H2A.Z regulates tumorigenesis, metastasis and sensitivity to cisplatin in intrahepatic cholangiocarcinoma

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Abstract. Intrahepatic cholangiocarcinoma (ICC) is a fatal, malignant tumor of the liver; effective diagnostic biomarkers and therapeutic targets for ICC have not been identified yet. High expression of H2A histone family member Z (H2A.Z) is a high-risk factor for poor prognosis in patients with breast cancer and primary hepatocellular cancer. However, the significance of H2A.Z and its expression in ICC remains unknown. The present study demonstrated that H2A.Z is overexpressed in ICC and expression of H2A.Z correlated with poor prognosis in patients with ICC. H2A.Z regulated cell proliferation *in vitro* and *in vivo* via H2A.Z/S-phase kinase-associated protein 2/p27/p21 signaling. Inhibition of H2A.Z reduced cell proliferation and induced apoptosis in ICC. In addition, down-regulation of H2AZ reduced tumor metastasis by repressing epithelial-mesenchymal transition and enhanced the antitumor effects of cisplatin in the treatment of ICC. Overall, H2A.Z promoted cell proliferation and epithelial-mesenchymal transition in ICC, suggesting that H2A.Z may be a novel biomarker and therapeutic target for ICC.

Introduction

Intrahepatic cholangiocarcinoma (ICC) and extrahepatic cholangiocarcinoma (EHCC) are two subtypes of cholangiocarcinoma (CC), which is the most common and deadly malignant tumor in the biliary tract. ICC originates form the liver parenchyma, and, worldwide, it is the most common hepatic malignant tumor after hepatocellular carcinoma (HCC). The incidence and mortality rates of ICC have increased during the last three decades (1-3). Unfortunately, there are no effective molecular targets for ICC, as is the case for other cancers, such as lung and breast cancer (4). Thus, it is necessary to identify new biomarkers and therapeutic targets for ICC.

H2A histone family member Z (H2A.Z) is a variant of histone H2A, serving an important role in regulating chromatin remodeling, gene expression and other basic cellular processes. The expression of H2A.Z was first reported in normal mouse tissues, human HeLa cells and chicken erythrocytes (5). H2A.Z is a key molecule in DNA replication, chromosome segregation and maintenance of the heterochromatic status (6-8), and has a pivotal role in regulating cell cycle transition in yeast (9). Furthermore, H2A.Z is crucial in the proliferation and differentiation of distributed stem cells (DSCs) (10,11), and is essential in the early development of mammals (12). H2A.Z is overexpressed in multiple malignant tumors, including breast cancer (13), prostate cancer (14), bladder cancer (15) and malignant melanoma (16). H2A.Z1, an isoform of H2A.Z, is upregulated in both HCC specimens and cell lines, and depletion of H2A.Z1 induces the expression of cell cycle inhibitors cyclin-dependent kinase inhibitor 1A (CDKN1A, also known

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as p21) and cyclin-dependent kinase inhibitor 1B (CDKN1B, also known as p27) in HCC (17). S-phase kinase-associated protein 2 (Skp2) is part of the E3 ubiquitin ligase complex that regulates many substrate proteins, including p27 and p21, through ubiquitination (18). This protein is encoded by *skp2*, an established proto-oncogene in many cancers that promotes cancer progression and metastasis (19,20). However, the relationship between ICC and H2A.Z, and whether H2A.Z regulates p27/p21 via Skp2 in ICC remains unclear.

In the present study, H2A.Z was demonstrated to be highly expressed in ICC tissues compared with normal tissues and high expression of H2A.Z correlated with poor prognosis in patients with ICC. H2A.Z promoted ICC progression by regulating cell cycle transition, reducing cell apoptosis and inhibiting epithelial-mesenchymal transition (EMT). The results also demonstrated that reduction of H2A.Z enhanced the antitumor effect of cisplatin on ICC cells. The present results suggest that H2A.Z may serve as a diagnostic biomarker and an effective therapeutic target in ICC.

Materials and methods

Research involving human participants and animals. For this type of study, informed consent was obtained from all patients at the original time of collection (2009-2012) for the storage and use of their tissue. The Clinical Specimens Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) approved the present research.

All animal experiments were performed according to the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, 2011). All experiments were approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China), and all procedures performed on animals were in accordance with the ethical standards of the First Affiliated Hospital of Zhejiang University School of Medicine.

ICC tissue samples. In the present study, 28 samples of ICC tissues with matching peritumoral tissues were collected between 2009 and 2012 at the First Affiliated Hospital, Zhejiang University School of Medicine (Hangzhou, China). Immunohistochemistry was performed as previously described (21), and data obtained were used to perform survival analysis. Eight samples of ICC tissues with paired peritumoral tissues were randomly selected to measure the expression of H2A.Z. The assessment of the staining was based on the following formula: Total score = the score of the % of positively stained cells over total x the score of the staining intensity. The scores of the % of positively stained cells were as follows: 0, ≤5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; 4, >75%. The staining intensity scores were as follows: 1, low intensity; 2, medium intensity; 3, high intensity. A total score ≤6 was considered indicative of low expression of H2A.Z, while a total score >6 was considered indicative of high expression of H2A.Z.

Cell culture. Four ICC cell lines (CCLP-1, HCCC-9810, RBE and HuCCT-1) and a normal human intrahepatic biliary epithelial cell line (HIBEC) were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences, (Shanghai, China). Cells were cultivated according to the protocols from their supplier. All cell lines were grown in RPMI-1640 complete medium (Biological Industries, Kibbutz Beit-Haemek, Israel) supplemented with 10% fetal bovine serum (FBS; Moregate Biotech, Brisbane, Australia), and were cultured in an incubator of 37°C and 5% CO_2.

Antibodies. For western blotting and immunohistochemistry analyses, the following antibodies were purchased from Abcam (Cambridge, UK): anti-H2A.Z (cat no. ab150402; dilution 1:1,000 for western blot analysis and dilution 1:300 for immunohistochemistry), anti-p21/WAF1/Cip1 (cat. no. ab109520), anti-p27/Kip1 (cat. no. ab32034), anti-Skp2 (cat. no. ab124799), anti-cyclinA (cat. no. ab181591) and matrix metalloproteinase (MMP)2 (cat. no. ab37150). The following antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA): anti-cyclin-dependent kinase (CDK) 4 (cat. no. 12790), anti-CDK6 (cat. no. 3136) and anti-CDK2 (cat. no. 2546). Ki67 (cat. no. ab15580; Abcam) was used for the immunohistochemical analysis of mouse tumor tissue. The following antibodies were purchased from Epitomics (Burlingame, CA, USA): anti-β-actin (cat. no. 1854-1), and anti-GAPDH (cat. no. 5632-1). A kit with antibodies targeting EMT-associated proteins (E-cadherin, N-cadherin, Slug, Snail, and Vimentin) was obtained from Cell Signaling Technology, Inc. (cat, no. 9782S). Antibodies, recognizing total (#9665) and cleaved caspase-3 (#9664), caspase-9 (#9508), Bcl-2 homologous antagonist/killer (Bak) (#12105) and B-cell lymphoma 2 (Bcl-2) (#15071) were obtained from Cell Signaling Technology, Inc.

