L-Type Cav 1.2 Calcium Channel-α1C Regulates Response to Rituximab in Diffuse Large B-Cell Lymphoma

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Abstract

Purpose: One third of patients with diffuse large B-cell lymphoma (DLBCL) succumb to the disease partly due to rituximab resistance. Rituximab-induced calcium flux is an important inducer of apoptotic cell death, and we investigated the potential role of calcium channels in rituximab resistance.

Experimental Design: The distinctive expression of calcium channel members was compared between patients sensitive and resistant to rituximab, cyclophosphamide, vincristine, doxorubicin, prednisone (RCHOP) regimen. The observation was further validated through mechanistic in vitro and in vivo studies using cell lines and patient-derived xenograft mouse models.

Results: A significant inverse correlation was observed between CACNA1C expression and RCHOP resistance in two independent DLBCL cohorts, and CACNA1C expression was an independent prognostic factor for RCHOP resistance after adjusting for International Prognostic Index, cell-of-origin classification, and MYC/BCL2 double expression. Loss of CACNA1C expression reduced rituximab-induced apoptosis and tumor shrinkage. We further demonstrated direct interaction of CACNA1C with CD20 and its role in CD20 stabilization. Functional modulators of L-type calcium channel showed expected alteration in rituximab-induced apoptosis and tumor suppression. Furthermore, we demonstrated that CACNA1C expression was directly regulated by miR-363 whose high expression is associated with worse prognosis in DLBCL.

Conclusions: We identified the role of CACNA1C in rituximab resistance, and modulating its expression or activity may alter rituximab sensitivity in DLBCL.

Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of non-Hodgkin lymphoma characterized by distinct genetic abnormalities, biological features, and prognosis (1, 2). The addition of rituximab, a humanized chimeric anti-CD20 monoclonal antibody, to the standard CHOP chemotherapy (RCHOP) has significantly improved overall survival (OS) of DLBCL patients (3). However, 30% to 40% of patients experience incomplete response during treatment or progression after the completion of RCHOP treatment (4). Salvage therapy and/or autologous stem cell transplantation do not improve the dismal prognosis, especially in patients with prior rituximab treatment and early relapse (5). It is often regarded that rituximab resistance plays a vital role in treatment failure, thus requires further understandings to improve clinical efficacy of rituximab. Rituximab induces cell death via antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), direct apoptotic signaling, and possible vaccinal effects (6). It can inhibit the expression of the antia apoptotic genes BCL2/BCL-XL by downregulating the survival pathways (refs. 7–9; i.e., p38MAPK, NF-kB, ERK 1/2, and PI3K-AKT). The resistance to rituximab is either tumor-intrinsic (i.e., loss of CD20 expression; ref. 10) and/or host-associated factors (FcRIII-α polymorphism at amino acid position 158) that inhibit ADCC action (11). Novel anti-CD20 antibodies (e.g., Obinutuzumab) have been developed to exceed rituximab action (12, 13), but with limited improvement for DLBCL prognosis.

CD20 belongs to the M84A gene family, having a tetraspanning membrane structure with an N- and C-terminal cytoplasmic domain involved in B-cell proliferation (14). CD20 has been...
Translational Relevance

B cells express a wide and diverse pool of ion channels, including components of the Cav1 subfamily of voltage-gated L-type calcium channels. L-type calcium channels can be manipulated by many small molecules and are capable of mediating calcium influx, which is required to trigger apoptosis. Crosslinking of CD20 molecules by rituximab can induce calcium influx leading to apoptosis. In this study, we identified the role of L-type Cav1.2 Calcium Channel-alpha 1C (CACNA1C) in regulating response to rituximab in diffuse large B-cell lymphoma. These observations provide a rationale that modulating L-type Cav1.2 calcium channel may increase the clinical efficacy of rituximab. Actually, more attention has now been paid to calcium channels due to their involvement in the proliferation and invasion of cancer cells and their potential modulation by pharmaceuticals.

