Epigenetic silencing of ZCCHC10 by the lncRNA SNHG1 promotes progression and venetoclax resistance of acute myeloid leukemia

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Abstract. The gene encoding the tumor suppressor p53 is the most frequently mutated gene in cancers. However, p53 mutation is rare in acute myeloid leukemia (AML), and p53 is inactivated predominantly by aberrant expression of p53 regulators (such as MDM2). A previous study by the authors revealed that the ZCCHC10 protein suppressed MDM2-mediated degradation of the p53 protein in lung cancer. However, the expression and role of the ZCCHC10 gene in AML have not been investigated. In the present study, it was found that ZCCHC10 expression was downregulated in bone marrow samples of AML patients and that ZCCHC10 expression was significantly and negatively correlated with the expression of the lncRNA SNHG1. Suppression of SNHG1 decreased ZCCHC10 promoter methylation and increased ZCCHC10 expression. Notably, there is a putative binding motif in SNHG1 with full complementarity to five sites surrounding the CpG island in the ZCCHC10 promoter. Overexpression of wild-type SNHG1 promoted ZCCHC10 methylation, but overexpression of SNHG1 with deletion of the binding motif did not. Further study identified that SNHG1 simultaneously bound to the ZCCHC10 promoter and the DNA methyltransferases DNMT1 and DNMT3B. These results indicated that SNHG1 recruits DNMT1 and DNMT3B to the ZCCHC10 promoter, resulting in hypermethylation of the ZCCHC10 promoter. Kaplan-Meier survival analysis showed that ZCCHC10 expression was positively associated with overall survival in AML patients. *In vitro* experiments demonstrated that ZCCHC10 increased p53 expression and suppressed AML cell proliferation and survival. In the xenograft mouse model, ZCCHC10 decreased the proliferation of leukemic cells, improved the survival of leukemic mice, and increased sensitivity to the BCL inhibitor venetoclax. In conclusion, ZCCHC10 expression is suppressed by SNHG1-induced DNA methylation in AML. Downregulation of ZCCHC10 decreases p53 activation, promotes cell proliferation and survival, and thereby accelerates AML progression and the acquisition of venetoclax resistance. The present study identified a SNHG1/ZCCHC10/p53 signaling axis in AML that may be a therapeutic target in this malignancy.

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Abbreviations: AML, acute myeloid leukemia; BMMC, bone marrow mononuclear cell; ChIP, chromatin immunoprecipitation; DNMT, DNA methyltransferase; IC50, half maximal inhibitory concentration; PBMC, peripheral blood mononuclear cell; RT-qPCR, reverse transcription-quantitative PCR; RIP, RNA immunoprecipitation; wtp53, wild-type p53

Introduction

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by clonal expansion of immature myeloid progenitor cells in bone marrow, peripheral blood, or other tissues (1). It is the most common form of acute leukemia in adults and has the lowest survival rate among leukemias (5-year survival=24%) (2). The pathogenesis of AML is heterogeneous and complex. Various cytogenetic and molecular genetic abnormalities are involved in the conversion of myeloid progenitor cells into leukemic blasts, leading to different therapeutic responses and clinical outcomes (1). Therefore, the

study of the molecular mechanism underlying leukemogenesis is crucial for the development of precision therapies for AML.

The TP53 gene encodes the critical tumor suppressor p53, which plays a role as a transcription factor (3). It is the most frequently mutated gene in human cancers; ~50% of human tumors carry a mutation in the TP53 gene. Compared with solid tumors, AMLs have a lower rate of TP53 genomic alterations, and the overall rate of TP53 mutation in all AML patients is 8% (4). TP53 mutations occur mainly in patients with therapy-related AML and are rare in *de novo* AMLs (4). However, p53 dysfunction is present in almost all AML subtypes. Non-mutated wild-type p53 (wtp53) is inactivated by abnormal expression of proteins that regulate p53 stability and function (5,6). The negative p53 regulator MDM2 is frequently overexpressed in samples that retain wtp53 and contributes to p53 inactivation and the pathogenesis of AML (5,6). Therefore, reactivating the function of p53 by pharmacologically disrupting the protein interaction between MDM2 and p53 is a promising therapeutic strategy for AML with wtp53 (7,8). For example, activation of p53 by an MDM2 inhibitor (RG7388) overcomes resistance to a Bcl-2 inhibitor (venetoclax) in AML (9).

Recently, it was revealed by the authors that ZCCHC10 exerts a tumor-suppressive effect by inhibiting MDM2-mediated degradation of p53 in lung cancer (10). Remarkably, the ZCCHC10 gene is located on chromosome 5q31.1, a small region that is frequently deleted (11) or epigenetically silenced (12,13) in AML, suggesting that this region may contain myeloid tumor suppressor genes. Several genes at 5q31.1 have been shown to play a suppressive role in AML, including PDLIM4 (also called RIL) (13), VTRNA2-1 (12), IRF1 (14) and IL4 (15). PDLIM4 is methylated in 80% of AML cases (13), the non-coding RNA VTRNA2-1 is silenced by hypermethylation in AML, and patients with a low level of methylation have a favorable prognosis (12). The ZCCHC10 gene is located between PDLIM4 and VTRNA2-1, 0.3 Mb and 3 Mb away from the PDLIM4 and VTRNA2-1 genes, respectively. Therefore, it was investigated whether ZCCHC10 is also silenced by hypermethylation in AML and the role of ZCCHC10 in AML was explored.

Materials and methods

Bone marrow specimens. Bone marrow samples were obtained from adults with AML (n=20; median age, 61.2; age range, 24-83; 11 male patients, 9 female patients) and non-leukemic patients (n=10; median age, 51.2; age range, 31-82; 5 male patients, 5 female patients) recruited at the Chongzuo People's Hospital of Youjiang Medical University for Nationalities (Chongzuo, China) between July 2021 and December 2022. The present study was approved (approval no. 2022-03-10) by the Ethics Committee of Youjiang Medical University for Nationalities (Baise, China). Written informed consent was obtained from all patients involved in the study.

Data collection and bioinformatics analysis. The mRNA profiling data and clinical information of AML patients were downloaded from cBioPortal (http://www.cbioportal.org/), Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/gds), and The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga). The cBioPortal dataset contains data for 451 AML patients, and RNA sequencing (RNA-seq) was performed on bone marrow mononuclear cells (BMMCs) or peripheral blood mononuclear cells (PBMCs) (16). The GSE71014 dataset contains clinical information and microarray-based mRNA expression data for 104 AML patients with normal karyotypes (17). RNA-seq data and clinical information for 151 AML patients are available in the TCGA cohort. Kaplan-Meier survival analysis was used to analyze the difference in survival between patients with high- and low-expression of ZCCHC10 mRNA using the 'survival' package in R software (version 4.1.2) (https://www.r-project.org).

Chemicals, antibodies and cell lines. All chemicals and antibodies used in the present study are listed in Table SI. All cell lines were obtained from American Type Culture Collection (ATCC) and cultured according to ATCC instructions. All cell lines were free of mycoplasma contamination and authenticated using short tandem repeat profiling by Yubo Biological Technology Co., Ltd.

