lncRNA CHROMR promotes the expression of the CNNM1 gene by adsorbing hsa-miR-1299 to obtain drug resistance in diffuse large B lymphoma cells

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Research Article

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Abstract

Background: Long-chain non-coding RNA (LncRNA) is differentially expressed in diffuse large B cell lines with varying degrees of resistance to rituximab, which plays a regulatory role in the cell cycle, and has a cancer-promoting effect.

Methods: This study investigated the role of lncRNA CHROMR in the diffuse large B lymphoma cells producing rituximab resistance and explored its underlying mechanism. We searched the GEO database for lncRNA-related expression data of rituximab resistance in the diffuse large B lymphoma cell line and tested it in cell lines. It was found that CHROMR was differentially expressed in different types of samples, and then the effect of CHROMR in the acquisition of drug resistance of cells was verified through experiments such as cell cycle, cell apoptosis, and cell proliferation. We use bioinformatics analysis to predict that there may be a CHROMR/miR-1299/CNNM1 signaling pathway that enables the diffuse large B lymphoma cells to acquire drug resistance. We first use dual-luciferase analysis to verify the interaction between pathway molecules. We use bioinformatics analysis to predict that there may be a CHROMR/miR-1299/CNNM1 signaling pathway that enables the diffuse large B lymphoma cells to acquire drug resistance. We first use dual-luciferase analysis to verify the interaction between pathway molecules. The cells were co-transfected with or without sh-CHROMR and miR-1299 inhibitor, CHROMR - over and miR-1299 mimic, whose proliferation, apoptosis, and cell cycle were determined subsequently.

Results: The results show that restoring the expression of miR-1299 can offset the effects of changes in CHROMR expression.

Conclusions: CHROMR promotes the expression of CNNM1 by adsorbing miR-1299 to cause changes in the cell cycle of the diffuse large B lymphoma cells to produce drug resistance.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is a type of tumor composed of medium to large B lymphoid cells. The nucleus of tumor cells is equal to or exceeds that of normal macrophages, or it is more than twice the nucleus of normal lymphocytes, showing a diffuse growth pattern. Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin's lymphoma in hematological diseases, accounting for 30%-40% of all cases of non-Hodgkin's lymphoma of which of adult NHL in Western countries 25%~35%, which can be as high as 45% in developing countries[1]. Diffuse large B-cell lymphoma shows apparent heterogeneity in cell origin, morphology, immunohistochemical phenotype, molecular genetics, involved location, chemotherapy response, and survival rate[2]. DLBCL can be a primary disease, or it can be transformed from other low-invasive lymphomas. The lesions mainly occur in the lymph nodes, and some can be found in the gastrointestinal tract, bones, and central nervous system[3, 4]. In patients with DLBCL, the early lymph nodes enlarge rapidly, while the DLBCL outside the lymph nodes becomes tumor-like, accompanied by fibrosis or not. As DLBCL progresses, the lesions grow aggressively, leading to poor treatment effects[5, 6]. Although advances in medicine have promoted

tremendous advances in the treatment of DLBCL, up to 40% of patients still die from relapse. At present, the molecular mechanism of DLBCL pathogenesis and drug resistance is still unclear, which seriously hinders the treatment of DLBCL[7, 8].

Long non-coding RNA (lncRNA) is involved in many aspects of cell metabolism, such as cell tumorigenesis, proliferation, apoptosis, and drug resistance[9–13]. At present, many lncRNAs have been intensely studied by many workers and used in clinical diagnoses, such as MALAT1, H19, BANCR, HOTAIR, Etc., verified as essential participants in cancer progression and can be used as new biomarkers in Some cancers' Early diagnosis or treatment[14–17]. However, the role of lncRNA in cancer remains to be explored. At present, some lncRNAs are considered to be essential factors for cancer to acquire drug resistance. BCAR4 is highly expressed in prostate cancer. It can bind to the promoter region of GLI2 and activate downstream genes of GLI2, making prostate cancer cells less sensitive to androgen stimulation[18]. In contrast, Lnc-SNHG17 releases CD51 by adsorbing miR-144, thereby enhancing the proliferation and migration of prostate cancer cells, avoiding drug killing[19]. lncRNA CCAT1 acts on miR-24-3p to up-regulate FSCN1, making cancer cells resistant to paclitaxel[20].

