubated without drugs were set at 100% survival and were used to calculate the concentration of each cytostatic drug lethal to 50% of the cells (half maximal inhibitory concentration, IC50). ADM was obtained from commercial sources and dissolved according to the manufacturer's instructions. After 48 h of transfection in 96-well plates, freshly prepared medium containing several final concentrations of ADM was added to the wells, with three replicate wells for each concentration. After incubation for an additional 48 h, cell viability was measured using CCK-8 according to the manufacturer's instructions. For colony formation assay, cells were treated with different concentrations of ADM for 7 days and stained with the addition of 0.005% crystal violet (Sigma).

Flow cytometry

P-gp efflux activity was determined by intracellular accumulation of rhodamine 123 (Rho123, Beyotime, China), a substrate of P-gp. After 48 h of transfection, cells were incubated with 4 μg/ml Rho123 for 1 h or 10

min at 37°C in the dark, then washed twice with PBS and subsequently analyzed using a flow cytometry (BD).

Cell apoptotic analysis were assessed by double-staining with Annexin V-FITC and propidium iodide (PI, Vazyme, Nanjing, China). Briefly, cells were harvested after 48 h of ADM treatment, washed with PBS, resuspended in 500 µl of binding buffer, added with 5 µl of Annexin V-FITC and 5 µl of propidium iodide, incubated for 10 min in the dark, and analyzed using a flow cytometer. The Annexin V-positive cells were considered apoptotic cells and analyzed using the FlowJo software. All of assays were carried out in triplicate in three independent experiments.

In vivo xenograft tumor model

All animal experiments were approved by the IACUC (Institutional Animal Care and Use Committee) of China Pharmaceutical University (Ethical approval number: 2017-04-023). Female BALB/c nude mice aged 6 weeks were purchased from Model Animal Research Center of Nanjing University. To assess the effects of BHLHE40 expression on tumor formation *in vivo*, the BHLHE40- or empty vector- expression stable K562/A and MCF-7/A cells, the BHLHE40-shRNA- or scramble (SC)- expression stable K562 and MCF-7 cells were injected subcutaneously into the right armpit of mice. When the average tumor size reached approximately 50 mm^3, 5.0 mg/kg ADM were subsequently injected through tail vein every other day. Tumor volume was calculated as $V = A * B^2/2$ (mm^3), where A is the largest diameter, and B is the perpendicular diameter. The weights of tumors were also documented simultaneously. The developing tumors were observed during the following 5 weeks, after which the mice were sacrificed.

Luciferase reporter and chromatin-immunoprecipitation (ChIP) assay

Cells were seeded in 24-well plates at 3.5×10^4 cells per well. After an overnight incubation, the cells were transfected with a DNA mixture including pGL3-ABCB1 promoter-luciferase, BHLHE40-expression vector or empty vector, as well as the internal β-galactosidase (β-gal) plasmids. Luciferase activity was measured by a Glomax 96 luminometer (Promega, Madison, WI, USA). Luciferase activity was normalized to β-gal activity. Each experiment was performed in triplicate and repeated independently for three times.

ChIP analysis was performed using a ChIP Assay Kit (Millipore) according to the manufacturer's protocols. The immunoprecipitated and input DNA were used as templates for RT-PCR analysis, and the primers were listed in Supplementary Table 2.

Statistical analyses

Data were analyzed using SPSS 16.0 software. All the experiments were repeated independently for at least three times. Data were expressed as mean ± SD. Data analysis between two groups was performed using Student's *t*-test. Two-way ANOVA was used to compare the growth inhibition rate of the groups, followed by Bonferroni multiple comparisons. P<0.05 was considered as statistically significant.

Results

BHLHE40 occupancy in the proximal promotor of ABCB1 reveals a function to suppress ABCB1 in ADM resistant tumor cells

BHLHE40 is a transcription factor that usually represses the expression of target genes by binding to the promoters and/or enhancers. Therefore, it is postulated that BHLHE40 may act as a transcriptional repressor for the ABC efflux transporter genes, and therefore affect chemoresistance. By analyzing an informative ChIP-seq dataset on mouse cell with BHLHE40 specific antibody in the GEO database, we found *ABCB1* was the only one ABC pump gene which bound to BHLHE40 in the proximal promoter, while other representative chemoresistance-associated genes didn't show occupancy, including *ABCC1∼4* (GSE125729) (Fig 1A). Similarly, BHLHE40 also occupied the promoter of *ABCB1* in homo sapiens K562, HepG2 and GM12878 cell lines as evidenced by the occupancy profiling using high throughput sequencing, indicating the conserved regulation of ABCB1 by BHLHE40 (GSM935616, GSM935566 and GSE106000) (Fig 1B). We further analyzed these ChIP-seq datasets and obtained a cluster of 671 genes which were occupied by BHLHE40 in all the three cell lines (Fig 1C). Gene ontology analysis showed that the genes with BHLHE40 occupancy were significantly enriched for circadian regulation of gene expression, histone modification and translation, underlying the established functions of BHLHE40 (Fig 1D). Notably, ABCB1 outstood from other known drug transporters by showing up in the cluster of genes with BHLHE40 occupancy in all the three cell lines (Fig 1C). It implies that *ABCB1* is the target gene for BHLHE40 in the regulation of chemosensitivity.

After the prolonged treatment of ADM, the establishment of resistant tumor cells were confirmed by drug sensitivity assay. The IC50 of ADM in chemoresistant cells (K562/A or MCF-7/A) was much higher than that in the control cells (K562 or MCF-7). Resistance index was calculated as IC50 of ADM-resistance cells divided by IC50 of parental cells. The resistance indexes were 10.28 for K562/A and 17.01 for MCF-7/A, indicating that both cell lines were ADM-resistant (Fig 1E-F). Then, the efflux activity of P-gp was analyzed by Rho123 intracellular accumulation assay with flow cytometry (Fig 1G). Intracellular Rho123 was significantly decreased in the chemoresistant cells (K562/A or MCF-7/A) compared with corresponding parental cells (K562 or MCF-7). To determine the role of BHLHE40 in ADM resistance, the expression of BHLHE40 and *ABCB1* (P-gp) in both sensitive (K562 or MCF-7) and resistant (K562/A or MCF-7/A) cells were compared. It was revealed that the expression of BHLHE40 had a negative correlation with that of ABCB1 in the cancer cells, which was compatible with the cells' sensitivity to ADM. In the chemosensitive cells (K562 or MCF-7), the expression of *ABCB1* (P-gp) was negligible, while BHLHE40 expression was high in both mRNA and protein level. The scenario of these two genes expression was opposite in ADM-resistant cells (K562/A or MCF-7/A), as shown with high expression of *ABCB1* (P-gp) and low of BHLHE40 (Fig 1H-I). The data suggest a regulatory role of BHLHE40 on the expression and activity of *ABCB1* (P-gp).

