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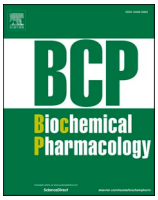

BCL6 confers resistance to HDAC inhibitors in DLBCL

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ABSTRACT

Diffuse large B-cell lymphoma (DLBCL) is an aggressive non-Hodgkin lymphoma with limited response to chemotherapy. Histone acetylation is reduced in DLBCL. Chidamide, a histone deacetylase inhibitor, shows promise in lymphomas but needs further investigation for DLBCL. Our study indicated that chidamide effectively suppresses DLBCL both in vitro and in vivo. High-throughput RNA sequencing analysis provided comprehensive evidence that chidamide markedly influences crucial signaling pathways in DLBCL, including the MAPK, MYC and p53 pathway. Additionally, we observed substantial variability in the sensitivity of DLBCL cells to chidamide, and identified that elevated expression of BCL6 might confer resistance to chidamide in DLBCL. Moreover, our investigations revealed that BCL6 inhibited chidamide-induced histone acetylation by recruiting histone deacetylase (HDACs), leading to drug resistance in DLBCL cells. Furthermore, we found that lenalidomide targeted BCL6 degradation through the ubiquitination pathway and restore the sensitivity of drug-resistant DLBCL to chidamide. Collectively, these findings provided valuable insights into the global impact of chidamide on DLBCL and highlight the potential of targeting HDACs as a therapeutic strategy for DLBCL. Identifying BCL6 as a biomarker for predicting the response to chidamide and the combination therapy with BCL6 inhibition has the potential to lead to more personalized and effective treatments for DLBCL patients.

1. Introduction

Diffuse large B-cell lymphoma (DLBCL) is an aggressive type of non-Hodgkin lymphoma, and although the standard of care for first-line treatment is rituximab in combination with chemotherapy (R-CHOP) [1,2], a significant number of patients either relapse after the initial response or do not respond to treatment [3,4]. Therefore, there is an urgent need to develop new therapeutic approaches for DLBCL.

Histone acetylation is a crucial epigenetic modification that affects gene expression by altering DNA accessibility to transcription factors and other regulatory proteins [5–7]. Studies have shown that DLBCL is characterized by a global decrease in histone acetylation levels, which may contribute to disease pathogenesis [8,9]. Hence, the development of new therapies targeting altered histone acetylation is promising.

Chidamide is a histone deacetylase inhibitor that has shown promising results in the treatment of certain types of lymphomas, including peripheral T-cell lymphoma (PTCL) and follicular lymphoma [10,11]. However, its use in diffuse large B-cell lymphoma (DLBCL), the most common type of non-Hodgkin's lymphoma, is still under investigation. Several clinical trials (NCT03373019, NCT05115409, NCT03201471), including ongoing study (NCT05690191), are evaluating the safety and efficacy of chidamide in combination with standard chemotherapy regimens for DLBCL. Early results from these trials indicate promising response rates and tolerability of the combination therapy.

Abbreviations: DLBCL, diffuse large B-cell lymphoma; HDACs, histone deacetylase; R-CHOP, rituximab in combination with chemotherapy; PTCL, peripheral T-cell lymphoma; SD, standard deviation; CI, combination index; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, Gene Set Enrichment Analysis; CCLE, Cancer Cell Line Encyclopedia; GDSC, Genomics of Drug Sensitivity in Cancer; HAT, histone acetyltransferase; C2H2, Cys2-His2; PCA, principal component analysis; TAMs, tumor-associated macrophages.

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Although data on the use of chidamide in DLBCL is limited, the preliminary results from ongoing clinical trials are encouraging. Further research is necessary to fully evaluate the efficacy of chidamide in DLBCL and determine its optimal use in combination with other treatments.

Our study aimed to investigate the impact of chidamide on DLBCL cells and identify biomarkers that could predict a favorable response to chidamide treatment in clinical settings. Furthermore, we aim to identify drugs that could enhance the sensitivity of DLBCL to chidamide, helping develop personalized treatment strategies for DLBCL patients.

2. Materials and methods

2.1. Compounds

Chidamide (selleck, S8567, Houston, TX, USA), Lenalidomide (selleck, S1029, Houston, TX, USA), Decitabine (selleck, S1200, Houston, TX, USA), and Ibrutinib (selleck, S2680, Houston, TX, USA).

2.2. Cell lines

The SU-DHL-8, SU-DHL-2, and FARAGE cell lines were cultured in RPMI-1640 medium (Hyclone) supplemented with 10 % fetal bovine serum at 37 °C in an atmosphere containing 5 % CO2. Chidamide resistant DLBCL cells (SU-DHL-8-re, SU-DHL-2-re) were developed through gradually increasing chidamide treatment. The resistant cell strains were eventually established by gradually increasing chidamide concentrations for 6 months. Then, the two cell lines were maintained in a complete medium containing chidamide. These cell lines underwent short tandem repeat (STR) marker analysis by Genetic Testing Biotechnology Corporation (Suzhou, China) to verify their identity.

2.3. Antibodies

The following antibodies were used: Anti-BCL6 antibody Proteintech (1:1000, Proteintech, Cat# 21187-1-AP, Chi, USA), Anti-HDAC1 antibody (1:1000, Proteintech, Cat# 10197-1-AP, Chi, USA), Anti-FLAG antibody (1:1000, Proteintech, Cat# 66008-4-Ig, Chi, USA), Anti-Ubiquitin antibody (1:1000, Proteintech, Cat# 80992-1-RR, Chi, USA), anti-H3 Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A2348, Wuhan, China), anti-H3K9ac Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A7255, Wuhan, China), anti-H3K18ac Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A7257, Wuhan, China), anti-H3K27ac Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A7253, Wuhan, China) and anti-GAPDH Rat Monoclonal Antibody (1:1000, Proteintech, Cat# 60004-1-Ig, Chi, USA).

2.4. Western blotting

The total proteins of cancer cells were extracted by lysis buffer containing phosphatase and protease inhibitors, then centrifuged at 4 °C at 12000g for 5 min. The protein concentration was determined by the enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were loaded onto 10 %–12 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China) for electrophoresis and then transferred to polyvinylidene difluoride membranes (Millipore, MA, USA).