RNA silencing. H2A.Z short interfering (si) RNA (two different sequences tested; siA, GGAACAUUCUGCAGUAUAAAGG GAG; and siB, GGACUCUAAAUACUCUAACAGCUGT) and negative control siRNA (UUCUCCGAACGUGUCACGU) were purchased from Origene Technologies, Inc. (Rockville, MD, USA). siRNA (10 nM) was transfected with 5 μl/ml Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Firstly, cells (2×10^5 per well) were seeded in 6-well plates and incubated at 37°C for 24 h. A total of 5 μl siRNA was added into 250 μl RPMI-1640, and 5 μl Lipofectamine 2000 was added to another 250 μl RPMI-1640, and the two solutions were incubated at 24°C for 5 min. Next, the two solutions were mixed together and incubated at 24°C for 20 min. The supernatant of the wells was removed, and the transfection solution and 1.5 ml RPMI-1640 medium were added and incubated at 37°C for 8 h. After 8 h, the supernatant was removed and 2 ml RPMI-1640 medium with 10% FBS were added, and the following experiments were performed at 24 h after transfection.

For stable H2A.Z knockdown, H2A.Z short hairpin RNA (shRNA) lentiviral vectors (lenti-shRNA/H2A.Z) and negative control lentivirus [expressing green fluorescent protein (GFP)] were purchased from GeneChem Co., Ltd. (Shanghai, China). Target cells were infected with lentivirus of shH2A.Z and negative control groups at the concentration of 1×10^8 TU/ml. Cells (2×10^5 per well) were seeded in 6-well plates and incubated for 24 h. Next, the supernatant was removed, and 1 ml RPMI-1640 was mixed with 1×10^8

TU lentivirus and 1μl polybrene and incubated at 37˚C for 6 h. After 6 h, the supernatant was changed with culture medium. The concentration of lentivirus and the conditions of infection were according to the manufacturer's instructions. Cells expressing shH2A.Z were selected in culture medium containing puromycin (3 μg/ml). The H2A.Z shRNA target sequence was ACTTGAACTGG CAGGAAAT.

Western blotting. CC tissues and cells were lysed using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and sonicated. Lysates containing soluble proteins were collected and stored at -80˚C. Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal amounts (30 μg) of proteins were separated by 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes for 1.5 h. The membranes were washed one time with TBS/0.1% Tween-20 (TBST) buffer, and incubated with a solution containing the primary antibody (1:1,000) at 4˚C overnight. Then, the membranes were washed three times with TBST, and incubated with a solution containing the horseradish peroxidase (HRP)-conjugated secondary antibody (1:3,000) for 1.5 h at 20-25˚C. Following incubation, the membranes were washed three times with TBST. Enhanced chemiluminescence (ECL) (Guge Biotechnology, China) was used to detect the immunoreactive bands, according to the manufacturer's recommendations.

Cell viability assay. A Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to assess cell viability. Cells ($5x10^3$ cells/well) were seeded in 96-well plates, and incubated in a humidified incubator for 24, 48, 72 or 96 h. The supernatant in each well was then replaced with 90 μl medium and 10 μl CCK-8 solution, and the cells were incubated at 37˚C for 1 h. The absorbance was detected at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Colony formation assay. Cells (1,000 cells/well) were seeded onto 6-well plates and incubated at 37˚C in a humidified incubator. The medium was changed every 4 days. After two weeks, the cells were washed with PBS, fixed with 100% methanol for 30 min at room temperature, and stained with 0.2% crystal violet for 15 min at room temperature. Following staining, the cells were washed with PBS three times and colonies were observed under a light microscope and counted.

Ethynyl deoxyuridine (EdU) assay. Control or H2A.Z knockdown cells ($5x10^4$) were plated in confocal dishes (2 cm diameter), and incubated for 24 h. An EdU cell proliferation assay kit (cat. no. C10310-1; Ribobio Co., Ltd., Guangzhou, China) was used for the EdU assays, according to the manufacturer's recommendations.

Cell cycle and cell apoptosis assay. Control or H2A.Z knockdown cells ($2x10^5$) were seeded into 6-well plates, and incubated for 48 h. The cells were then harvested and fixed in 75% ethanol at -20˚C for 24 h. Following fixation, the cells were resuspended and washed one time with PBS. Next, 200 μl DNA PREP Stain (Beckman Coulter, Inc., Brea, CA, USA) was added and the suspension was incubated in the dark for 20 min at room temperature. Cell cycle analysis was performed by flow cytometry, using a BD LSR II instrument (BD Biosciences, San Jose, CA, USA). Modfit LT version 5 was used for the analysis.

Cell apoptosis assays were performed as previously described (22). Specifically, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays were performed to evaluate apoptosis, according to the instructions provided by the manufacturer (Promega, Madison, WI, USA).

Wound-healing assays. Cells ($1x10^5$/well) were seeded in 24-well plates, and wounds were generated by making a scratch on the plate using a sterile tip. Cells were washed with PBS and incubated in culture medium without serum. After the indicated time, the distance between the two margins of the wound was measured. The data for the CCLP-1 cells were measured at 0, 48 and 96 h, and the data for the HCCC-9810 cells were measured at 0, 48 and 120 h, respectively.

Cell migration and invasion assays. Cell migration and invasion assays were performed using Millicell Cell Culture Inserts (24-well plates; 8 μm pore size; Merck KGaA, Darmstadt, Germany). Stably transduced cells were used for these assays. For the migration assay, $3x10^4$ CCLP-1 cells or $5x10^4$ HCCC-9810 cells in serum-free medium were seeded on the upper chambers. For the invasion assays, the membranes of the upper chambers were coated with 8 μl Matrigel (BD Biosciences) in 32 μl RPMI-1640 medium for 3 h in a humidified incubator. The cells were then seeded in the coated upper chambers. RPMI-1640 medium containing 10% FBS was added into the lower chambers. CCLP-1 cells and HCCC-9810 cell were incubated for 24 or 48 h for the migration assays, and 48 or 72 h for the invasion assays, respectively. Then, the cells on the lower membranes were stained using a Wright-Giemsa Stain kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and observed at x100 magnification. Five fields were randomly chose and cells were counted upon observation under a light microscope, the number of cell of average per field was calculated finally.

