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H22954, a novel long non-coding RNA down-regulated in AML, inhibits cancer growth in a BCL-2-dependent mechanism

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ABSTRACT

Long non-coding RNAs (lncRNAs) are important in cancer biology. In this study, we analyzed differentially expressed genes in CD34$^+$ hematopoietic cells and identified a novel lncRNA, H22954, which was down-regulated in acute myeloid leukemia (AML) patients. In cultured AML cells and mouse xenograft models, H22954 expression inhibited cell proliferation and tumor growth, respectively. Bioinformatic analysis and RNA antisense purification assay indicated that H22954 targeted the 3' untranslated region of the BCL2 gene. In luciferase assays, H22954 expression inhibited BCL2 expression. In transfected K562 cells and mouse xenograft tumors, H22954 overexpression reduced BCL-2 protein levels and promoted cell death. In AML patients, H22954 expression inversely correlated with BCL-2 protein levels in bone marrow cells, blast cell numbers and disease prognosis. These results indicate that H22954 is a novel regulator of BCL-2 and that reduced H22954 expression may play an important role in the pathogenesis of AML.

1. Introduction

Non-coding RNAs (ncRNAs) such as microRNAs play a key role in controlling gene expression [1]. Long non-coding RNAs (lncRNAs), *i.e.* > 200 nucleotides in length, are an important class of ncRNAs that regulate gene expression, protein structure-function and cell-cell signaling [2,3]. To date, lncRNAs have been implicated in diverse biological processes [4,5]. In cancers, dysregulated lncRNA expression is a critical part of pathology [6–10]. LncRNAs may contribute to cancer development and progression by enhancing cell proliferative signaling, evading cell growth suppression, increasing angiogenesis, and preventing cell apoptosis [11–16]. In many cancers, the mechanisms by which individual lncRNAs contribute to cancer still remain incompletely understood [11–16].

Acute myeloid leukemia (AML) is a common malignant hematological disease characterized by uncontrolled clonal proliferation of poorly differentiated myeloid precursors in the bone marrow [17,18]. To date, various molecules and genetic alterations have been identified as contributing factors in AML [19–22]. In many AML patients, however, the underlying disease mechanisms remain unclear, indicating that additional pathological factors are yet to be identified [23–26].

To understand the molecular mechanism underlying AML, we conducted a microarray study to examine differentially expressed genes in AML and identified an expressed sequence tag (EST), H22954, which was down-regulated in CD34$^+$ cells and bone marrow mononuclear cells (BMNCs) in AML patients. Bioinformatic analysis indicated that H22954 was most likely an lncRNA targeting the 3' untranslated region (UTR) of the *BCL2* gene. Here we report our studies of H22954 expression in AML patients and effects of H22954 expression on cell proliferation and apoptosis *in vitro* and in mouse tumor xenograft models. Our data indicate that H22954 is a novel regulator of BCL-2 expression and that dysregulated H22954 expression may be an underlying mechanism in AML.

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2. Materials and methods

2.1. AML patient samples

This study was approved by the ethics committee at the First Affiliated Hospital of Soochow University and was conducted in accordance with the Declaration of Helsinki. AML classification was based on the French-American-British guidelines [27]. All patient data were reviewed by at least two independent hematologists. Bone marrow samples were obtained from patients with written informed consents. Bone marrow mononuclear cells (BMNCs) were isolated by Ficoll-Hypaque density gradient centrifugation. $CD34^+$ cells were selected from the BMNCs by immunomagnetic sorting, as described previously [28]. $CD34^+$ cells from one healthy individual and eight patients [two refractory anemia (RA), one refractory anemia with ring sideroblasts (RAS), two refractory anemia with excess blasts (RAEB), two AML, and one chronic aplastic anemia (CAA)] were used for gene expression microarray analysis. Additional BMNC samples were obtained from AML patients for subsequent studies.

2.2. Gene expression microarray analysis

Total RNAs were extracted from $CD34^+$ cells using TRIzol reagents (Invitrogen) [28]. RNAs were hybridized to Affymetrix U133A Plus 2.0 Arrays (Santa Clara, CA). Sample preparation and microarray procedures were based on the Affymetrix protocols. Data were normalized by the Quantile regression algorithm. Limma R package was used to select differentially expressed genes with > 2-fold changes and p values < 0.05. Hierarchical clustering was used to visualize differentially expressed genes identified by Limma. Genes of interest were compared with those in public BMNC databases. Genes with similar expression profiles in our study and public databases were selected for further studies.

2.3. Bioinformatic analysis of miRNAs

The full-length H22954 sequence was used in BLASTN analysis to identify stem-loop sequences in the miRBase database (version 21) (http://www.mirbase.org/search.shtml). After miRNAs homologous to H22954 were found, the TargetScanHuman 6.0 database were searched to identify potential target genes by the miRNAs (Supplementary Table S1). The genes identified were compared with those found in the microarray analysis to select overlapping genes. To identify specific H22954 target sites, mature miRNAs in the miRBase database (version 21) were searched for homologous sequences to H22954. Identified mature miRNAs were analyzed against the TargetScanHuman database to locate H22954 target sites in particular genes.

2.4. Cell culture

Human myeloid leukemia cell lines, including K562, NB4, SHI, Kasumi-1, and U937 cells, were from the American Type Culture Collection and cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS) at 37 °C in humidified incubators with 5% CO_2 and 95% air.

2.5. RT-PCR, real-time PCR and plasmid constructs

Plasmids for overexpression were pcDNA3.0-based. Plasmids for knockdown experiments were pLKO.1-based. Total RNAs from BMNCs or cell lines were isolated, treated with RNase-free DNase, and used to make cDNAs. RT-PCR to amplify H22954 mRNA was done with a forward primer, 5′-CCC GAA GTG CTG GGA TTA-3′, and a reverse primer, 5′-TCA GTT TCA GTA GAT GGA GGT A-3'. H22954 expression levels in AML patient-derived BMNCs were quantified by real-time PCR [28]. The plasmid pcDNA-H22954 was constructed by inserting human H22954 cDNA into the pcDNA3.0 vector (Invitrogen). The full-length H22954 cDNA from normal human BMNCs was amplified by RT-PCR using Phusion polymerase and primers 5′-CGG GAT CCC TTT TAA GTA GAG ATG GG-3′ and 5′-GGA ATT CTG ATG GGA AAT TTC AGA C-3'.

2.6. Cell transfection and Western blotting

K562 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) [29]. After 24–48 h, the cells were lysed in a solution with 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% (v/v) Triton X-100, and a protease inhibitor mixture (1:100 dilution; Sigma). Proteins were analyzed by SDS-PAGE and Western blotting, as described previously [30]. For stable cell lines, G418 (500 μg/mL) was added to the culture medium 24–48 h after the plasmid transfection. After 3–4 weeks, individual cell clones were selected and expanded for further studies.

2.7. Cell proliferation assay

Cell Counting Kit-8 (CCK-8) assay (Dojindao Laboratories) was used to measure cell proliferation in 96-well plates. Cells in each well were incubated with 10 μL of CCK-8 solution at 37 °C for 2 h. Optical absorbance was monitored in a plate-reader at 450 nm wavelength. Each data point was assayed in triplicate. Each experiment was performed at least three times, as described previously [31].

2.8. Apoptosis assay

Serum-starved (6–8 h) cells were washed in PBS and suspended in 100 μL of staining solution containing annexin-V fluorescein and propidium iodide (PI) in HEPES buffer (Boehringer-Mannheim). After 15 min at room temperature, the cells were analyzed by flow cytometry. Apoptotic cells were indicated by annexin-V staining [28].