Long non-coding RNA also plays a vital role in the occurrence, progression, resistance, and prognosis of DLBCL[21–23]. LINC00857 can promote the occurrence and progression of DLBCL[24]. SNHG14 can make DLBCL immune escape and promote proliferation[25]. We analyzed the data set GSE159852 in the GEO database and obtained Differential Expression of lncRNAs in Rituximab Sensitive and Resistant DLBCL Cell Lines. We found that lncRNA CHROMR is highly expressed in drug-resistant cell lines.

The main research direction of lncRNA CHROMR currently focuses on cholesterol diseases. However, there is no report on cancer. Therefore, this article will explore the mechanism by which lncRNA CHROMR acquires drug resistance in DLBCL.

Materials And Methods

Cell Culture and Treatment

human DLBCL cell lines, including SU_DHL_4, SU_DHL_8 (ATCC, Virginia, USA), were cultured in RIMI-1640 medium (Thermo Fisher Scientific, Massachusetts, USA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin with an atmosphere of 5% CO2 at 37°C.When necessary, 17nM Rituximab (MCE) were added to the medium to Induce cell death.

Cell transfection

According to the manufacturer's instructions, miR-1299 mimic and its negative control (NC) (100 nM, Guangzhou all-perfect Biological Technology Co., Ltd.), pcDNA3.1-CHROMR, and pcDNA-NC (50 nM, Guangzhou all -perfect Biological Technology Co., Ltd.) vectors were transfected into SU_DHL_4 cells

using Lipofectamine™ 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. Perform related functional tests 48 hours after transfection. Psicheck2.0-CHROMR-WT and psicheck2.0-CHROMR-MUT were co-transfected with miR-1299 mimic and mimic NC into 293T cells, and the fluorescence intensity was detected 48 hours after transfection.

Psicheck2.0-CNNM1-WT and psicheck2.0-CNNM1-MUT were co-transfected with miR-1299 mimic and mimic NC into 293T cells, and the fluorescence intensity was detected 48 hours after transfection.

Flow Cytometry analysis for apoptosis

Cell apoptosis was assessed by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Beyotime, Shanghai, China). Briefly, cells were collected, washed with phosphate buffered saline (PBS), and then stained with APC-conjugated anti-Annexin V antibody and propidium iodide (PI) according to the manufacturer's protocol. The percentage of Annexin V+ PI+ cells was determined by using a flow cytometer (Becton Dickinson, New Jersey, USA).

Flow Cytometry analysis for cell cycle

cell cycle was assessed by flow cytometry using the Cell Cycle and Apoptosis Analysis Kit (Beyotime, Shanghai, China). Cells with medium only were used for the control. The cells were collected and then fixed in 70% ice-cold ethanol at 4 °C for overnight. Cells were rewashed by cold PBS and suspended in PBS containing 0.1 mg/mL RNase A and 50 μg/mL PI for 30 min at room temperature. DNA contents of cells were determined with flow cytometry within an hour. Cell cycles were resolved by NovoExpress software (Agilent Technologies,Inc.).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

The RNAiso plus kit (Takara, Dalian, China) was used to extract total RNA from SU_DHL_4, SU_DHL_8. The total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Takara). RT-qPCR was performed on a LightCyclerR 96 PCR machine (Roche Diagnostics, Basel, Switzerland) using the SYBR-Green PCR kit (Takara Bio). The reaction conditions were pre-denaturation at 95°C for 10 min, denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec, and extension at 72°C for 34 sec, a total of 40 cycles. U6 or GAPDH served as the internal reference. Quantitative expression was calculated using the 2−ΔΔCT method (16). The primers used are shown in Table I.

Table 1

Primer sequences for RT-qPCR.