Tumor xenograft experiments. A total of 40 BALB/c male nude mice (8 weeks old) were purchased from Shanghai X-B Animal Ltd., Shanghai, China. All mice were kept in pathogen-free cages in 26-28˚C and 50% humidity. CCLP-1 expressing shH2A.Z or control cells were resuspended in 100 μl PBS and subcutaneously injected into the right side of nude mice ($5x10^6$ cells/mouse, 10 nude mice per group). Tumor volumes were measured after 7 days, and every 2 days afterwards. Tumor volume was calculated using the formula: $V (mm^3) = width^2 (mm^2) \times length (mm)/2$. All the mice were sacrificed 3 weeks after the injection and tumors were harvested for analysis.

To generate a pulmonary metastasis mouse model, $2.5x10^6$ CCLP-1 cells expressing shH2A.Z or control shRNA were resuspended in 100 μl PBS and injected into another set of BALB/c nude mice through the tail vein (10 nude mice per group). The lungs of the mice were collected after 7 weeks. Each lung was disposed into 30 sections, and H&E staining was performed to analyze the tumor clusters in the lung tissues. All tumors were examined.

Table I. Association between H2A.Z expression and clinico-pathological features.

| Variable | H2A.Z expression | | P-value |
	Low (n=8)	High (n=20)	
Sex			
Male	1	5	0.432
Female	7	15	
Age (years)			
≤54	1	8	0.17
>54	7	12	
Tumor size (cm)			
≤5	4	4	0.131
>5	4	16	
Metastasis			
Yes	5	13	0.615
No	3	7	
TNM stage			
I-II	6	5	0.022
III-IV	2	15	
Tumor number			
≤1	7	15	0.432
>1	1	5	
CA-19-9 (U/ml)			
≤40	3	7	0.615
>40	5	13	

H2A histone family member Z; TNM, tumor, node and metastasis; CA, cancer antigen.

Cisplatin treatment and H2A.Z knockdown assays. CCLP-1 cells were used for this experiment. The cells were divided into four groups: normal control (NC), H2A.Z knockdown (H2A.Z-KD), NC treated with cisplatin, and H2A.Z-KD treated with cisplatin. The cells were first seeded in 6-well plates for 24 h, and then cisplatin (4 μg/ml) was added into the cell culture medium for 48 h. Cells were then processed as described.

Statistical analysis. The SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used for statistical analyses. Analysis of variance was used for multiple comparison among groups, and Student-Newman-Keuls test was used as a post hoc test. Comparisons between two groups were assessed using a two-tailed Student's t-test. The Kaplan-Meier method was used to assess the overall survival rate of patients. Data were presented as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

Results

H2A.Z is highly expressed in ICC tissues and is associated with poor prognosis in patients with ICC. First, immunohis-

tochemistry analysis was performed on the 28 pairs of ICC specimens. The clinicopathological features of the patients that the specimens were derived from are listed in Table I. H2A.Z was overexpressed in the cancer tissues, compared with their paired non-cancer samples (Fig. 1A and B). Additionally, H2A.Z expression was significantly associated with the tumor, node and metastasis (TNM) stage of the samples (P=0.022; Table I). Eight pairs of ICC samples were randomly chosen to determine the protein expression levels of H2A.Z by western blotting. As expected, the results of the western blot analysis also revealed that the expression of H2A.Z in ICC tissues was markedly higher compared with their paired non-cancerous tissues (Fig. 1C). To elucidate the association between H2A.Z expression and overall survival, the 28 patients whose ICC samples were analyzed in the present study were divided into two groups: H2A.Z high expression group (n=18) and H2A.Z low expression group (n=10). Kaplan-Meier analysis indicated that the overall survival rate of the H2A.Z high expression cluster was higher compared with the H2A.Z low expression cluster (P=0.045; Fig. 1D).

Knockdown of H2A.Z inhibits cell proliferation of ICC cells in vitro. To better understand the role of H2A.Z in ICC, the expression of H2A.Z was investigated in HIBEC cells and ICC cell lines (CCLP-1, HuCCT-1, RBE and HCCC-9810). The expression of H2A.Z in the ICC cell lines was higher compared with the normal HIBEC cells (Fig. 2A). The two cell lines with significantly higher H2A.Z expression (CCLP-1 and HCCC-9810) were selected to perform additional experiments. To better explore the functional role of H2A.Z in ICC, H2A.Z expression was silenced in CCLP-1 and HCCC-9810 cells. The effect of knockdown was confirmed by western blot analysis (Fig. 2B and C). CCK-8 and colony formation assays were performed to examine the role of H2A.Z in the proliferation of ICC cells. H2A.Z knockdown inhibited cell viability and colony formation ability in both CCLP-1 and HCCC-9810 cells (Fig. 2D-G). These results indicated that H2AZ affects the proliferation of ICC cells.

Knockdown of H2A.Z induces cell cycle arrest in ICC cells in vitro. Cell cycle arrest, apoptosis or cellular senescence could explain the effect mediated by the H2A.Z knockdown. In EdU proliferation assays, the nuclei were weakly stained in the H2A.Z knockdown group in both CCLP-1 and HCCC-9810 cells (Fig. 3A). In the flow cytometric cell cycle analyses, the results demonstrated a significant increase in the % of cells arrested in the G1 and a decrease of the % of cells in S and G2 phases, in the H2A.Z-silenced group compared with control group (P<0.05; Fig. 3B and C). Through western blot analysis, CDK2, CDK4, cyclinA and Skp2 were demonstrated to be downregulated following H2A.Z knockdown, whereas p21 and p27 were upregulated in both CCLP-1 and HCCC-9810 cells; however, no change was observed in the level of MMP2 after H2A.Z knockdown (Fig. 3D and E). These data strongly suggested that H2A.Z knockdown results in the suppression of Skp2, upregulation of p21 and p27, and downregulation of CDK2, CDK4, CDK6 and cyclinA in ICC cells.