2.9. $CD15^+$ induction

Three stable K562 cell lines from single colonies expressing H22954 were cultured without FBS for 6–8 h and switched to medium containing 10% FBS with or without 12-O-tetradecanoylphorbol-13-acetate (TPA) (5 nM, Sigma). After 36–48 h at 37 °C, the cells were stained with a phycoerythrin (PE)-conjugated anti-CD15 antibody and analyzed by flow cytometry (FACScan; Becton Dickinson) to detect $CD15^+$ cells.

2.10. Cell cycle analysis

Serum-starved (6–8 h) cells were cultured with RPMI-1640 medium containing 10% FBS at 37 °C for 18 h. The cell cycle was analyzed by flow cytometry, as described previously [28].

2.11. Mouse xenograft tumor model

Six-week-old athymic female BALB/c mice (n = 6 per group) were randomized and injected subcutaneously on the right dorsal flank with K562 cells (5×10^6) stably transfected with pcDNA-H22954 or a control vector. Every 3 d, tumor sizes in these mice were measured using a slide gauge. Tumor volume was calculated using the following equation: volume (v) = tumor mass length × $\text{width}^2/2$ [31]. All animal experimentation were carried out in accordance with NIH guidelines and under protocols approved by the Institutional Animal Care and Use Committee of Soochow University.

2.12. Tumor histology and TUNEL assay

Xenograft tumor tissues were fixed in 10% formalin and embedded in paraffin. Sections (5-μm in thickness) were cut, stained with hematoxylin and eosin (H&E), and examined under a light microscope

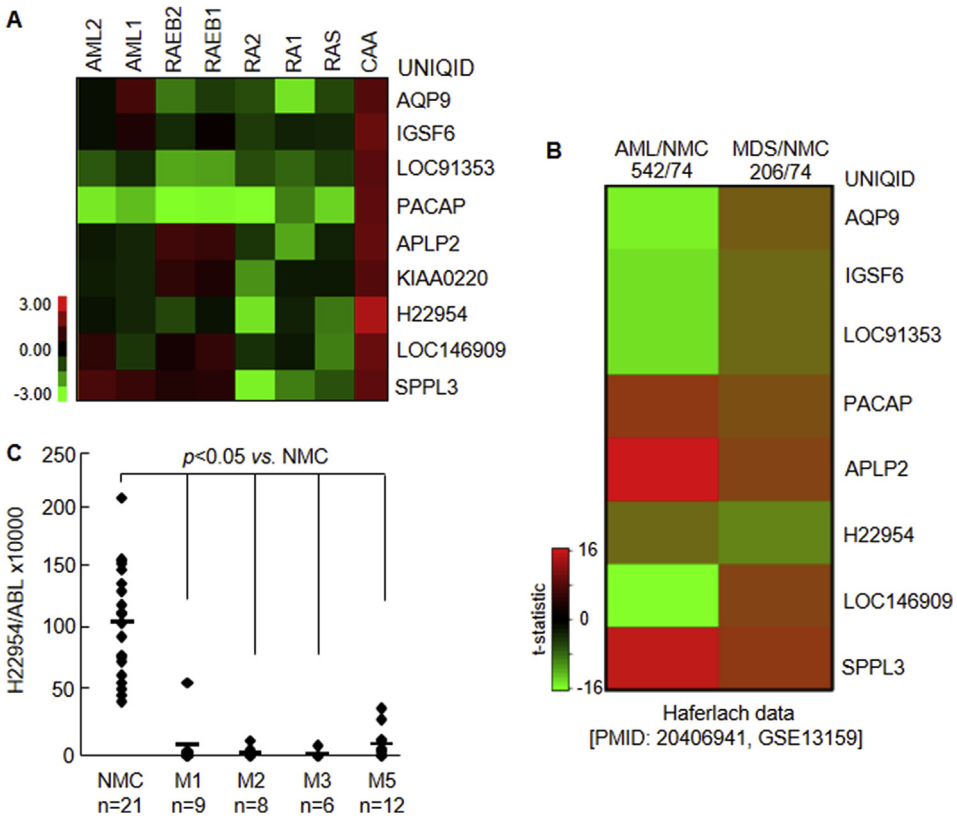

Fig. 1. H22954 is down-regulated in AML. A, Hierarchical clustering of a selected set of genes differentially expressed in $CD34^+$ cells selected from BMNCs from AML, refractory anemia with excess blasts (RAEB), refractory anemia (RA), refractory anemia with ring sideroblasts (RAS) and chromic aplastic anemia (CAA) patients. Each column represents an individual sample, and each row represents a gene in microarray. UNIQID: gene symbol. Patient samples are arranged based on similar gene expression patterns. Red and green colors in the image indicate expression levels of greater or less than the mean value. The color intensity indicates the magnitude of deviation from the mean. **B**, Differential expression of selected genes in BMNCs between AML, MDS and non-malignant control samples. Differential expression is represented as the t-statistic of genes. The data is derived from the analysis of a public database [20]. **C**, H22954 mRNA levels in BMNCs from non-malignant controls (NMC) and AML patients of M1, M2, M3, and M5 subtypes, who were newly diagnosed and untreated. H22954 mRNA levels were quantified by quantitative real-time RT-PCR and the data were normalized using *ABL* as a reference in each sample. Sample numbers in each group are indicated. The data are from non-parametric analysis. All p values are < 0.05 *vs.* NMC. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

[32,33]. A TUNEL assay was used to examine apoptotic cells in tissue sections. Frozen sections from the Xenograft tumors were fixed, permeabilized and incubated with TUNEL reagents (Roche Diagnostics) at 37 °C for 1 h. The stained sections were examined under a fluorescence microscope.

2.13. Luciferase assay

K562 and SHI cells were transfected with 1 μg of the H22954 plasmid or a control vector together with 1 μg of a luciferase reporter vector. To normalize for the transfection efficiency in each experiment, 50 ng of pRLTK plasmid (Promega) was used in each well. Luciferase activity was measured by the Dual-Luciferase Reporter Assay System (Promega).

2.14. FISH assay

To perform the FISH assay [16], cells were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100 in PBS at 4 °C. After 5 min, the cells were washed with PBS and hybridized with a FISH probe in a dark moist chamber at 37 °C overnight, according to manufacturer's protocol (Ribobio Tech, China). Cell images were examined under a confocal microscope (Olympus FV1200).

2.15. Primary cell culture

Bone marrow-derived primary BMNCs from AML patients were isolated by Ficoll-Hypaque density gradient centrifugation. The cells were divided into two equal aliquots and infected with a lentiviral vector expressing H22954 and a control vector, respectively. After 72–96 h at 37 °C, the cells were analyzed by flow cytometry (FACScan; Becton Dickinson) for their immunophenotypes.

2.16. RNA antisense purification (RAP) assay

RAP was performed based on published methods [34,35]. A graph to illustrate the RAP assay is shown in Supplementary Fig. S1 [35]. Heat-denatured biotinylated oligonucleotide probes, complementary to target RNAs, were incubated at 37 °C with pre-cleared cell lysates in a hybridization solution (20 mM Tris-HCl, pH 7.5, 7 mM EDTA, 3 mM EGTA, 150 mM LiCl, 1% NP-40, 0.2% N-lauroylsarcosine, 0.1% sodium deoxycholate, 3 M guanidine thiocyanate and 2.5 mM TCEP). After 2 h, Streptavidin magnetic beads were added and incubated at 37 °C for 30 min. After washing, the beads were magnetically separated and washed with RNase H elution buffer (50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 3 mM $MgCl_2$, 0.125% N-lauroylsarcosine, 0.025% sodium deoxycholate and 2.5 mM TCEP). Eluted RNA complexes were used in qPCR to quantify RNA yield and enrichment.