Anti-BCL6 antibody Proteintech (1:1000, Proteintech, Cat# 21187-1-AP, Chi, USA), Anti-HDAC1 antibody (1:1000, Proteintech, Cat# 10197-1-AP, Chi, USA), Anti-FLAG antibody (1:1000, Proteintech, Cat# 66008-4-Ig, Chi, USA), Anti-Ubiquitin antibody (1:1000, Proteintech, Cat# 80992-1-RR, Chi, USA), anti-H3 Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A2348, Wuhan, China), anti-H3K9ac Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A7255, Wuhan, China), anti-H3K18ac Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A7257, Wuhan, China), anti-H3K27ac Rabbit Polyclonal Antibody (1:1000, Abclone, Cat# A7253, Wuhan, China) and anti-GAPDH Rat Monoclonal Antibody (1:1000, Proteintech, Cat# 60004-1-Ig, Chi, USA) were used as primary antibodies. The membranes were incubated with corresponding secondary antibodies and detected using enhanced chemiluminescence (ECL) immunoblotting detection reagent (Tanon, Shanghai, China). The western blot data has been gone through a densitometry analysis compared to reference protein (GAPDH) and has been reported relative to their corresponding controls. Gels and Western blots were performed three times.

2.5. RT-qPCR assays

RT-qPCR was performed using the SYBR® Green master mix (Bio-Rad, USA) on an ABI7500 system (Foster City, CA, USA). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was regarded as endogenous controls. Reaction cycles were as follows: 95 °C for 5 min, 95 °C for 30 s with 40 cycles, 60 °C for 45 s, 72 °C for 5 min. All data were analyzed using $2 - \Delta\Delta CT$ method to quantify the relative expression of genes. Each sample was performed with three replicates and every experiment was repeated three times. The primer sequences are described in Table 1.

2.6. Cytotoxicity assay and apoptosis analysis

A CCK-8 assay was performed to measure the cell inhibition rate of the drug. The apoptosis levels of DLBCL cell lines were analyzed by FCM. The cells were stained with annexin V-FITC and propidium iodide and analyzed by FCM.

2.7. RNA-seq and analysis of differentially expressed genes

Prepared libraries were sequenced on an Illumina HiSeq X Ten platform (RRID: SCR_016385). The abundances of transcripts (including mRNAs, pseudogenes, noncoding RNAs, and other predicted RNAs) were calculated and normalized in fragments per kilobase of transcript per million mapped reads, as described above, from the raw RNA-seq data and used for GSEA (Broad Institute, RRID: SCR_003199). Differential gene expression evaluation was analyzed using DESeq2 software (v1.30.0,RRID: SCR_015687; ref. 69). The transcriptome sequencing was conducted by OE Biotech, Inc., Shanghai, China.

2.8. In vivo tumor xenograft model

The animal experiments were conducted according to the institutional guidelines for the use of laboratory animals and after permission was acquired from the local ethical committee for animal experimentation. Five-week-old female NOD-SCID mice were purchased from Gempharmatech Company. The NOD-SCID were injected with FARAGE cell line in 50 % Matrigel subcutaneously on the left flank and were randomly divided into four groups receiving daily treatments: (1) control group; (2) chidamide group (4 mg/kg); (3) lenalidomide group (15 mg/kg); (4) combination group (4 mg/kg chidamide and 15 mg/kg lenalidomide).

Table 1
The sequences of primers used in RT-qPCR.

Primers	Sequences
GAPDH/F	5′-GGAGCGAGATCCCTCCAAAAT-3′
GAPDH/R	5′-GGCTGTTGTCATACTTCTCATGG-3′
CRBN/F	5′-TCTGCCGACATCACATACATACCT-3′
CRBN/R	5′-TGGAAGAACTGGAATCACCTGACA-3′
BCL6/F	5′-TCGTGAGGTGGTGGAGAACAA-3′
BCL6/R	5′-GAGAAGAGGAGGCTGCTGACA-3′

2.9. Cell transfection

Cells were transfected with siRNAs targeting BCL6 (GenePharma) using Neon Electroporation (Invitrogen). We generated Cas9-stable expressed cell lines, FARAGE-ko, either by stable integration or by transduction with Cas9 lentivirus (Cas9-Puro or Cas9-Blast), followed by puromycin or blasticidin selection. The sequences of siRNA and gRNA are described in Table 2.

2.10. Statistical analysis

Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA). Results are shown as mean plus or minus standard deviation (SD) of values obtained in independent experiments. The paired Student *t* test was used to compare the differences between paired samples. Differences were considered significant at P value below 0.05. The combination index (CI) was calculated for a 2-drug combination using compusyn program. A CI of 1 indicates an additive effect; a CI above 1, an antagonistic effect; and a CI below 1, a synergistic effect.

3. Results

3.1. Global impact of chidamide on DLBCL: Insights from high-throughput RNA sequencing analysis

To investigate the global impact of chidamide on diffuse large B-cell lymphoma (DLBCL), we conducted high-throughput RNA sequencing on DLBCL cells treated with either chidamide or a vehicle control. A heat map displayed the cluster analysis of these differentially expressed genes impacted by chidamide in DLBCL cells (Fig. 1B). Following chidamide treatment, 155 genes were consistently upregulated, and 3 genes were consistently downregulated across the three cell lines (Fig. 1C, Table 3). Gene Ontology (GO) functional annotations and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed the signaling pathways affected by chidamide (Fig. 1D, 1E). Gene Set Enrichment Analysis (GSEA) further revealed that chidamide treatment regulated the MYC pathway, p53 signaling pathway, and apoptosis (Fig. 1F). These results suggested that chidamide exerted a significant impact on various biological processes in DLBCL cells.