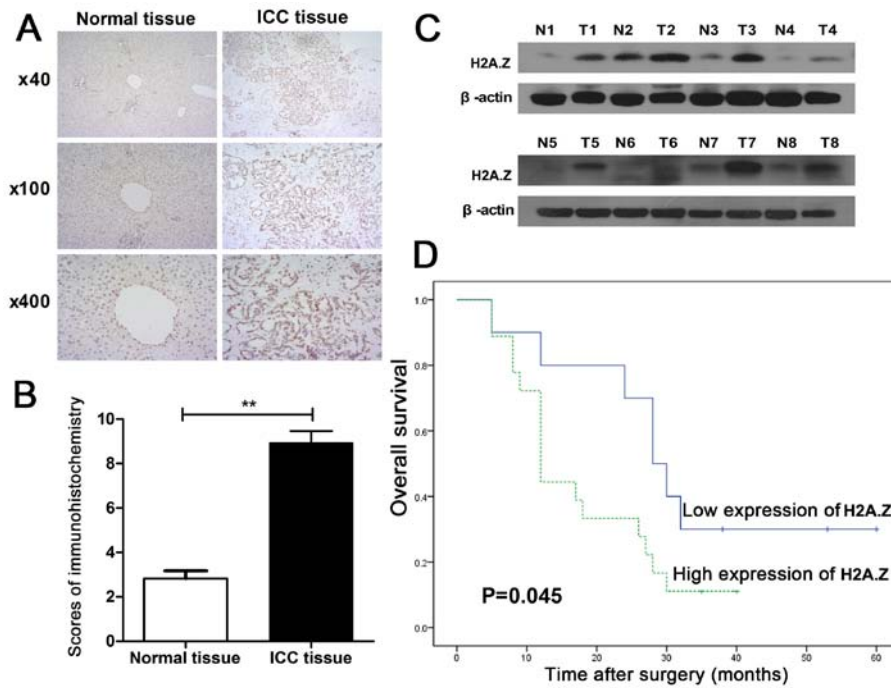To elucidate the association between H2A.Z and ICC cell apoptosis, flow cytometry analyses were conducted in

Figure 1. H2A.Z is highly expressed in ICC tissues and associated with poorer prognosis of ICC patients. (A) Representative images from immunohisto-chemical analysis of H2A.Z expression in paired ICC and normal tissue. (B) Total staining scores from the H2A.Z immunohistochemistry results in paired and peritumoral tissues. (C) H2A.Z protein expression levels were examined by western blotting in 8 pairs of ICC and normal peritumoral tissues. (D) Association between H2A.Z expression and overall survival in patients with ICC. $^{**}P<0.01$. H2A.Z, H2A histone family member Z; ICC, intrahepatic cholangiocarcinoma; N, normal; T, tumor.
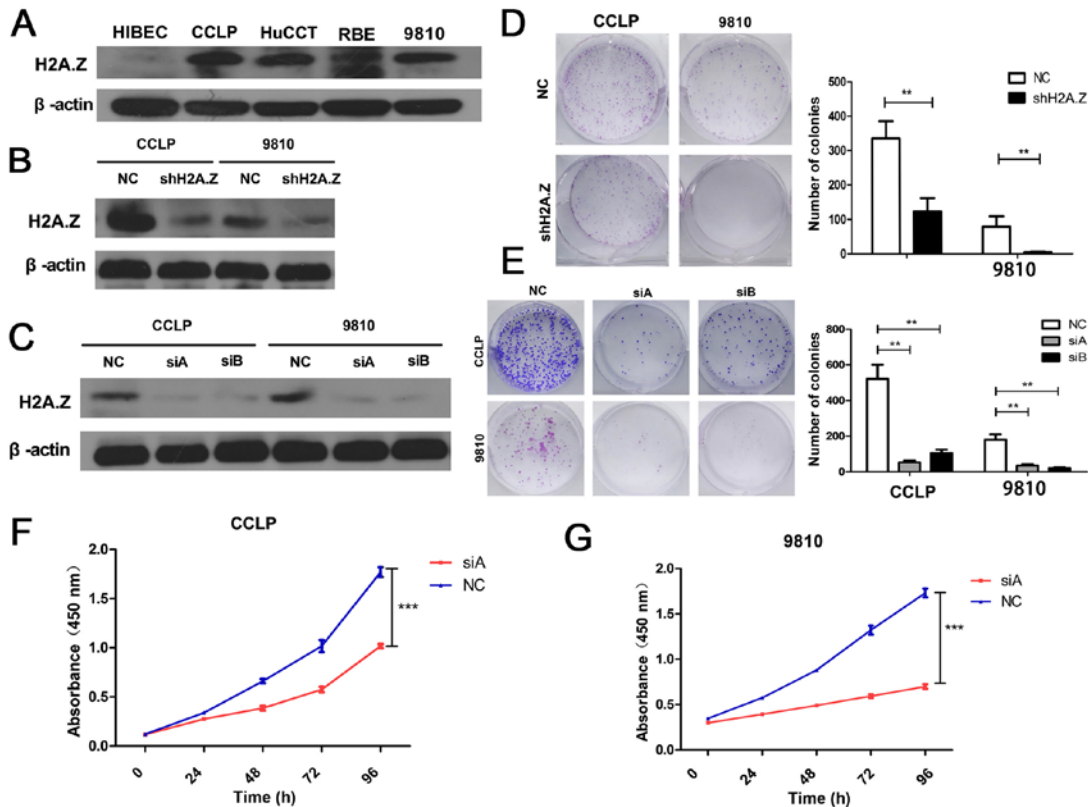