2.17. Statistical analyses

All data are presented as means ± S.D. Statistical analysis between two groups was done using Student's *t*-test with the SPSS 15 software. Comparisons among three or more groups were done by 1-way ANOVA or Kruskal-Wallis test. Correlation analysis was done using Person and Spearman correlation coefficients between 2 variables. Comparisons between relapse rates were presented in Kaplan-Meier curves. Statistical significance was considered when p values were < 0.05.

3. Results

3.1. Identification of a novel EST, H22954, that is down-regulated in AML

To identify differentially expressed genes in $CD34^+$ hematopoietic stem/progenitor cells in AML patients, we conducted a microarray study using the Affymetrix Human Genome U133 Plus 2.0 Array chips (GSE107968). We identified a novel EST, H22954, that was down-regulated in $CD34^+$ cells from two AML and five myelodysplastic

syndrome (MDS) patients, compared with that in normal controls (Fig. 1A). In contrast, H22954 expression was up-regulated in $CD34^+$ cells from chronic aplastic anemia (CAA) patients (Fig. 1A). We also analyzed public databases with BMNC gene profiles from normal controls and leukemia patients, in which H22954 expression data were available. We found similar results of H22954 down-regulation in AML and MDS patients in the dataset deposited by Haferlach et al. (PMID: 20406941, GSE13159) [36] (Fig. 1B).

The full-length H22954 cDNA consists of 432 nucleotides that have no homologies with any known genes [37,38] (Supplementary Fig. S2A). The gene encoding H22954 is on chromosome 8 (Supplementary Fig. S2B). We did RACE experiments but found no additional cDNA sequences. The longest possible open-reading frame (ORF) of H22954 is of 47 amino acids (Supplementary Fig. S2A) with no similarities to any proteins in public databases and no identifiable domains such as signal peptide, suggesting that H22954 is likely a novel lncRNA but not a protein-coding gene.

To understand the significance of H22954 down-regulation in human myeloid leukemia, we examined H22964 expression in BMNCs from patients with different AML subtypes. Consistent with the microarray finding, H22954 mRNA levels were markedly decreased in all AML subtypes examined, including AML without maturation (M1) (n = 9), AML with maturation (M2) (n = 8), acute promyelocytic leukemia (M3) (n = 6), and acute monoblastic and monocytic leukemia (M5) (n = 12), all of which were newly diagnosed and untreated (Fig. 1C).

We also examined H22954 mRNA levels in BMNCs from five M2 AML patients before and after the NCCN recommended chemotherapy (7 days of cytarabine, 100–200 mg/m^2, infusion and 3 days of idarubicin 12 mg/m^2). In RT-PCR, H22954 mRNA levels were undetectable in all of the five untreated patients but increased when the patients were in remission after the treatment, which inversely correlated with levels of the *AML-ETO* fusion gene, an AML marker (Fig. 2A). The results suggest that H22954 expression may be a blast marker associated with AML progression.

To verify this hypothesis, we examined H22954 expression in 46 BMNC samples from 28 M2 AML patients. We found an inverse correlation between H22954 mRNA levels and blast cell counts ($r = -0.311$, $p < 0.05$) (Fig. 2B), and a positive correlation between *AML-ETO* levels and blast cell counts ($r = 0.558$, $p < 0.05$) (Fig. 2C). Patients with low H22954 levels appeared more likely to relapse. Among 15 M2 AML patients who received one course of the chemotherapy, 5/8 (62.5%) patients with below median H22954 mRNA levels relapsed within 30 months, whereas all seven patients with above median H22954 mRNA levels remained in remission (5/8 *vs.* 0/7, $p < 0.05$) (Fig. 2D). These results suggest that the down-regulation of H22954 expression may play an important role in AML.

3.2. H22954 expression inhibits AML cell proliferation in vitro

RT-PCR detected high H22954 mRNA levels in SHI and U937, but not K562 and NB4, cells. Low H22954 mRNA levels also were detected in Kasumi-1 cells (Supplementary Fig. S3A). To test the effect of H22954 expression on cell growth, we transfected K562 cells, which had no detectable endogenous H22954 expression, with a plasmid expressing H22954 and observed an inhibitory effect on cell proliferation that was associated with G2/M phase arrest and increased apoptosis (Supplementary Figs. S3B–D). In Kasumi-1 cells, the number of apoptotic cells was also increased when H22954 was overexpressed (Supplementary Fig. S3E).

We extended the study and established three single colony-derived stable K562 cell lines expressing H22954 and three single colony-derived control K562 stable cell lines. H22954 mRNA expression in these stable cells were verified by RT-PCR (Fig. 3A). Consistent with findings from the transiently transfected cells (Supplementary Figs. S3C and D), high percentages of G2/M-phase (Fig. 3B) and apoptotic (Fig. 3C) cells were found in the stable cells expressing H22954, compared with those in the control cells. In a control rescue experiment, apoptotic cell numbers were reduced when a BCL2 plasmid was transfected in the H22954-expressing cells (Fig. 3F). Moreover, more $CD15^+$-cells were

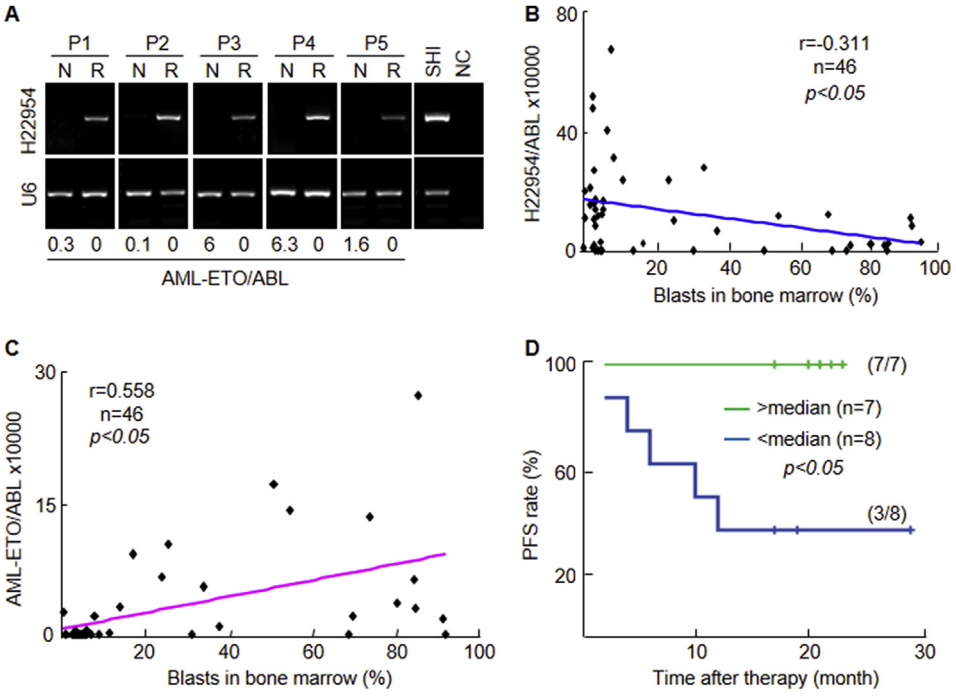

Fig. 2. H22954 expression levels inversely correlate with AML progression. A, RT-PCR analysis of H22954 mRNA expression in BMNCs from five M2 AML patients (P1-5) before chemotherapy (N) and in remission (R). SHI cells were used a positive control and reactions without mRNAs were used as a negative control (NC). U6 expression was used as an internal control for RT-PCR. *AML-ETO* fusion gene expression levels were also analyzed and normalized to *ABL* expression levels. Numbers under lower panels indicate relative expression levels in each sample. **B**, Correlation between H22954 expression levels and percentages of bone marrow blasts in 46 M2 AML patients. The data were analyzed by Pearson correlations. **C**, Correlation between *AML-ETO* expression levels and percentages of bone marrow blasts in the same set of M2 AML patients, as analyzed by Pearson correlations. **D**, Progression-free-survival (PFS) rates in M2 AML patients with H22954 expression levels above (green) or below (blue) the median value. All patients received one course of standard chemotherapy. Five of 8 patients with H22954 levels below the median value relapsed within three years, whereas none of 7 patients with H22954 levels above the median value relapsed during the same period. Statistical analysis was based on Survival analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

