

Overexpression of c-Myc-dependent heterogeneous nuclear ribonucleoprotein A1 promotes proliferation and inhibits apoptosis in *NOTCH1*-mutated chronic lymphocytic leukemia cells

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Abstract

Background: *NOTCH1* mutation is an essential molecular biologic aberration in chronic lymphocytic leukemia (CLL). CLL patients with *NOTCH1* mutation have shown an unfavorable survival and a poor response to chemoimmunotherapy. This study aims to present the mechanisms of adverse prognosis caused by *NOTCH1* mutation from the perspective of the splicing factor heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1).

Methods: The microarray data in Gene Expression Omnibus datasets were analyzed by bioinformatics and the function of hnRNPA1 was checked by testing the proliferation and apoptosis of CLL-like cell lines. Afterward, quantitative reverse transcription-polymerase chain reaction and Western blotting were applied to explore the relationship among *NOTCH1*, c-Myc, and hnRNPA1.

Results: RNA splicing was found to play a vital part in *NOTCH1*-mutated CLL cells; hence, hnRNPA1 was selected as the focus of this study. Higher expression of hnRNPA1 validated in primary *NOTCH1*-mutated CLL samples could promote proliferation and inhibit apoptosis in CLL. The expression of hnRNPA1 increased when *NOTCH1* signaling was activated by transfection with *NOTCH1* intracellular domain (NICD)-overexpressed adenovirus vector and declined after *NOTCH1* signaling was inhibited by *NOTCH1*-shRNA. Higher expression of c-Myc was observed in NICD-overexpressed cells and hnRNPA1 expression was downregulated after applying c-Myc inhibitor 10058-F4. Moreover, in NICD-overexpressed cells, hnRNPA1 expression decreased through c-Myc inhibition.

Conclusion: Overexpression of c-Myc-dependent hnRNPA1 could promote proliferation and inhibit apoptosis in *NOTCH1*-mutated CLL cells, which might partly account for the poor prognosis of patients with *NOTCH1* mutation.

Keywords: Chronic lymphocytic leukemia; *NOTCH1* mutation; c-Myc; Heterogeneous nuclear ribonucleoprotein A1

Introduction

Chronic lymphocytic leukemia (CLL) is a common type of mature B-cell chronic lymphoproliferative disease in adults with heterogeneous clinical courses.^[1] Many patients have more than one molecular biologic or cytogenetic aberration.^[2] *NOTCH1* mutation is an essential molecular biologic change in CLL. Previous studies reported that patients with *NOTCH1* mutation have shorter treatment-free survival (TFS), progression-free survival, and overall survival (OS), implying *NOTCH1* mutation as an adverse prognostic factor.^[3,4]

NOTCH1 gene located in chromosome 9q34.3 encodes *NOTCH1* protein, whose extracellular receptor binding to its corresponding ligand would trigger two sequential cleavages and eventually release *NOTCH1* intracellular domain (NICD), which could form transcriptional activation complexes to activate target gene transcription along with other transcription factors. A majority of *NOTCH1* mutations (eg, c.7541_7542delCT) could increase NICD stability by truncating the domain recognized by ubiquitin ligase F-box and WD repeat-containing protein 7 (FBXW7), thus causing sustained *NOTCH1* signaling activation and target gene transcription.^[5]

Access this article online	
Quick Response Code: 	Website: www.cmj.org
	DOI: 10.1097/CM9.0000000000002037

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Chinese Medical Journal 2022;135(8)

Received: 19-05-2021; Online: 15-02-2022 Edited by: Peng Lyu

Up to now, there has been little research on the mechanisms of poor prognosis caused by *NOTCH1* mutation. Arruga *et al*^[6] found that sustained *NOTCH1* signaling activation caused by *NOTCH1* mutation resulted in increased expression of genes associated with B cell receptor signaling, which, in turn, enhanced *NOTCH1* translation. Consequently, *NOTCH1* and B cell receptor signaling synergistically facilitated the development of CLL. In addition, *NOTCH1* mutation could upregulate surface C-C chemokine receptor 7 of CLL cells by inhibiting *DUSP22*, thereby facilitating the chemotaxis response to chemokine C-C motif ligand 19 (CCL19). CCL19 would guide CLL cells to lymph nodes and spleen, where the CLL cells would find ideal micro-environments for their proliferation.^[7] Pozzo *et al*^[8] discovered that a higher proliferation rate in *NOTCH1*-mutated CLL cells was related to overexpression of c-Myc-related nucleophosmin 1. Moreover, *NOTCH1* mutation could downregulate CD20 on the surface of CLL cells by epigenetic dysregulation and cause a poor response to anti-CD20 antibodies.^[9]

Different gene expression patterns between *NOTCH1*-mutated and *NOTCH1*-wild-type (wt) CLL samples were analyzed by using Gene Expression Omnibus (GEO) dataset GSE75122. It was found that the expression of genes associated with RNA splicing increased in *NOTCH1*-mutated cells, including the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1). hnRNPA1 had been proved to affect proliferation, apoptosis, and metabolism in several solid tumors, verifying its importance as a regulator in the development of cancer.^[10,11] However, its function in *NOTCH1*-mutated CLL remains unknown. Therefore, investigating its effect on proliferation and apoptosis and exploring the key mediator between *NOTCH1* and hnRNPA1 in CLL were set to be the focus of the present research.

Methods

Primary CLL cells

Seven *NOTCH1*-mutated and eight *NOTCH1*-wt CLL peripheral blood samples were collected from the patients who were admitted to our department. Detailed characteristics of patients were shown in Supplementary Table 1, <http://links.lww.com/CM9/A9>. The diagnosis was based on the International Workshop on CLL-National Cancer Institute criteria. Ficoll-Hypaque solution (Tbdscience, Tianjin, China) and B-CLL cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) were applied to obtain purified B cells (>95%). *NOTCH1* mutation was detected by Sanger sequencing or next-generation sequencing. Moreover, this study was approved by the hospital ethics committee (No. 2019-SR-307) and the informed consent of all patients was provided in line with the *Helsinki Declaration* of 1975.

Cell lines and cell culture

CLL-like cell lines MEC-1 and JVM-3 were purchased from Cobioer Biological Technology Co., Ltd. (Nanjing, China). Short tandem repeat authentication was con-

ducted by Shanghai Biowing Applied Biotechnology Co., Ltd. (Shanghai, China). MEC-1 was established from the peripheral blood of a 61-year-old Caucasian man with chronic B cell leukemia. JVM-3 was constructed from the peripheral blood of a 73-year-old man with B-prolymphocytic leukemia. Neither of these two cell lines harbored *NOTCH1* mutation. The culture medium of MEC-1 consisted of 90% Iscove's modified Dulbecco's Modified Eagle's Medium (Gibco, Invitrogen, Grand Island, NY, USA) and 10% fetal bovine serum (Gibco). JVM-3 was cultured in 90% RPMI 1640 culture medium (Gibco) and 10% fetal bovine serum (Gibco). All the cells were incubated in a 5% CO₂ humidified incubator at 37°C. All experiments were conducted with mycoplasma-free cells.