Figure 2. H2A.Z knockdown inhibits cell proliferation in ICC cells *in vitro*. (A) H2A.Z protein expression levels were examined by western blotting in various ICC cell lines (CCLP-1, HuCCT-1, RBE and HCCC-9810) and the normal HIBEC cell line. (B) H2A.Z knockdown stable cell lines were constructed in CCLP1 and HCCC-9810 cells by shRNA lentiviral transduction. (C) The efficacy of H2A.Z silencing by siRNA transfection in CCLP1 and HCCC-9810 cells was confirmed by western blotting. (D and E) Colony formation assay results for the H2A.Z knockdown (conducted both by shRNA and siRNA) or negative control CCLP1 and HCCC-9810 cells. (F and G) Cell Counting Kit-8 assay results for either siRNA or control-transfected CCLP1 and HCCC-9810 cells. Every experi-ment was repeated three times. $^{**}P<0.01$ and $^{***}P<0.001$ compared with NC. H2A.Z, H2A histone family member Z; ICC, intrahepatic cholangiocarcinoma; HIBEC, human intrahepatic biliary epithelial cell; sh, short hairpin; si, small interfering; NC, negative control.
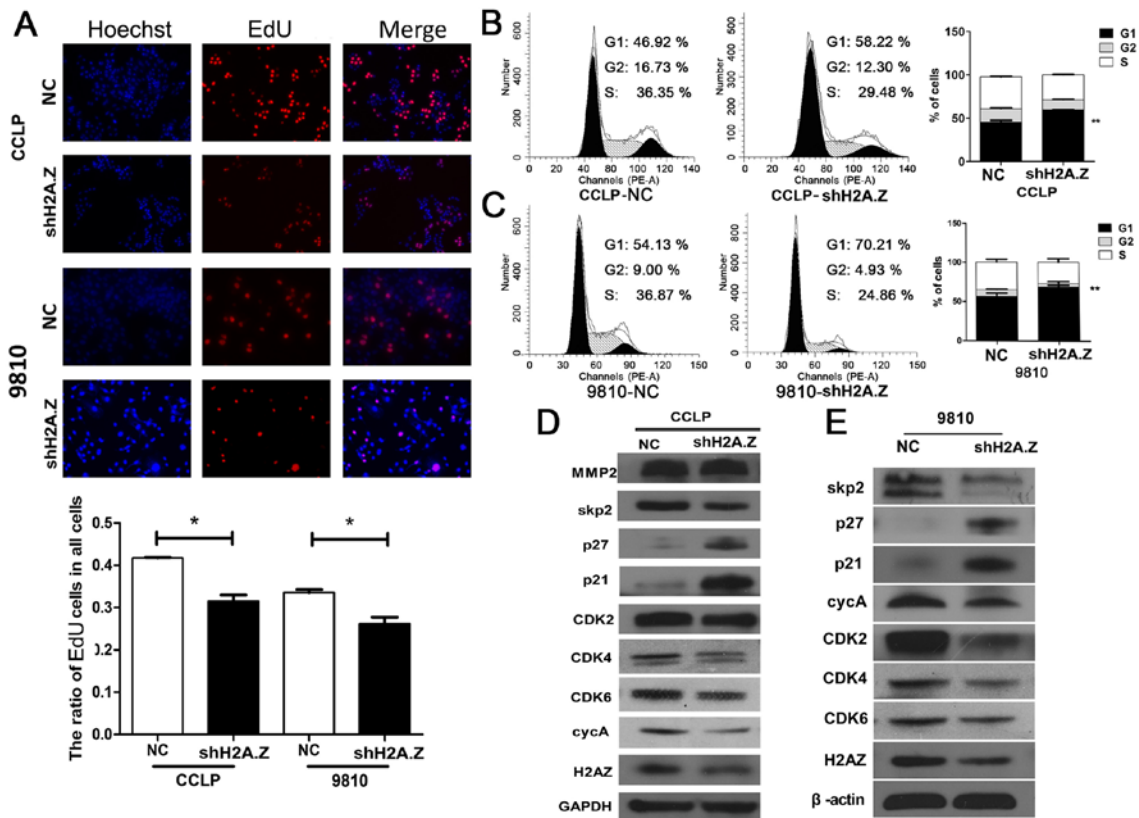
Figure 3. H2A.Z knockdown induces cell cycle arrest in ICC cells. (A) Cell proliferation was detected by EdU assay (red signal). Hoechst (blue signal) was used to counterstain the cell nuclei. Representative images and quantification are shown. (B and C) The effect of H2A.Z knockdown on cell cycle arrest was examined by flow cytometry. (D and E) CCLP-1 and HCCC-9810 cells were transfected with H2A.Z shRNA by lentivirus. The cell lysates were used to perform western blot analysis with the indicated antibodies and β-actin was used as a loading control. Every experiment was repeated three times. *P<0.05 and, **P<0.01 compared with NC. H2A.Z, H2A histone family member Z; ICC, intrahepatic cholangiocarcinoma; EdU, ethynyl deoxyuridine; sh, short hairpin; NC, negative control; Skp2, S-phase kinase-associated protein 2; p27, cyclin-dependent kinase inhibitor 1B; p21, cyclin-dependent kinase inhibitor 1A; CDK, cyclin-dependent kinase.

CCLP-1 and HCCC-9810 cells. Compared with the negative control group, the cells in which H2A.Z was knocked down displayed a significantly increased percentage of apoptotic cells (Fig. 4A). The analysis of expression of apoptosis-related proteins by western blotting revealed that H2A.Z knockdown resulted in the downregulation of Bcl-2, and the upregulation of Bak, caspase-9, and both total and cleaved caspase-3 (Fig. 4B and C).

Knockdown of H2A.Z inhibits EMT in ICC cells in vitro. To investigate whether H2A.Z has a role in cell migration and invasion, Transwell assays were performed. H2A.Z knockdown significantly decreased the motility and invasion ability of CCLP-1 and HCCC-9810 cells (Fig. 5A and B). Similarly, wound healing assays revealed that H2A.Z silencing was associated with decreased wound healing in ICC cells (Fig. 5C). To elucidate the molecular mechanism of H2A.Z in EMT, western blot analysis was performed for EMT-associated proteins in ICC cells. Notably, H2A.Z knockdown induced E-cadherin expression, while it inhibited the expression of N-cadherin, Slug and Snail in CCLP-1 and HCCC-9810 cells (Fig. 4B and C).

H2A.Z knockdown inhibits tumor growth and metastasis in vivo. Next, to demonstrate the effect of H2A.Z on ICC growth *in vivo*, H2A.Z-silenced or negative control CCLP-1 cells were injected subcutaneously into nude mice (10 mice per group). Compared with control, tumors derived from the H2A.Z knockdown cells had significantly reduced tumor growth rate, average volume and weight (Fig. 6A).

Tumors from the two experimental groups were then immunostained for H2A.Z, Ki67 and p21. H2A.Z knockdown tumors exhibited decreased Ki67 staining, but increased p21 staining (Fig. 6B). In addition, in a pulmonary experimental metastasis assay, it was demonstrated that H2A.Z knockdown cells resulted in decreased number of metastases in the lung (Fig. 6C). H2A.Z knockdown induced apoptosis in ICC cell lines (Fig. 4A). Therefore, TUNEL assays were performed in sections from the harvested tumors in order to investigate whether H2A.Z had the same effect on cell apoptosis *in vivo*. As expected, H2A.Z knockdown was associated with a higher percentage of TUNEL-positive cells in the tumors, compared with the control group (Fig. 6D).