3.2. BCL6 confers resistance to chidamide in DLBCL

We investigated the effects of chidamide on three DLBCL cell lines of varying subtypes: SU-DHL-8 (GCB subtype), FARAGE (GCB subtype), and SU-DHL-2 (ABC subtype). The sensitivity of DLBCL cells to chidamide varies greatly, with SU-DHL-8, FARAGE, and SU-DHL-2 cells exhibiting IC50 values of 371 nM, 5872 nM, and 1556 nM, respectively (Fig. 2A). The subtype did not appear to be related to sensitivity. To identify biomarkers that could predict favorable response to chidamide treatment in clinical settings, we performed correlation analysis between gene expression levels obtained from the Cancer Cell Line Encyclopedia (CCLE) and IC50 values of four HDAC inhibitors (AR-42, Belinostat, MS-275, SAHA), obtained from the Genomics of Drug Sensitivity in Cancer (GDSC) across various cancer cell lines. The analysis showed that the expression of five genes, IRF4, LRMP, IL-16, BCL6, and NEK6, were associated with the sensitivity to HDAC inhibitors (Fig. 2B). Among these genes, BCL6 was found to be closely related with HDAC. BCL6 is a transcriptional repressor by binding to specific DNA sequences and recruiting HDACs to remove acetyl groups from histones, thereby leading to gene repression [12,13]. This interaction between BCL6 and HDAC is critical for the regulation of B-cell development and differentiation [12,14]. Our study revealed that the expression of BCL6 was significantly higher in FARAGE cells compared to the other two DLBCL cells (Fig. 2C), while FARAGE cells were found to be the least sensitive to chidamide. To confirm the crucial role of BCL6 in determining chidamide sensitivity, we knocked down BCL6 in FARAGE cells using CRISPR-Cas9. The result showed that the knockdown of BCL6 sensitized FARAGE cells to chidamide (Fig. 2D). Furthermore, the chidamide-resistance SU-DHL-2 and SU-DHL-8 were established by gradually increasing the concentration and continuing induction (Fig. 2E). Compared with wild-type cells, the expression of BCL6 increased in chidamide-resistant cells (Fig. 2F). Taken together, our results suggested that BCL6 played a crucial role in determining chidamide sensitivity in DLBCL cells, and that high expression of BCL6 may confer resistance to chidamide treatment.

Table 2
The sequences of siRNA and Grna.

siRNA/gRNA	Sequences
BCL6 (siRNA-1) Sense	5′-CCAUGCCAGUGAUGUUCUUTT-3′
BCL6 (siRNA-1) Antisense	5′-AAGAACAUCACUGGCAUGGTT-3′
BCL6 (siRNA-2) Sense	5′-CCAGUUGAAAUGCAACCUUTT-3′
BCL6 (siRNA-2) Antisense	5′-AAGGUUGCAUUUCAACUGGTT-3′
BCL6 (gRNA) Sense	5′-CACCCTGTAAAGATGCTATAGAAC-3′
BCL6 (gRNA) Antisense	5′-CGACATTTCTACGATATCTTGCAAA-3′
siRNA control Sense	5′-UUCUCCGAACGUGUCACGUTT-3′
siRNA control Antisense	5′-ACGUGACACGUUCGGAGAATT-3′

3.3. BCL6 inhibition of chidamide-mediated histone acetylation levels in DLBCL cells

Next, our research aimed to investigate the underlying mechanism by which BCL6 affected the sensitivity of DLBCL cells to chidamide. As depicted in Fig. 3A, FARAGE with higher expression of BCL6 had lower histone H3 acetylation levels. The level of histone H3 acetylation is determined by the balance between the activities of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes [6]. We therefore measured the enzymatic activities of HATs and HDACs in both FARAGE and SU-DHL-8 cell lines. The results showed that the enzymatic activity of HATs in FARAGE was significantly higher than that of SU-DHL-8, while the enzymatic activity of HDACs was similar in both cell lines (Fig. 3B). Compared to the sensitive cell line SU-DHL-8, FARAGE cells had a higher ratio of HATs/HDACs enzyme activity, but paradoxically exhibited lower levels of histone acetylation. One hypothesis is that BCL6 might recruit HDACs to bind with histone H3, and that this interaction could reduce the inhibitory effect of chidamide on HDACs. To test this hypothesis, we treated both cell lines with chidamide at the same concentration and measured the histone acetylation levels. The results showed that the histone acetylation levels were significantly increased in SU-DHL-8 cells with low expression of BCL6, while the histone acetylation levels in FARAGE cells with high expression of BCL6 only slightly increased (Fig. 3C). Further interference of BCL6 by siRNA led to a significant increase of histone acetylation levels in the response of FARAGE cells to chidamide (Fig. 3D). Similar results were obtained in chidamide-resistant SU-DHL-2 cells, where the ability of chidamide to upregulate histone H3 acetylation levels was weakened with the upregulation of BCL6 (Fig. 3E, 3F). Furthermore, HDAC1 binds to histone H3 in FARAGE cells, and the binding ability of HDAC1 to histone H3 was decreased after interference with BCL6 expression (Fig. 3G). These results indicated that BCL6 inhibited chidamide-mediated histone acetylation level upregulation by recruiting HDACs to promote their binding with histone H3, resulting in resistance of DLBCL cells to chidamide.

3.4. Lenalidomide targets BCL6 degradation and restores the sensitivity of drug-resistant DLBCL to chidamide

Next, we aimed to enhance the sensitivity of DLBCL to chidamide by inhibiting BCL6. Previous studies have shown that patients with BCL6-dependent DLBCLs may represent the best candidates for therapeutic trials of BCL6 inhibitors [15–17]. The role of BCL6 as an oncogene in DLBCL and its association with aberrant proliferation have been established in previous studies [18].Our study found that interfering with