In vitro treatment with a c-Myc inhibitor

To inhibit c-Myc expression, cells were treated with c-Myc inhibitor 10058-F4 (100 mmol/L, Selleck, Shanghai, China) for 24 hours, which could inhibit c-Myc-max heterodimerization and transcription of c-Myc target genes.^[12] As ethanol was chosen as the solvent for 10058-F4, it was chosen as the control in the experiments of this study.

Vector constructs, virus production, and cell transfection

Commercially available adenovirus vectors were applied to construct the HBAD-*NOTCH1*-NICD-Null-GFP vector and corresponding negative control vector (Hanbio, Shanghai, China). Commercially available lentiviral vectors were applied to construct LV-*NOTCH1*-RNAi vector (Genechem, Shanghai, China), HBLV-h-HNRNPA1-3xflag-ZsGreen-PURO vector (Hanbio), HBLV-h-HNRNPA1 shRNA-ZsGreen-PURO vector (Hanbio), and corresponding negative control vectors. These constructions were verified by DNA sequencing before being used. An appropriate multiplicity of infection was chosen to infect MEC-1 and JVM-3. Stable cell lines were screened by adding 2 µg/mL puromycin (InvivoGen, San Diego, CA, USA) for 2 days. Transfection efficiency was confirmed by flow cytometry and expression was validated by real-time quantitative polymerase chain reaction and Western blot.

Total RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was extracted from purified B cells or cell lines applying an RNA-quick purification kit (Yishan Biotec, Shanghai, China) in line with instructions. Complementary DNA was generated by using PrimeScriptTM RT Master Mix (Takara, Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) experiments were conducted using AceQ qPCR SYBR Green Master Mix (Vazyme, Nanjing, China). The relative messenger RNA (mRNA) expression levels of target genes were calculated through the 2^{-ΔΔCt} method. Primers for qRT-PCR were as follows: *NOTCH1* forward: 5'-GGACGTCAGACTT-GGCTCAG-3'; reverse: 5'-ACATCTTGGGACGCAT-CTGG-3'; *NICD* forward: 5'-TGCACACTATTCTGCCC-CAG-3'; reverse: 5'-ACTTGAAGGCCTCCGGAATG-3'; *c-Myc* forward: 5'-TTCGGGTAGTGAAACCAG-3'; reverse: 5'-AGTAGAAATACGGCTGCACC-3'; *hnRNPA1* forward: 5'-GCTCACGGACTGTGTGGTAA-3'; reverse:

5'-GCAAAGTATTGGCCTCCACC-3'; *HES1* forward: 5'-CTGAGCACAGACCCCAAGTGT-3'; reverse: 5'-GAGTGCACACCTCGGTATTA-3'; and β -*Actin* forward: 5'-AGC-GAGCATCCCCCAAAGTT-3'; reverse: 5'-GGGCAC-GAAGGCTCATCATT-3'.

Western blot

Total proteins extracted from purified B cells or cell lines were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China) and transferred to polyvinylidene fluoride (PVDF, Millipore, Billerica, Massachusetts, USA) membranes. Fast SDS-PAGE running buffer (Cwbio, Taizhou, China) and Tris-Glycine transfer buffer (Cwbio) were purchased from CoWin Biosciences. The membranes were blocked with Quickblock™ blocking buffer for Western blot (Beyotime) for 30 minutes followed by successive incubations with primary antibodies overnight at 4°C and corresponding secondary antibodies for 1 h. Protein expression levels were visualized using ECL Plus (Millipore) and Bio-Imaging System. The primary and secondary antibodies used in our experiments are NOTCH1 (#3608, CST, Danvers, Massachusetts, USA), cleaved NOTCH1 (#4147, CST), hnRNPA1 (#8443, CST), c-Myc (ab32072, Abcam, Boston, MA, USA), β -Actin (#3700, CST), GAPDH (#2118, CST), HRP-conjugated anti-mouse IgG (ZB-2305, ZSGB-BIO, Beijing, China), and HRP-conjugated anti-rabbit IgG (ZB-5301, ZSGB-BIO). Immunoblot bands were quantified through densitometry and ImageJ software (NIH, Bethesda, MD, USA). Relative folds were normalized to β -actin levels.

Cell count Kit-8 assay

Cell Count Kit-8 (CCK-8, Dojindo, Kumamoto, Japan) assay was applied to evaluate cell proliferation. A total of 1.0×10^4 cells/wells were seeded into 96-well plates with 100 μ L corresponding medium (MEC-1: IMDM; JVM-3: RPMI 1640). 10 μ L CCK-8 solution was added into specific wells at 0, 24, 48, and 72 hours followed by incubation in a 5% CO₂ humidified incubator at 37°C for 4 h. Afterward, the plates were taken out and had their optical density value measured at wavelength 450 nm in Synergy H1 microplate reader (BioTek, Agilent, Santa Clara, CA, USA) immediately.

Cell apoptosis analysis

Cell apoptosis was tested by Annexin V-allophycocyanin (APC)/7-amino-actinomycin D (7-AAD) apoptosis kit (KeyGen Biotech, Nanjing, China). A total of 1.0×10^6 cells/tubes were stained with 5 μ L Annexin V-APC and 5 μ L 7-AAD in the dark at room temperature for 15 min. Afterward, apoptotic cells were measured with BD FACSCanto, and data were analyzed via FlowJo (X 10.0.7r2, Treestar, Ashland, OR, USA).