H2A.Z knockdown strengthens the antitumor effect of cisplatin in ICC cells. Unresectable primary CC is fatal, and the median survival of these patients without treatment is ~3-6 months (23). Cisplatin is an effective antineoplastic drug in the treatment of multiple neoplasms (24,25). In specific, the hydrolysis product of cisplatin can react with DNA, producing
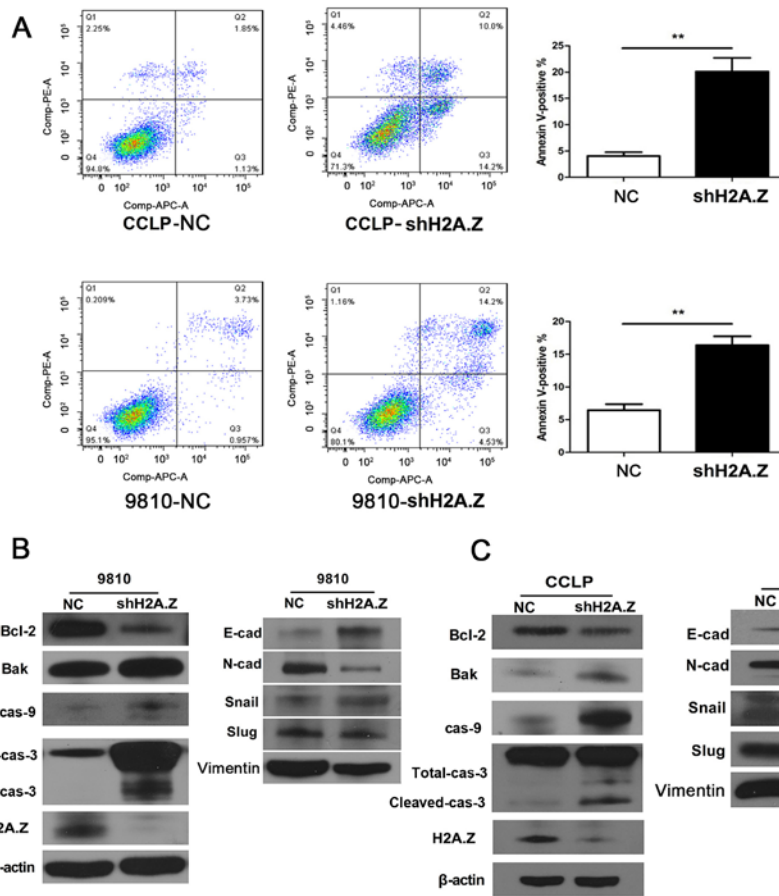
Figure 4. H2A.Z knockdown induces cell apoptosis in ICC cells. (A) The effect of H2A.Z knockdown on cell apoptosis was examined by flow cytometry, and the % of apoptotic cells was calculated in each experimental group. (B and C) CCLP-1 and HCCC-9810 cells were transfected with H2A.Z shRNA. The lysates were used to perform western blot analysis with the indicated antibodies and β-actin was used as a loading control. Every experiment was repeated three times. **P<0.01 compared with NC. H2A histone family member Z; ICC, intrahepatic cholangiocarcinoma; sh, short hairpin; NC, negative control; Bcl-2, B-cell lymphoma 2; Bak, Bcl-2 homologous antagonist/killer; cas, caspase; cad, cadherin.
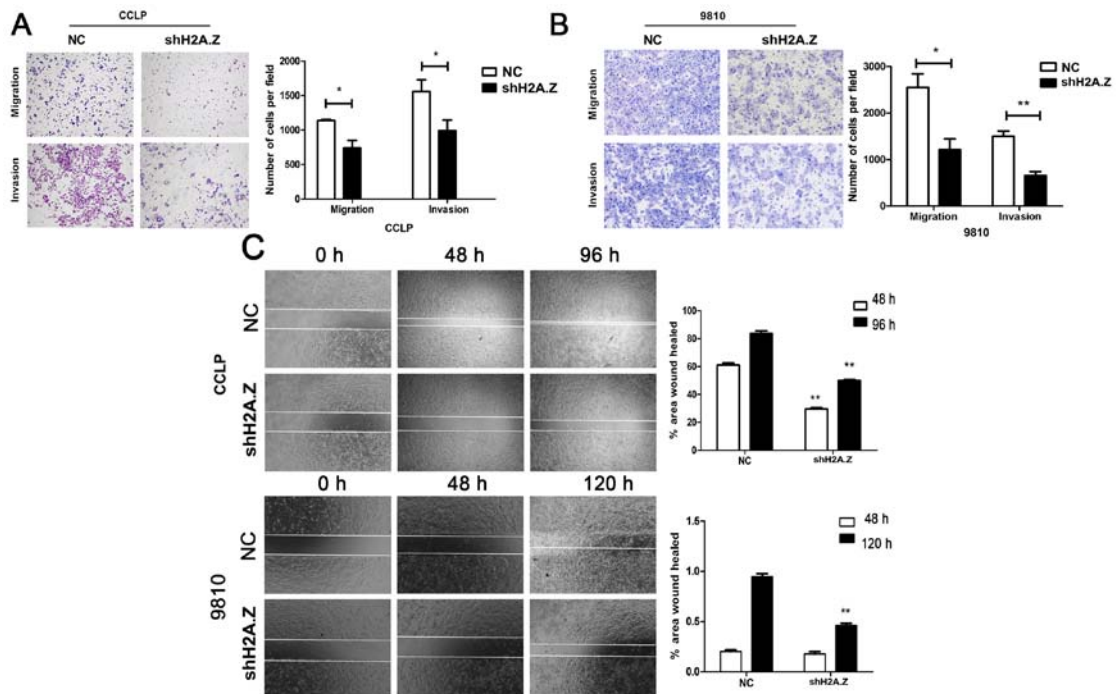