Primer	Sequences (5'-3')	Accession
CHROMR	F:TTCCCTGCACTCCAAACACAGA	NR_110204.1
	R:AAGTCTTCCATCTGTCAGCGCC	
Hsa-miR-1299	F:TTCTGGAATTCTGTGTGAGGGA	MIMAT0005887
	R:TTTTTTTTTTTTTTTTTTTTTTTT	
GAPDH	F:ATGGTTTACATGTTCCAATATGA	NM_001256799
	R:TTACTCCTTGGAGGCCATGTGG	
U6	F:CGCTTCGGCAGCACATATAC	NR_004394
	R:AATATGGAACGCTTCACGA	

Western blot analysis

The cells were lysed in RIPA buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 0.1 mM PMSF, and 2 mg/ml aprotinin. Protein concentration was measured using the BCA kit (Beijing Solarbio Science & Technology Co., Ltd.). Then, the proteins (50 μg/lane) were separated with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Beyotime Biotech) and transferred onto nitrocellulose membranes. The membranes were blocked with 3% blocking buffer (Beyotime) for 1 h at room temperature and cultured with the primary antibodies CNNM1 (1:1,000, ab122648, Abcam) , BAX (1:1,000, ab32503,Abcam) ,bate-actin(1:1000,ab6276,Abcam)and BCL2 (1:1000,ab32124,Abcam) overnight at 4°C. Subsequently, the membranes were cultured with the secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000, ab205718, Abcam) for 2 h. Next, the membranes were visualized using an enhanced chemiluminescence reagent (Millipore). Protein blotting was analyzed using the Image Pro Plus 6.0 software (National Institutes of Health), with bate-actin as the internal reference. The experiment was repeated three times.

Dual-luciferase reporter gene assay

After 48 hours of transfection, discard the cell culture medium, wash with PBS once, add 200ul of reporter gene cell lysate, fully lyse the cells, centrifuge at 10,000-15,000g for 3-5 minutes, and save the supernatant. Add 65ul of supernatant to a 96-well plate, and add 100ul of firefly luciferase detection reagent to each well for detection. The detection method is luminescence detection. Add 100ul Renilla luciferase detection reagent again for detection, the detection method is luminescence detection. The fluorescence detection value of Renilla was used as the benchmark to calibrate the detection value of firefly. Compare the difference in luminous value of each group.

Statistical analysis

All data were processed using the IBM SPSS Statistics version 21.0 software (IBM Corp). Data were first verified to show normal distribution and homogeneity of variance. Data were expressed as means ± standard deviation or counts. An independent samples t-test was used to compare two groups. P<0.05 indicated a statistically significant difference.

Results

CHROMR is highly expressed in Rituximab Resistant DLBCL Cell Lines

DLBCL is the most common histological subtype of NHL and generally responds well to treatment with rituximab. However, some patients are resistant to rituximab, making the treatment effect poor. We found that CHROMR was significantly highly expressed in rituximab-resistant cell lines through bio-information analysis and speculated a CHROMR/miR-1299/CNNM1 pathway that may be related to the acquisition of drug resistance in DLBCL cell lines. We selected Rituximab Sensitive DLBCL Cell Lines (SU_DHL_4) and Rituximab Resistant DLBCL Cell Lines (SU_DHL_8) for RT-qPCR detection. It was found that CHROMR and CNNM1 were significantly high in SU_DHL_8, and miR-1299 was low in SU_DHL_8. (All P<0.001) (Fig. 1A-C). We predicted the targets of CHROMR and miR-1299 and CNNM1 and miR-1299 in Starbase (Fig. 1D) and constructed a vector for the dual-luciferase Target verification experiment. The results show that miR-1299 can reduce the firefly expression of psicheck2.0-CHROMR-WT and psicheck2.0-CNNM1-WT, while psicheck2.0-CHROMR-MUT and psicheck2.0-CNNM1-MUT are not affected (Fig. 1E, F); this shows that CHROMR and CNNM1 do have targets for miR-1299. All in all, we proved that CHROMR, miR-1299, and CNNM1 have significant differences in expression in cells with different sensitivity to rituximab, and there is a correlation between these three.