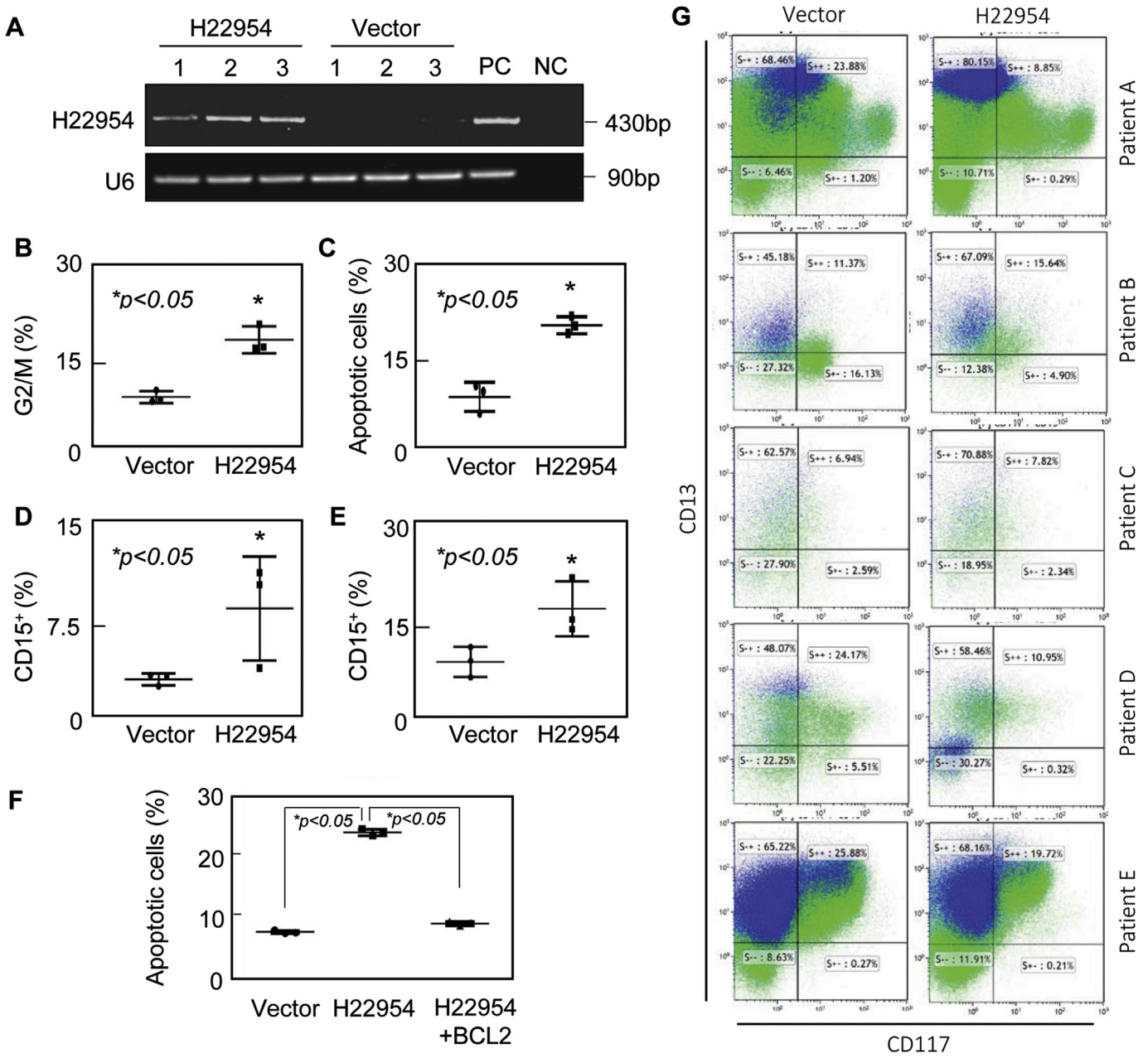

Fig. 3. Effects of H22954 expression on stably transfected K562 cells and primary AML cells. A, RT-PCR analysis of H22954 mRNA expression in individual K562 cell lines stably transfected with the H22954-expressing plasmid or a control vector. Normal BMNCs and reactions without mRNAs were used as positive control (PC) and negative control (NC), respectively. U6 expression was used as another control. **B-C**, Percentage of cells in the G2/M phase (**B**) and apoptotic cells (**C**) in K562 cells stably transfected with H22954-expressing plasmid or a control vector. **D-E**, Percentages of $CD15^+$ cells in K562 cells stably transfected with the H22954-expressing plasmid or a control vector and treated without (**D**) or with (**E**) TPA. **F**, BCL2 expression reduced H22954-induced apoptosis in K562 cells. **G**, Flow cytometric analysis of CD117 and CD13 markers in primary BMNCs from 5 AML patients (patients A-E), which were transduced with an H22954-expressing lentiviral vector or a control lentiviral vector. Cells in the low right area were $CD117^+CD13^{low}$ and in the up left area were $CD13^+CD117^{low}$.

detected in the H22954-expressing cells than the control cells, when the cells were cultured without (Fig. 3D) or with (Fig. 3E) 12-O-tetradecanoylphorbol-13-acetate (TPA), a myelomonocytic differentiation inducer. Cyclin A2 expression, known to be reduced in the G2 phase, was also lower in the H22954-expressing cells (Supplementary Fig. S4A). These results indicate that H22954 expression promoted K562 cell differentiation in culture.

We verified these results in primary BMNCs from five AML patients (Supplementary Table S2). As shown by flow cytometry, H22954 overexpression decreased $CD117^+CD13^{low}$ (myeloid progenitor) cells and increased $CD13^+CD117^{low}$ (relatively mature myeloid) cells in the primary BMNCs from all five patients studied (Fig. 3G). In contrast, H22954 overexpression did not alter $CD34^+$ cell population in these primary BMNCs (Supplementary Fig. S5). The results are consistent with the K562 cell-derived results, indicating that H22954 promoted the differentiation of cultured primary BMNCs from AML patients.