RNA preparation and reverse transcription-quantitative PCR (RT-qPCR). Mononuclear cells were extracted from bone marrow or peripheral blood using human lymphocyte separation medium (Applygen Technologies Inc.). Total RNA was isolated from mononuclear cells or AML cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then reverse-transcribed into cDNA using the All-in-One™ First-Strand cDNA Synthesis Kit (GeneCopoeia, Inc.) according to the manufacturer' instructions. SYBR Green-based real-time PCR (GeneCopoeia, Inc.) was performed in an ABI 7900 thermocycler (Thermo Fisher Scientific, Inc.) according to the manufacturer' instructions. Primers were designed by PrimerBank (https://pga.mgh.harvard.edu/primerbank) and are listed in Table SII. The thermocycling conditions were as follows: Initial denaturation at 94°C for 10 min, followed by 40 cycles of denaturation at 94°C for 10 sec, annealing at 60°C for 30 sec, and extension at 72°C for 30 sec. All reactions were examined in technical triplicate. The 18S rRNA was used as the internal reference, and the relative gene expression was estimated using $2^{-\Delta\Delta Cq}$ method (18).

Western blotting. Proteins were extracted with radioimmunoprecipitation assay lysis buffer (Cell Signaling Technology, Inc.), protein concentration was determined by bicinchoninic acid. A total of 30-50 µg protein was loaded per lane and separated by 10-12% SDS-PAGE. After separation, proteins were transferred onto PVDF membranes, and the membrane were blocked with 5% non-fat milk for 1 h at room temperature. After sequential incubation with primary antibodies overnight at 4°C and appropriate horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:1,0000 (cat. nos. 5450-0010 and 5220-0338, respectively; KPL, Inc.) for 1 h at room temperature, the membranes were exposed to Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and images were captured using a gel imaging system (Tanon 4600SF; Tanon Science and Technology Co., Ltd.). The software ImageJ (version 1.53n; National Institutes of Health) was used for densitometric analysis.

Lentiviral transduction. All lentiviruses were purchased from Shanghai GeneChem Co., Ltd. The lentiviruses overexpressing ZCCHC10 cDNA and expressing ZCCHC10 shRNA were previously described by the authors (10). The target sequences of SNHG1 shRNA were previously described by Sun *et al* (19) (shSNHG1-1: GGCCAGCACCTTCTCTCT AAA, shSNHG1-2: GGTTTGCTGTGTATCACATTTCT). Cells were infected with lentiviral particles at a multiplicity of infection of 10 for 8 h, then cultured with fresh medium for 72 h. At 72 h after infection, infected cells were selected with puromycin (2.5 μg/ml).

Nested methylation-specific PCR. DNA was modified with the EpiTect bisulfite kit (cat. no. 59104; Qiagen, Inc.) for conversion of all unmethylated cytosines to uracil. The converted DNA was first amplified with an outer primer pair specific for converted DNA without distinguishing between methylated and unmethylated DNA, and then amplified with primers specific for unmethylated and unmethylated DNA. PCR products were detected by 2% agarose gel electrophoresis and visualized using ethidium bromide. Primers were designed using MethPrimer software (http://www.urogene. org/cgi-bin/methprimer/methprimer.cgi) (Table SII).

Chromatin immunoprecipitation (ChIP). The ChIP experiment was performed using a kit (cat. no. Bes5001) from Guangzhou BersinBio Co. Ltd. Briefly, cells were subjected to crosslinking with 1% formaldehyde and lysed, and chromatin was then sheared with sonication until DNA fragments of 200-1,000 bp were obtained. The cross-linked chromatin was precipitated with normal IgG, an anti-DNA methyltransferase (DNMT)1 antibody and an anti-DNMT3B antibody. The amount of precipitated DNA was evaluated by semi-quantitative PCR. In preliminary experiments, the thermal cycling program was selected for linear amplification. The final PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide. Primers are listed in Table SII.

RNA immunoprecipitation (RIP). The RIP assay was performed using the RIP kit (cat. no. Bes5101; Guangzhou BersinBio Co. Ltd.). A total of $5x10^7$ cells were lysed using 2.5 ml lysis buffer, 0.1 ml lysates were used for input, the remaining lysates were evenly divided into three parts and precipitated with IgG, an anti-DNMT1 antibody and an anti-DNMT3B antibody, respectively. Input and precipitated RNA were purified and reverse-transcribed into cDNA. SYBR Green-based real-time PCR was then performed with specific primers (Table SII) as aforementioned.

Methylcellulose colony formation assay. Cells were seeded in 24-well plates at a density of $1x10^3$ cells/well in human methylcellulose complete medium (R&D Systems Europe, Ltd.) according to the manufacturer's instructions. After 20 days of incubation at 37°C and 5% CO_2, colonies with at least 50 cells were counted under an inverted light microscope.

Cell viability assay. Cells were cultured in 96-well plate (2,000 cells per well) at 37°C and 5% CO_2 for indicated time. Cell viability was determined according to the manufacturer's instructions using a Cell Counting Kit-8 (CCK-8) assay (cat. no. C0037; Beyotime Institute of Biotechnology). Briefly, 10 μl CCK-8 solution was added to each well and cultured at 37°C and 5% CO_2 for 4 h, and the absorbance was measured at 450 nm wavelength.

Cell cycle and apoptosis assays. Cell cycle and apoptosis assays were performed by flow cytometry as previously described (10).

AML xenograft models. NOD/SCID mice (male, ~7-8 weeks old) were obtained from Hunan Slac Jingda Laboratory Co., Ltd. (Changsha, China) and housed in groups of 5 per cage with water and food provided *ad libitum*, in a specific-pathogen-free room with filtered air and controlled light/dark cycle (12/12 h), temperature (24±2°C) and relative humidity (45-65%). All mice were pretreated with an intraperitoneal injection of 20 mg/kg busulfan (APeXBIO Technology LLC) 24 h before inoculation and then injected intravenously (lateral tail vein) with $5x10^6$ cells in 100 μl serum-free medium (OCI-AML3-Vec, OCI-AML3-Zh10, OCI-AML3-shNC, or OCI-AML3-shZh10). Each treatment group included at least 10 mice; three of the mice were euthanized through cervical dislocation 30 days after inoculation, and the rest were monitored for survival. The tibias, femurs and spleens of the mice were harvested at sacrifice. Spleens, livers and kidneys were fixed with 10% formalin at room temperature for 24 h and processed for staining with hematoxylin and eosin or an anti-human CD45 antibody. Bone marrow (mixed from tibias and femurs) was minced in PBS and processed into single-cell suspensions for analysis by flow cytometry. All mice were maintained under standard conditions in accordance with institutional animal welfare guidelines. The animal experiments were approved (approval no. 2020-020) by the Animal Ethics Committee of Hunan Normal University (Changsha, China).

Evaluation of the effect of ZCCHC10 on the efficacy of venetoclax in AML xenograft mice. NOD/SCID mice were pretreated with busulfan and injected intravenously with MOLM13-Vec or MOLM13-Zh10 cells according to the aforementioned methods. Each treatment group included 10 mice. A total of eight days after inoculation, each of the aforementioned groups was divided into two subgroups (5 animals/subgroup) and treated with vehicle or venetoclax via oral gavage for three weeks (5 days/week). As recommended by Selleck Chemicals, venetoclax was prepared by the formulation in 5% DMSO, 50% PEG300 and 5% Tween-80. Mice were euthanized and dissected as aforementioned.