BHLHE40 increases ADM sensitivity by inhibiting the expression and export activity of ABCB1

As a well-known transcription repressor, it was hypothesized that BHLHE40 might suppress the expression of *ABCB1* to confer chemosensitivity of cancer cells based on the above data. Thus, the chemoresistant cells (K562/A or MCF-7/A) were transfected with the BHLHE40 expression vector or empty vector, and chemosensitive cells (K562 or MCF-7) were transfected with BHLHE40-specific shRNA (BHLHE40-shRNA) or scramble control shRNA (SC). The expression of *ABCB1* (P-gp) and BHLHE40 were evaluated subsequently. It was notable that the mRNA (Fig 2A-B) of *ABCB1* and protein (Fig 2C) of P-gp expression were decreased when BHLHE40 was overexpressed in chemoresistant tumor cells (K562/A or MCF-7/A). Meanwhile, *ABCB1* and P-gp expression were increased when BHLHE40 was knocked down in the chemosensitive parental tumor cells (Fig 2D-F). Then, we used flow cytometry analysis to assess the effect of BHLHE40 on P-gp efflux activity. Intracellular Rho123 accumulation was higher with the forced expression of BHLHE40 in ADM-resistant K562/A and MCF-7/A cells than the empty vector transfected cells (Fig 2G). Whereas BHLHE40 knockdown in the K562 and MCF-7 cells significantly decreased intracellular Rho123 compared with the SC group (Fig 2H). These data indicate that BHLHE40 negatively regulates the expression and activity of *ABCB1* (P-gp).

To delineate the role of BHLHE40 on reversing chemoresistance, CCK-8 assay and colony formation analysis were then performed to examine cell sensitivity and proliferation induced by ADM with the manipulation of BHLHE40. IC50 values for ADM in response to BHLHE40 up-

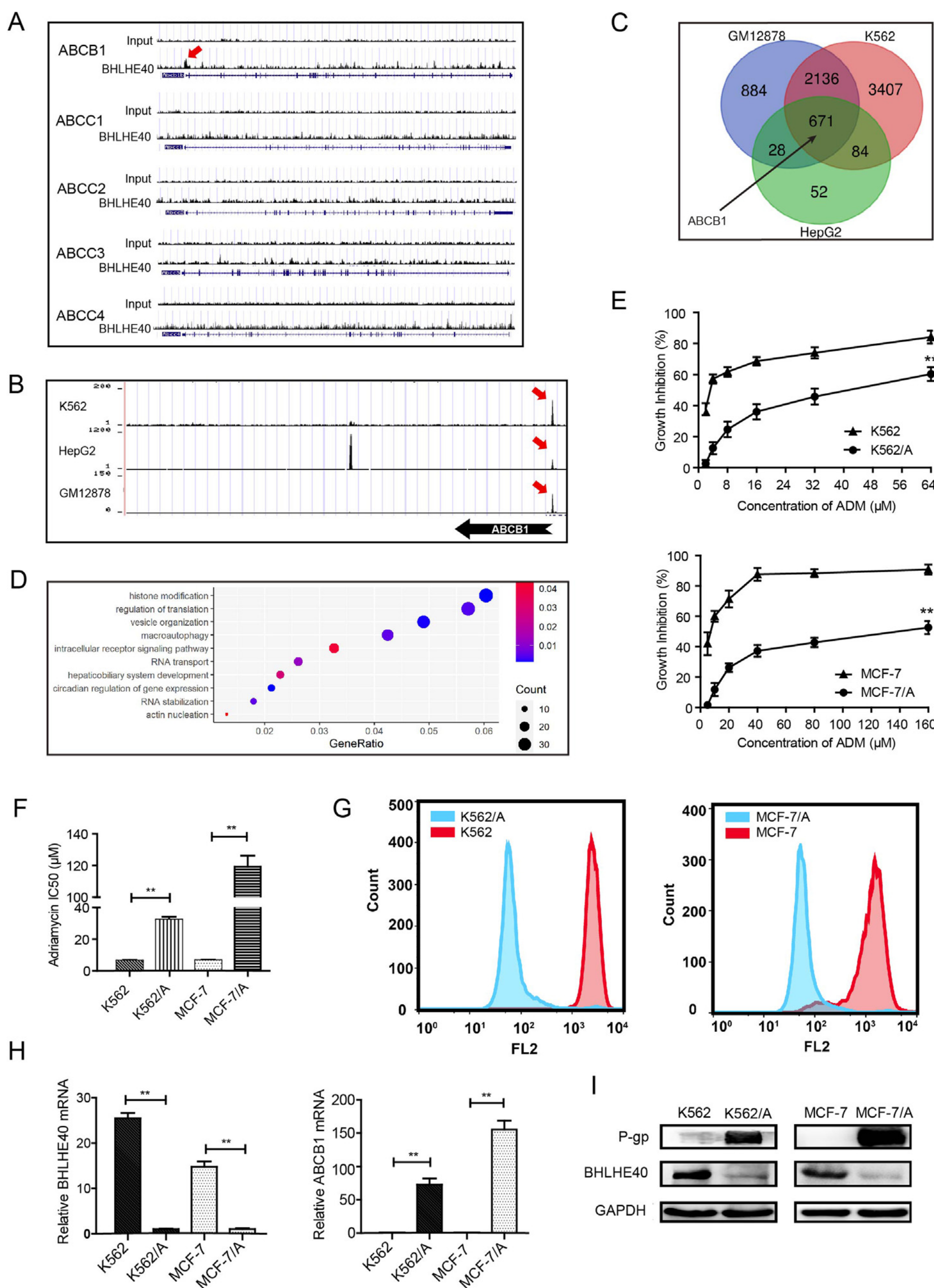

Fig. 1. BHLHE40 and ABCB1 expression are negatively correlated in ADM resistant and their parental cells. (A) BHLHE40 occupancy in the promoter of MDR-related genes was analyzed in a ChIP-seq dataset on mouse cell (GSE125729). (B) BHLHE40 occupancy in the promoter of ABCB1 using ChIP-seq datasets on human K562, HepG2 and GM12878 cell lines (GSM935616, GSM935566 and GSE106000). (C) The gene counts with BHLHE40 occupancy in the promoter in the ChIP-seq datasets of all the three human cell lines. (D) Gene ontology enrichment analysis of the above gene cluster overlapping in the three cell lines. (E) The inhibitory effect of ADM on K562 (or MCF-7) and K562/A (or MCF-7/A) cells was detected by CCK8 assay. After treatment with various concentrations of ADM for 48 h, growth inhibition was assessed. (F) Half maximal inhibitory concentration (IC50) of ADM was calculated in the respective cells. (G) The accumulation of Rho123 was measured by flow cytometric analysis after cells were incubated with 4 μg/ml Rho123 for 1 h at 37°C. (H) The mRNA levels of *BHLHE40* and *ABCB1* in ADM resistant or parental cells were detected by RT-PCR. (I) The protein expression of BHLHE40 and P-gp was assessed by Western blot analysis. Data are presented as means ± SD (n = 3). **P <0.01 vs. control.