3.3. H22954 expression inhibits tumor growth in a mouse xenograft model

To test whether H22954 has an anti-tumor growth activity *in vivo*, we established a xenograft tumor model in athymic BALB/c mice using the stable K562 cells expressing H22954. Compared with the vehicle-transfected control cells, the K562 cells expressing H22954 grew slower, as indicated by smaller tumor volumes (Fig. 4A–C). In tissue

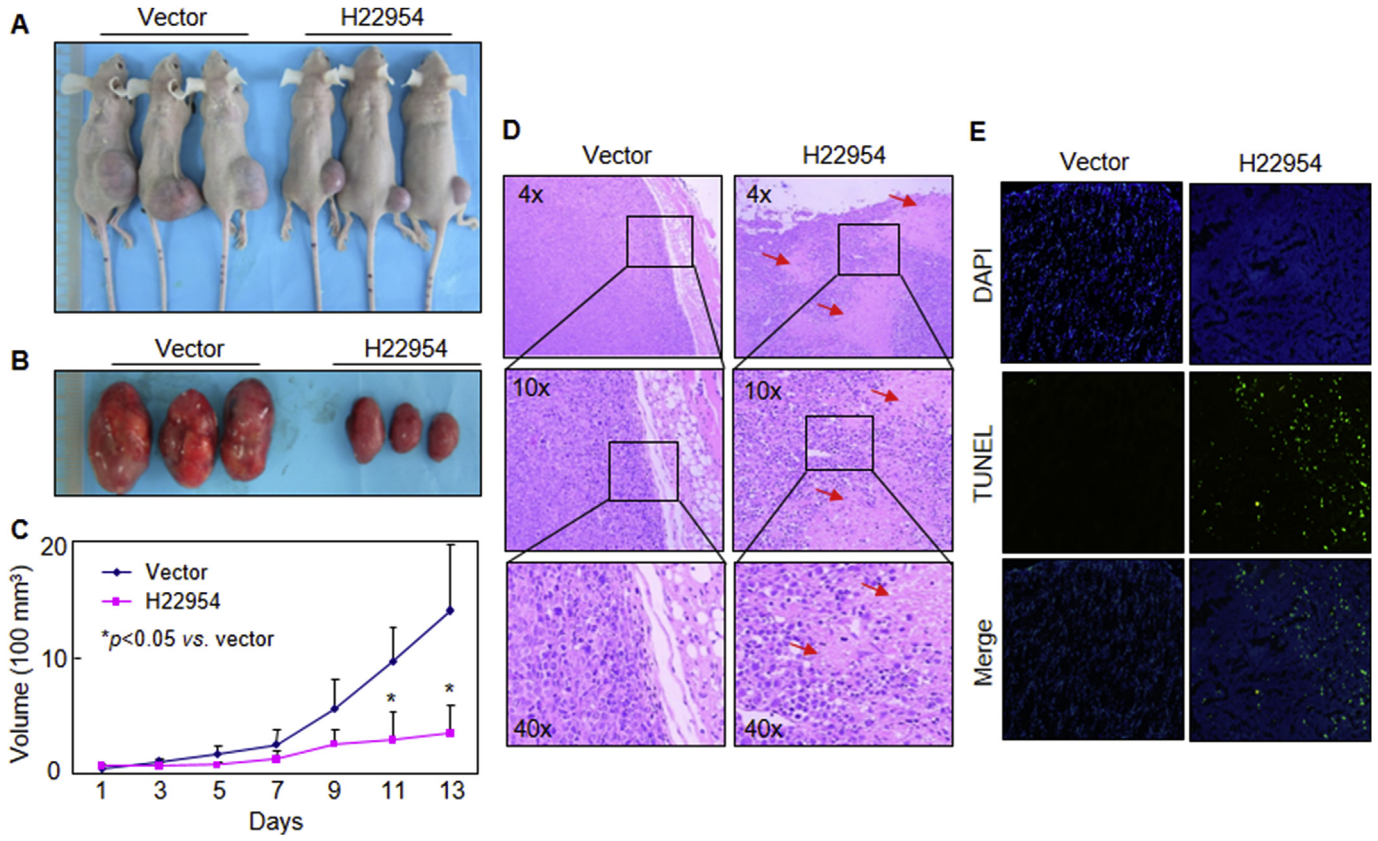

Fig. 4. H22954 expression inhibits tumor growth in a mouse xenograft model. A, Images of mice receiving K562 cells stably transfected with a control vector or the H22954-expressing plasmid. The images were taken on day 14 after cell implantation. **B**, Images of isolated tumors form the mouse xenograft model. **C**, Tumor volumes in mice receiving K562 cells stably transfected with a control vector or the H22954-expressing plasmid. $*p < 0.05$ *vs.* vector control of the same time point by Student's *t*-test (n = 3). **D-E**, H&E staining (**D**) and TUNEL-positive cells (**E**) in tumor sections from mice receiving K562 cells stably transfected with a vector or the H22954-expressing plasmid. Red arrows in **D** indicate necrotic lesions. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

sections, more necrotic lesions (Fig. 4D), TUNEL-positive cells (Fig. 4E), and $CD15^+$ cells (Supplementary Fig. S6) were found in the tumors from the H22954-expressing K562 cells. These results indicate that H22954 expression promoted apoptosis and inhibited tumor growth *in vivo*.

3.4. *BCL2 is a gene targeted by H22954*

To understand the mechanism underlying the anti-tumor activity of H22954, we compared gene expression profiles in the stable K562 cells with or without H22954 expression. In microarray, 1,613 differentially expressed genes were found that had > 2-fold changes and p values < 0.05 (Fig. 5A). In parallel, BLAST analysis identified 1,518 human genes whose 3′UTRs may be targeted by H22954 (Fig. 5A). Eleven genes were present in the both sets. Six of them (*BCL2, XIAP, LAPTM5, ADORA, THBS1* and *PDGFA*) had reduced expression levels in the microarray analysis (Fig. 5A). We examined the expression levels of these genes in BMNCs from four M2 patients before and after remission. By RT-PCR, increased H22954 and decreased *BCL2* mRNA levels were found in all of the four patients after remission (Fig. 5B). In comparison, *LAPTM5* and *ADORA* mRNA levels were unchanged, whereas both increased and decreased *XIAP, THBS1,* and *PDGFA* mRNA levels were observed before and after remission (Fig. 5B). By analyzing public databases, we found a similar negative correlation between H22954 and *BCL2* expression in AML-derived BMNCs ($r = -0.26$, $p = 2^{e-9}$, n = 521) in a dataset deposited by Taskesen et al. (PMID: 21177436, GSE14468) [39] (Fig. 5C). We analyzed BMNCs samples from five additional M2 patients before chemotherapy and in remission for AML-ETO, H22954 and BCL2 expression. Decreased AML-ETO and BCL2 expression and increased H22954 expression were observed (Supplementary Fig. S7). These results suggest that *BCL2* may be an H22954 target.

3.5. *H22954 inhibits BCL2 expression*

To test if H22954 inhibits *BCL2* expression, we examined BCL-2 protein levels in the H22954-expressing stable K562 cells. In Western blotting, BCL-2 protein was detected in the control cells, but not in the H22954-expressing cells (Fig. 5D, and Supplementary Fig. S8). We also examined proteins involved in apoptosis by Western blotting and found increased levels of cleaved caspase 9, poly(ADP-ribose) polymerase (PARP) and caspase 7 forms in the H22954-expressing cells (Fig. 5D and Supplementary Figs. S8–9). Consistently, BCL-2 protein levels were also lower in mouse xenograft tumors from the H92254-expressing cells than in the controls (Fig. 5E, Supplementary Fig. S10). In SHI cells, which expressed high levels of endogenous H22954, knocking down H22954 expression by shRNA increased BCL-2 expression (Supplementary Figs. S11A and B). In K562 cells, H22954 expression reduced the half-life of *BCL2* mRNA (Supplementary Fig. S12). These results indicate that H22954 inhibited *BCL2* expression and promoted apoptosis in cultured AML cells and xenograft tumors in mice.