Statistical analysis. Statistical analysis was performed using Excel 365 software (Microsoft) and R software. The data are presented as the mean ± SD values. The difference between the means of two groups was analyzed using unpaired Student's t-test. The log-rank test was used to compare two survival curves. Relationships between two variables were evaluated using Pearson's correlation coefficient analysis, and paired Student's t-test was used to assess if the correlation was statistically significant. $P<0.05$ was considered to indicate a statistically significant difference.
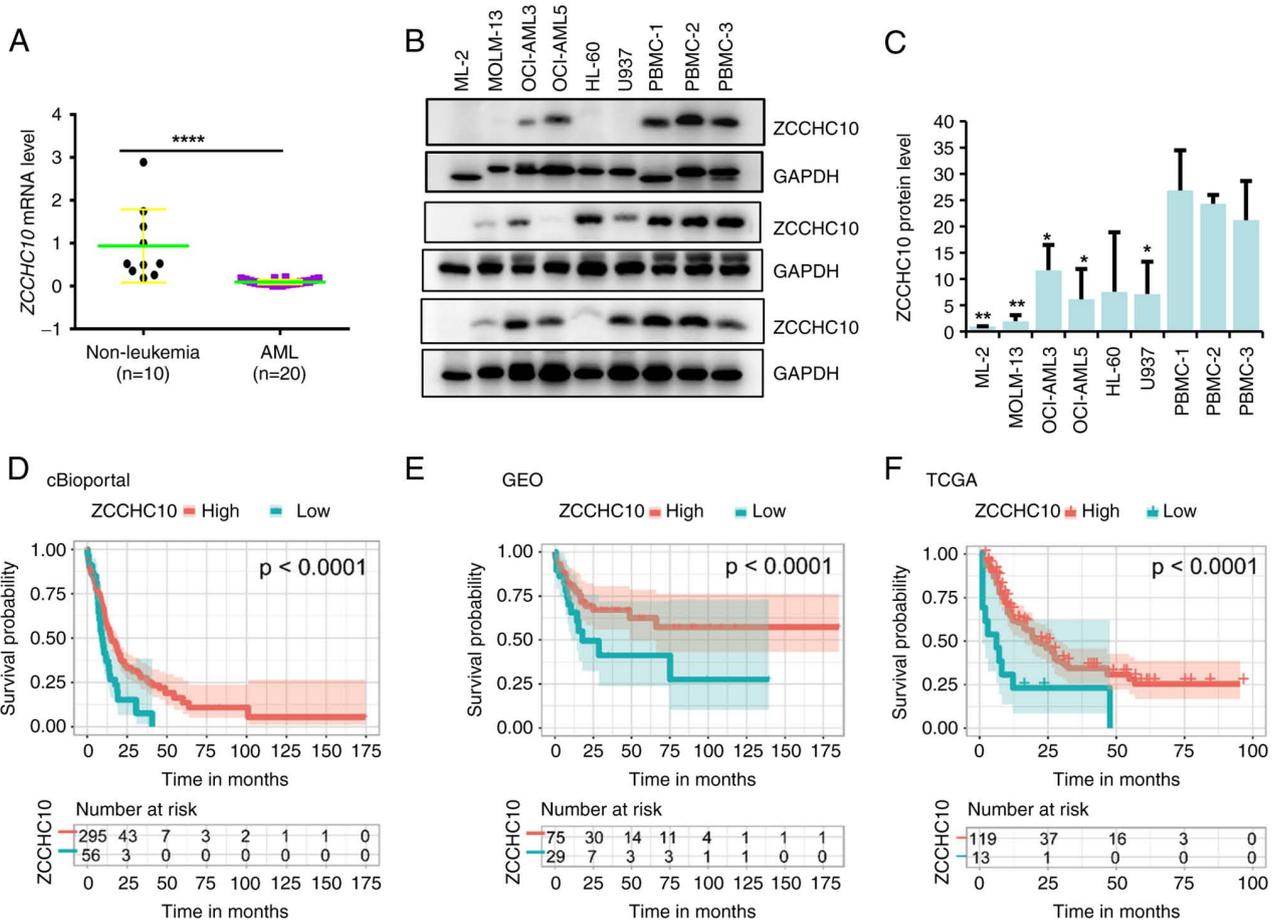
Figure 1. Decreased expression of ZCCHC10 is associated with shorter overall survival time in patients with AML. (A) The mRNA expression of ZCCHC10 was measured by reverse transcription-quantitative PCR in nonleukemic and AML patients, ****P<0.0001. (B and C) Expression of the protein encoded by the ZCCHC10 gene in AML cell lines and PBMCs from three healthy donors was determined using western blot analysis (performed in triplicate). The relative band intensity of ZCCHC10 normalized to that of GAPDH is shown as the mean ± SD value. *P<0.05 and **P<0.01 vs. PBMC-3. (D-F) Kaplan-Meier survival curves showing the difference in overall survival between patients with high and low ZCCHC10 expression in the cBioPortal, GEO and TCGA cohorts. AML, acute myeloid leukemia; PBMC, peripheral blood mononuclear cells; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas.

Results

Decreased expression of ZCCHC10 is associated with shorter overall survival times in AML patients. RT-qPCR was performed to measure ZCCHC10 gene expression in BMMCs from 20 AML patients and 10 non-leukemic patients and it was found that ZCCHC10 mRNA expression was significantly decreased in AML patients compared with non-leukemic patients (Fig. 1A). Western blot analysis showed that the protein level of ZCCHC10 was significantly lower in AML (ML2, MOLM-13, OCI-AML3, OCI-AML5, and HL-60) and acute monocytic leukemia (U937) cell lines than in PBMCs (Fig. 1B and C). To assess the prognostic significance of ZCCH10 expression, Kaplan-Meier survival analysis was performed based on clinical and gene expression data from AML patients in cBioPortal, GEO and TCGA cohorts. In all three cohorts, AML patients with higher ZCCHC10 expression had longer overall survival times (Fig. 1D-F). These results suggested that ZCCHC10 expression is a favorable prognostic factor in AML.

ZCCHC10 is downregulated by SNHG1-mediated DNA methylation. ZCCHC10 expression in blood leukocytes has been shown to be regulated by DNA methylation (20). Therefore,