Bioinformatics analysis

GSE75122 in the GEO database was selected as the dataset for our research and analysis. The testing platform for this dataset was Agilent-014850 Whole Human Genome Microarray 4x44K G4112F. All the probe names were converted to gene names in line with the instructions of the platform. GEO2R (www.ncbi.nlm.nih.gov/geo/)

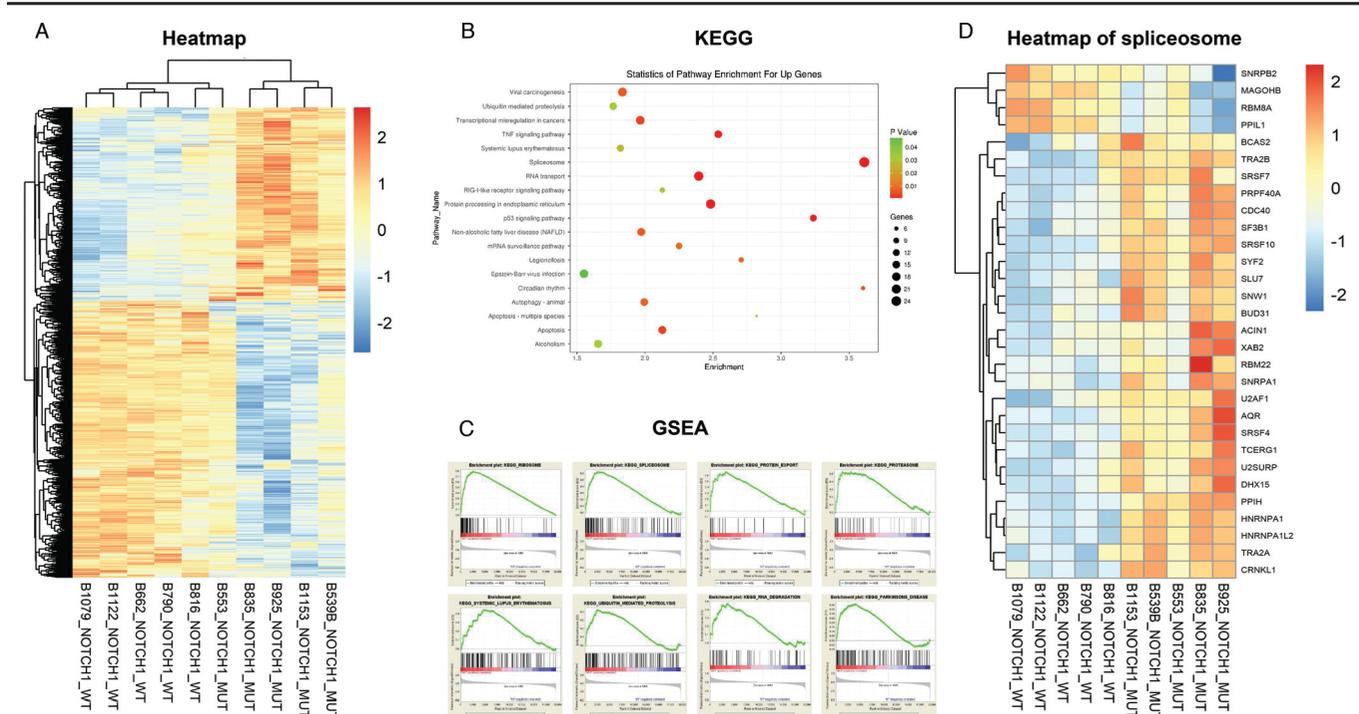


Figure 1: Bio-informatics analysis of *NOTCH1*-mutated and *NOTCH1*-wt CLL cells in GSE75122. (A) The heatmap of five *NOTCH1*-mutated and five *NOTCH1*-wt CLL samples. (B) KEGG analysis of DEGs whose expressions were upregulated in *NOTCH1*-mutated CLL samples. (C) GSEA analysis of five *NOTCH1*-mutated and five *NOTCH1*-wt CLL samples. (D) The heatmap of genes involved in spliceosome. CLL: Chronic lymphocytic leukemia; DEGs: Differentially expressed genes; GSEA: Gene set enrichment analysis; KEGG: Kyoto Encyclopedia of Genes and Genomes.

geo2r) was applied to analyze differentially expressed genes (DEGs). The standard of DEGs was defined as $P < 0.05$ and $|\log_2[\text{fold change}]| > 1$. Heatmap was generated by supervised analysis. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was conducted in The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (<https://david.ncifcrf.gov/>, version 6.8).^[13] The level of statistical significance in KEGG analysis was expressed as $P < 0.05$. Gene Set Enrichment Analysis (GSEA) was conducted via GSEA software (<http://software.broadinstitute.org/gsea/download.jsp>, UC San Diego and Broad Institute, USA). The statistical significance in GSEA analysis^[14] was expressed as false discovery rate < 0.05 and nominal P value < 0.05 . Figures were drawn via R software (Version 3.5.3, The R Foundation for Statistical Computing, Vienna, Austria).

Statistical analysis

The cutoff value was determined by the use of X-tile,^[15] a software that allowed users to move a cursor across the grid to test multiple divisions and accept the best P value. Data were analyzed by applying SPSS 23 software (IBM Corporation, Armonk, NY, USA) and presented as mean \pm standard error of the mean. The student's t -test was applied to compare data between two groups. One-way analysis of variance was applied to compare data between multiple groups. $P < 0.05$ was defined as statistical significance. Graphs

were made via GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Bioinformatics analysis of NOTCH1-mutated CLL cells

To explore different gene expression patterns between *NOTCH1*-mutated and *NOTCH1*-wt CLL cells, GSE75122 in the GEO database was analyzed and key signal pathways and downstream genes were identified. This dataset included five *NOTCH1*-mutated and five *NOTCH1*-wt CLL samples. Heatmap was generated by supervised analysis [Figure 1A]. A total of 2570 genes met the standard of DEGs with 1037 genes upregulated in *NOTCH1*-mutated CLL cells and 1533 downregulated. KEGG analysis indicated that highly expressed genes mainly contributed to spliceosome, protein processing in the endoplasmic reticulum, RNA transport, tumor protein 53 signaling pathway, tumor necrosis factor signaling pathway, apoptosis, etc. [Figure 1B]. GSEA revealed that genes involved in the ribosome, spliceosome, proteasome, protein export, ubiquitin-mediated proteolysis, RNA degradation, systemic lupus erythematosus, and Parkinson's disease were enriched in *NOTCH1*-mutated CLL cells [Figure 1C].