Materials and Methods

Patients and cell lines

The study was carried out in a retrospective series of 48 DLBCL cases with cryopreserved tissues and 63 cases with formalin-fixed paraffin-embedded tissues. Basic clinical characteristics of patients are presented in Table 1. The diagnosis of DLBCL was confirmed by at least two pathologists in accordance to the World Health Organization classification (25). All patients were treated with the RCHOP regimen, and involved-field radiotherapy was performed as consolidation treatment in 5 cases in the primary therapy. Responses to treatment were evaluated by CT scans or PET/CT following the response criteria for lymphoma as defined by Cheson and colleagues (26). The study was reviewed and approved by hospital review boards with informed consent of the patients and was conducted in accordance with the Declaration of Helsinki. Another independent DLBCL cohort (27) was used to validate the findings.

OCI-ly7, OCI-ly8, and OCI-ly3 DLBCL cell lines were cultured in IMDM (Gibco) with 10% FBS, 100 U/mL penicillin, 100 mg/ml streptomycin, and 0.2% beta mercaptoethanol (Invitrogen). These cell lines were gifted from Department of Pathology and Microbiology, University of Nebraska Medical Center. All of them were genotyped by short tandem repeat analysis and were confirmed to be negative for mycoplasma at the time of testing with the mycoplasma detection Kit (Lanza).

RNA isolation and gene expression profiling

Total RNA for gene expression profiling (GEP) was extracted using the Qiagen all prep RNA and DNA isolation Kit (Qiagen). We used HG-U133 plus 2 arrays (Affymetrix) for GEP, and RNA processing, hybridization, and image processing were performed according to the protocol described previously (28). The expression of miR-363 for these cases has been quantitated in a previous study (24).

qRT-PCR

For qRT-PCR, total RNA was isolated from tissues and cells using the miRNeasy Mini Kit (Qiagen). Note that 2 μg RNA was transcribed with the High Capacity cDNA Reverse Transcription...
Kits (ABI) according to the manufacturer's instructions. The qRT-PCR reactions were set up in triplicate using Brilliant II SYBR Green qPCR Master Mix (Toyobo) and ran on an ABI PRISM7300 Real-Time PCR system (Applied Biosciences) with the specific primers (Supplementary Table S1).

**IHC assay**

Indirect immunoperoxidase assays were performed on 5-μm thick paraffin sections using antibodies against CACNA1C (Omnimabs, 1:100), CD20 (Abcam, 1:50), BCL2 (Abcam, 1:250), or C-MYC (Abcam, 1:250). Antigen retrieval was performed by high pressure heating for 1 minute in PH 6.0 buffer for CACNA1C and in PH 9.0 buffer for CD20, BCL2, and C-MYC. Both CACNA1C and CD20 were located at plasma membrane, and the positive one was defined to have more than 30% of lymphoma cells stained.

**Flow cytometry assay**

The cells were fixed with 80% methanol for 5 minutes and then permeabilized with PBST (0.2% Tween-20) for 20 minutes. The cells were incubated in 10% normal goat serum and followed by antibody against CACNA1C (Omnimabs, 1 μg/10^6 cells) for 30 minutes at 22°C. The goat anti-mouse IgG (H+L) antibody conjugated with FITC (ThermoFisher, 1:200) was added for 30 minutes at 22°C in the dark. The fluorochrome-conjugated antibody against CD20 (BD, 1:20) was incubated for 30 minutes at 22°C in the dark. Run and analyze on flow cytometry.

**Apoptosis assay**

After drug treatment, 1 × 10^6 cells were washed with PBS and resuspended in the 500 μL of 1 × binding buffer containing Annexin-V–PE (Calbiochem). After incubation for 15 minutes at room temperature in the dark, cells were pelleted and resuspended in 500 μL binding buffer containing 10 μL 7-AAD, and analyzed on a FACScan. The lower right-hand and the upper right-hand quadrant cells were considered apoptotic.

**Immunofluorescence assay**

After being cultured with or without rituximab (50 μg/mL), OCI-ly7 cells were incubated with antibodies against CD20 (Abcam, 1:50) and CACNA1C (Omnimabs, 1:50) at 4°C overnight. Then the secondary antibodies Alexa Fluor 647 (Thermo Fisher Scientific, 1:1,000) and Cy3 (Thermo Fisher Scientific, 1:1,000) were incubated in the dark for 1 hour at room temperature, following 1 μg/mL DAPI being incubated in the dark for 1 minute. Finally, samples were added a drop of Dako fluorescence mounting medium (Dako) and mounted with coverslips. Images were collected with Nikon confocal microscope system.