methylation-specific PCR was performed to determine the methylation status of the ZCCHC10 promoter in BMMCs from AML and non-leukemic patients. As demonstrated in Fig. 2A, methylated PCR products were more abundant than unmethylated products in 16 of the 20 AML samples (Nos. 1, 2, 4, 5, 6, 7, 8, 10, 11, 12, 13, 14, 15, 18, 19 and 20), but the ZCCHC10 promoter was hypomethylated in all nonleukemic samples except sample no. 6. Moreover, treatment with 5-aza-2'-deoxycytidine (decitabine), a DNA methylation inhibitor, significantly increased the ZCCHC10 protein level in AML cell lines (Fig. 2B). These results suggested that ZCCHC10 expression is suppressed by hypermethylation in AML. The lncRNA SNHG1 has been shown to be upregulated in AML (21,22). Accordingly, in the present study, SNHG1 was found to be overexpressed in the BMMCs of AML patients (Fig. 2C). Correlation analysis revealed that SNHG1 expression was negatively correlated with the ZCCHC10 mRNA level (Fig. 2D). In addition, the correlation between ZCCHC10 and SNHG1 was analyzed using the expression data from the cBio-Portal database and an inverse correlation was found between SNHG1 and ZCCHC10 (Fig. 2E). SNHG1 has been reported to be mainly distributed in the nucleus, where it is involved in transcriptional regulation by interacting with transcription
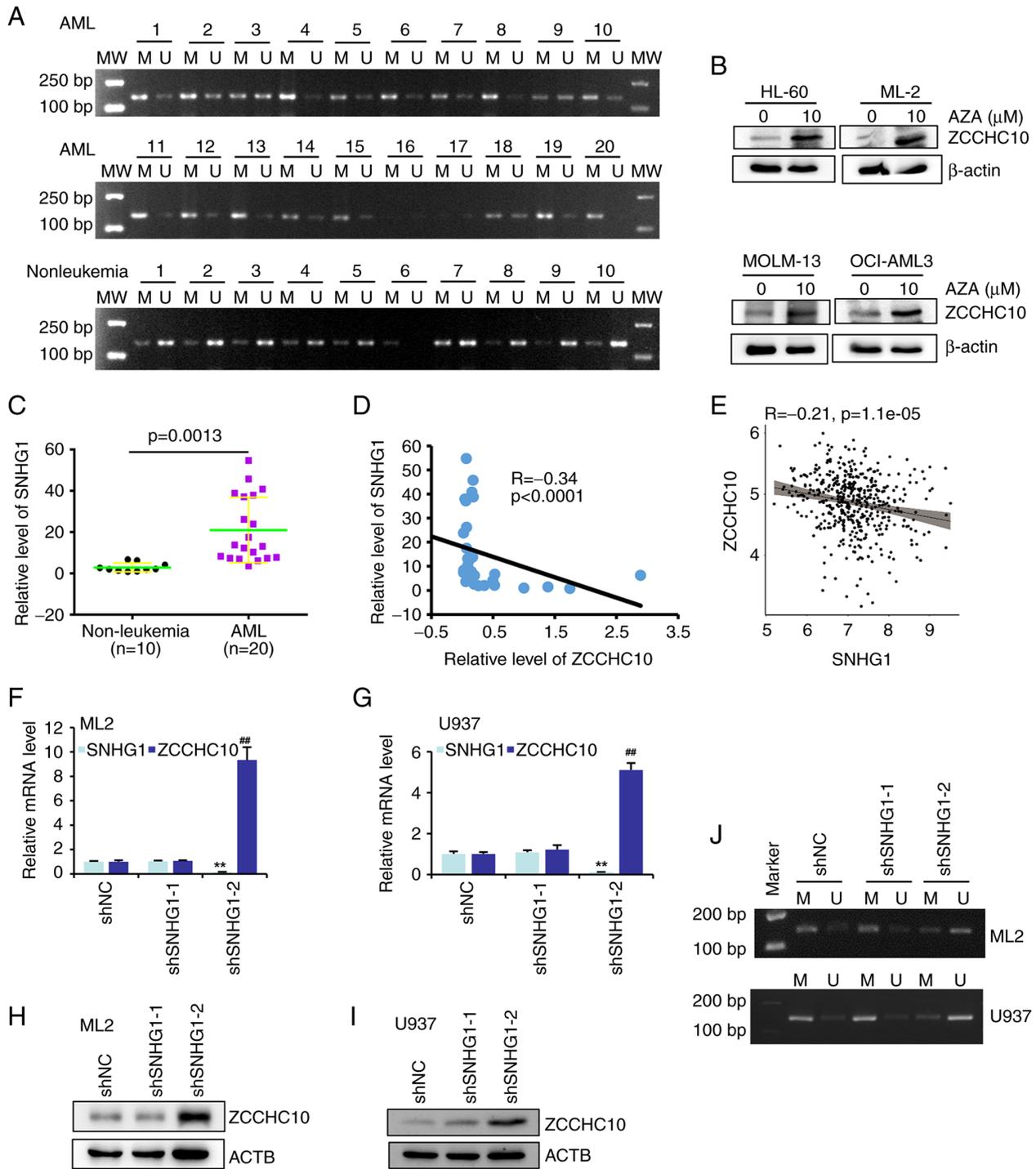
Figure 2. ZCCHC10 is downregulated by SNHG1-mediated DNA methylation in AML. (A) The methylation status of ZCCHC10 was determined by nested methylation-specific PCR. Bisulfite-modified DNA was first amplified with an outer primer pair specific for converted DNA and then amplified with primer pairs specific for methylated (M) or unmethylated (U) DNA. Each Arabic numeral indicates the number of samples. (B) Western blotting was performed 48 h after treatment with or without AZA. (C) SNHG1 expression was measured by RT-qPCR in non-leukemic and AML patients. (D) Pearson correlation analysis between ZCCHC10 and SNHG1 expression in nonleukemic and AML patients. (E) Pearson's correlation analysis between ZCCHC10 and SNHG1 expression in AML patients based on expression data from the cBioPortal database. (F-I) The mRNA and protein levels of SNHG1 and ZCCHC10 in ML2 and U937 cells were determined by (F and G) RT-qPCR and (H and I) western blotting, respectively, 72 h after infection with lentiviral particles expressing scrambled shRNA (shNC), or SNHG1 shRNA (shSNHG1-1, shSNHG1-2). **P<0.01 (SNHG1) and ##P<0.01 (ZCCHC10) compared with the shNC group. (J) The methylation status of ZCCHC10 was determined by nested methylation-specific PCR 72 h after infection with lentiviral particles expressing shNC, shSNHG1-1 or, shSNHG1-2. AML, acute myeloid leukemia; MW, molecular marker; AZA, 5-aza-2'-deoxycytidine; RT-qPCR, reverse transcription-quantitative PCR; sh-, short hairpin; NC, negative control.

factors (19) or histone methyltransferases (23). Therefore, it was investigated whether SNHG1 affects the expression and methylation of ZCCHC10. Compared with transduction of