Based on the results of KEGG and GSEA analysis, it was found that spliceosomes played an essential role in

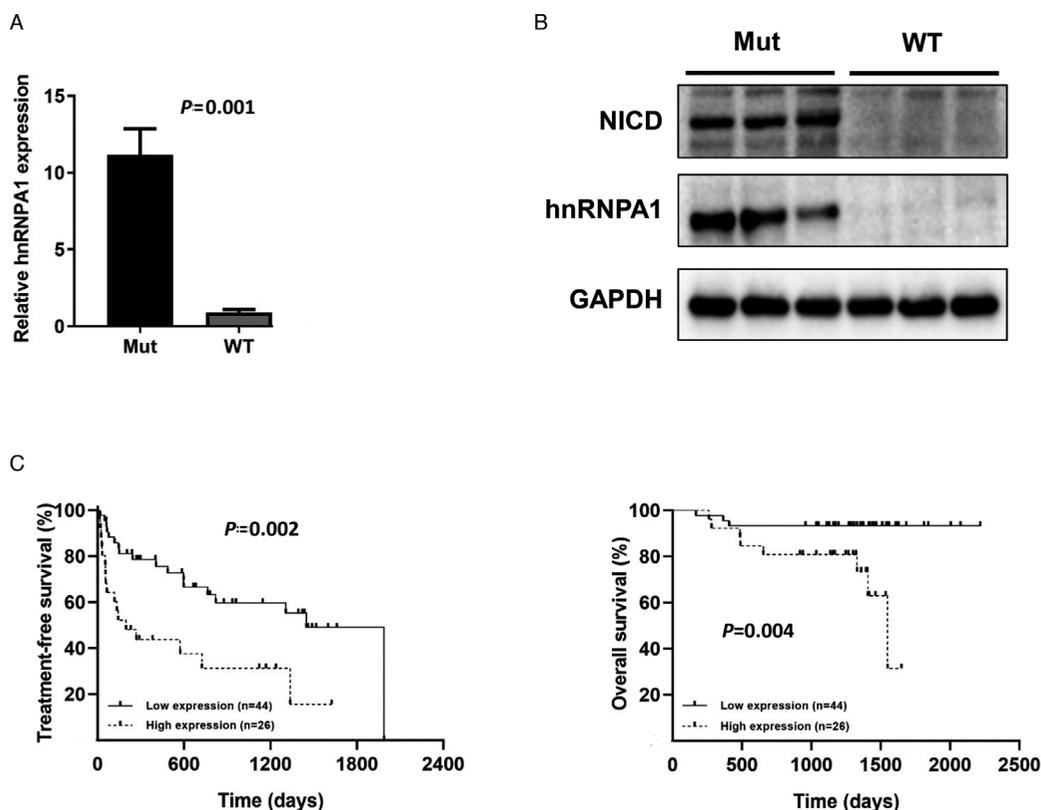


Figure 2: The expression of hnRNPA1 in CLL and its prognostic value. (A) The transcript level of *hnRNPA1* in primary *NOTCH1*-mutated ($n = 7$) and *NOTCH1*-wt ($n = 8$) CLL samples was detected by qRT-PCR. (B) The protein level of NICD and hnRNPA1 in *NOTCH1*-mutated ($n = 3$) and *NOTCH1*-wt ($n = 3$) CLL samples was detected by Western blot. (C) Kaplan–Meier curves of TFS (left panel) and OS (right panel) for different mRNA level of hnRNPA1. CLL: Chronic lymphocytic leukemia; hnRNPA1: Heterogeneous nuclear ribonucleoprotein A1; mRNA: Messenger RNA; NICD: NOTCH1 intracellular domain; OS: Overall survival; qRT-PCR: Quantitative real-time polymerase chain reaction; TFS: Treatment-free survival.

NOTCH1-mutated CLL cells. The heatmap of DEGs involved in spliceosome was presented in Figure 1D.

RNA splicing had been known to play a key part in various malignancies, but its functions in *NOTCH1*-mutated CLL cells remain unclear. Thus, *hnRNPA1* was chosen for further examination due to its high expression in *NOTCH1*-mutated CLL cells and involvement in carcinogenesis in several solid tumors, hoping that it would bring insight into the role RNA splicing plays in these instances.

Expression and prognostic value of *hnRNPA1* in primary CLL

We first validated the results of bioinformatics analysis in our primary samples. The mRNA level of *hnRNPA1* was determined in seven *NOTCH1*-mutated samples (six c.7541_7542delCT and one c.7443delC) and eight *NOTCH1*-wt CLL samples by qRT-PCR. The results revealed that *hnRNPA1* was highly expressed in *NOTCH1*-mutated CLL samples [Figure 2A]. Increased NICD and *hnRNPA1* protein levels in *NOTCH1*-mutated CLL cells were confirmed by Western blot [Figure 2B]. Afterward, another dataset GSE22762 was utilized to

study the prognostic value of *hnRNPA1* mRNA level in CLL patients. This dataset contained the data of three platforms and GPL570 had larger amounts of cases than the other two platforms, hence GPL570 was chosen to conduct the survival analysis. Some missing data were deleted and 70 cases were reserved eventually. X-tile was applied to determine the cutoff value, according to which patients were divided into two groups, that is, high expression (exceeding cutoff value) and low expression (below or equal to cutoff value). Survival curves constructed through the Kaplan–Meier method revealed that patients with high mRNA level of *hnRNPA1* had inferior TFS and OS [Figure 2C].

Effects of *hnRNPA1* on proliferation and apoptosis of CLL

In vitro assays were conducted to investigate the effects of *hnRNPA1* in the carcinogenesis of CLL. CLL-like cell lines MEC-1 and JVM-3 were selected to construct *hnRNPA1*-overexpressed cell lines. The transcript [Figure 3A] and protein level [Figure 3B] were confirmed through the corresponding methods as previously mentioned. Over-expressed *hnRNPA1* led to a higher proliferation rate and a lower apoptosis rate of MEC-1 and JVM-3 [Figure 3C and 3D].