or downregulation were measured. Compared with ADM-resistant cells (K562/A or MCF-7/A) transfected with empty vector, viability of cells overexpressed with BHLHE40 were significantly reduced (Fig 3A-B). Compared with ADM-sensitive cells (K562 or MCF-7) transfected with SC, viability of cells transfected with BHLHE40-shRNA were remarkably increased (Fig 3C-D). Then, colony formation assays revealed that cell proliferation was significantly suppressed in BHLHE40-overexpressing ADM-resistant cells (K562/A or MCF-7/A) compared with empty vector transfected cells, when exposed to ADM (16 μM or 50 μM respectively) for 7 days (Fig. 3E). Cell proliferation was consistently increased in BHLHE40-downregulated ADM-sensitive cells (K562 or MCF-7) compared with SC-transfected cells, when exposed to 4 μM Adriamycin for 7 days (Fig. 3F). Further, we used flow cytometry analysis to assess the effect of BHLHE40 on ADM-induced cell apoptosis. Similarly, compared with negative control, overexpression of BHLHE40 resulted in increased cell apoptosis proportion of K562/A or MCF-7/A cells when exposed to ADM (16 μM or 50 μM respectively) for 48 h (Fig 4A and C). Likewise, down-regulation of BHLHE40 caused decrease in apoptosis proportion of K562 or MCF-7 cells compared with the SC group treated with ADM (4 μM) for 48 h (Fig 4B and D). It supports that BHLHE40 endorses the sensitivity of CML and breast cancer cells to ADM *in vitro*. These integrated data illustrate a modulating role of BHLHE40 on chemosensitivity by regulating the expression and activity of P-gp.

Overexpression of BHLHE40 improves chemosensitivity to ADM in vivo

To confirm BHLHE40 in suppressing the chemoresistance of tumor cells to ADM *in vivo*, we inoculated nude mice with ADM-resistant K562/A or MCF-7/A cells stably transfected with BHLHE40 expression vector or empty vector and sensitive K562 or MCF-7 cells stably transfected with BHLHE40-shRNA or SC. These cells were subcutaneously injected into mice followed by treatment with ADM (5 mg/kg) every day when tumor formation reached average size of 50 mm^3. Tumors derived from BHLHE40-overexpressed K562/A or MCF-7/A cells grew significantly slower than empty vector controls following ADM treatment (Fig 5A). In consistence, tumors formed from BHLHE40-shRNA

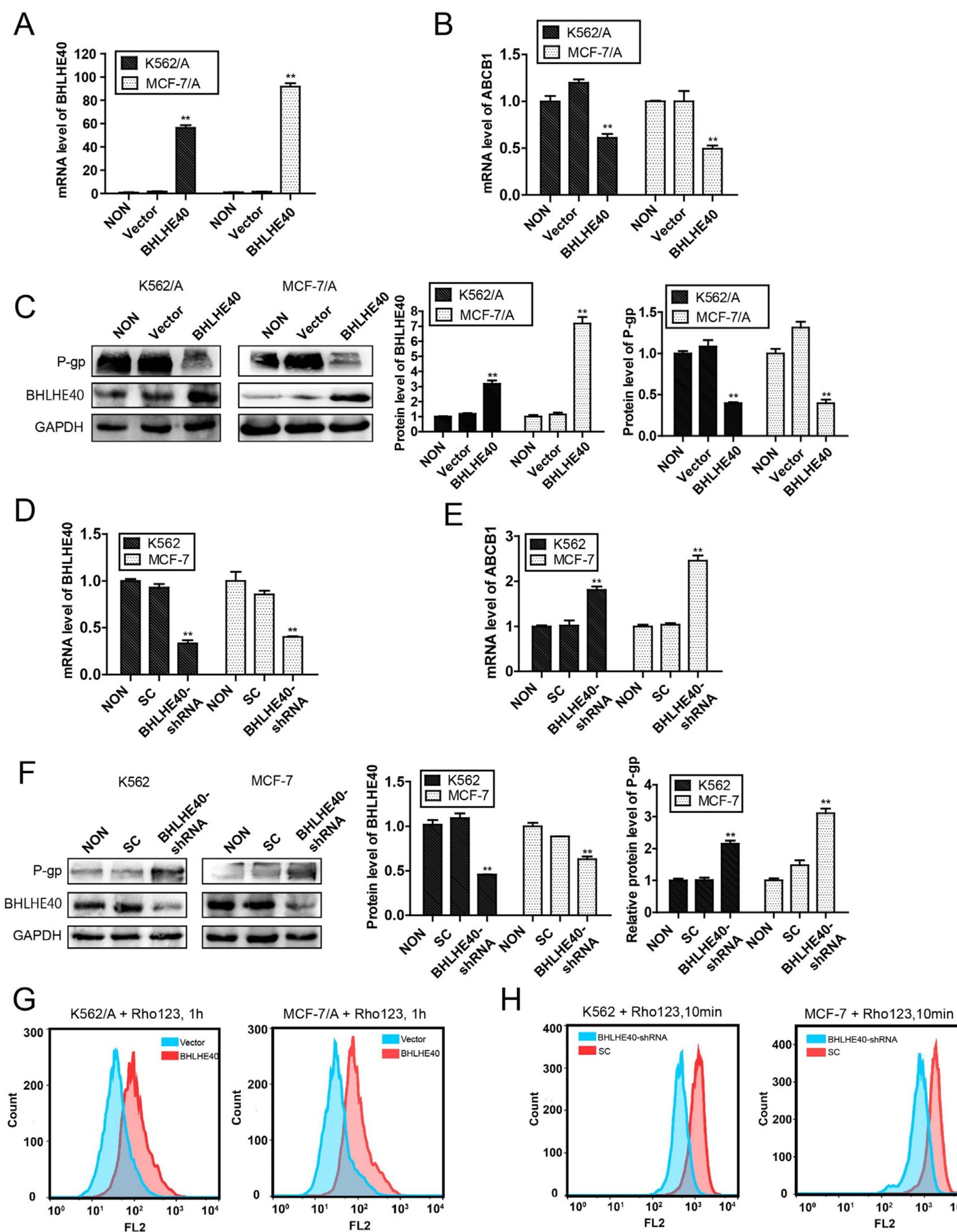

Fig. 2. BHLHE40 negatively regulates expression of *ABCB1* (P-gp) in both RNA and protein level. (A, B, C) The mRNA and protein levels of BHLHE40 and *ABCB1* (P-gp) were detected after overexpression of BHLHE40 for 48 h in resistant cells (K562/A or MCF-7/A) by RT-PCR and Western blot analysis. (D, E, F) The mRNA and protein levels of BHLHE40 and *ABCB1* (P-gp) were analyzed after knockdown of BHLHE40 for 48 h in the parental cells (K562 or MCF-7). (G, H) Intracellular accumulation of Rho 123 was measured by flow cytometric analysis after cells were transfected for 48 h and then incubated with 4 μg/ml Rho123 for another 1 h (G) or 10 min (H) at 37°C. Data are presented as means ± SD (n = 3). $^{**}p<0.01$ *vs*. control.

transfected K562 or MCF-7 cells grew faster compared with SC groups after ADM exposure (Fig 5B). Mice were sacrificed after 4 weeks of ADM injection. Tumor volumes and weights significantly decreased with the overexpression of BHLHE40 (Fig 5C-D), and markedly increased in the BHLHE40 knockdown group (Fig. 5E-F). Taken together, these data indicate that BHLHE40 plays a role of reversing ADM-resistance *in vivo*.