Overexpression of CHROMR increases the Resistance of DLBCL cells to rituximab

In order to test our hypothesis，the pcDNA3.1-CHROMR vector was transfected into SU_DHL_4 cells and treated with 17nM rituximab. We used qPCR and WB to detect the expression of CHROMR, miR-1299, and CNNM1 before and after transfection. The expression of CHROMR and CNNM1 increased significantly (P<0.05), and the expression of miR-1299 decreased significantly (P<0.001). (Fig. 2 A, B) Subsequently, we tested by flow cytometry and found that under the condition of 17nM rituximab treatment, after the cells overexpressed CHROMR, the proportion of cells in the G2 phase increased by about three times with that before overexpression, while apoptosis decreased by 6% (P<0.001). (Fig. 2 C, D)We also detected the expression of apoptosis-related proteins BAX, BCL2, and caspase-3 by Western Blot and found that the expression of BAX and caspase-3 was significantly reduced (P<0.05), and the expression of BCL2 was

significantly increased (P<0.01). (Fig. 2 B)In addition, we also transfected SU_DHL_4 cells with pcDNA3.1-CHROMR vector and cultured them usually. Through the cck8 experiment, we found that after cells overexpress CHROMR, the level of cell proliferation increased (P<0.01). (Fig. 2 E)The above result shows that CHROMR can increase the resistance of DLBCL cells to rituximab by regulating the cell cycle to promote cell proliferation and reduce the level of apoptosis.

CHROME/miR-1299/NCAM1 signaling pathway reduces the sensitivity of DLBCL cells to rituximab

We have determined that CHROMR can enhance the resistance of DLBCL cells to rituximab, and we have also confirmed that there are interaction targets among CHROMR, miR-1299, and CNNM1. So does CHROMR regulate the cell cycle and apoptosis level of DLBCL cells through the CHROMR/miR-1299/CNNM1 pathway? We transfected pCDNA-CHROMR, pCDNA-CHROMR and miR-1299 mimic mixture, pCDNA-CHROMR and mimic NC mixture into SU_DHL_4 cells respectively, gave them 17nM rituximab stimulation, and tested them 48 hours later. First, we confirmed that pCDNA-CHROMR and miR-1299 played a role in the cell through qpcr and western Blot, and confirmed that there is a CHROMR/miR-1299/CNNM1 pathway. (Fig. 3 A and B) Then use flow cytometry to detect changes in the cell cycle and the level of apoptosis. We found that the simple CHROMR overexpression cell group was used as the control group, compared with miR-1299 mimic can increase the apoptosis level of CHROMR overexpression SU_DHL_4 cells (P<0.01) and reduce the proportion of cells in the G2 phase (P <0.01), which prevents cells from undergoing the proliferation cycle, but mimic NC has no such effect.(Fig. 3 C, D) The above results show that increasing the expression of miR-1299 can eliminate the rituximab resistance caused by the high expression of CHROMR, and also shows that the CHROMR/miR-1299/CNNM1 pathway can regulate the rituximab resistance of cells.

Discussion

In cancer, cholesterol levels have also been confirmed to be related to cancer progression. Studies have shown that cholesterol levels in cancer cells are often higher than normal cells. What does this represent? There have been many studies in this area. In terms of epidemiological studies, studies have shown that for every 10 mg/dl increase in cholesterol levels, the recurrence rate of prostate cancer will increase by 9%, and the use of lipid-lowering drugs (statins) is related to 13. In addition, there is a link between a slight reduction in cancer-related mortality of different cancer types. Still, some studies indicate that there is no link between cholesterol and cancer[26–29]. In terms of molecular mechanism, studies have shown that as cholesterol levels increase, the ability of anti-tumor cells TC9 cells polarized by CD8 + T cells to secrete IL9 is impaired, which is because cholesterol can bind to LXRs and reduce the binding of LXRs Sumoylation to P65 and IL9 promoter sequences[30]. So, in addition to acting on immune cells and weakening the lethality of tumor cells, does cholesterol affect tumor cells themselves? In this paper, through GEO analysis, we obtained the lncRNA difference data between rituximab-resistant DLBCL cells and rituximab-sensitive DLBCL cells. We found that the cholesterol-related lncRNA CHROMR was significantly high in expression. CHROMR is cholesterol-induced and metabolic regulation related. The current research on lncRNA is relatively more in atherosclerosis-related diseases. It has been confirmed to

be elevated in the plasma and atherosclerotic plaques of patients with coronary artery disease and is positively correlated with cholesterol levels[31]. What role does CHROMR play in tumors, especially in the production of rituximab resistance in tumor cells? We overexpressed lncRNA CHROMR in rituximab-sensitive DLBCL cells SU_DHL_4 and then stimulated it with rituximab. We found that under rituximab killing, compared with SU_DHL_4 cells that generally express CHROMR, SU_DHL_4 cells with high CHROMR expression have more cells to enter the cell proliferation cycle, the proliferation ability is enhanced, and the level of apoptosis is reduced. The result indicates that lncRNA CHROMR may reduce the killing of rituximab on tumor tissues by promoting cell proliferation.