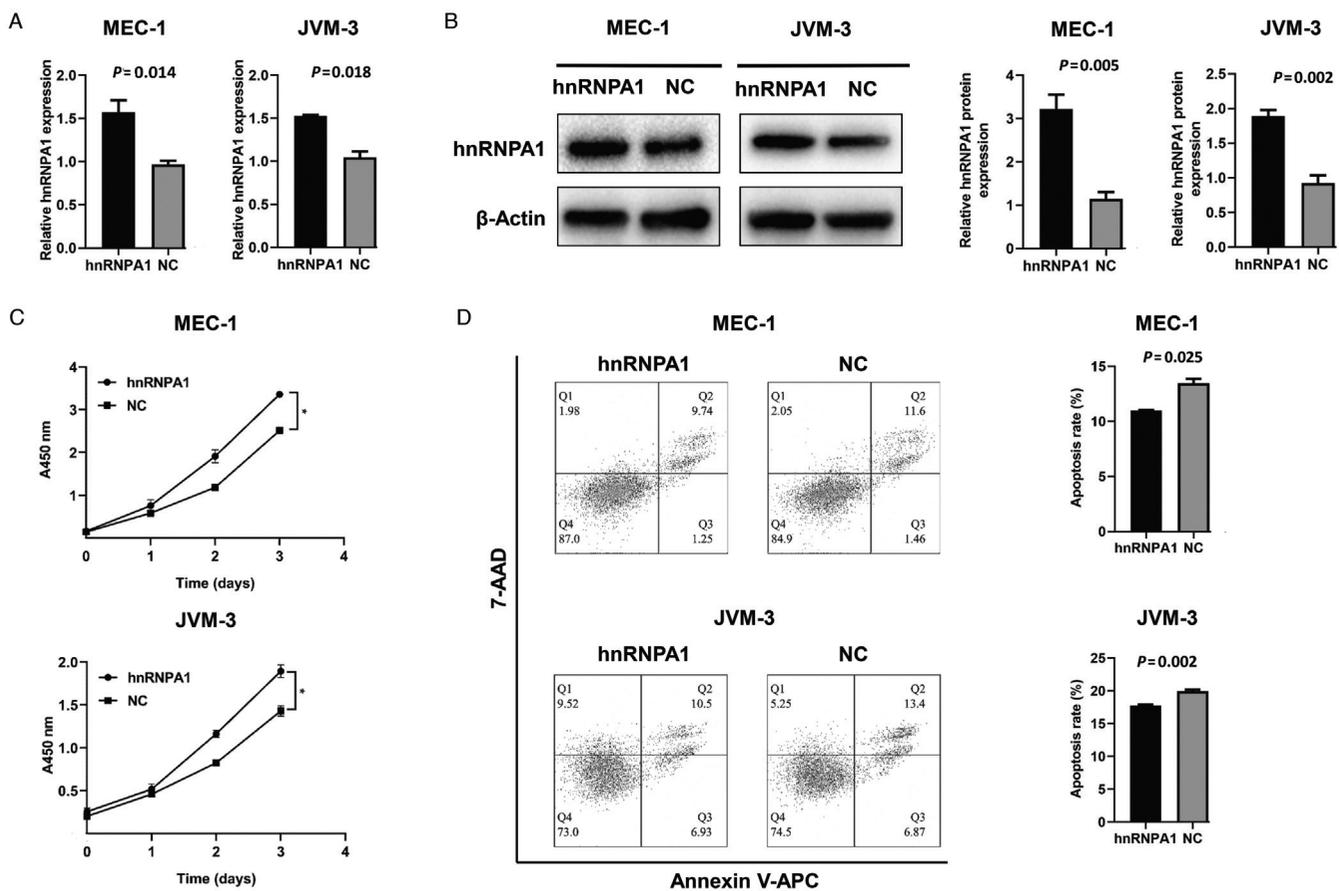


Figure 3: The characteristics of *hnRNPA1*-overexpressed CLL cells. (A) The transcript level of *hnRNPA1* in *hnRNPA1*-overexpressed MEC-1 and JVM-3 was verified by qRT-PCR. (B) The protein level of *hnRNPA1* in *hnRNPA1*-overexpressed MEC-1 and JVM-3 was verified by Western blot. (C) The proliferative ability of *hnRNPA1*-overexpressed MEC-1 as well as JVM-3 and corresponding negative control tested by CCK-8 assay. (D) The apoptosis of *hnRNPA1*-overexpressed MEC-1 as well as JVM-3 and corresponding negative control detected by Annexin V-APC/7-AAD apoptosis kit. Data are representative of three independent experiments. $P < 0.05$. CCK-8: Cell count kit-8; CLL: Chronic lymphocytic leukemia; *hnRNPA1*: Heterogeneous nuclear ribonucleoprotein A1; qRT-PCR: Quantitative real-time polymerase chain reaction.