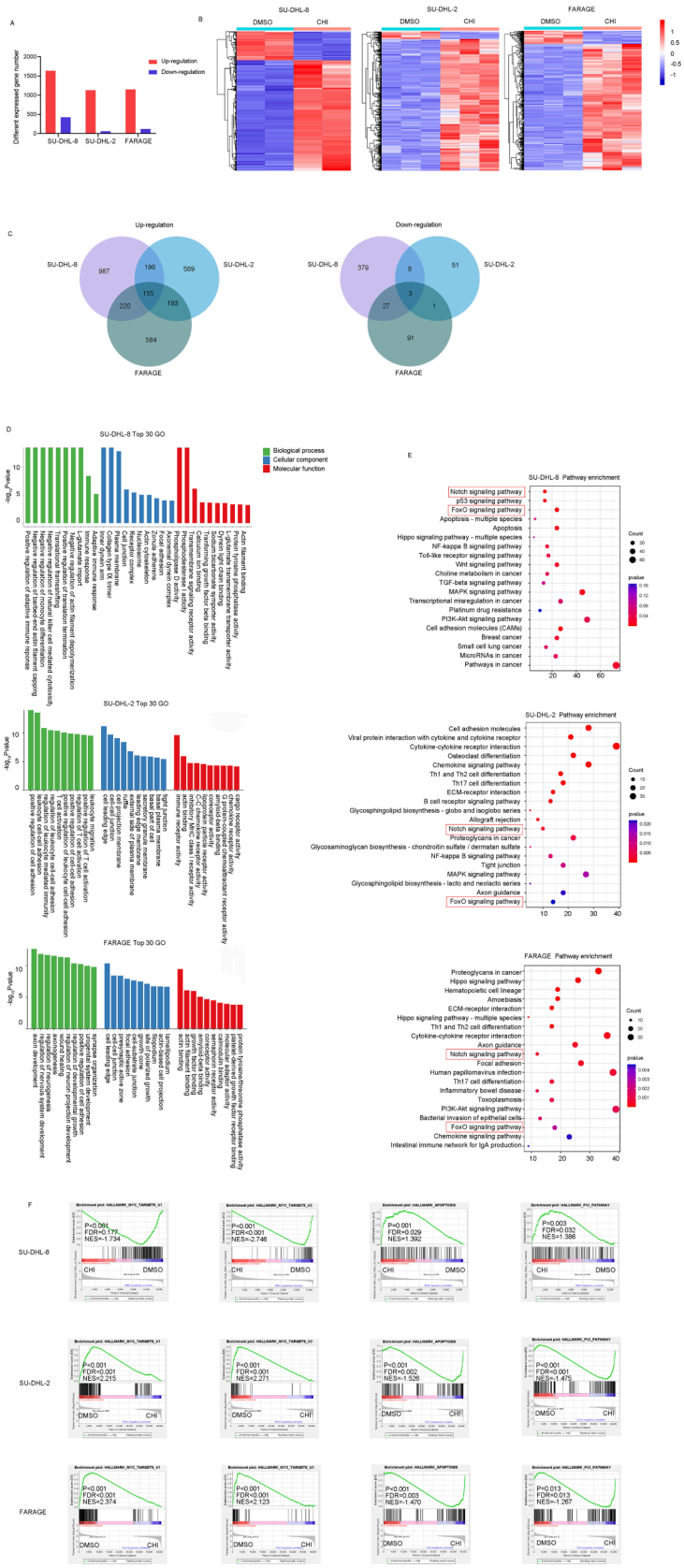

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Fig. 1. Global impact of chidamide on DLBCL: Insights from high-throughput RNA sequencing analysis. A The statistical analysis showed differentially expressed genes following treatment of DLBLC cells with chidamide. B Heatmap of differentially expressed gene in the RNA-seq analysis of DLBCL cells treated with chidamide for 12 h(N = 2). C Venn diagram showed the co-upregulated and co-down-regulated genes of the three cell lines. D GO analysis showed that chidamide significantly affected many biological pathways of DLBCL cells. The enriched pathway is meaningful when $P<0.05$. E KEGG pathway analysis indicated differentially expressed genes were enriched in cancer-related pathways. The enriched pathway is meaningful when $P<0.05$. F Differential genes were enriched in pathways. The enriched pathway is meaningful when FDR<0.25, $P<0.05$. FDR, false discovery rate, NES normalized enrichment score.

Table 3
Genes consistently regulated by chidamide treatment in three DLBCL cell lines.

up										down
ABCA1	C3AR1	CDON	DUSP4	GSTA4	MAST4	PARM1	RARG	SMIM14	TMEM178B	KIAA0753
ABCA3	C5	CEACAM1	DUSP5	HELZ2	MED12L	PCDH9	RASL11A	SNAI3	TMEM52B	CCDC34
ACBD7	CACNB3	CEMIP2	EHD3	HES7	MOXD1	PCGF2	REPS2	SRCIN1	TMEM74	DNAJC2
ADAM28	CAMKK1	CEND1	EIF4E3	HEY1	MS4A7	PDE4A	RGS8	STON2	TPRG1	
ADAMTS6	CAND2	CGN	ELL2	HIP1	MYBPC2	PDE4DIP	RNF175	STX3	TTC21A	
ADGRB2	CAPN14	CLEC7A	ETV7	IER3	MYO1D	PELI2	RRAGD	STXBP1	WDR31	
ADGRL1	CARMIL1	CLIP2	FAM171A2	IFIT3	NABP1	PHLDA1	SATB1	SUCNR1	XIRP1	
ADRB2	CASKIN2	COL1A1	FAM43A	IL2RB	NAPSA	PHLDB2	SCN8A	SYP	YPEL2	
AGRN	CASZ1	CORO2A	FGD4	JARID2	NEO1	PLEKHH3	SDC3	SYT11	ZBTB46	
AIG1	CAV1	CR2	FMN1	KIAA0513	NGFR	PPFIA3	SERPINE2	TBC1D16	ZNF385A	
ANK3	CCDC71L	CTTN	FSD1	KLF5	NOL4L	PPFIBP1	SERPINI1	TBX21	ZNF774	
APBB1	CCNO	CUEDC1	GAB2	KSR1	NUMBL	PPM1H	SESN3	TESK2		
ASAP3	CD69	CXXC4	GABBR1	LAMP3	NXPH4	PPM1J	SFXN3	TEX19		
ATP10D	CDC42BPG	CYSLTR1	GBGT1	LILRB4	OTULINL	PRKCB	SKIDA1	THEMIS2		
BCAS1	CDKL5	DLL1	GIPR	LRP1	PALLD	PTGER4	SLC17A7	TLR7		
BRSK1	CDKN1A	DOCK4	GLIPR2	LYRM9	PAPLN	PTMS	SMAD3	TMCC2		

BCL6 (Fig. 4A, 4B) inhibited proliferation (Fig. 4C), induced cell cycle arrest (Fig. 4D) and apoptosis (Fig. 4E) in FARAGE cells, but not in SU-DHL-8 and SU-DHL-2. Subsequently, to identify drugs that could enhance the sensitivity of DLBCL to chidamide by inhibiting BCL6, we conducted a search from small molecule drugs (decitabine, lenalidomide, ibrutinib) currently undergoing clinical investigation for various cancers. Combination treatment with lenalidomide and chidamide showed the best synergistic cytotoxicity against FARAGE cells (Fig. 4F).