We found the differential miRNA data of DLBCL through the HMDD v3.2 database, used the RegRNA database to predict the potential relationship between them and CHROMR, and found that miR-1299 and CHROMR have potential targets. Hsa-miR-1299 is a tumor suppressor, which has been confirmed in many cancers[32]. miR-1299 can act on the EGFR/PI3K/AKT signaling pathway to inhibit non-small cell lung cancer development[33]. miR-1299 can act on NOTCH3 to make the ovarian cancer cell cycle stay in the G0G1 phase[34]. In hepatocellular carcinoma, miR-1299 targets CDK6 to inhibit cell proliferation[35]. miR-1299 is not only a tumor suppressor; it has also been reported to be associated with tumor drug resistance, reducing miR-1299 to promote paclitaxel resistance in ovarian cancer cells and breast cancer cells[36, 37]. In this study, we tested the expression of miR-1299 in different rituximab-resistant cell lines. miR-1299 was highly expressed in the rituximab-sensitive cell line SU_DHL_4. After CHROMR was overexpressed, the expression of miR-1299 decreased in SU_DHL_4 cells. Through the dual-luciferase experiment, we also verified that there is an interaction target between miR-1299 and CHROMR, and we confirmed that miR-1299 is the downstream target of CHROMR through the recovery experiment. CHROMR makes DLBCL cells resistant to rituximab by adsorbing miR-1299.

CNNM1 is a cell cycle-related protein, which has been shown to be related to tumor development in prostate cancer and hepatocellular carcinoma, and can regulate cell cycle and proliferation[38]. In this study, we have proved through experiments that CHROMR is overexpressed and CNNM1 is also expressed, but if the expression of miR-1299 is restored, the expression of CNNM1 will also be restored. Therefore, it is likely to constitute a ceRNA mechanism pathway with CHROMR and miR-1299. CHROMR increases the expression of CNNM1 by adsorbing miR-1299, thereby promoting the cycle progress of DLBCL cells and making DLBCL cells resistant to rituximab.

Conclusions

All in all, our study proved that there is an lncRNA CHROMR/miR-1299/CNNM1 pathway that makes DLBCL cells resistant to rituximab. This provides a new idea to solve rituximab resistance and better treat DLBCL.

Declarations

Ethics approval and consent to participate

Our study did not require an ethical board approval because it did not contain human or animal trials.

Consent for publication

Not Applicable

Availability of data and materials

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

Competing interests

The authors declare no conflict of interest.

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Authors' contributions

CW and MW made substantial contributions to the conception and design of the study, acquired, analyzed and interpreted the data, and drafted and revised the manuscript for important intellectual content. ZM performed the experiments and interpreted the data. All authors read and approved the final manuscript.