3.6. *H22954 interacts with the BCL2 3′UTR*

Some lncRNAs are known to act in cytoplasm to regulate targeting genes by interacting with their 3′UTRs [40]. To examine the subcellular

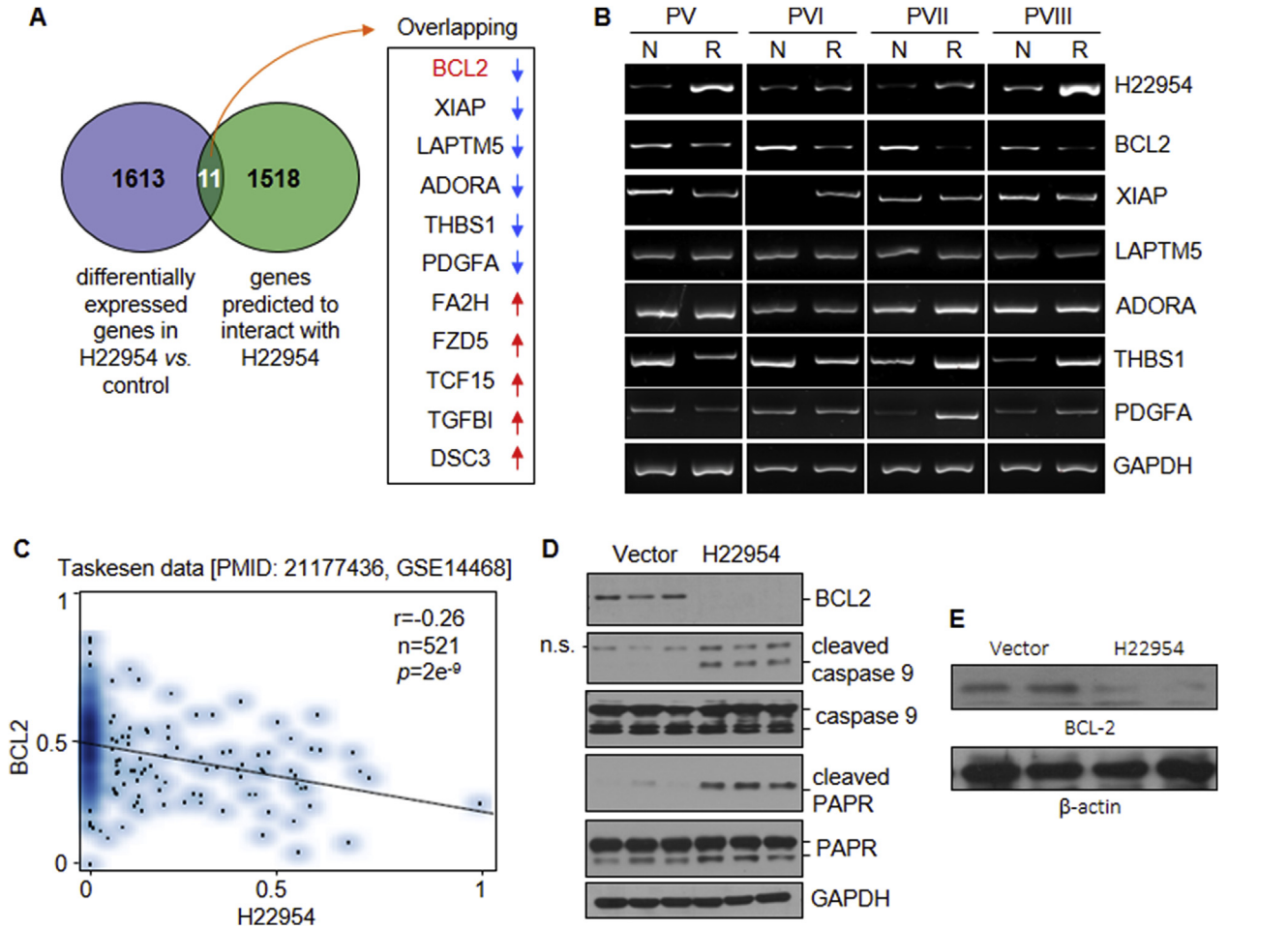

Fig. 5. *BCL2* is a potential H22954 target. A, Microarray analysis identified 1,613 genes differentially expressed in stable K562 cells with or without H22954 expression (left circle). Bioinformatic analysis identified 1,518 genes, whose 3′UTR may be targeted by H22954 (right circle). Eleven genes common in both sets are shown in the box. Up and down arrows indicate increased and decreased mRNA levels, respectively, in K562 cells stably transfected with the H22954-expressing plasmid *vs.* in vector-transfected control cells. **B**, RT-PCR analysis of *H22954*, *BCL2*, *XIAP*, *LAPTM5*, *ADORA*, *THBS1* and *PDGFA* expression in BMNCs from four M2 AML patients (PV-VIII) before chemotherapy (N) and after remission (R). *GAPDH* was a control. **C**, Correlation between *H22954* and *BCL2* expression based on the analysis of a public data set [50]. **D**, Western blotting of BCL-2, caspase9 and PARP proteins in K562 cells stably transfected with the H22954-expressing plasmid or a control vector. Cleaved caspase 9 and PARP forms are indicated. GAPDH was used as a control. Experiments repeated at least three times. **E**, Western blotting of BCL-2 protein in tumor tissues from xenograft mice receiving K562 cells stably transfected the H22954-expressing plasmid or a control vector. β-Actin was used as a control. Experiments repeated at least three times.

location of H22954, we did an RNA FISH experiment and detected positive H22954 staining in the cytoplasm of both K562 and SHI cells (Fig. 6A). In controls, 18S staining was in the cytoplasm, whereas U6 staining was in the nucleus (Fig. 6A). To test if H22954 directly interacts with the *BCL2* 3′UTR, we performed an RNA antisense purification (RAP) assay [34,35], in which *BCL2* 3′UTR and H22954 fragments were added in a reaction mixture and hybridized complexes were captured. Subsequent qPCR analysis indicated that the *BCL2* probe captured the H22954 fragment and the H22954 probe captured the *BCL2* fragment (Fig. 6B). Similar results were found when the RAP assay was done in K562 cells transfected with the H22954-expressing plasmid alone or with the plasmid expressing the *BCL2* 3′UTR. The *BCL2* probe captured more H22954 copies compared with those in the cells transfected with corresponding control plasmids (Fig. 6C). The results indicate a direct interaction between H22954 and the *BCL2* 3′UTR.

In bioinformatic analysis, hsa-miR-5095 and hsa-miR-619-5p were identified in the miRBase database that shares the sequence homology with H22954 and targets *BCL2* 3′UTR. Further analysis of the TargetScanHuman database pointed to the 8 nucleotide GCCTGTAA sequence, which was present in *BCL2* 3′UTR (nucleotides 1565 and 1572), as the H22954 targeting site in *BCL2* 3′UTR (Fig. 7A). To test this hypothesis, we inserted the *BCL2* 3′UTR fragment into the pGL3 vector with the luciferase activity (Fig. 7B). As controls, pGL3 vector alone or with a mutated H22954-targeting sequence in the *BCL2* 3′UTR (pGL3-BCL2m) were included (Fig. 7B). In K562 cells, co-transfection of H22954 inhibited the luciferase activity of the pGL3-*BCL2* 3′UTR plasmid, but not those of pGL3 and pGL3-BCL2m plasmids (Fig. 7C). In SHI cells with high levels of endogenous H22954, the luciferase activity of pGL3-BCL2 was lower than that of the control plasmid pGL3 (Fig. 7D). The activity was further reduced when H22954-expressing plasmid was co-transfected (Fig. 7D). The inhibitory effect of H22954 on pGL3-BCL2 plasmid was shown to be dose-dependent (Fig. 7E). Such an effect was not observed when H22954-expressing plasmid was co-transfected with pGL3-BCL2m plasmid lacking the H22954-targeting sequence. These results support the idea that H22954 inhibits *BCL2* expression by interacting with its 3′UTR.