BHLHE40 downregulates the transcriptional expression of ABCB1 by directly binding to its promoter

As a transcription factor, we further analyzed whether BHLHE40 directly regulated ABCB1 at the transcriptional level. To test this hypothesis, bioinformatics analysis using JASPAR database was used and five binding regions were chosen as candidates in homo sapiens *ABCB1* promoter (-5052 to +120 bp) (Fig 6A). After co-transfected pGL3-ABCB1-A ∼ E plasmids into HEK-293T cells with BHLHE40 expression plasmid or empty vector, luciferase reporter assay revealed that overexpression of BHLHE40 significantly reduced the activity of the *ABCB1* promoter and the fourth binding region (-1605 to -1597 bp) was the key regions required for BHLHE40 binding (Fig 6B). To further investigate BHLHE40 binding region in the *ABCB1* promoter, pGL3-ABCB1-A and -D were chosen and respective constructs with site-directed mutagenesis of binding regions were generated (Fig 6C). The result showed that the relative luciferase activity was significantly suppressed with mutant binding region compared with the ones with wild type binding region (Fig 6D). This result was confirmed by a ChIP-PCR assay, we determined that the

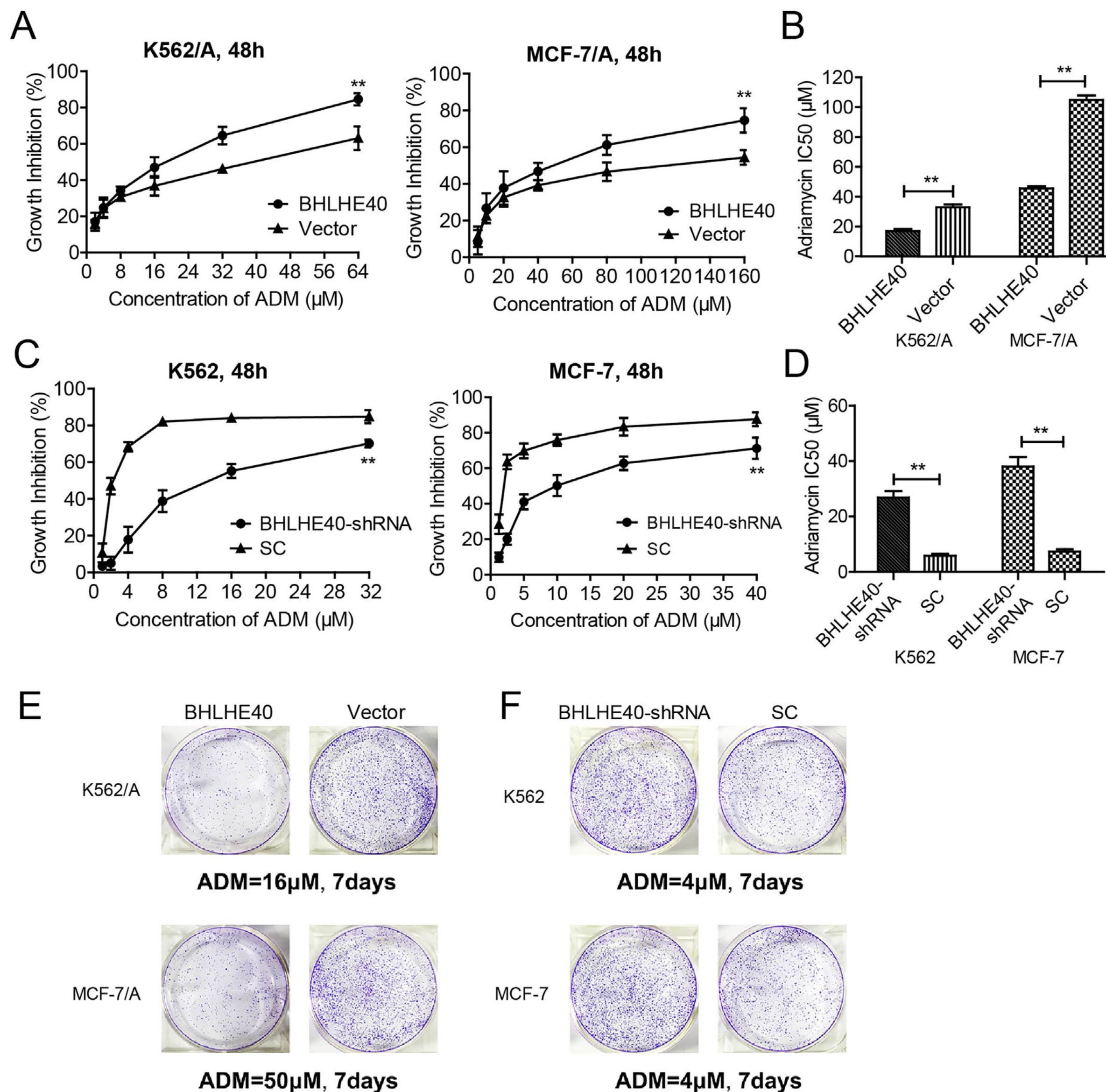

Fig. 3. Manipulation of BHLHE40 alters ADM sensitivity *in vitro*. (A) ADM resistant cells were transfected with BHLHE40 or empty vector, and cell viability was evaluated using the CCK-8 assay after the exposure to various concentrations of ADM for 48 h. (B) IC50 of ADM was calculated in the resistant cells. (C) The parental cells were transfected with shRNA-BHLHE40 or shRNA-SC, and cell viability was measured using CCK-8 assay after the exposure to the different concentrations of ADM for 48 h. (D) IC50 of ADM was calculated in the parental cells. (E) Cell proliferation in response to ADM was examined using colony formation analysis after exposed to the indicated concentrations of ADM for 7 days. Data are presented as means ± SD (n = 3). $^{**}P$ <0.01 *vs*. control.

crucial binding region resided within -1605 to -1597 bp (cgtgacgtg) of the *ABCB1* promoter (Fig 6E-F). These findings demonstrate that ABCB1 is a direct BHLHE40 target gene.

BHLHE40 expression is negatively associated with ABCB1 in cancer cell lines and tumor samples from cancer patients

High-throughput RNA sequencing (RNA-seq) has provided a useful platform for the transcriptomic analysis of cancer samples. To determine the correlation between BHLHE40 and ABCB1 in human cancer samples, RNA-seq data from the Cancer Genome Atlas (TCGA) of GEPIA online database has been analyzed [24]. In breast invasive carcinoma (BRCA) patients, *ABCB1* was significantly decreased, while *BHLHE40* increased in tumor versus non-tumor. The expression of the two genes was conversely consistent in acute myeloid leukemia (LAML) with increased *ABCB1* and decreased *BHLHE4*0 in tumors (Fig 7A). Further, the expression of *BHLHE40* was compared with that of *ABCB1* in tumor tissues of well-known MDR-associated cancer types, including breast BRCA, LAML, colon adenocarcinoma (COAD), rectum adenocarcinoma (READ) and liver hepatocellular carcinoma (LIHC) [25–28]. It exhibited that *BHLHE40* was negatively correlated with *ABCB1* in human cancer tumor samples (Fig 7B). Furthermore, higher expression of *BHLHE40* was positively correlated with better overall survival in breast cancer patients (Fig 7C). Using ProteomicsDB (www.ProteomicsDB.org) to analyze the protein expression of ABCB1 and BHLHE40 in cell lines, it was supported that the above two were negatively corelated in a broad range of cancer cells (Fig 7D). Next, we studied the relationship between mRNA expression of *ABCB1* and *BHLHE40* versus clinical outcome using a Kaplan–Meier plotter (https://kmplot.com/analysis/). *ABCB1* high expression was found to be correlated to significantly lower overall survival rate for all liver cancer patients, while *BHLHE40* high expression was correlated to higher overall survival rate for most liver cancer pa-