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Figures

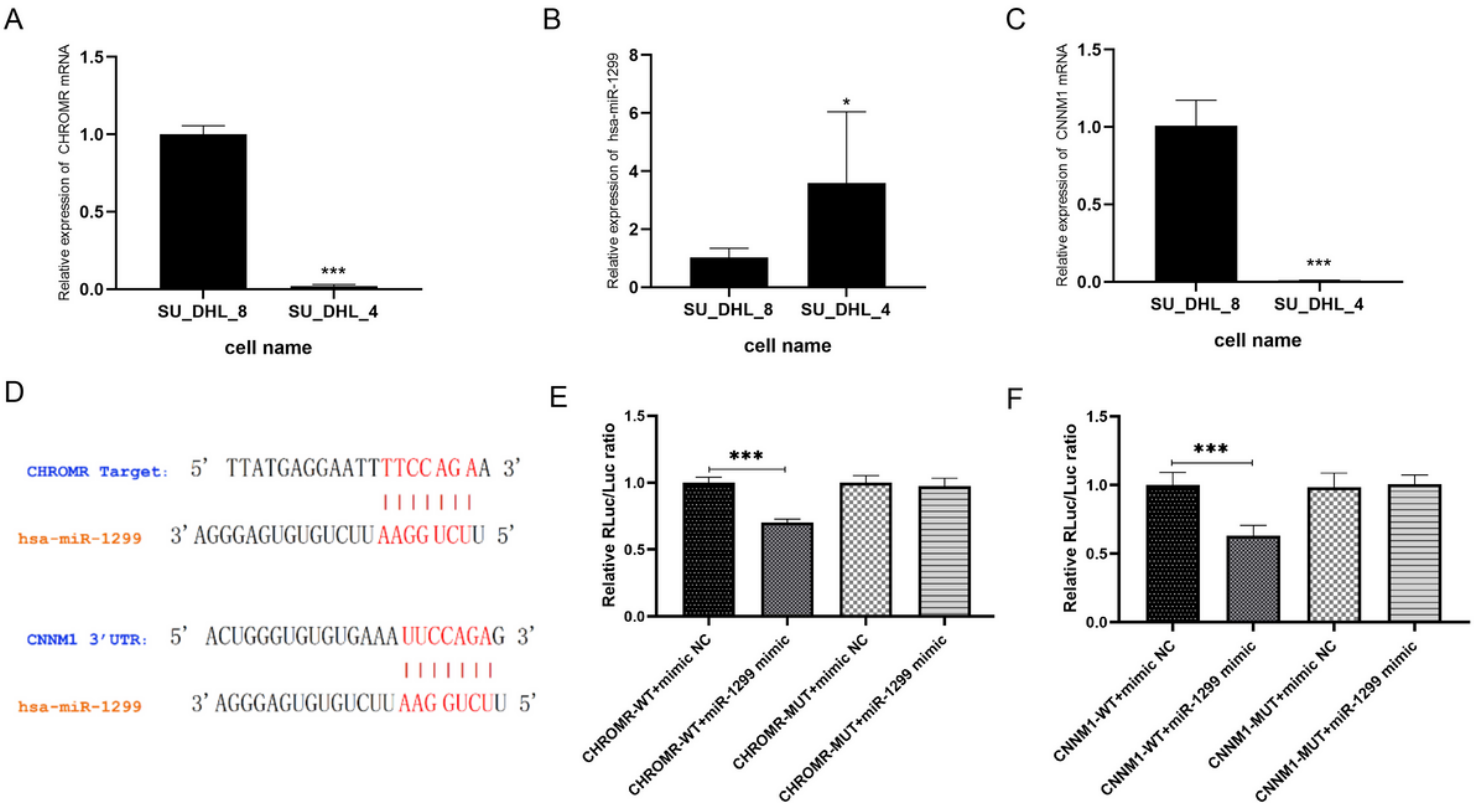

Figure 1

CHROMR, miR-1299, CNNM1 expression in different resistant cell lines and verification of their targets. (A) The expression of CHROMR in different cell lines of DLBCL was detected by qPCR. (B) The expression of miR-1299 in different cell lines of DLBCL was detected by qPCR. (C) The expression of CNNM1 in different cell lines of DLBCL was detected by qPCR. (D) Predictive targets of CHROME and miR-1299, miR-1299 and NCAM1. (E) Verification of the target of CHROMR and miR-1299 by dual luciferase experiment. (F) Verification of the target of CNMM1 and miR-1299 by dual luciferase experiment.

Figure 2

The role of CHROME in DLBCL cell line. (A) After CHROME overexpression, detect CHROMR, miR-1299, CNNM1 expression changes in SU DHL 4 cell line by qpcr. (B) After CHROME overexpression, detect apoptosis-related genes and CNNM1 expression by Western Blot. (C) In the case of rituximab killing, flow cytometry detected the proportion of cells in the G2 phase of each group. (D) In the case of rituximab killing, the proportion of apoptosis in each group was detected by flow cytometry. (E) After CHROME overexpression, detect cell proliferation ability of each group by cck8.