**Calcium flux assay**

OCI-ly3 and OCI-ly7 cells were suspended at a density of 1 × 10^6 cells/mL with the Hank's Balanced Salt Solution (HBSS) containing 1% BSA(W/V), and 2 μmol/L Fluo-4 AM was dispatched into the 5 mL Falcon cytometry tubes for 30 minutes at 37°C in the dark. After being washed twice with HBSS, the cells were resuspended in saline solution containing 10 mmol/L HEPES, 1 mmol/L Na2HPO4, 137 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L CaCl2, 0.5 mmol/L MgCl2, 5 mmol/L Glucose, and 0.1% BSA(W/V). After adding 50 μmol/L rituximab, calcium flux assays were performed on the Becton-Dickinson FACS AriaII flow cytometry. The Flu-4 bound cytoplasmic free calcium and emit a green fluorescence (peak at 526 nm) detected using FL1.

**Western blotting assay**

Total proteins were extracted with NP40 lysis buffer (Beyotime). Western blotting assay was carried out using standard protocols with antibodies against CACNA1C (Omnimabs, 1:500), CACNA1D (Omnimabs, 1:200), CACNA1F (Abcam, 1:1,000), CACNA1S (Abcam, 1:2,000), and CD20 (Abcam, 1:2,000). Protein bands were visualized using the enhanced chemiluminescence system (Millipore) according to the manufacturer's instruction.

**Coimmunoprecipitation**

Total proteins from OCI-ly7 cells were extracted with NP40 lysis buffer (Beyotime). Anti-CD20 antibody (Abcam, 1:50) or control immunoglobulin (anti-IgG; Santa Cruz Biotechnology, 1:250) was incubated with cell lysate. Antigen–antibody complex was slowly shaken on rotating shaker overnight at 4°C followed by protein A/G PLUS-Agarose (Santa Cruz Biotechnology, 20 μL/500 μL) incubation for 3 hours at 4°C. The pellets were washed 3 times with NP40 lysis buffer (Beyotime). The pulled-down proteins were examined by Western blotting as described above.

**Construction of CACNA1C shRNA and miR-363 lentiviral vectors and transduction**

The shRNA to suppress the expression of CACNA1C was inserted into the Ptko.1-puro vector digested by AgeI/EcoRI according to the manufacturer's instruction. The lentiviral vector of miR-363 was generated after BamH1/EcoRI cloning and under the control of the Ubc promoter (Supplementary Fig. S1A). 293T cells were transfected with 12 μg of plasmid containing the desired construct and 8 μg of packing vector pSPAX2 and 4 μg of envelope vector pMD2G in a 10 cm dish for the production of lentivirus. After 48 hours, culture supernatant was filtered through the 0.45 μm filter and virus concentrated with PEG 6000 method. Lymphoma cells were incubated with the virus preparation overnight, and the culture medium was replaced with 2 mL fresh medium. We used puromycin (2 μg/mL) to select infected cells.

**CRISPR/Cas9 system to knock out miR-363**

The single-guide RNA (sgRNA) was designed nearest to the seed region of miR-363-3p and inserted into the pSpCas9 (BB)-2A-GFP (PX458) vector. A single-stranded DNA oligonucleotide (ssODN) of 99 bp was also designed in which the seed region of miR-363 (ATTGCAC) was replaced by EcoRI sequence (GAATTC). The sgRNA and ssODN were cotransfected into OCI-ly3 cell line using the Lonza Nucleofector system (Supplementary Fig. S1B). EGFP-positive cells were sorted by flow cytometry after transfection for 48 hours and then diluted into 96-well to generate single clones. All single clones were examined by DNA sequencing and qRT-PCR before further application.