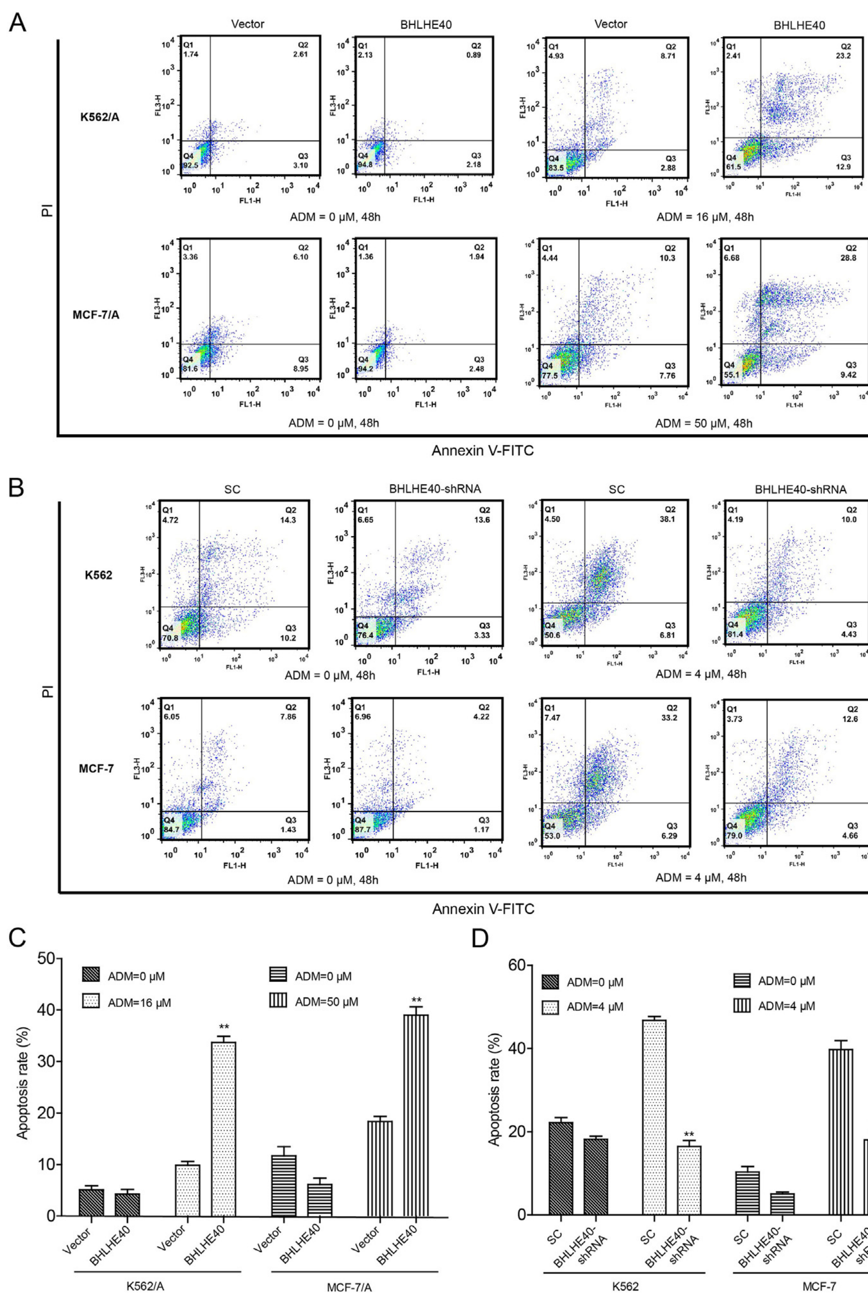

Fig. 4. BHLHE40 promotes cell apoptosis in response to ADM by regulating the activity of P-gp. (A, C) Effects of overexpressing BHLHE40 on apoptosis in resistant cells (K562/A or MCF-7/A) were analyzed after exposed to ADM for 48 h using Annexin V-FITC and propidium iodide staining assay. (B, D) Effects of BHLHE40 downregulation on apoptosis in parental (K562 or MCF-7) cells after exposed to ADM for 48 h. Data are presented as means ± SD (n = 3). $^{**}p<0.01$ *vs*. control.

tients (Fig 7E-F). It indicates an inverse effect on the clinical outcome between the two genes.

Following, the expression level of *BHLHE40* and *ABCB1* was compared with cellular sensitivities to the treatment of ADM in a gene expression dataset of human cancer cell lines [29]. Oncomine analysis of ADM sensitivity *vs*. ADM resistance in Shimokuni Cellline 2 Dataset (20 samples) showed that the expression of *BHLHE40* was decreased in ADM resistant cell lines, while *ABCB1* was increased, compared with ADM sensitive cell lines (Fig 7G-H). These results confirm that *BHLHE40* negatively regulates the expression of *ABCB1* in human cancer samples and subsequently reverses chemoresistance to ADM.

Discussion

MDR remains a major cause of treatment failure in cancer patients, despite the availability of numerous chemotherapeutics. Strategies to overcome P-gp-mediated MDR have been partially explored, including the design of novel drugs that evade recognition, inhibitors for ATP hydrolysis, as well as small molecules that are selectively lethal to P-gp-expressing cells [30–34]. The development of more specific or potent P-gp inhibitors is facilitated by the recent understanding of structural interaction between P-gp and some small-molecule compounds [35]. However, the above programs are still halted in clinical trials mostly due to high toxicity, low potency and lack of translatability including verapamil, quinidine and elacridar [36–39]. Thus, it is necessary to further clarify the mechanisms regulating ABCB1 activity to find a new applicable treatment target.

In all the factors that may alter the transportation capacity of ABC transporters, regulated expression contributes the most [40]. The identification of transcription factors to regulate the expression of ABCB1 is therefore important for manipulating its activity. In our previous study, we find BHLHE40 acts as a repressor for the expression of target genes including drug metabolizing enzyme CYP3A4 [41]. By analyzing the ChIP-seq results on mouse and human cells from GEO datasets