Thalidomide analogs induce the ubiquitination and proteasomal degradation of Ikaros (IKZF1) and Aiolos (IKZF3) by recruiting a Cys2-His2 (C2H2) zinc finger domain to Cereblon (CRBN). Previous studies have shown that thalidomide analogs target a specific family of proteins called Cys2-His2 (C2H2) zinc fingers, which includes BCL6 as a member [19]. Therefore, we investigated whether BCL6 was the target of lenalidomide in FARAGE cells. Our results showed that lenalidomide reduced the protein level of BCL6, but not the RNA level, in FARAGE cells (Fig. 4G). Lenalidomide induced the ubiquitination and proteasomal degradation of the target proteins by recruiting a C2H2 zinc finger domain to Cereblon (CRBN) [20]. We found that chidamide did not change the CRBN expression level in FARAGE cells (Fig. 4H). To confirm that lenalidomide reduces the expression of BCL6 protein through ubiquitination, we used the MG-132 proteasome inhibitor to block the degradation of ubiquitinated proteins. The results showed that the administration of MG-132 partially reversed the degradation of BCL6 protein induced by lenalidomide. Additionally, we detected the level of ubiquitination modification on the BCL6 protein through immunoprecipitation technology and found that the level of ubiquitination on the BCL6 protein significantly increased after lenalidomide treatment in FARAGE cells (Fig. 4I).

Furthermore, we pretreated FARAGE cells and SU-DHL-2-re cells with lenalidomide to decrease the BCL6 protein level and determine whether the synergistic effect of lenalidomide and chidamide still existed after BCL6 protein depletion. The results showed that the synergistic effect of lenalidomide and chidamide weakened when BCL6 protein was decreased in both cells (Fig. 4J).

We also noticed that BCL6 expression levels significantly increased according to RNA-seq data (Fig. 4K), which was confirmed by subsequent qPCR validation in three DLBCL cells (Fig. 4L). We then tested the synergistic effects of chidamide and lenalidomide in SU-DHL-8 and SU-DHL-2. The results showed that chidamide and lenalidomide had a synergistic effect in both cell lines, albeit smaller than in FARAGE cells (Fig. 4M).

These results indicated that lenalidomide can target BCL6 degradation through the ubiquitination pathway, thus restoring the sensitivity of drug-resistant DLBCL cell lines to chidamide.

3.5. *Combination of chidamide and lenalidomide shows synergistic effect in reducing DLBCL burden in vivo*

In order to evaluate the therapeutic potential of combining chidamide and Lenalidomide in DLBCL in vivo, we established DLBCL mice models. Tumor-bearing mice were treated with DMSO, lenalidomide, chidamide or a combination of lenalidomide and chidamide, respectively. Results showed that the combination of chidamide and lenalidomide resulted in a synergistic effect (Fig. 5A). The combination treatments significantly reduced the burden of disease compared to monotherapy groups, as assessed by tumor volumes (Fig. 5B). These findings suggest that the combination of chidamide and lenalidomide holds promise as a synergistic therapeutic approach in reducing DLBCL burden.

4. Discussion

Even though rituximab combined with chemotherapy is currently the recommended treatment for first-line therapy, many DLBCL patients either don't respond to it or experience a relapse after initially responding [21]. Hence, there is a pressing need to discover alternative therapeutic strategies for DLBCL.

In this study, we investigated the impact of chidamide, a histone deacetylase inhibitor, on DLBCL cells and identified biomarkers that could predict a favorable response to chidamide treatment in clinical settings. We also aimed to identify drugs that could enhance the sensitivity of DLBCL to chidamide, helping develop personalized treatment strategies for DLBCL patients.

Through RNA sequencing analysis, we discovered 2066 genes that were impacted by chidamide. Gene Set Enrichment Analysis (GSEA) revealed that chidamide treatment had a significant effect on various biological processes in DLBCL cells, including enhancing the MYC