Figure 5. H2A.Z knockdown inhibits migration and invasion in ICC cells. (A and B) H2A.Z knockdown significantly suppressed migration and invasion compared with control cells, as detected by Transwell assays. (C) Similar effects were observed in cell motility by wound healing assays. Every experiment was repeated 3 times. *P<0.05 and, **P<0.01 compared with NC. H2A histone family member Z; ICC, intrahepatic cholangiocarcinoma; sh, short hairpin; NC, negative control.
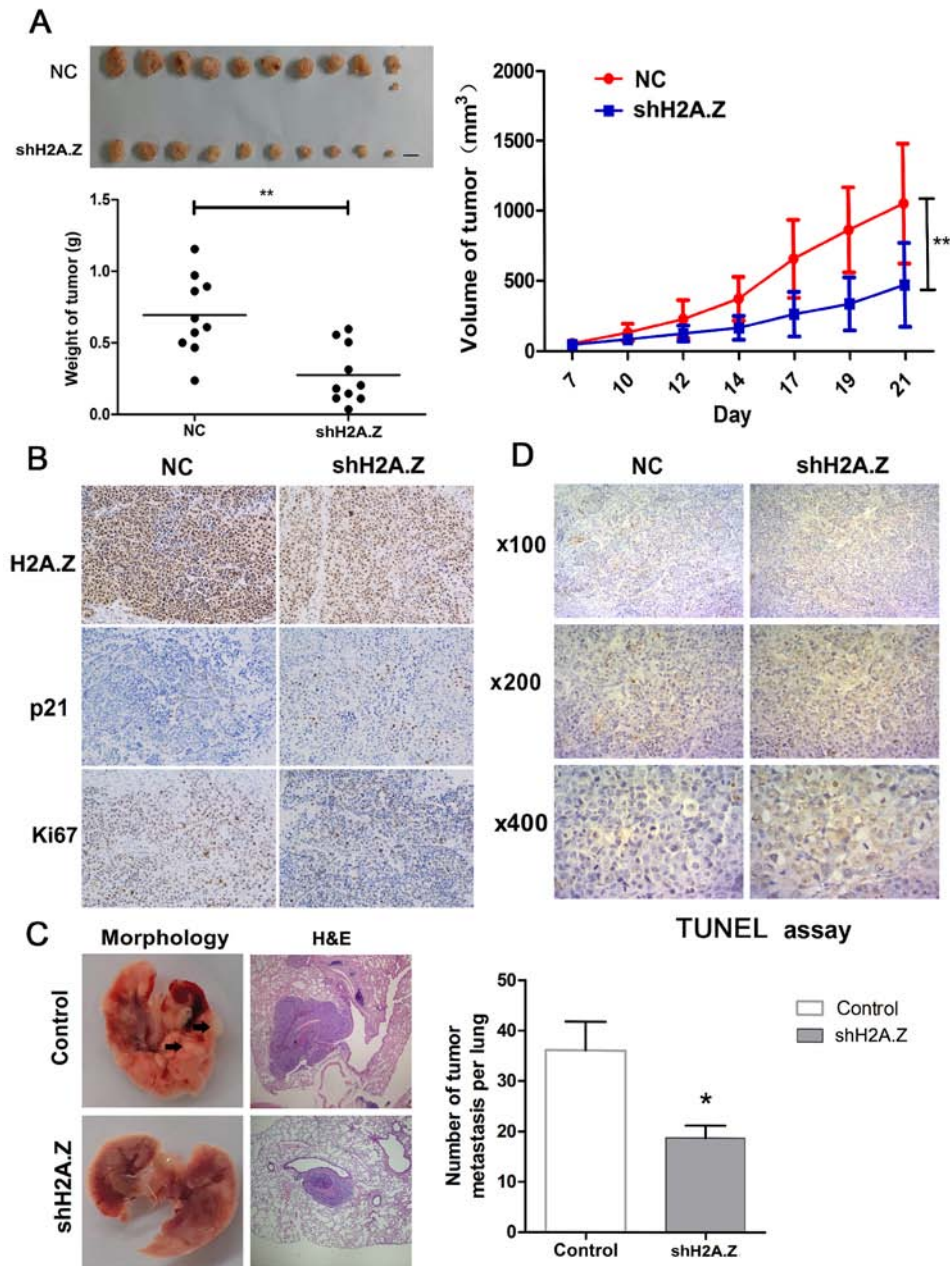
Figure 6. H2A.Z knockdown inhibits tumor growth and metastasis *in vivo*. (A) Photographic images of the xenograft tumors from the two experimental groups (knockdown and control). Tumor weight and volumes were measured. (B) Representative images from immunohistochemistry analysis of tumor sections for p21, Ki67 and H2A.Z protein expression. (C) Representative images of the lungs and of H&E-stained lung sections from mice that underwent experimental metastasis assay. Metastatic tumors in lungs are marked with arrows. The numbers of metastases per lung were calculated. (D) Apoptotic cells (brown staining) in sections from the xenograft tumors were detected by TUNEL assay in the control and the H2AZ knockdown groups. $^{*}P<0.05$ and, $^{**}P<0.01$ compared with NC. H2A histone family member Z; p21, cyclin-dependent kinase inhibitor 1A; H&E, hematoxylin and eosin; NC, negative control; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

crosslinks which can destroy transcription and replication of DNA. Combination of gemcitabine and cisplatin is the first line chemotherapy for unresectable CC (26).