**Xenograft mouse model assay**

Five- to 7-week-old SCID mice (Charles River) were injected with 1 × 10^6 OCI-ly7 cells that had been transduced with c-GFP or CACNA1C shRNA lentiviral vector, or DLBCL cells from patient sample in the posterior flank subcutaneously. When the tumor approached about 100 mm³, mice were randomly divided in groups of seven mice each. Saline (control), rituximab (25 μg/g),...
nimodipine (2 μg/g), Bay K8644 (2 μg/g), rituximab (25 μg/g) plus nimodipine (2 μg/g), and rituximab (25 μg/g) plus Bay K8644 (2 μg/g) were injected i.p. into patient-derived xenograft (PDX) mice once every other day for 10 times, respectively; saline (control) and rituximab (25 μg/g) were injected i.p. into OCI-ly7–derived mouse xenografts once every other day for 7 times. Tumor volume was measured every 3 days with a caliper and calculated using the formula: (Length x Width^2) x 0.5. Animals were maintained in accordance with the principles of laboratory animal care under an Institutional Animal Care and Use Committee–approved protocol.

Statistical analysis
Statistical analyses were performed using the Statistical Software Package for the Social Sciences (SPSS version 13.0 for Windows) and TIGR TM4 software (version 4.8.1). OS and event-free survival (EFS) were estimated using the Kaplan–Meier method, and differences were assessed using the log-rank test. We used the probe sets (242973_at and 238636_at) for estimating the CACNA1C mRNA expression and rituximab efficacy association (Supplementary Fig. S2). Multivariate logistic regression examined the effect of CACNA1C mRNA expression on RCHOP resistance. Differences among groups were considered statistically significant at P values below 0.05 (*).

Results
Inverse correlation between CACNA1C expression and RCHOP resistance
DLBCL patients treated with RCHOP regimen were divided into sensitive (n = 30) and resistant (n = 18) groups according to treatment response. Resistant patients were defined as not achieving complete remission or developing rapid disease progression (less than 6 months) after 6 to 8 cycles of RCHOP administration. Using GEP, we found that members of L-type calcium channel [i.e., CACNA1 (-C, -D, -F, -S), CACNA2 (-D1, -D2, -D3, -D4), CACNB (-1, -2, -3, -4), CACNG (1 to 8)] were expressed in both sensitive and refractory subgroups. Of them only CACNA1C mRNA expression presented significant difference with lower levels in resistant patients (P = 0.009), whereas CD20 mRNA expression did not show significant difference (Fig. 1A; Supplementary Table S2). The CACNA1C expression was not significantly different between germinal center (GC) B-cell (n = 26) and non-GC B-cell (n = 22) subtypes (P = 0.797). Using IHC, we observed that loss of CACNA1C protein was significantly associated with RCHOP resistance (44% vs. 76%, P = 0.001; Table 1). Two cases which represented the distinctive expression were presented in Fig. 1B. Univariate analysis revealed the correlation of CACNA1C expression (P = 0.001), double expression (P = 0.044), and international prognostic index (IPI) score (P = 0.017) with resistant disease. Multivariate analysis showed the independent association of CACNA1C expression and IPI score with resistance, excluding double expression.

The results were confirmed in an independent DLBCL cohort with RCHOP treatment (27). There was no correlation between CACNA1C expression and cell-of-origin classification (P = 0.94; Fig. 1C). Of the normal B-cell subsets, centrocytes and plasmablast cells showed comparatively lower expression of CACNA1C mRNA (Fig. 1D). When CACNA1C expression was divided into quartiles, the first quartile (Q1) with the lowest expression showed independent association with RCHOP resistance (OR = 2.897, P = 0.014). We also demonstrated the independent correlation of the lowest quartile of CACNA1C expression with inferior OS (HR = 1.974; P = 0.020; Fig. 1E). Similar trends were observed with EFS analysis (HR = 1.686, P = 0.053). In patients treated by CHOP regimen, we did not observe the relation of CACNA1C expression with outcome (Supplemental Fig. S3).

Loss of CACNA1C expression leading to rituximab resistance
To demonstrate the significance of CACNA1C expression in rituximab resistance, we used three DLBCL cell lines OCI-ly3, OCI-ly7, and OCI-ly8 for in vitro assays, including qRT-PCR, flow cytometry, and Western blotting assays. A good concordance was observed between mRNA and protein expression for CACNA1C and CD20 with OCI-ly3 showing the lowest and OCI-ly7 showing the highest expression (Supplementary Fig. S4A–S4E). When these cell lines were treated with rituximab (50 μg/mL) for 48 hours, we observed a positive relationship between CACNA1C expression and rituximab sensitivity. As shown in Supplementary Fig. S4F–S4G, cell lines with high expression of CACNA1C produced significant apoptosis (27.59% ± 2.41% in OCI-ly7, 18.84% ± 2.63% in OCI-ly8) compared with low expressing cell line (1.54% ± 1.39% in OCI-ly3; P < 0.05).