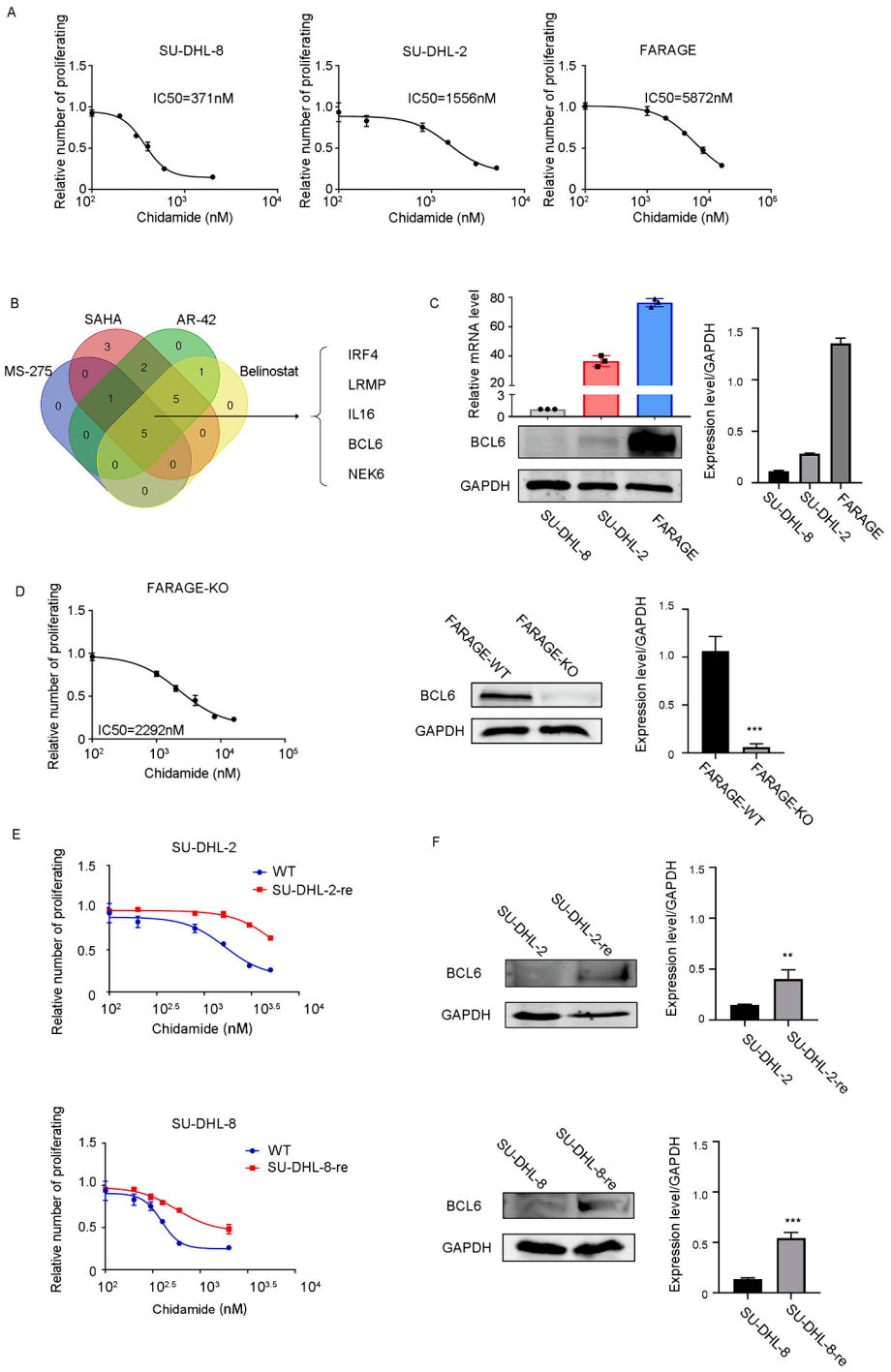

Fig. 2. BCL6 confers resistance to chidamide in DLBCL. A SU-DHL-8, SU-DHL-2, and FARAGE were treated with chidamide at different concentrations for 72 h. Cell viability were measured by using cck-8 assay, and IC50 of chidamide were calculated (N = 3). B Venn diagram showed the related genes of HDAC inhibitor resistance screened by correlation analysis. There is a correlation at $P<0.05$. C Changes of BCL6 were examined by qPCR in SU-DHL-8, SU-DHL-2 and FARAGE treated with chidamide at IC50 concentration for 12 h (N = 3). GAPDH was used as loading control. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with negative control group. D The expression level of BCL6 in three DLBCL cells was detected by qPCR and Western blotting (N = 3). GAPDH was used as loading control. E Chidamide resistant DLBCL cells (SU-DHL-8-re, SU-DHL-2-re) were developed through gradually increasing chidamide treatment. SU-DHL-8, SU-DHL-8-re, SU-DHL-2 and SU-DHL-2-re were treated with chidamide at different concentrations for 72 h. Cell viability were measured by using cck-8 assay (N = 3). F The expression level of BCL6 in SU-DHL-8, SU-DHL-8-re, SU-DHL-2 and SU-DHL-2-re was detected by Western blotting. GAPDH was used as loading control.

pathway, p53 signaling pathway, and apoptosis.

We also found that BCL6 confers resistance to chidamide in DLBCL. BCL6, a transcriptional repressor, is known to recruit HDACs to repress gene expression by removing acetyl groups from histones [22]. This interplay between BCL6 and HDACs is essential for B-cell development and differentiation [23]. We found that DLBCL cells, which expressed higher levels of BCL6, were less responsive to chidamide. These findings indicate that BCL6 expression may serve as a key determinant of chidamide sensitivity in DLBCL.

To overcome chidamide resistance in DLBCL cells, we explored the synergistic effects of three small-molecule targeted drugs in combination with chidamide. Among them, the combination of lenalidomide and