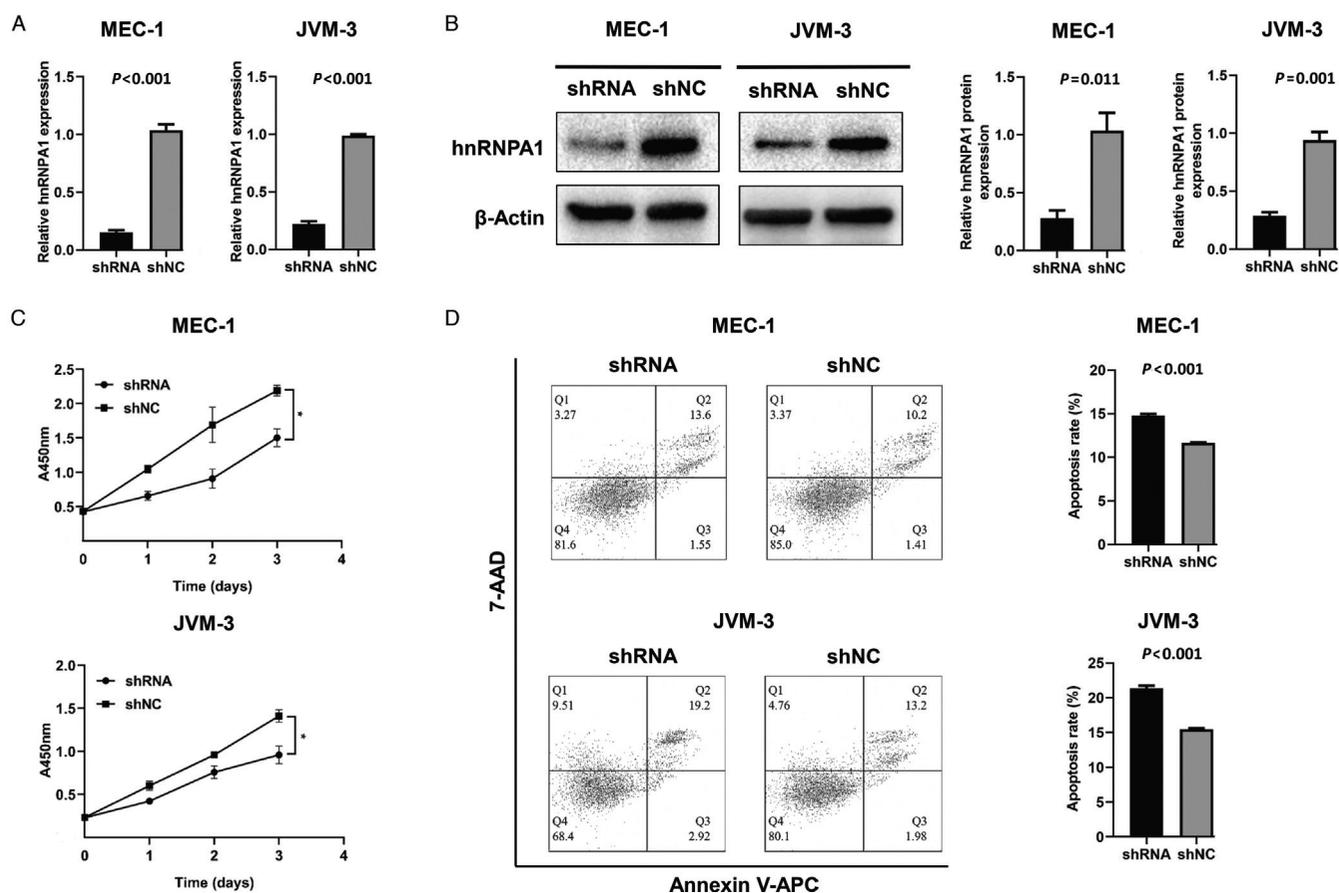


Figure 4: The characteristics of hnRNPA1-knockdown CLL cells. (A) The transcript level of *hnRNPA1* in hnRNPA1-knockdown MEC-1 and JVM-3 was verified by qRT-PCR. (B) The protein level of hnRNPA1 in hnRNPA1-knockdown MEC-1 and JVM-3 was verified by Western blot. (C) The proliferative ability of hnRNPA1-knockdown MEC-1 as well as JVM-3 and corresponding negative control tested by CCK-8 assay. (D) The apoptosis of hnRNPA1-knockdown MEC-1 as well as JVM-3 and corresponding negative control detected by Annexin V-APC/7-AAD apoptosis kit. Data are representative of three independent experiments. **P* < 0.05. CCK-8: Cell count kit-8; CLL: Chronic lymphocytic leukemia; hnRNPA1: Heterogeneous nuclear ribonucleoprotein A1; qRT-PCR: Quantitative real-time polymerase chain reaction.

In addition, hnRNPA1-knockdown cell lines were also constructed. The transcript and protein levels were shown in Figure 4A and 4B, respectively. On the contrary, hnRNPA1-knockdown MEC-1 and JVM-3 indicated a lower proliferation rate [Figure 4C] and a higher apoptosis rate [Figure 4D].

NOTCH1 signaling and hnRNPA1 expression in CLL

NOTCH1 mutation would cause impaired degradation of NICD and eventually cause continuous NOTCH1 signaling activation.^[5] To evaluate the relationship between NOTCH1 signaling and hnRNPA1 expression in CLL, an adenovirus vector was applied to construct NICD-overexpressed cell lines. The transcript level of *HES1* was an indicator for NOTCH1 signaling activation.^[16] In NICD-overexpressed MEC-1 and JVM-3, the mRNA level of *NICD*, *HES1*, and *hnRNPA1* was significantly higher than those in negative control cells [Figure 5A]. Similarly, the protein level of hnRNPA1 was upregulated in NICD-overexpressed cells [Figure 5B]. In addition, NOTCH1-knockdown MEC-1 and JVM-3 were constructed by using NOTCH1-shRNA to inhibit NOTCH1 signaling. Consequently, the mRNA level of *NOTCH1*, *HES1*, and *hnRNPA1* was substantially downregulated in those cell lines [Figure 5C]. Similarly,

the protein level of NOTCH1 and hnRNPA1 also decreased [Figure 5D].

c-Myc as a key regulator between NOTCH1 and hnRNPA1 in CLL

Previous research confirmed the direct NOTCH1 binding to the c-Myc promoter in CLL, which illustrated that c-Myc was a transcriptional target of NOTCH1.^[8] In addition, a positive correlation was observed in the expression of c-Myc and hnRNPA1 in several solid tumors.^[10,17] Thus, c-Myc was speculated as a vital regulator between NOTCH1 and hnRNPA1 in CLL. To verify this speculation, the transcript level of c-Myc between seven *NOTCH1*-mutated samples and eight *NOTCH1*-wt CLL samples were compared first [Figure 6A] and no difference between these two groups was found, which was consistent with the results of many previous researches.^[8,18] Afterward, to explore the correlation of NOTCH1 signaling and c-Myc expression, the mRNA level of c-Myc in NICD-overexpressed and NOTCH1-knockdown cell lines was tested. The transcript level of c-Myc increased in NICD-overexpressed cells and declined in NOTCH1-knockdown cells [Figure 6B]. Similarly, NICD-overexpressed cells had higher c-Myc protein levels than the negative control [Figure 6C].