lentiviruses expressing scrambled shRNA, transduction of lentiviruses expressing shSNHG1-2 increased the ZCCHC10 mRNA and protein levels (Fig. 2F-I) while suppressing CpG
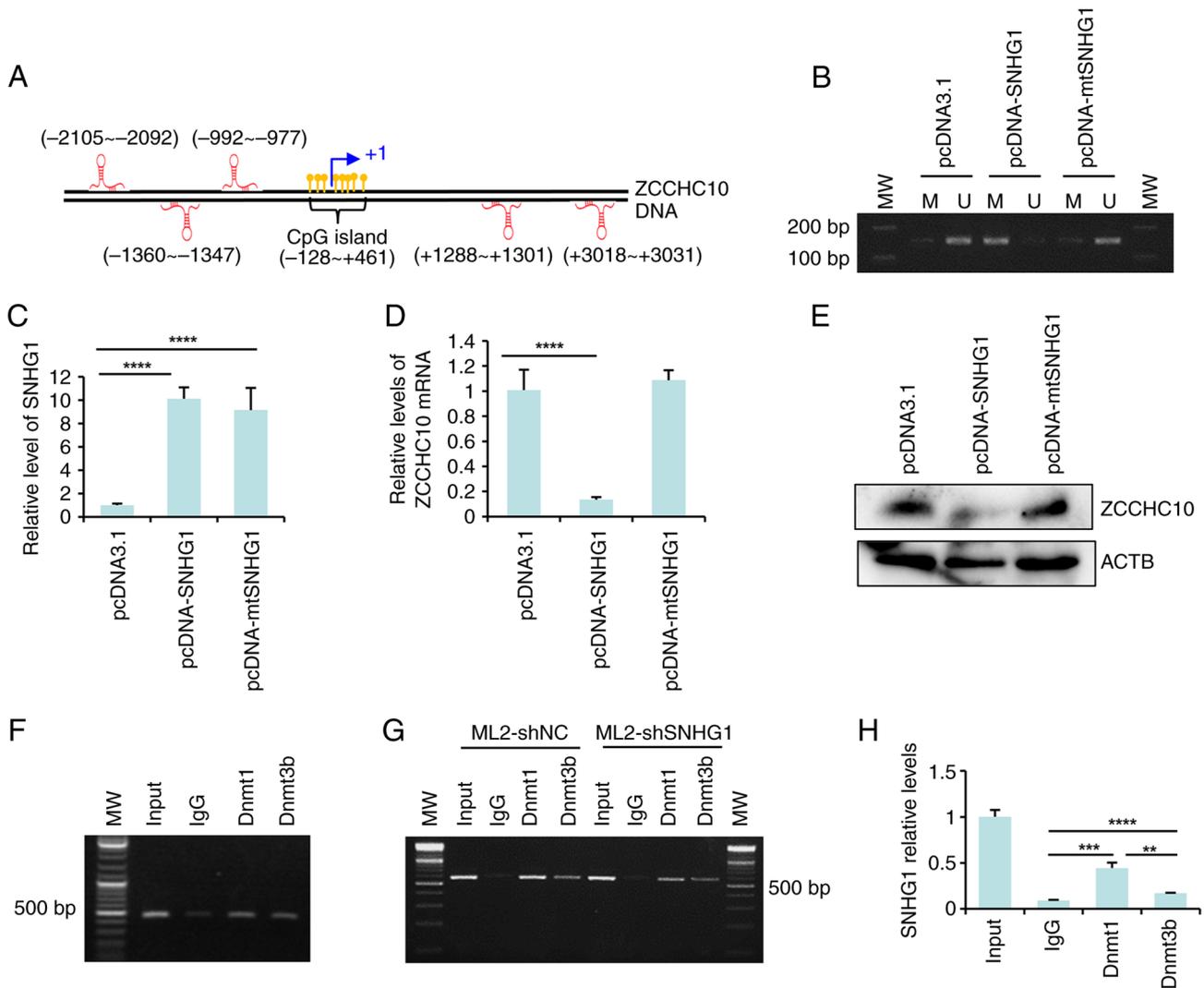
Figure 3. SNHG1 promotes ZCCHC10 promoter methylation by recruiting DNMT1 and DNMT3B in acute myeloid leukemia. (A) Schematic representation of the ZCCHC10 gene promoter. The CpG island was predicted by MethPrimer software and encompasses nucleotides -128 to +461 relative to the transcription start site (+1). The binding sites of SNHG1 in the sense or antisense strands of ZCCHC10 DNA are shown above and below the promoter representation, respectively. (B-E) 293 cells were transfected with empty vector, wild-type SNHG1 or mutant SNHG1. A total of 24 h post-transfection, the methylation status of ZCCHC10 was determined by (B) nested methylation-specific PCR, the mRNA levels of SNHG1 and ZCCHC10 were determined by (C and D) RT-qPCR, and the protein level of ZCCHC10 was determined by western blotting (E). (F and G) Chromatin immunoprecipitation assays were performed in (F) U937 and (G) ML2 cells expressing shNC or shSNHG1-2 using IgG (negative control), an anti-DNMT1 antibody, or an anti-DNMT3B antibody. Input and precipitated DNA were analyzed by semi-quantitative RT-qPCR. (H) RNA immunoprecipitation assays were performed in ML2 cells using IgG (negative control), an anti-DNMT1 antibody, or an anti-DNMT3B antibody. Input and precipitated RNA were reverse-transcribed into cDNA and analyzed by RT-qPCR. **P<0.01, ***P<0.001 and ****P<0.0001. DNMT, DNA methyltransferase; RT-qPCR, reverse transcription-quantitative PCR; sh-, short hairpin; NC, negative control.

island methylation in the ZCCHC10 gene (Fig. 2J). However, transduction of the ineffective shSNHG1-1 lentivirus had no significant effects (Fig. 2F-J). These results indicated that SNHG1 suppresses ZCCHC10 expression by promoting methylation of the ZCCHC10 promoter.

SNHG1 promotes methylation of the ZCCHC10 promoter by recruiting DNMT1 and DNMT3B. Notably, sequence analysis by BLAST identified a specific sequence (5'-AAAGUGCUG GGAUU-3') in SNHG1 with full complementarity to five sites surrounding the CpG island in the ZCCHC10 promoter (Fig. 3A). It was hypothesized that this sequence is a potential DNA-binding motif of SNHG1. To examine whether the predicted DNA-binding motif is required for the regulation of DNA methylation of the ZCCHC10 gene, wild-type and mutant SNHG1 expression

plasmids were transfected into 293 cells and the methylation status of the ZCCHC10 promoter was determined 24 h after transfection (Fig. 3B and C). The results of methylation-specific PCR showed that overexpression of wild-type SNHG1 increased the methylation of the ZCCHC10 promoter (Fig. 3B) and decreased the ZCCHC10 mRNA and protein levels (Fig. 3D and E). By contrast, overexpression of mutant SNHG1, whose DNA-binding motif was deleted, had no effect on the methylation or expression of ZCCHC10 (Fig. 3B, D and E).