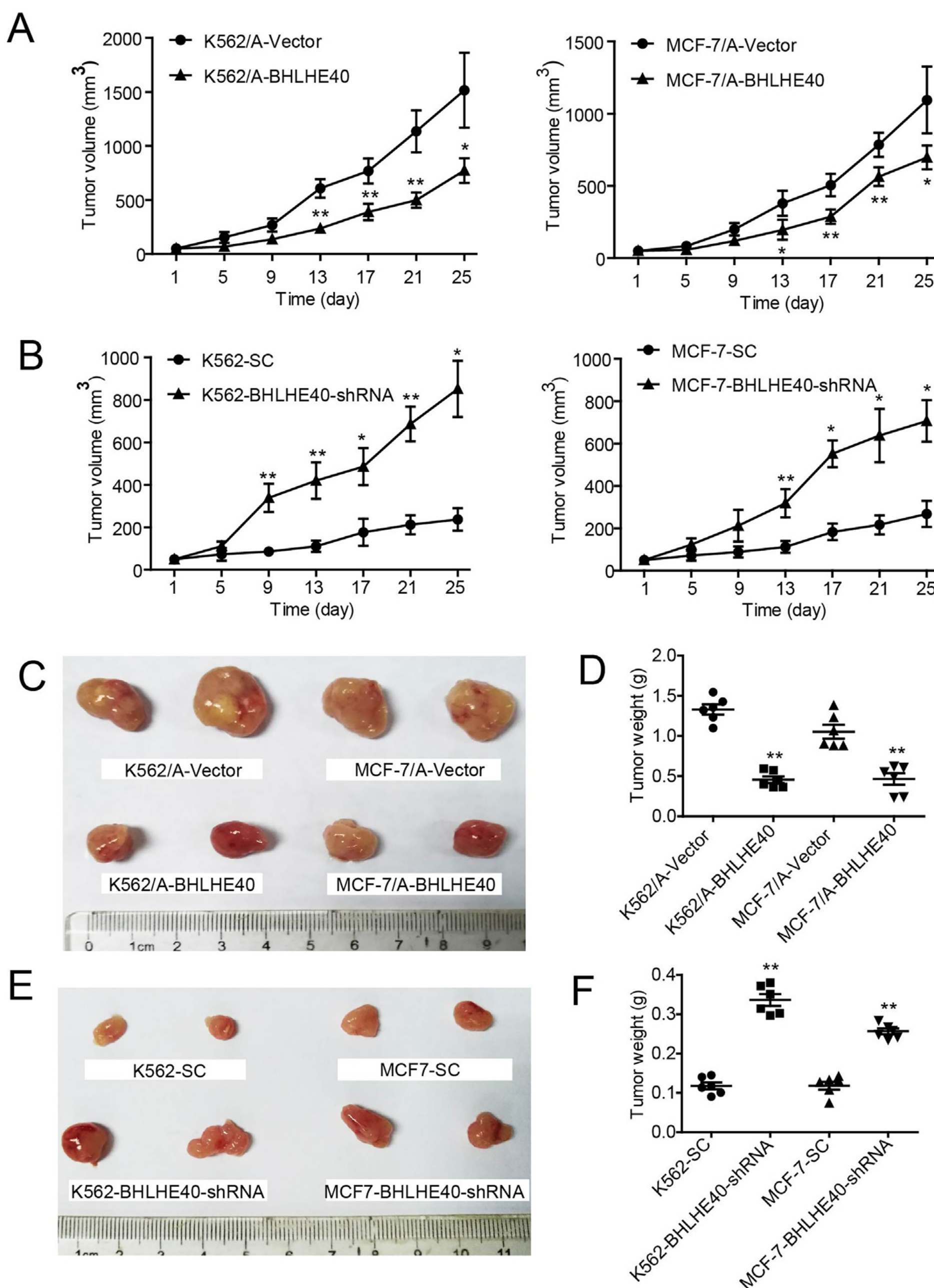

Fig. 5. BHLHE40 improves chemosensitivity to ADM *in vivo*. (A, B) Stably transfected cells were injected into nude mice. When volume of tumors reached 50 mm^3, mice were injected with ADM (5 mg/kg). Tumor volumes were measured with Vernier calipers at the indicated time periods. Each group included 6 mice. (C, E) Representative images of tumors obtained from mice which were sacrificed after 4 weeks drug exposure. (D, F) Weight of tumors in respective groups. Data are presented as means ± SD (n = 6). $^*p<0.05$, $^{**}p<0.01$ *vs*. control.

(GSE125729, GSM935616, GSM935566 and GSE106000), ABCB1 outstands from other representative chemoresistance-associated ABC transporters by binding to BHLHE40 in the promoter (Fig 1A-D). It rings a bell that BHLHE40 might directly repress the transcriptional regulation of *ABCB1* and therefore improve the response to chemotherapeutical strategies. It is confirmed by the negative correlation between BHLHE40 and *ABCB1* (P-gp) in the parental and chemoresistant cells (Fig 1H-I). By manipulating the expression level of BHLHE40, it is supported that knockdown restricts efficacy of ADM in the parental cells while overexpression reverses chemoresistance *in vivo* and *in vitro* (Fig 3-4). Further experiments identify the binding site of BHLHE40 in the promoter of *ABCB1* by luciferase reporter and ChIP-PCR assays (Fig 6A-F). The present study establishes a novel role of transcription factor BHLHE40 in negatively regulating the expression and activity of ABCB1. Conversely, several transcription factors are reported to stimulate the expression of ABCB1 by activating its promoter activity, including E2F1, MyoD, PEA3, Trps1 and BMI1 [42–45]. It is interesting that BMI1 positively regulates ABCB1 as a well-established transcription repressor, which is distinct with BHLHE40. How the above transcription factors coordinate to regulate the expression of ABCB1 with the application of chemotherapeutics in cancer scenario still need further elucidation.

The substrates of ABC transporters have low specificity regarding chemical structures, which includes a variety of endogenous or exogenous ones. Typically, P-gp has a wide range of substrates, including ADM, vinblastine and paclitaxel. Increased expression and activity of P-gp is reported to confer resistance to multiple anticancer drugs including but not limited to those mentioned drugs and tyrosine kinase inhibitors [46,47]. Therefore, the establishment of K562/A cells and MCF-7/A cells based on prolonged treatment of ADM on their parental cells is a frequently used model to evaluate multidrug resistance *in vitro* and *in vivo* [48]. Multiple mechanisms have been documented beneath the effects of tumor cell death induced by ADM, including topoisomerase II poison, casing DNA damage and cytotoxicity [49]. In the present study, we firstly found that the expression of BHLHE40 is negatively correlated