4. Discussion

AML is a malignant hematological disease that results from different genetic and epigenetic abnormalities [17–19]. In this study, we examined differentially expressed genes by microarray analysis and

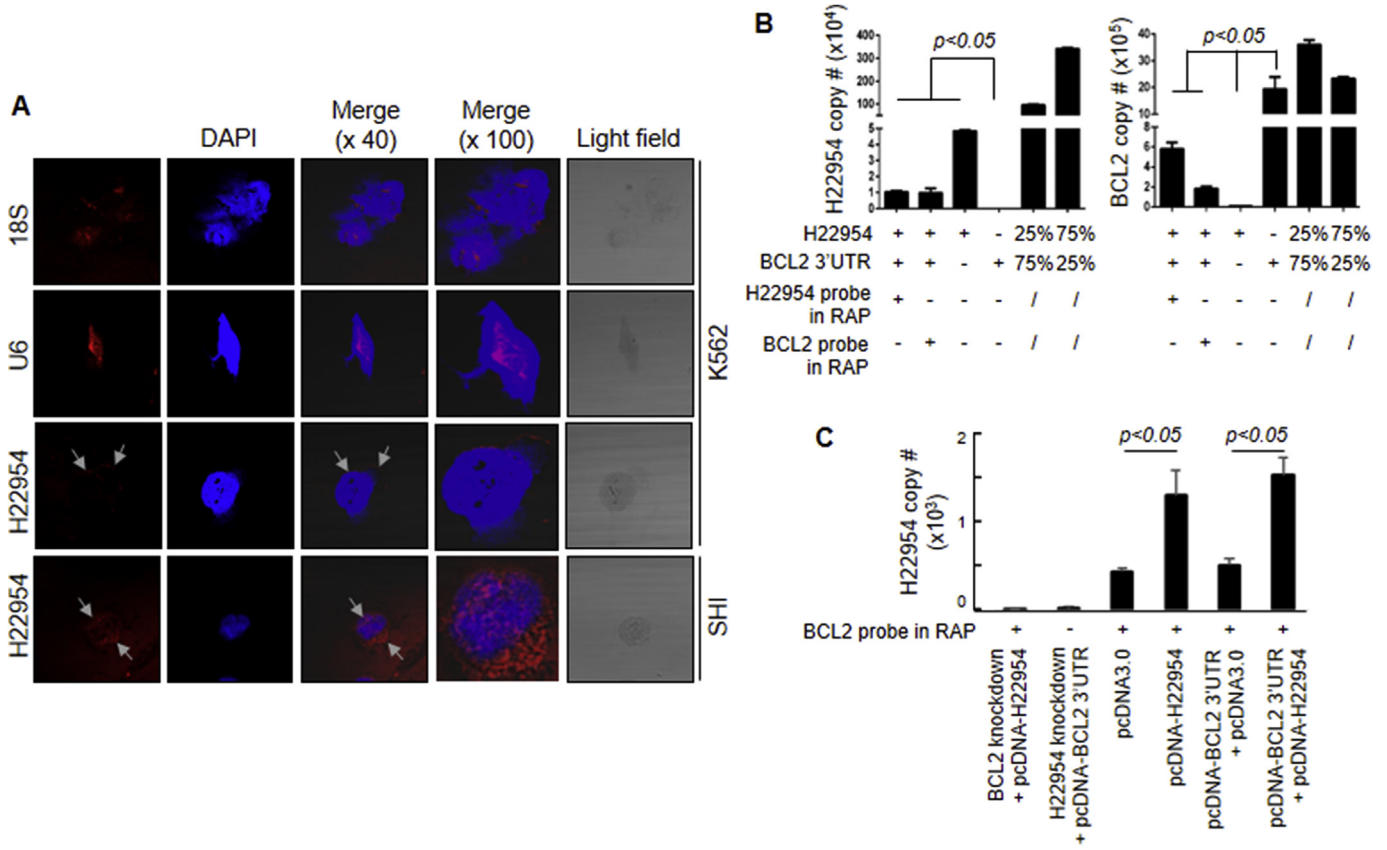

Fig. 6. H22954 targets the *BCL2* 3′UTR. A, Subcellular localization of H22954 in K562 and SHI cells analyzed by RNA FISH, in which 18S was a cytoplasm control and U6 was a nuclei control. H22954 staining was indicated by white arrows. **B**, RAP assay to detect H22954 and *BCL2* 3′UTR interaction in solution. H22954 and *BCL2* 3′UTR fragments were added in the GuSCN hybridization buffer. RAP assay was done with a *BCL2* probe to detect H22954 (left) or with a H22954 probe to detect *BCL2* (right) by quantitative real-time PCR. H22954 only and fragments of *BCL2* 3′UTR only were used as controls in RT-PCR. Data are mean ± S.D. (n = 3), $p < 0.05$ by one-way ANOVA. **C**, RAP assay to detect H22954 and *BCL2* 3′UTR interaction in K562 cells. K562 cells were transfected with pcDNA-H22954 or pcDNA-H22954 and pcDNA-*BCL2* 3′UTR. In controls, K562 cells were transfected with pcDNA-3.0 or pcDNA-3.0 and pcDNA-*BCL2* 3′UTR. Data are mean ± S.D. (n = 3), $p < 0.05$ by Student's *t*-test or one-way ANOVA.

verified our results by analyzing the data in public databases. We identified a novel EST, H22954, which was down-regulated in AML patients. We found that H22954 expression levels inversely correlated with the number of bone marrow blasts in AML patients and that the patients with low H22954 levels were more likely to relapse than those with higher H22954 levels. When the patients were in remission, H22954 expression levels in their BMNCs increased, suggesting that reduced H22954 expression may indicate the presence of AML blasts and hence disease progression and poor prognosis.

In functional studies, we showed that H22954 expression inhibited cell proliferation and induced G2/M phase arrest and apoptosis in transfected K562 cells. H22954 expression also promoted cell differentiation in primary BMNCs from AML patients. Moreover, H22954 expression increased tissue necrosis and apoptosis, and suppressed K562 cell-derived tumor growth in a mouse xenograft model. These results are consistent with the inverse correlation between H22954 expression and disease progression observed in AML patients, supporting the idea that H22954 expression inhibits AML development and/or progression.

If H22954 inhibits AML cell growth, what is the molecular basis underlying such a function? The full-length H22954 cDNA consists of 432 nucleotides. The longest possible ORF encoded by H22954 is of 47 amino acids in length, which shares no similarities with any known proteins and lacks an identifiable signal peptide sequence required for protein secretion. We analyzed EST databases for overlapping sequences and performed RACE experiments to verify the full-length H22954 sequence. Our results indicate that H22954 is most likely a novel lncRNA, although we cannot formally exclude the possibility that H22954 may be a fragment of an extended 3′UTR of an adjacent gene. We also want to point out that H22954 contains miRNA coding sequences. It has been shown that some lncRNAs may exert their functions in the form of miRNA precursors [41]. Between H22954 and prehsa-miR-619 where is a stretch of 39 bp homologous sequence. Although there is no evidence that H22954 acts as a miRNA precursor, such a possibility cannot be excluded. Curiously, the H22954 homolog gene does not appear in mice, making it challenging to verify H22954 function in knockout mouse models.