DNA methylation is mediated by DNMTs, including DNMT1, DNMT3A and DNMT3B. DNMT3A is frequently mutated in AML, whereas DNMT1 and DNMT3B are usually overexpressed in AML and contribute to the hypermethylation of tumor suppressor genes (24). Therefore, a ChIP assay was performed to investigate the binding of DNMT1 and DNMT3B
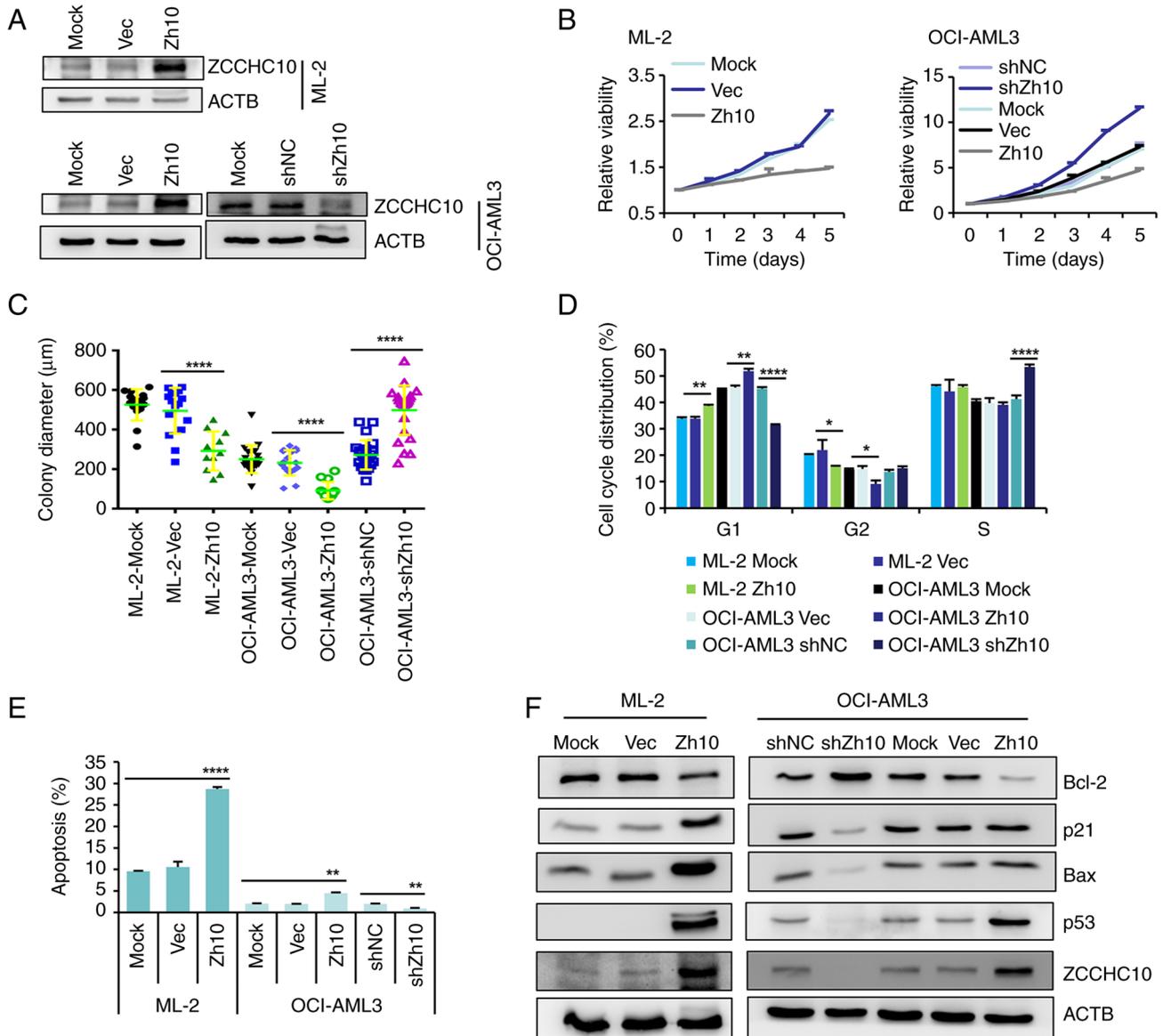
Figure 4. ZCCHC10 induces G1 arrest and apoptosis via p53 in AML cells. (A) Western blot analysis of ZCCHC10 protein expression in untreated cells (mock) and cells expressing empty vector (vec), ZCCHC10 cDNA (Zh10), scrambled shRNA (shNC) or ZCCHC10 shRNA (shZh10). (B) Cell viability was determined by a Cell Counting Kit-8 assay. (C) Diameter of colonies in triplicate experiments. (D) Distribution of the cell cycle in triplicate experiments, as determined by flow cytometry. (E) Apoptotic rate in triplicate experiments, as determined by flow cytometry. (F) Western blot analysis with the indicated antibodies. $^{*}P<0.05$, $^{**}P<0.01$ and $^{****}P<0.0001$. Representative images of colony formation and flow cytometry are shown in Figs. S1-S3. AML, acute myeloid leukemia; sh-, short hairpin; NC, negative control.

to the ZCCHC10 promoter in U937 acute monocytic leukemia cells. It was found that both DNMT1 and DNMT3B could bind to the ZCCHC10 promoter, but DNTM1 bound with slightly higher affinity than DNMT3B (Fig. 3F). Moreover, knockdown of SNHG1 reduced the interaction of DNMT1 and DNMT3B with the ZCCHC10 promoter (Fig. 3G). The RIP assay revealed that SNHG1 could bind to DNMT1 and DNMT3B (Fig. 3H). These results indicated that SNHG1 associates with both the ZCCHC10 promoter and DNMTs, and then recruits DNMTs to the ZCCHC10 promoter, leading to methylation of the ZCCHC10 promoter.

ZCCHC10 induces G1 arrest and apoptosis via p53. To elucidate the role of ZCCHC10 in AML, the lentivirus-mediated expression system was used to generate AML cell lines with stable overexpression or knockdown of ZCCHC10 gene expression (Fig. 4A). Cell proliferation and anchorage-independent growth were investigated using a CCK-8 assay and a methylcellulose colony formation assay, respectively. The CCK-8 assay revealed that overexpression of ZCCHC10 decreased the viability, whereas suppression of ZCCHC10 increased the viability (Fig. 4B). According to the results of the methylcellulose colony formation assay, cells stably expressing ZCCHC10 formed significantly less and smaller colonies than the cells expressing empty vector, whereas cells expressing ZCCHC10 shRNA formed more and larger colonies than those expressing scrambled shRNA (Figs. 4C and S1). Flow cytometric analysis showed that the proportion of G1 phase cells was increased among cells overexpressing ZCCHC10 whereas it was decreased among cells with ZCCHC10 knockdown, indicating
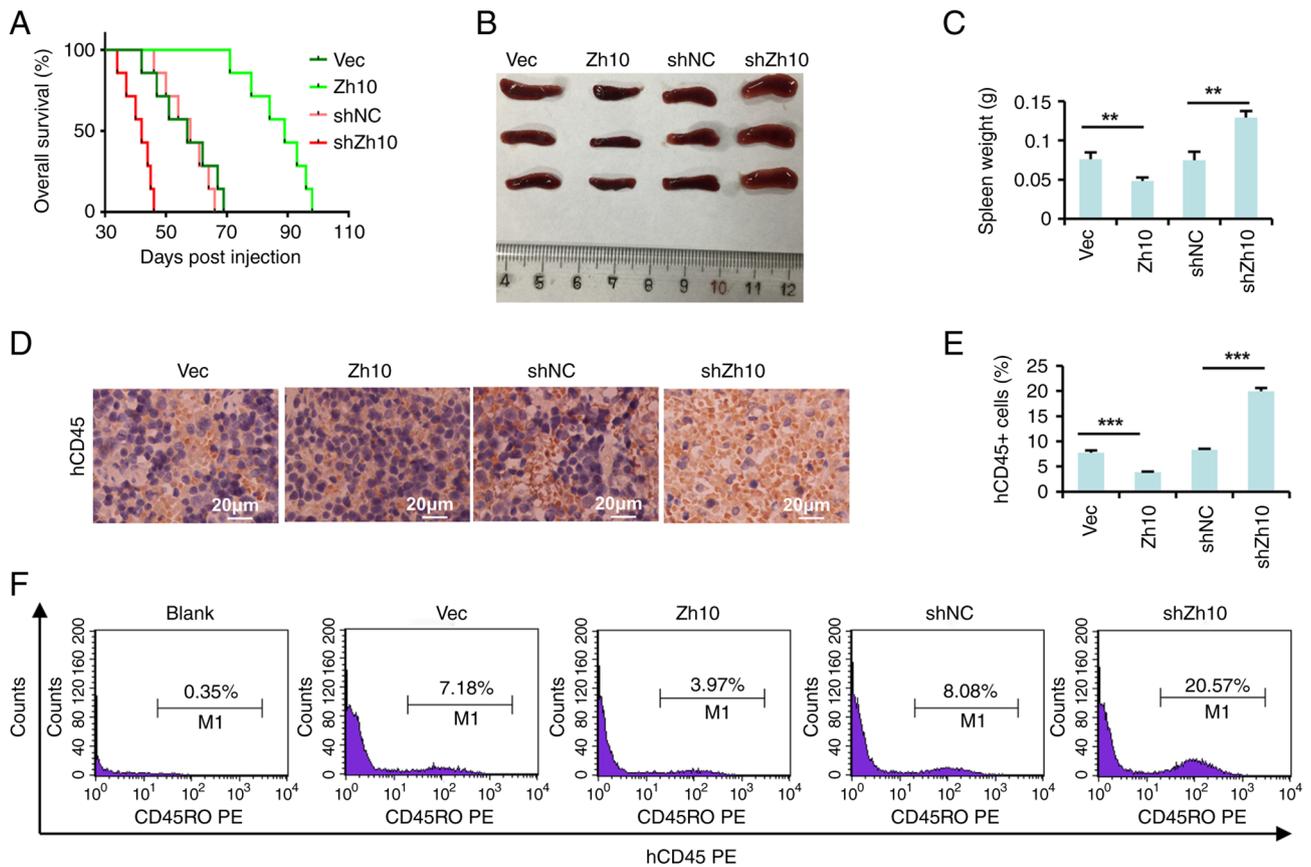
Figure 5. ZCCHC10 suppresses AML progression in AML xenografts. (A) Kaplan-Meier survival curve showing the overall survival rates of mice bearing xenografts derived from ML2 cells expressing empty vector (Vec), ZCCHC10 cDNA (Zh10), scrambled shRNA (shNC) or ZCCHC10 shRNA (shZh10). (B) Gross appearance of spleens. (C) Average weight of spleens. (D) Immunochemical assay of spleen sections with anti-human CD45 antibody. (E and F) Human CD45+ AML cells in bone marrow were analyzed by flow cytometry, bone marrow from control mice without transplantation of human AML cells as a negative control (blank). The data are presented as the mean ± SD of three mice (E) and representative images are shown (F). **P<0.01 and ***P<0.001. AML, acute myeloid leukemia; sh-, short hairpin; NC, negative control.