Because H2A.Z knockdown induced cell cycle arrest and apoptosis in ICC cell lines, it was hypothesized that H2A.Z knockdown might synergistically act with cisplatin in the therapy of ICC. Indeed, treatment with cisplatin in the H2A.Z-silenced CCLP-1 cells resulted in increased apoptosis compared with the normal control group (Fig. 7A and B). In cell proliferation assays, treatment with cisplatin combined with H2A.Z knockdown inhibited the proliferation of CCLP-1 cells more powerfully than the single treatments of either cisplatin or shRNA (P<0.01; Fig. 7C). Next, western blot analysis was performed to evaluate the molecular mechanism underlying the synergetic effect of H2A.Z knockdown and cisplatin in ICC cells. Separately, both H2A.Z knockdown and cisplatin treatment induced the expression of apoptotic markers. However, the treatment with cisplatin in H2A.Z-silenced cells resulted in a synergistically increased expression of apoptosis-related proteins (Fig. 7C). Taken together, these results suggested that H2A.Z knockdown increased the sensitivity of ICC cells to cisplatin.
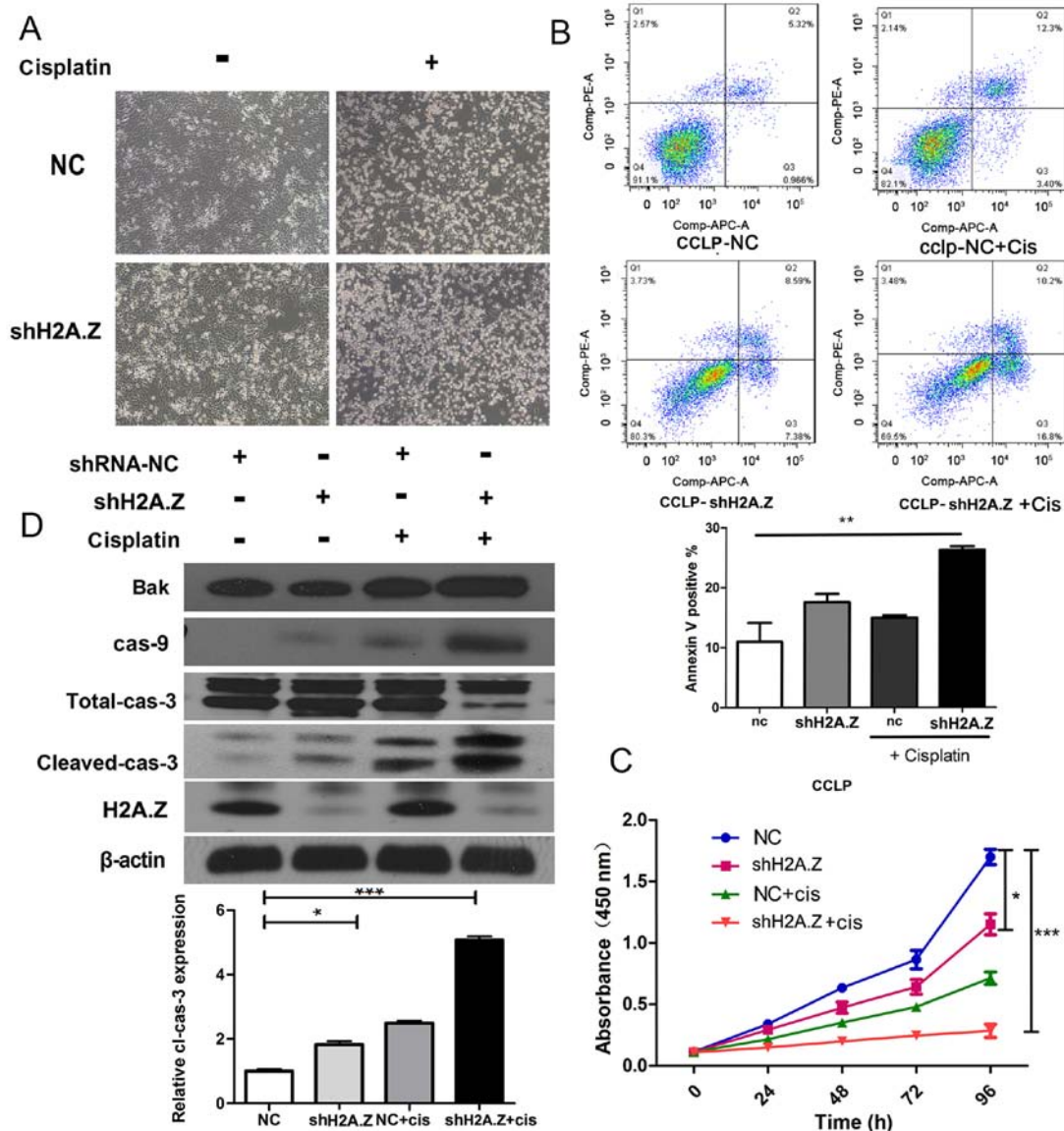
Figure 7. H2AZ knockdown strengthens the effect of cisplatin treatment in ICC cells. CCLP-1 cells were treated with shH2A.Z lentivirus. After 48 h, 4 μg/ml cisplatin was added in the medium and cells were incubated for another 24 h. (A) Cell morphology was captured by microscopy. (B) Apoptosis was examined by flow cytometry. (C) Cell viability was measured by Cell Counting Kit-8 assay. (D) Protein expression levels of apoptosis-related proteins were evaluated by western blotting. Every experiment was repeated three times. *P<0.05, **P<0.01 and ***P<0.001 compared with NC. H2A histone family member Z; ICC, intrahepatic cholangiocarcinoma; sh, short hairpin; NC, negative control; Cis, cisplatin; Bak, Bcl-2 homologous antagonist/killer; cas, caspase.

Discussion

In eukaryotes, the nucleosome is the smallest subunit of chromatin, consisting of histones H2A, H2B, H3, H4 and a segment of DNA. Nucleosome composition is highly variable and controls DNA transcription and gene silencing (27). Histone H2A.Z is a variant of histone H2A; it is an indispensable component of nucleosomes in a wide variety of organisms, especially vertebrates. H2A.Z is considered a key chromatin component that regulates DNA double-strand breaks, chromatin structure formation and DNA transcription (6,28,29). H2A.Z also regulates cell proliferation and viability of cancer cells (17,30,31). The present study demonstrated that H2A.Z regulated cell cycle and apoptosis and promoted the proliferation of ICC. Additionally, H2A.Z was demonstrated to promote ICC metastasis by inducing EMT.

Skp2 is an oncogene. According to a previous report, Skp2 is associated with the development of lymphomas (32). Skp2 contains an F-box domain, which is a component of the SKP1-cullin-F-box (SCF) complex, an E3 ubiquitin ligase complex that catalyzes the ubiquitination of multiple proteins (33). The SCF-Skp2 complex specifically controls the ubiquitination-mediated proteasomal degradation of p21 and p27. Thus, Skp2 controls the G1/S phase transition of the cell cycle (34,35). In the present study, knockdown of H2A.Z reduced the expression of Skp2, followed by upregulation of p21 and p27. These data confirmed that H2A.Z controls the cell cycle by regulating the expression of Skp2.

Although the therapeutic effect of chemotherapy is not always satisfactory, chemotherapy is the treatment of choice following the surgical resection of a primary tumor (4). Combination of cisplatin and gemcitabine is considered the

standard chemotherapy for the treatment of ICC. However, cisplatin treatment is associated with renal toxicity and other side-effects. Therefore, it would be beneficial to increase the antitumor effects of cisplatin while reducing its side effects. Cisplatin induces cancer cell apoptosis and inhibits cell proliferation. In the present study, knockdown of H2A.Z upregulated the expression of caspase-3 and caspase-9, and enhanced the effect of cisplatin on ICC cells.

In conclusion, H2A.Z was demonstrated to be highly expressed in ICC and to correlate with overall survival in patients with ICC. H2A.Z knockdown inhibited tumor growth and metastasis *in vivo* via regulation of cell proliferation and EMT. Furthermore, H2A.Z knockdown improved the efficacy of cisplatin treatment in a ICC xenograft mouse model. These findings suggest that H2A.Z may be a novel biomarker and therapeutic target for ICC.

Acknowledgements

Availability of data and materials

The analyzed datasets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

BY, JW and SZ designed the study. BY, RT, HL, JW and CD performed the experiments. BY, ZX and DC performed the tumor xenograft experiments. BY and ZX drafted the manuscript. BY, HX and LZ analyzed the results. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

For the human specimens, the Clinical Specimens Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) approved the present research. Informed consent was obtained from all patients for the storage and use of their tissue. All animal experiments were approved by the Animal Experimental Ethics Committee of the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China), and all procedures performed on animals were in accordance with the ethical standards of the First Affiliated Hospital of Zhejiang University School of Medicine.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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