The role of lncRNAs in cancer has been well documented [4,6–10]. Although lncRNAs may bind to proteins and regulate protein conformation and function [40], the most common mechanism underlying lncRNA function is to regulate gene expression by interacting with 3′UTRs [13]. In bioinformatic and gene expression profiling analyses, we identified *BCL2* as a possible H22954 target. In cells, H22954 was found mostly in the cytoplasm. In RAP assays, H22954 and the *BLC2* 3′UTR were shown to interact in test tubes and K562 cells. Co-transfection of H22954 and pGL3 constructs containing a *BCL2* 3′UTR sequence inhibited the luciferase activity, supporting the idea that H22954 acts as an lncRNA to inhibit *BCL2* expression. Consistently, stable K562 cells expressing H22954 had reduced BCL-2 protein levels and increased caspase 7, caspase 9 and PARP activation cleavages. The reduced BCL-2 level also was found in the mouse xenograft tumors from the K562 cells expressing H22954 and in the BMNCs from AML patients in remission. In SHI cells, *H22954* knockdown was associated with increased BCL-2 expression. Conversely, *BCL2* mRNA half-life was

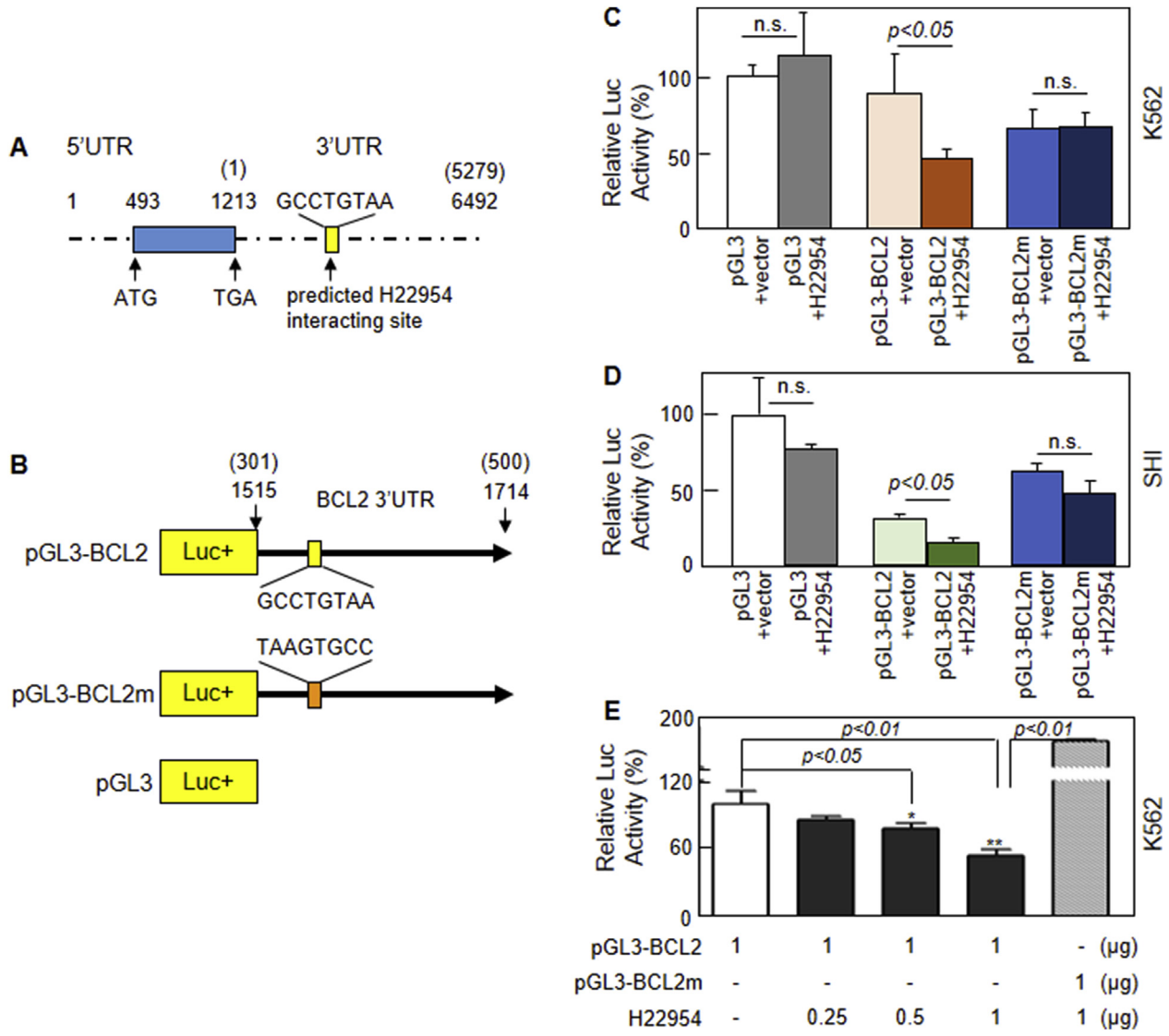

Fig. 7. H22954 inhibits the luciferase activity in K562 and SHI cells transfected with plasmids containing the *BCL2* 3′UTR. A, Illustration of the relative position of a H22954-targeting sequence in the human *BCL2* 3′UTR. **B**, Illustration of pGL3-based plasmids with the firefly luciferase (Luc) reporter gene and a wild-type (pGL3-BCL2) or a mutant (pGL3-BCL2m) *BCL2* 3′UTR sequence. **C-D**, Relative Luc activities in K562 (**C**) and SHI (**D**) cells transfected with pGL3-derived plasmids. One μg of the H22954 plasmid or a control vector together with 1 μg of a luciferase reporter vector. Data are mean ± S.D. (n = 3); $p < 0.05$ by Student's *t*-test; n.s.: not significant. **E**, H22954 inhibited the Luc activity in a dose-dependent manner in K562 transfected with the pGL3-BCL2 plasmid. Different concentrations of the H22954 plasmid were co-transfected with the pGL3-BCL2 plasmid (1 μg) in K562 cells. Data are mean ± S.D. (n = 3); $p < 0.05$ by Student's *t*-test.

decreased when H22954 was overexpressed. Moreover, H22954-induced apoptosis was inhibited by *BCL2* expression. These results indicate that the anti-tumor function of H22954 is mediated, at least in part, by inhibiting *BCL2* expression and inducing AML cell apoptosis. Additional studies are important to examine if there is a mechanistic association between AML1-ETO and H22954. How H22954 is down-regulated in AML. Whether a mutant H22954 in BCL2 targeted sequence could show lack of effect in inhibition of proliferation and induction of apoptosis, and If H22954 down-regulation occurs in other BCL-2 driven cancers, such as lymphomas and solid tumors.

To date, lncRNAs that affects cell death have been reported in cancers [42–46]. To our knowledge, H22954 is the first lncRNA that has been found to inhibit *BCL2* expression in cancer. It has been shown that BCL-2 is an important pro-cancer cell survival factor in human AML and that BCL-2 inhibition leads to selective killing of AML cells [47–50]. BCL-2 also contributes to chemotherapy resistance in AML [51]. These findings suggest a strategy to inhibit BCL-2 as a therapeutic approach to treat AML. Indeed, several studies have reported promising results of BCL-2 inhibitors in inducing AML cell death *in vitro* and prolonging survival in mouse AML models [52–54]. Thus, these data are in agreement with our findings that H22954 functions as an endogenous negative regulator of *BCL2* and that loss of such a function may contribute to AML development and/or progression in patients.

In summary, we have identified H22954 as a novel lncRNA, which is down-regulated in AML patients. H22954 expression negatively correlates with blast cell numbers and disease progression in AML patients. In cultured cells and mouse xenograft models, H22954 expression promotes cell death and inhibits tumor growth. The anti-tumor activity of H22954 is likely mediated by blocking *BCL2* expression. Our results indicate that H22954 is a novel *BCL2* regulator that plays a critical role in AML.

Conflicts of interest

The authors declare no competing financial interests.

Ethics approval and consent to participate

Bone marrow aspirates were obtained after informed consent in accordance with the Declaration of Helsinki from all human participants to this study. This study was approved by the ethics committee at the First Affiliated Hospital of Soochow University.

Consent for publication

Not applicable.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.055.

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