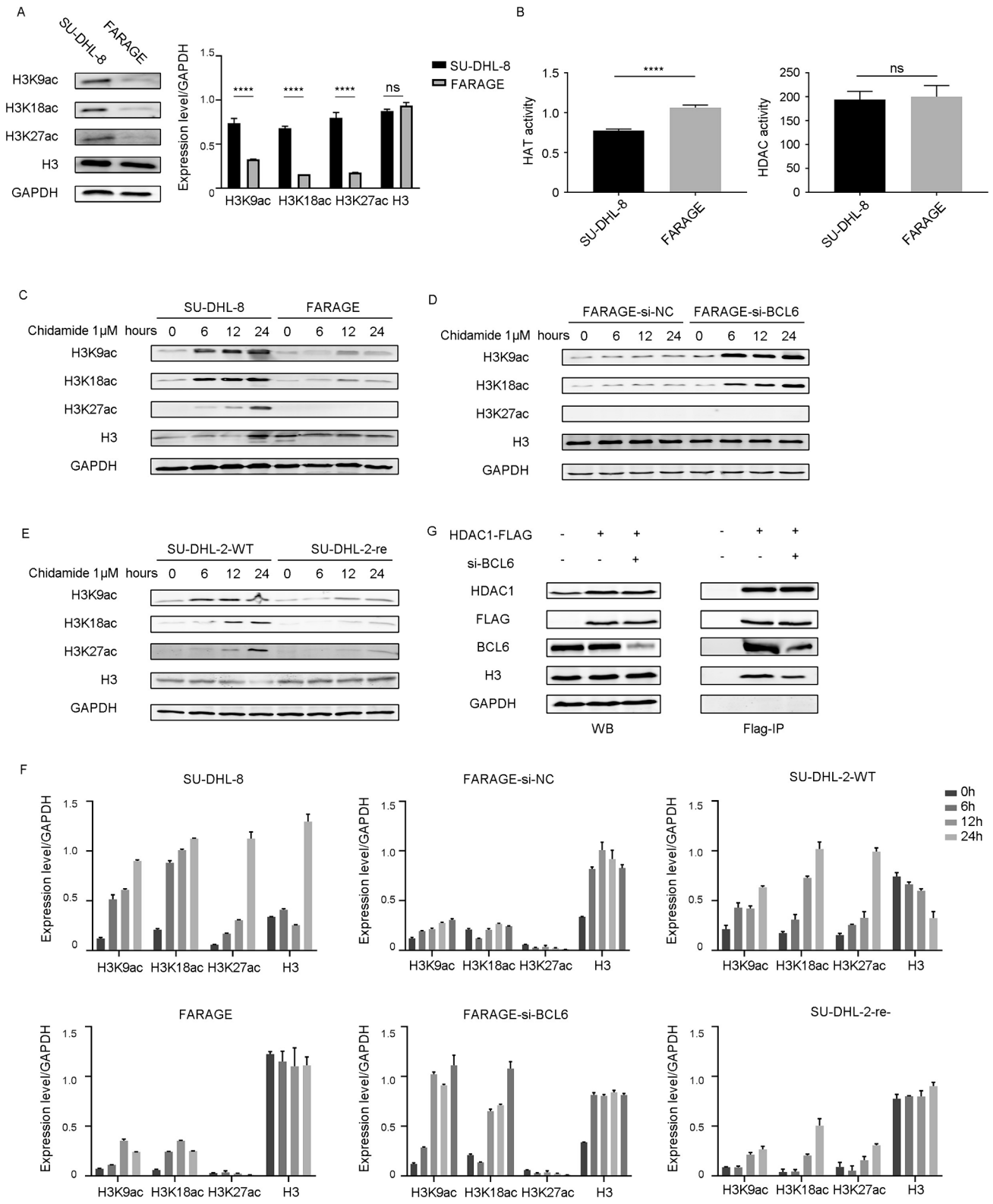

Fig. 3. BCL6 inhibition of chidamide-mediated histone acetylation levels in DLBCL cells. A The level of histone H3 acetylation in SU-DHL-8, SU-DHL-2, and FARAGE was detected by Western blotting. B HATs and HDACs enzyme activity were detected by extracting 50 μg nucleoprotein (N = 3). ****P<0.0001. C The acetylation levels of H3K9,H3K18, and H3K27 were detected by western blotting after the cells were treated with 1 μM chidamide for 0 h,6h,12 h, and 24 h. D The acetylation levels were detected by western blotting after chidamide. E-F Western blotting showed the acetylation levels after treated with 1 μM chidamide for 0 h,6h,12 h, and 24 h. GAPDH was used as loading control. G BCL6 siRNA and pcDNA3.1-HDAC1-FLAG overexpression vector were transfected into FARAGE cells by electro-transfection. After 48 h, cells were collected to enrich HDAC1 protein and other HDAC1-binding proteins with FLAG antibody, and the binding ability of HDAC1 to histone H3 was detected.