that ZCCHC10 led to cell cycle arrest in the G1 phase (Figs. 4D and S2). In addition, ZCCHC10 promoted apoptosis (Figs. 4E and S3).

It was previously demonstrated that ZCCHC10 plays a role in stabilizing the p53 protein in lung cancer (10). Consistent with this finding, the present study demonstrated that overexpression of ZCCHC10 increased the levels of p53, Bax and p21, but decreased the level of Bcl2; the effect of ZCCHC10 knockdown on p53 and its targets (p21, Bax and Bcl2) was opposite to that of ZCCHC10 overexpression (Fig. 4F). This suggested that ZCCHC10 activates the p53 pathway in AML cells.

ZCCHC10 suppresses AML progression in xenograft mice. NOD/SCID mice were injected with 5×10^6 cells (OCI-AML3-Vec, OCI-AML3-Zh10, OCI-AML3-shNC or OCI-AML3-shZh10) via the lateral tail vein, and the leukemia burden and survival of the NOD/SCID mice were compared. Kaplan-Meier survival analysis demonstrated that mice injected with OCI-AML3-Zh10 cells survived longer than mice injected with OCI-AML3-Vec cells. By contrast, mice injected with OCI-AML3-shZh10 cells had shorter survival times than mice injected with OCI-AML3-shNC cells (Fig. 5A). Leukemia burden was determined by assessing the spleen size and human hematopoietic (hCD45) chimerism in spleen and bone marrow. The mice bearing OCI-AML3-Zh10 cells had smaller and lighter

spleens than the mice bearing OCI-AML3-Vec cells, but the mice bearing OCI-AML3-shZh10 cells had larger and heavier spleens than the mice bearing OCI-AML3-Vec cells (Fig. 5B and C). IHC assays and flow cytometric analysis demonstrated that overexpression and knockdown of ZCCHC10 decreased and increased, respectively, the number of hCD45+ cells in the spleen (Fig. 5D) and bone marrow (Fig. 5E and F). These results indicated that ZCCHC10 can suppress the growth of AML cells in hematopoietic tissues in a xenograft mouse model.

ZCCHC10 increases sensitivity to venetoclax in AML. It has been reported that the antiapoptotic protein BCL2 is overexpressed in AML and that targeting BCL2 may be a successful therapeutic strategy in AML patients (25). A BCL2-specific inhibitor, venetoclax (ABT-199), has been approved by the FDA for use in AML. The aforementioned results revealed that overexpression of ZCCHC10 increased cleavage of PARP-1 and caspase 3, while treatment with venetoclax enhanced the effects of ZCCHC10 (Fig. 6A and B). Knockdown of ZCCHC10 decreased the cleavage of PARP-1 and caspase 3, but venetoclax treatment reversed the effect of ZCCHC10 knockdown (Fig. 6A and B). In addition, the effects of ZCCHC10 on venetoclax sensitivity were examined *in vitro* and *in vivo*. Overexpression of ZCCHC10 decreased the half maximal inhibitory concentration (IC_{50}) values of venetoclax in
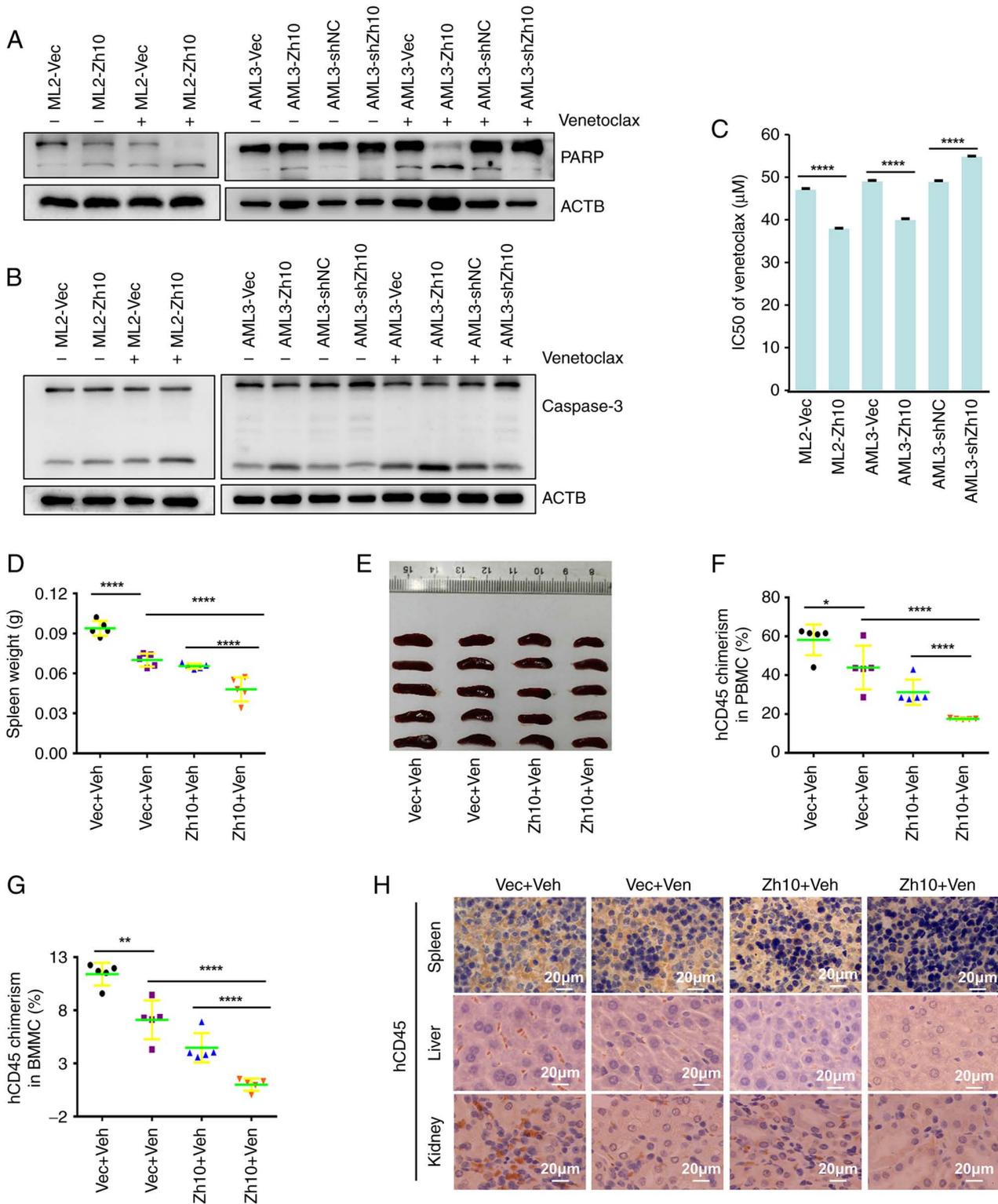
Figure 6. ZCCHC10 increases sensitivity to venetoclax in AML. (A and B) Western blot analysis of PARP1 and caspase 3 protein levels after 48 h of treatment with or without venetoclax. (C) Cell viability was determined by a Cell Counting Kit-8 assay after 48 h of treatment with venetoclax, and then the IC_{50} was calculated. (D) Average weight of the spleens of five mice. (E) Gross appearance of spleens. (F and G) The percentage of human CD45+ AML cells in (F) peripheral blood and (G) bone marrow were determined by flow cytometry. Representative images are shown in Fig. S4. The data are presented as the mean ± SD of five mice. (H) Immunochemical assay of spleen, liver, and kidney sections with anti-human CD45 antibody. $^*P<0.05$, $^{**}P<0.01$ and $^{****}P<0.0001$. AML, acute myeloid leukemia; IC_{50}, half maximal inhibitory concentration; sh-, short hairpin; NC, negative control.