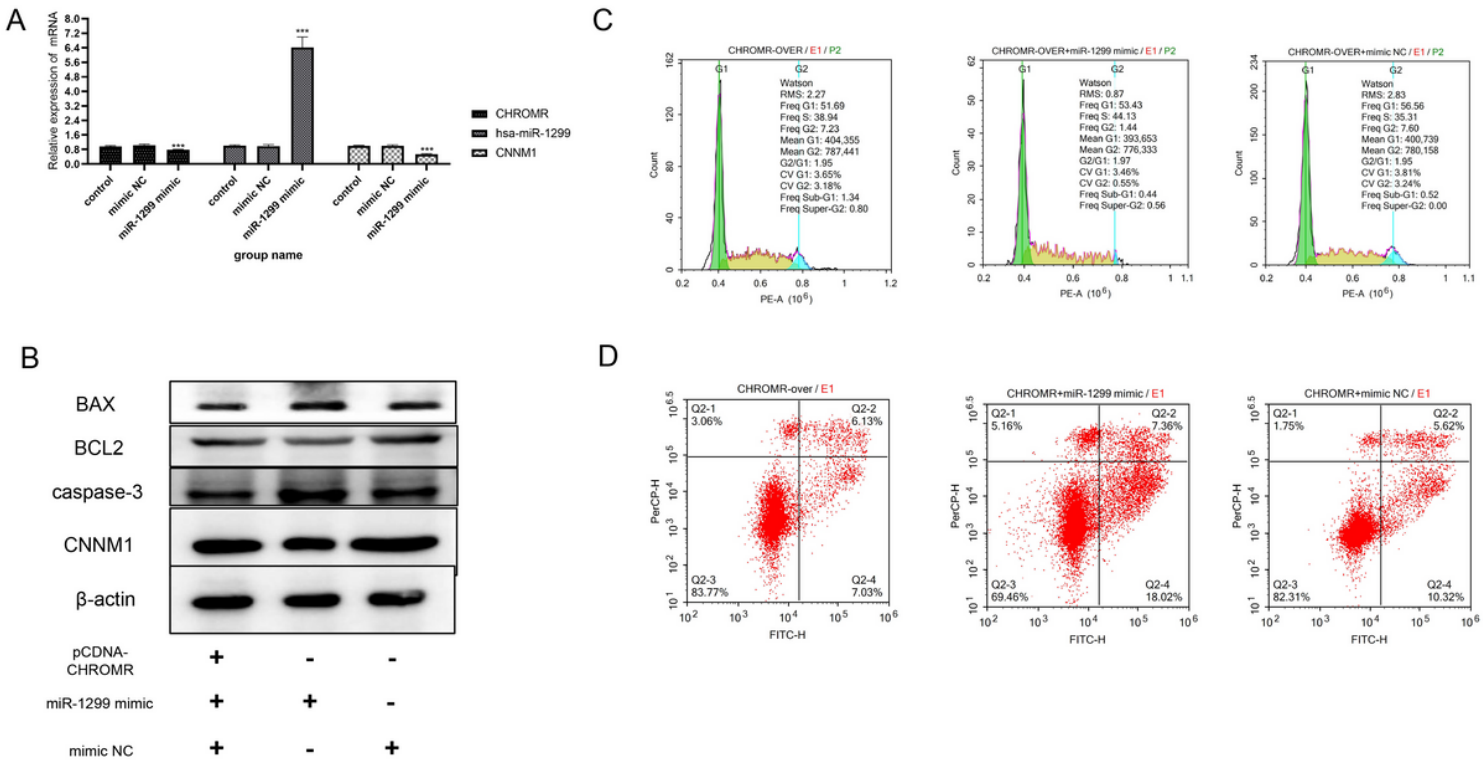

Figure 3

Verify the CHROME/miR-1299/CNMM1 pathway through cell function, PCR, and Western Blot experiments. (A) After transfection of CHROMR-over plasmid and miR-1299 mimic recovery,detect CHROMR, miR-1299, CNNM1 expression changes in SU DHL 4 cell line by qpcr. (B) After transfection of CHROMR-over plasmid and miR-1299 mimic recovery, detect apoptosis-related genes and CNNM1 expression by Western Blot. (C) In the case of rituximab killing, flow cytometry detected the proportion of cells in the G2 phase of each group. (D) In the case of rituximab killing, the proportion of apoptosis in each group was detected by flow cytometry.