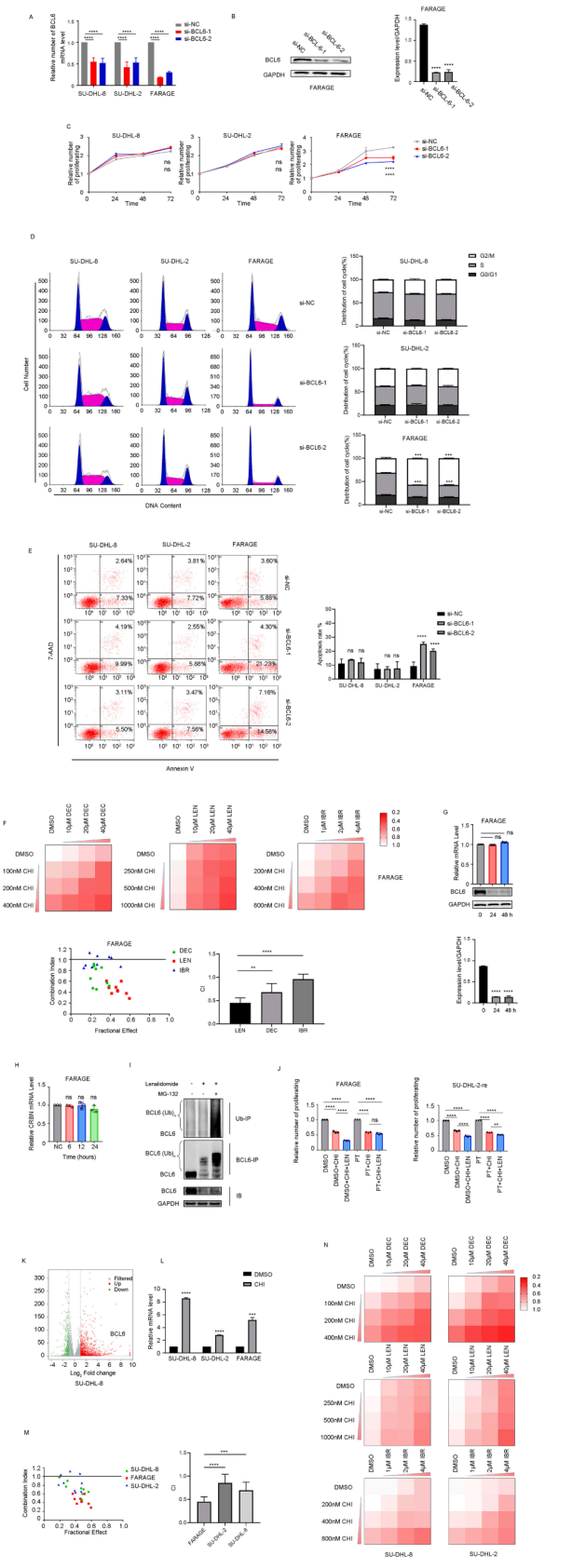

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Fig. 4. Lenalidomide targets BCL6 degradation and restores the sensitivity of drug-resistant DLBCL to chidamide. A Changes of BCL6 in three DLBCL cells was detected by qPCR (N = 3) B Changes of BCL6 in FARAGE was detected by Western blotting. C Changes of cell proliferation in DLBCL cells by inhibiting BCL6. D SU-DHL-8, SU-DHL-2, and Farage cells were transfected with siBCL6 for 48 h, stained with PI, and analyzed by flow cytometry. Percentages of G0/G1, S, and G2/M phases in the cell cycle are shown in the statistical graph. E Control siRNA or BCL6 siRNAs were transfected into cells, and cells were collected 48 h after transfection, double stained with 7-AAD and Annexin V-PE, and analyzed by flow cytometry.F FARAGE was treated with different concentrations of small molecule drugs and chidamide for 48 h. CCK-8 was used to detect cell proliferation inhibition and calculate cell proliferation inhibition rate. Percentage of cells alive was expressed using heat maps. CI plots were calculated based on the Chou–Talalay equation using CompuSyn software. T-test statistical analysis was conducted on the nine CI values for each different drug combination. G FARAGE was treated with 50 μM lenalidomide for 24 h and 48 h. The expression level of BCL6 was detected by qPCR and Western blotting (N = 3). H FARAGE was treated with 5 μM chidamide for 6 h, 12 h and 24 h, and the transcriptional changes were detected by qPCR (N = 3). I FARAGE was treated with 50 μM lenalidomide and 5 μM MG-132 for 48 h, and the expression of BCL-6 protein was detected by western blotting. The BCL6-FLAG fusion protein was overexpressed in FARAGE cells, FLAG and ubiquitination group antibody were used for immunocoprecipitation after 48 h. The effect of lenalidomide on ubiquitination level of BCL-6 protein was detected by western blotting. J The cells were divided into NC group and pre-treatment (PT) group. The pre-treatment group was treated with 50 μM lenalidomide for 48 h, and then the cells in the two groups were treated with chidamide alone or chidamide combined with lenalidomide for 48 h. CCK-8 was used to detect cell proliferation and calculate the inhibition rate of cell proliferation. K Volcano diagram displayed differential protein-coding genes after chidamide treatment was displayed and the expression of BCL-6 was significantly increased. Filtered: genes with no difference in expression; Up: up-regulated differential protein-coding genes; Down: down-regulated differential protein-coding genes. L Changes of BCL6 were examined by qPCR in SU-DHL-8, SU-DHL-2 and FARAGE treated with chidamide at IC50 concentration for 12 h (N = 3). M DLBCL cells were treated with different concentrations of small molecule drugs and chidamide for 48 h. CCK-8 was used to detect cell proliferation inhibition and calculate cell proliferation inhibition rate. T-test statistical analysis was conducted on the nine CI values for each different drug combination. All data are shown as the means ± SD based on triplicate measures.*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.N Percentage of cells alive was expressed using heat maps.

chidamide exhibited the most promising results, particularly in FARAGE cells. Notably, lenalidomide demonstrated the ability to target BCL6 degradation in DLBCL cells through the ubiquitination pathway. This mechanism contributed to the restoration of sensitivity in drug-resistant DLBCL cell lines when combined with chidamide. Several hypotheses have been proposed regarding the mechanisms of resistance to HDAC inhibitors [24,25]. It is plausible that other resistance mechanisms may exist in Farage cells, contributing to their higher resistance even after BCL6 knock-out.

In conclusion, our study provides new insights into the mechanisms underlying chidamide's therapeutic effects in DLBCL and identifies potential biomarkers that could predict a favorable response to chidamide treatment in clinical settings. Our findings highlight the need for personalized treatment strategies for DLBCL patients and provide a basis for further research into the optimal use of chidamide in combination with other treatments.

Ethical approval

All the animals were acclimated under standard laboratory conditions and had free access to standard water and food. All procedures were conducted in accordance with the "Guiding Principles in the Care and Use of Animals" (China) and were approved by the Laboratory Animal Ethics Committee of Soochow university.

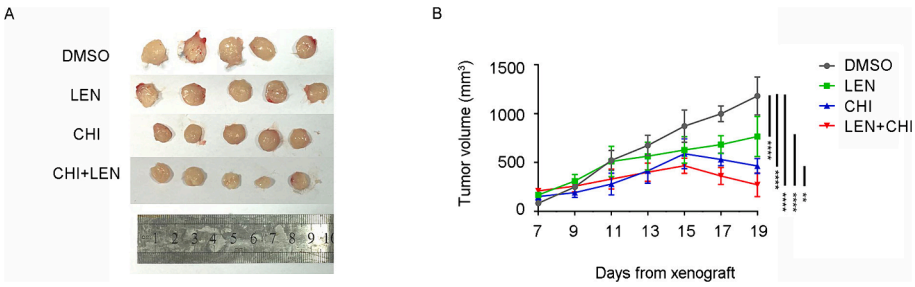

Fig. 5. Combination of chidamide and lenalidomide shows synergistic effect in reducing DLBCL burden in vivo. A Tumor specimens photographed with a high-definition digital camera. B Tumor volume was measured every 2 days.

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7. Authors' contributions

Wenzhuo Zhuang and Bingzong Li designed the research, Gao Fan and Yuchen Zhang performed research and analyzed data, Qi Li and Rong Rong wrote the paper, Si Chen and Lexin He modified the paper.

CRediT authorship contribution statement

Gao Fan: Formal analysis, Data curation. **Yuchen Zhang:** Formal analysis, Data curation. **Qi Li:** Data curation. **Rong Rong:** Data curation. **Si Chen:** Validation. **Lexin He:** Formal analysis. **Bingzong Li:** Writing – review & editing, Formal analysis, Data curation. **Wenzhuo Zhuang:** Funding acquisition, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

You may view our GSE230465 study at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE230465

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