ML2 and AML3 cells, but knockdown of ZCCHC10 increased the venetoclax IC_{50} values in AML3 cells (Fig. 6C), suggesting that ZCCHC10 enhanced sensitivity to venetoclax. In addition, sensitivity to venetoclax was examined *in vivo* in AML

xenograft mice. Venetoclax treatment significantly decreased splenomegaly (Fig. 6D and E) and engraftment of leukemic cells in the peripheral blood (Figs. 6F and S4), bone marrow (Figs. 6G and S4), spleen, liver, and kidney (Fig. 6H) in mice

bearing MOLM-13-Vec and MOLM13-Zh10 cell-derived xenografts. Moreover, venetoclax had a stronger anti-AML effect in mice bearing MOLM13-Zh10 cell-derived xenografts than in the corresponding control xenograft mice (Fig. 6D-H). These results indicated that ZCCHC10 enhances sensitivity to venetoclax in AML *in vitro* and *in vivo*.

Discussion

Hypermethylation of tumor suppressor genes is frequently observed in AML and plays a role in AML pathogenesis (24). Therapeutic agents targeting DNA methylation, such as the DNMT inhibitors azacitidine and decitabine, are widely used for the treatment of myelodysplastic syndrome and AML (26,27). The ZCCHC10 gene is located on chromosome 5q31.1 between PDLIM4 and VTRNA2-1, both of which play suppressive roles and are silenced by methylation in AML (12,13). The present study demonstrated that ZCCHC10 was also downregulated by DNA methylation in AML. Further study showed that ZCCHC10 expression was negatively associated with SNHG1 expression and that silencing SNHG1 suppressed the methylation of the ZCCHC10 promoter and increased the expression of ZCCHC10. SNHG1 is an oncogenic lncRNA that is overexpressed in various cancers, including AML (28,29). It was found to be distributed mainly in the nucleus, with only a small amount observed in the cytoplasm (19,23). In the cytoplasm, SNHG1 acts as a competing endogenous RNA by sponging microRNAs (21,23,29). In the nucleus, SNHG1 is involved in transcriptional regulation by interacting with transcription factors (19) or histone methyltransferases (23). The present study demonstrated that SNHG1 can recruit DNMT1 and DNMT3B to the ZCCHC10 promoter and then promote DNA methylation of the ZCCHC10 promoter. These results indicated that ZCCHC10 is epigenetically regulated by SNHG1-induced DNA methylation in AML. To the best of our knowledge, this is the first study reporting that SNHG1 regulates DNA methylation. In AML, hypermethylation of tumor suppressor genes is mediated mainly by DNMT1 or DNMT3B, whereas DNMT3A is frequently inactivated by mutation (24). Therefore, the role of DNMT3A was not investigated. However, the possibility that DNMT3A may act as a regulator of ZCCHC10 in the cases without DNMT3A mutation cannot be ruled out.

A common feature of all human cancers is loss of p53 function by either mutation or inactivation (30). Several studies have shown that p53 is rarely mutated in AML but is functionally inactivated by overexpression of MDM2 (5,6). ZCCHC10 can maintain the stability of p53 by disrupting the MDM2-p53 interaction (10). Therefore, downregulation of ZCCHC10 may be one of the reasons for p53 inactivation in AML. It was previously demonstrated that ZCCHC10 exerts a tumor-suppressive function by activating p53 in lung cancer (10). ZCCHC10 has also been shown to be a tumor suppressor in melanoma (31) and colorectal cancer (32). In line with previous studies, the present study demonstrated that overexpression of ZCCHC10 in AML cell lines triggered p53 activation, suppressed AML cell proliferation and survival, reduced the leukemia burden, and prolonged the survival of AML mice. These results indicated that ZCCHC10 plays a tumor-suppressive role in AML.

The BCL2 inhibitor venetoclax has been shown to induce apoptosis and is approved for clinical use in the treatment of AML. Bcl2 and p53 are the major negative and positive regulators of apoptosis, respectively. Reactivation of p53 by treatment with the MDM2 antagonist idasanutlin was found to significantly augment the antitumor effect of the Bcl-2 inhibitor venetoclax in *in vitro* and *in vivo* in xenograft models of human AML (33). According to the results of the present study, ZCCHC10 can activate p53 and reduce BCL2 expression. Therefore, the effect of ZCCHC10 on the efficacy of venetoclax was investigated *in vivo* in xenograft models of human AML. As expected, overexpression of ZCCHC10 enhanced sensitivity to venetoclax in the AML mouse model. Since the expression of ZCCHC10 can be increased by treatment with hypomethylating agents or interference with SNHG1, hypomethylating agents and/or small interfering RNA against SNHG1 can be used to treat AML or to improve the efficacy of venetoclax.

In conclusion, ZCCHC10 is epigenetically silenced by SNHG1-induced DNA methylation in AML. Suppression of ZCCHC10 decreases the stability and activation of p53, while inactivation of p53 promotes cell proliferation and survival and then accelerates the progression of AML and the acquisition of venetoclax resistance. The present study identified an SNHG1/ZCCHC10/p53 signaling axis in AML progression that may be a therapeutic target for AML.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HZ conducted the experiments, analyzed the data and wrote the manuscript. QZ analyzed the data. WH and CH collected and analyzed the clinical samples. CZ interpreted the data and revised the manuscript. JZ conceived the study, interpreted the data and wrote the manuscript. YN designed and conducted the experiments, analyzed the data and wrote the manuscript. HZ, YN and JZ confirm the authenticity of all the raw data. All authors reviewed, read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Animal Ethics Committee of Hunan Normal University (approval no. 2020-020;

Changsha, China) and the Ethics Committee of Youjiang Medical University for Nationalities (approval no. 2022-03-10; Baise, China). Written informed consent was obtained from all subjects involved in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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