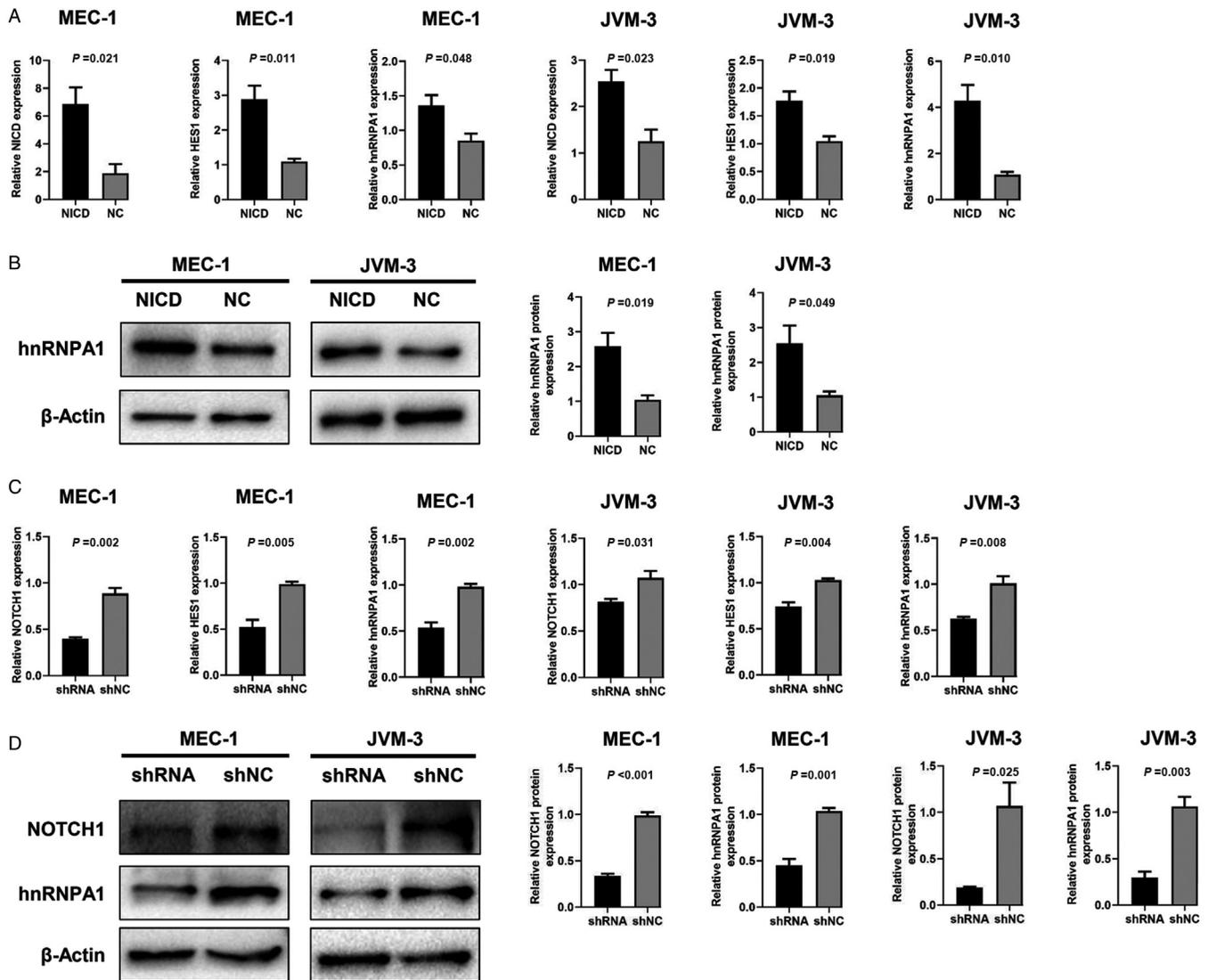


Figure 5: The relationships between NOTCH1 signaling and hnRNPA1 in CLL. (A) The transcript level of *NICD*, *HES1*, and *hnRNPA1* in NICD-overexpressed MEC-1 and JVM-3 was tested by qRT-PCR. (B) The protein level of hnRNPA1 in NICD-overexpressed MEC-1 and JVM-3 was tested by Western blot. (C) The transcript level of *NOTCH1*, *HES1*, and *hnRNPA1* in NOTCH1-knockdown MEC-1 and JVM-3 was tested by qRT-PCR. (D) The protein level of NOTCH1 and hnRNPA1 in NOTCH1-knockdown MEC-1 and JVM-3 was tested by Western blot. CLL: Chronic lymphocytic leukemia; hnRNPA1: Heterogeneous nuclear ribonucleoprotein A1; NICD: NOTCH1 intracellular domain; qRT-PCR: Quantitative real-time polymerase chain reaction.

Furthermore, the relationship between c-Myc and hnRNPA1 expression was investigated and it was found that the protein level of c-Myc and hnRNPA1 simultaneously declined after treatment with 10058-F4 [Figure 6D]. Finally, a rescue experiment was conducted to verify the relationship among NOTCH1, c-Myc, and hnRNPA1. After 10058-F4 was added, the expression of c-Myc and hnRNPA1 was substantially downregulated in both NICD-overexpressed and corresponding negative control cell lines. The highest and lowest expression was observed in NICD-overexpressed cells treated with ethanol (as a control) and negative control cells treated with 10058-F4, respectively [Figure 6E].

Discussion

NOTCH1 mutation is one of the most essential molecular biological aberrations in CLL.^[2] In the era of chemioimmunotherapy, patients with *NOTCH1* mutation

showed a poor response to treatment and had an adverse prognosis.^[19] In addition, NOTCH1 and B cell receptor signals could support each other to promote the development of CLL. *NOTCH1* mutation enhanced the expression of the B cell receptor signal by activating the NOTCH1 pathway, which in turn facilitated the translation and activation of the NOTCH1 signal. The efficacy of ibrutinib was negatively correlated with NOTCH1 pathway activation. Based on this, *NOTCH1* mutation was associated with ibrutinib resistance.^[6] Therefore, comprehending the changes caused by *NOTCH1* mutation allows us to provide better treatment for those patients. After analyzing the gene expression profile of five *NOTCH1*-mutated and five *NOTCH1*-wt samples, we found that several genes involved in RNA splicing were upregulated in *NOTCH1*-mutated samples, one of which was hnRNPA1. In this research, we investigated the role of hnRNPA1 in *NOTCH1*-mutated CLL cells. We validated the higher mRNA and protein level of hnRNPA1 in our