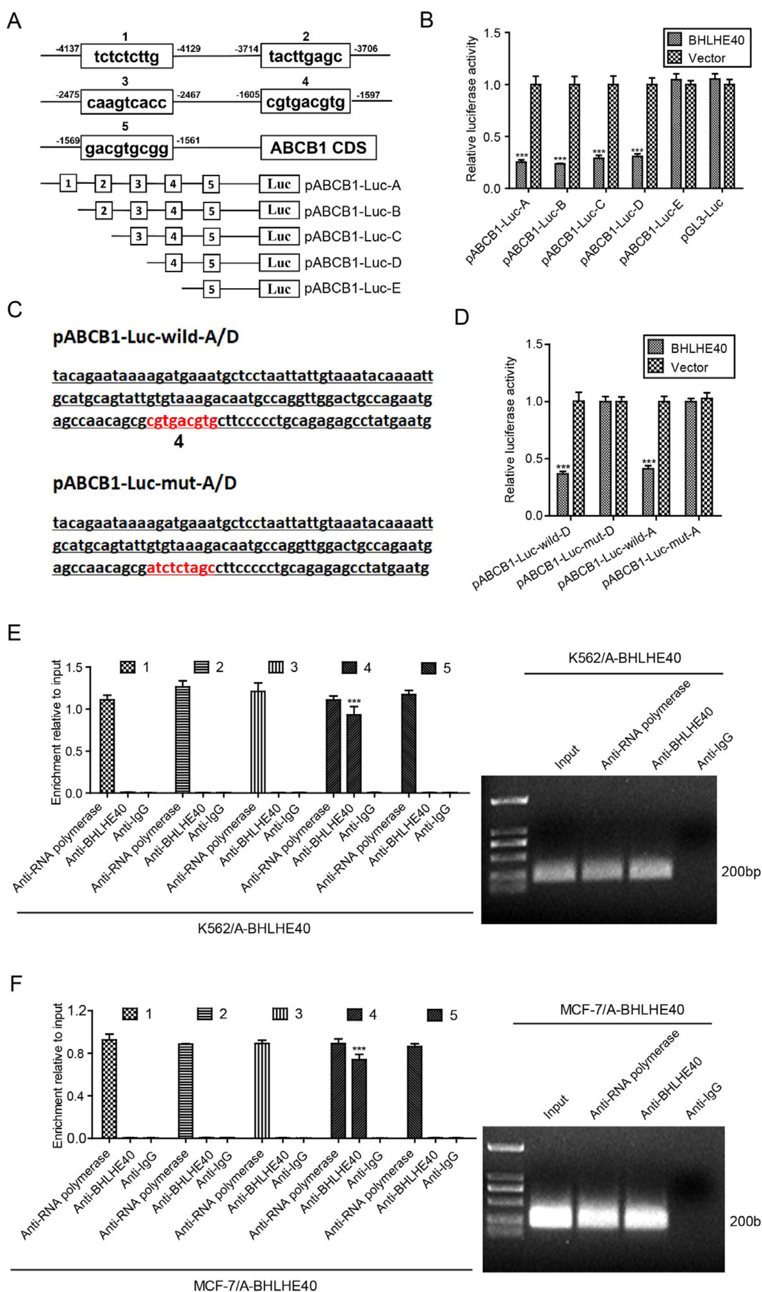

Fig. 6. BHLHE40 suppresses ABCB1 transcription by directly binding to its promoter. (A) Different lengths of ABCB1 promoter with predicted sites for BHLHE40 binding were cloned into a luciferase reporter construct based on the prediction using JASPAR database. (B) Luciferase reporter assay confirms direct binding site of BHLHE40 on the ABCB1 promoter. Luciferase activity was measured and normalized to β-galactosidase activity. (C, D) Wild type and mutant binding sites of BHLHE40 on the ABCB1 promoter were constructed and analyzed using luciferase reporter assay. (E, F) ChIP-PCR assay was performed to demonstrate the direct binding sites of BHLHE40 on the ABCB1 promoter. PCR analysis on the specific binding sites were performed using primers as shown in supplementary table 2. Anti-RNA polymerase antibody was used as positive control and normalized to IgG. Data are presented as means ± SD (n = 3), $^{***}p<0.001$ *vs*. control.

with the expression level of ABCB1 in the parental and chemoresistant cells (Fig 1H-I). As ABCB1 is a major transporter for ADM, it decreases the concentration of ADM intracellularly by exporting and its increased expression results in the resistance to ADM. Although we didn't investigate the mechanisms of ADM involved in the effect of BHLHE40, we observed significantly altered anti-cancer efficacy of ADM in cell viability and colony formation with the manipulation of BHLHE40 (Fig 3-4). We speculate that all the above mechanisms of ADM should be connected with its altered anti-cancer activity resulting from the regulated expression and activity of ABCB1 by BHLHE40 (Fig 2), since the application of ADM has been reported to induce DNA damage and topoisomerase II poison in both K562 and MCF7 cell lines [50,51]. Our findings are also supported by the report that ABCB1 protects the cancer cells from DNA damage induced by ADM [52].

BHLHE40 is involved in a variety of physiological processes of tumor cells with obscure and diverse mechanisms. Some papers documented that the expression of BHLHE40 is elevated in patients and correlated positively with tumor growth [53]. To the contrary, no significant changes in BHLHE40 expression was reported between cancer and normal tissues in human endometrial cancer and non-small-cell lung cancer [54]. There are also some studies on the expression profiles of oral, lung, liver and esophageal cancer showing that BHLHE40 expression inversely correlated with the tumor stage or differentiation grade. These findings suggest that strongly expressed BHLHE40 indicate a better prognosis [55–57]. Specifically, the overexpression of BHLHE40 was demonstrated to directly inhibit expression of CCND1 and TWIST1, therefore suppress cell proliferation, migration or invasion [55,58]. Inconsistently, BHLHE40 knockdown significantly reduced primary tumor growth and lung metastasis in orthotopic xenograft and experimental metastasis models of breast cancer [59]. Some literatures indicate the transactivation of oncogenic factors, activation of tumorigenic signaling and suppression of cell death pathway induced by BHLHE40 [60–62], which suggest a carcinogenic activity of this transcription factor. However, it is also reported to inhibit cell cycle progression of