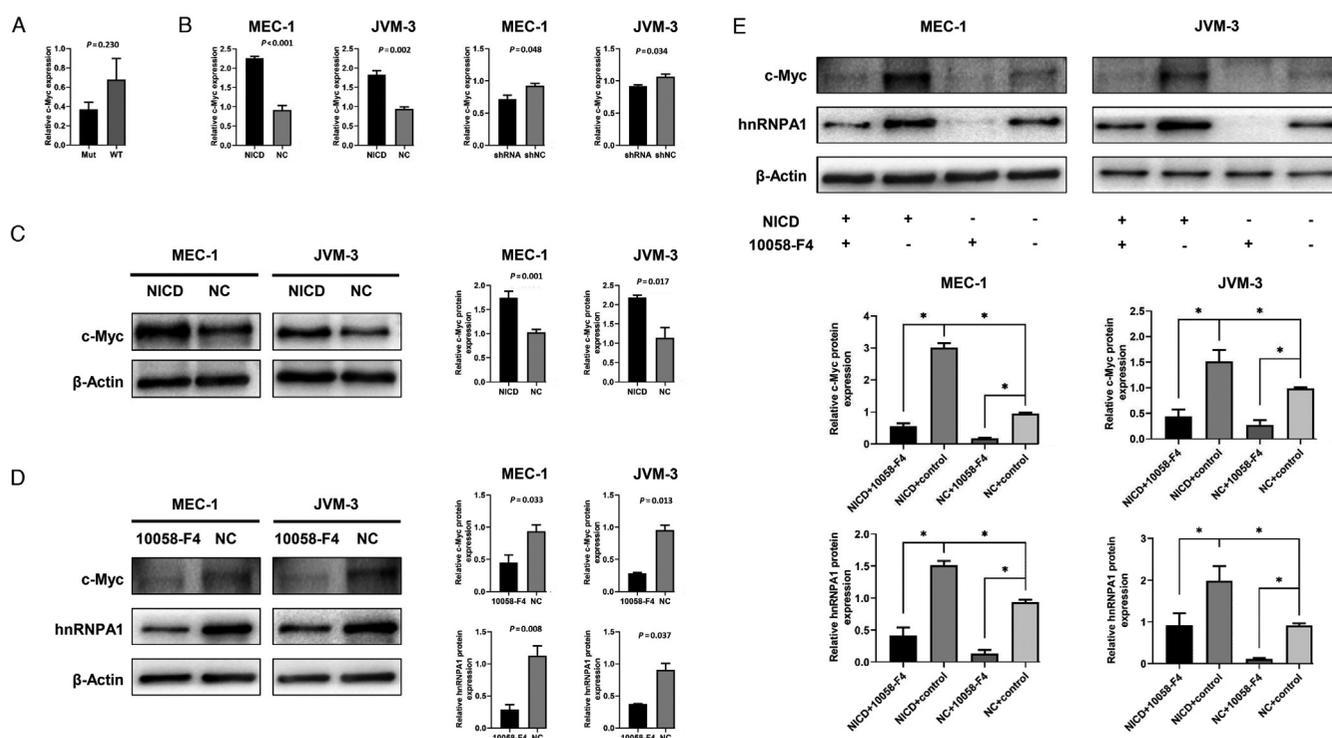


Figure 6: The relationships among NOTCH1, c-Myc, and hnRNPA1 in CLL. (A) The transcript level of *c-Myc* in primary *NOTCH1*-mutated ($n = 7$) and *NOTCH1*-wt ($n = 8$). CLL samples were detected by qRT-PCR. (B) The transcript level of *c-Myc* in NICD-overexpressed and NOTCH1-knockdown MEC-1 as well as JVM-3 detected by qRT-PCR. (C) The protein level of c-Myc in NICD-overexpressed MEC-1 and JVM-3 was tested by Western blot. (D) The protein level of c-Myc and hnRNPA1 after treatment with c-Myc inhibitor 10058-F4 in MEC-1 and JVM-3 tested by Western blot. (E) The protein level of c-Myc and hnRNPA1 after treatment with c-Myc inhibitor 10058-F4 in NICD-overexpressed MEC-1 and JVM-3 tested by Western blot. $P < 0.05$. CLL: Chronic lymphocytic leukemia; hnRNPA1: Heterogeneous nuclear ribonucleoprotein A1; NICD: NOTCH1 intracellular domain; qRT-PCR: Quantitative real-time polymerase chain reaction.

primary *NOTCH1*-mutated CLL samples, which were consistent with gene expression profile data. The functions of hnRNPA1 were explored by the CCK-8 assay and the cell apoptosis analysis in hnRNPA1-overexpressed and knockdown cell lines. These results illustrated that hnRNPA1 overexpression could promote proliferation and inhibit apoptosis in CLL cells, which might partly account for the poor prognosis of *NOTCH1* mutation. However, the reasons why hnRNPA1 overexpression led to CLL development remain unknown. As stated in previous studies, hnRNPA1 could regulate the splicing of pyruvate kinase by promoting the formation of pyruvate kinase isozymes M2 rather than M1 to affect metabolism.^[20] In that case, hnRNPA1 mediated metabolism changes might be an underlying mechanism for CLL development. Hence RNA sequencing or microarray is urgently needed to further study the role of hnRNPA1 in *NOTCH1*-mutated CLL cells.

NOTCH1 mutation leads to impaired NICD degradation, which eventually results in sustained NOTCH1 signaling activation.^[5] In this study, the mRNA and protein level of hnRNPA1 increased after NOTCH1 signaling was over-activated by transfection with NICD-overexpressed adenovirus vector. On the contrary, the expression of hnRNPA1 declined after NOTCH1 signaling was inhibited by transfection with NOTCH1-shRNA lentiviral vector. These results indicated the positive correlation between NOTCH1 signaling and hnRNPA1 expression.

Previous research reported that c-Myc was a direct transcriptional target of the NOTCH1 activation complex in CLL confirmed by chromatin immunoprecipitation assay.^[8] In addition, c-Myc could regulate the expression of hnRNPA1 in prostate cancer and gliomas.^[17,21] However, the relationship among NOTCH1 signaling, c-Myc, and hnRNPA1 in CLL remains unclear. Consistent with earlier studies, we failed to find a higher mRNA level of c-Myc in *NOTCH1*-mutated samples, which might be due to the rapid turnover of c-Myc.^[8,22] Activating NOTCH1 signaling by overexpressing NICD could upregulate both transcript and protein levels of c-Myc. Due to low c-Myc protein expression in CLL cells, we only examined mRNA level in NOTCH1-knockdown cells and proved that c-Myc mRNA expression declined after the NOTCH1 signaling was inhibited. These results confirmed the previously mentioned relationship between NOTCH1 signaling and c-Myc expression in CLL.

It is known that c-Myc is a vital transcription factor that could regulate several target genes expression.^[23] In the present research, inhibiting c-Myc by adding c-Myc inhibitor 10058-F4, which could interfere with c-Myc-Max interaction, could downregulate hnRNPA1 expression. Moreover, in NICD-overexpressed cells, the protein level of hnRNPA1 decreased after 10058-F4 was applied.

All these results demonstrated that c-Myc was a key regulator between NOTCH1 and hnRNPA1 in CLL.

Nevertheless, there are still certain limitations in our research. Wang *et al*^[24] found that the untranslated part in NICD caused by *NOTCH1* mutation exerted some unique functions beyond recognition and degradation. Therefore, applying full-length NICD to simulate *NOTCH1* mutation status might cause a certain level of deviation. In this study, we only utilized one method to activate or inhibit *NOTCH1* signaling. In further research, we could add ethylene diamine tetra-acetic acid and gamma-secretase inhibitor to activate and inhibit *NOTCH1* signaling, respectively. Meanwhile, primary CLL cells could better reflect the real status and are more suitable for research compared with cell lines.^[8]

RNA splicing has aroused increasing attention in recent years. With the accumulation of knowledge, the relationship between RNA splicing and tumor development is being gradually revealed.^[25-27] Our research aimed to explore the molecular mechanisms of *NOTCH1* mutation which focuses on splicing factors. In summary, we found that overexpression of c-Myc-related hnRNPA1 could promote proliferation and inhibit apoptosis in *NOTCH1*-mutated CLL cells. Nevertheless, further researches are needed to study which genes hnRNPA1 could change and what functions they could exert to better understand *NOTCH1* mutation in CLL from the perspective of RNA splicing.

Funding

This work was supported by the National Natural Science Foundation of China (Nos. 81700193, 81970146, 82170186, and 81720108002), National Major Science and Technology Projects of China (No. 2018ZX09734-007), China Postdoctoral Science Foundation (No. 2021M691336), and Jiangsu Postdoctoral Science Foundation (No. 2021K083A).

Conflicts of interest

None.

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How to cite this article: Zou Y, Tang H, Miao Y, Zhu H, Wang L, Fan L, Fu J, Xu W, Li J, Xia Y. Overexpression of c-Myc-dependent heterogeneous nuclear ribonucleoprotein A1 promotes proliferation and inhibits apoptosis in *NOTCH1*-mutated chronic lymphocytic leukemia cells. *Chin Med J* 2022;135:920–929. doi: 10.1097/CM9.0000000000002037