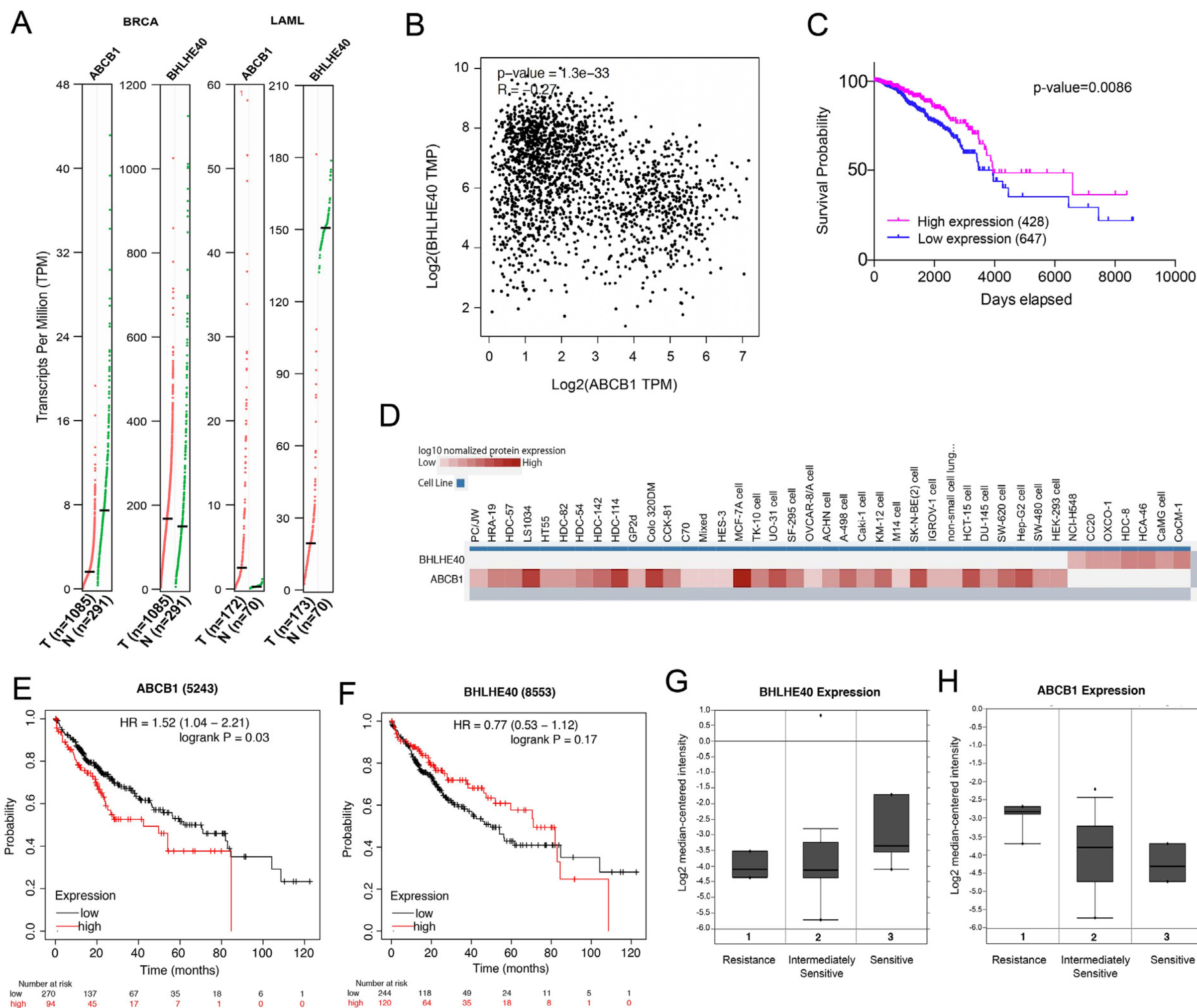

Fig. 7. BHLHE40 expression is negatively associated with ABCB1 in cancer cell lines and tumor samples from cancer patients. (A) The expression of BHLHE40 and ABCB1 in tumor and non-tumor tissue of breast invasive carcinoma (BRCA) and acute myeloid leukemia (LAML) patients. (B) The correlation between BHLHE40 and ABCB1 in the tumors of MDR-associated cancer types, including BRCA, LAML, colon adenocarcinoma (COAD), rectum adenocarcinoma (READ) and liver hepatocellular carcinoma (LIHC), was analyzed using TCGA database. (C) Overall survival curves from Kaplan-Meier plot on the analysis of a published microarray dataset from patients of breast cancer with sole chemotherapy were shown based on the expression level of BHLHE40. (D) The protein expression of ABCB1 and BHLHE40 was analyzed in cell lines using ProteomicsDB (www.ProteomicsDB.org). (E, F) The prognostic value of ABCB1 and BHLHE40 expression in liver cancer patients using Kaplan–Meier analysis. (G, H) Box plots derived from gene expression data in Oncomine comparing expression of BHLHE40 and ABCB1 in Shimokuni Cellline 2 Dataset.

breast cancer cells by stabilizing cyclin E in the cytosol to suppress the growth of breast cancer cells *in vitro* and *in vivo* [63]. The findings indicate a tumor-suppressing role of BHLHE40 through the interaction with proteins in cytosol, distinct with its oncogenic role in nucleus. As a transcription factor, BHLHE40 expresses in the nucleus and binds to the hexanucleaotide E-box (CANNTG) sequences, or SP1 domains in the promoters of target genes. Both nuclear and cytoplasmic compartments are stained for BHLHE40 in various cancer tissue samples, the subcellular localization thus largely determines its function [64]. However, the mechanisms modulating the subcellular localization of BHLHE40 is still unclear. In addition, the availability of the cooperative transcription factors or coregulators in various cancers cells and tissues might be another possible explanation for the contradictory findings regarding the function and regulation of BHLHE40. By revealing the role of BHLHE40 reversing chemoresistance in the present study, we have lifted the mist a bit more to the clearer understanding of this transcription factor. However, we can not exclude the involvement of other mechanisms besides ABCB1 in the chemosensitivity mediated by BHLHE40, which deserves better elucidation in the future.

BHLHE40 is regulated by many chemicals or drugs, therefore, it is considered as a potential drug target for cancer treatment, according to previous findings from both our and other groups. However, the current evidence approaches to an agreement with a dual function of BHLHE40 [64], thus the regulating mechanisms are of great importance to expand its potential as a drug target in the future. Paclitaxel upregulates BHLHE40 and increases the expression of p53 through positive feedback mechanisms, leading to the activation of mitochondrial apoptotic pathways in MCF-7 cells [65]. Conversely, our group have reported that overexpression of BHLHE40 partially abolishes the decrease of survivin and the activation of caspase-3 induced by 8-Methoxypsoralen (8-MOP), a naturally occurring compound [66]. Overexpression of BHLHE40 also promoted EMT process via suppressing expression of epithelial protein and enhancing expression of mesenchymal proteins, while potently antagonized the regulation of EMT-associated genes by 8-MOP [67]. As shown in previous study, simple overexpression of BHLHE40 doesn't significantly alter the apoptotic rate in HCC cells. Consistently, BHLHE40 doesn't induce apoptosis in K562/A and MCF7/A cells without the combination of ADM either, but obviously elevates the apoptotic rate after exposure to ADM (Fig 3C, E). Above all, it suggests that BHLHE40 play

a crucial role for regaining sensitivity to ADM in resistant cells through the downregulation of ABCB1, besides being a potential target for hepatocellular carcinoma by promoting EMT process.

Recently, it is highlighted that BHLHE40 is required for the mitochondrial fitness and epigenetic programming of $CD8^+$ tissue-resident memory (Trm) cells and tumor-infiltrating lymphocytes (TIL). BHLHE40 is important for the development and functionality of Trm cells and TILs, as its deficiency impairs the production of metabolites required for Acetyl-CoA synthesis and results in the altered status of histone acetylation for the expression of functional factors [21]. BHLHE40 also mediates mitochondrial dysfunction induced by PD-1 [68]. It is thus speculated that BHLHE40 has potential to be developed as a strategy to achieve more effective cancer immunotherapy. Based on the present study, we proposed a hypothesis that the activation of BHLHE40 could be synergistically beneficial to the cancer patients by elevating the efficacy of immunotherapies, as well as the reversal of chemoresistance.

In conclusion, we have shown that BHLHE40 is an important modulator for ADM resistance by negatively regulating the expression of ABCB1. Manipulation of BHLHE40 is sufficient to alter the transportation capability of P-gp, leading to the induction or reversal of drug resistance to ADM in the parental or resistant cells, respectively. Furthermore, our data provide solid evidence that BHLHE40 inhibits *ABCB1* gene transcription by directly binding to its promoter. Therefore, it is speculated that BHLHE40 serves as a potential therapeutic target against MDR based on the present findings. The properties of BHLHE40 in clinical MDR warrant full elucidation in the future.

Declaration of Competing Interest

None.

CRediT authorship contribution statement

Yongmei Yin: Investigation, Visualization, Writing – review & editing. **Yu Xin:** Investigation, Data curation, Writing – original draft. **Feng Zhang:** Investigation, Data curation. **Donghao An:** Investigation, Data curation. **Hui Fan:** Validation, Visualization. **Mengyao Qin:** Validation. **Jinxin Xia:** Validation. **Tao Xi:** Resources, Writing – review & editing. **Jing Xiong:** Conceptualization, Data curation, Visualization, Resources, Writing – original draft, Writing – review & editing, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2023.100891.

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