To further validate mechanistically, we generated CACNA1C-deficient stable cell lines using shRNAs (sh1C-1 and sh1C-2) in OCI-ly7 and OCI-ly8 cell lines, which resulted in significant loss of CACNA1C protein expression, and observed significant decrease in percentage of apoptosis in transduced cells after rituximab treatment (OCI-ly7: 23.05% ± 2.12%, sh1C-1 9.85% ± 1.32%, P = 0.042; sh1C-2 3.70% ± 1.03%, P = 0.022; and OCI-ly8: 17.86% ± 2.32%, sh1C-1 4.80% ± 1.06%, P = 0.035; sh1C-2 4.84% ± 1.08%, P = 0.038; Fig. 2A and B). To validate the finding in vivo, xenografts mice models were established using transduced OCI-ly7 cell line (with and without sh1C-1 vector) and treated with rituximab. As expected, xenografts with control vector showed significant tumor shrinkage in tumor volume (P = 0.035) and weight (P = 0.024), whereas those with sh1C vector was not significantly reduced in tumor volume (P = 0.273) and weight (P = 0.278; Fig. 2C). These data suggested that loss of CACNA1C significantly reduced rituximab-induced tumor inhibition.

Direct interaction of CACNA1C with CD20 during rituximab action
Using confocal microscopy, we observed that CD20 molecules located in the plasma membrane exhibited a gradual polarization (green light) after rituximab treatment at 0, 2, and 12 hours (Fig. 3A). CACNA1C molecule mainly confined to the plasma membrane with modest cytoplasmic and nuclear expression, and showed similar polarized distribution (red light) after rituximab treatment. The colocalization of CD20 and CACNA1C was observed in the merged image with overlapping distribution (yellow light) in the plasma membrane, which suggested possible interaction. To validate their interaction, CACNA1C protein was coimmunoprecipitated with either anti-CD20 or anti-IgG antibody after rituximab treatment of 2 hours in OCI-ly7 cells. CACNA1C protein was detected in anti-CD20 pull-down proteins, suggesting the association between CD20 and CACNA1C molecules (Fig. 3B).
Because rituximab performs its action via the ligation with cell surface CD20 molecule, we further explored the role of CACNA1C in regulating rituximab action. Knockdown of CACNA1C expression in DLBCL cell lines downregulated CD20 protein expression without any significant change in CD20 mRNA levels (Fig. 3C). Other forms of α1 subunits, such as CACNA1D, CACNA1F, and CACNA1S, showed no obvious alteration, indicating the specific effect of CACNA1C on CD20 expression. When these cells were treated with the proteasome inhibitor bortezomib (20 nmol/L) for 6 hours, CD20 expression was restored suggesting that CACNA1C may play a role in proteasome-dependent CD20 degradation, thus affecting efficacy of rituximab (Fig. 3D).

Modulators of L-type calcium channel affecting rituximab efficacy

Because there was not a specific modulator of CACNA1C, antagonist (nimodipine) and agonist (Bay K8644) of pan-L-type calcium channel were used to evaluate the effect of on rituximab efficacy. We observed significant reduction of apoptosis in cell lines when treated with rituximab (50 μg/mL) and nimodipine (2 μmol/L) for 48 hours compared with rituximab alone (OCI-ly7: 26.02% vs. 3.12%, P < 0.01; OCI-ly8: 17.65% vs. 1.89%, P < 0.01). When rituximab combined with agonist Bay K8644 (10 nmol/L), significant increase in apoptosis was observed in the above cell lines (OCI-ly7: 26.02% ± 3.12% vs. 37.36% ± 3.18%, P < 0.01; OCI-ly8: 17.65% ± 1.54% vs. 33.89% ± 2.98%, P < 0.01).
vs. 35.02% ± 2.58%, P < 0.01), and even in OCI-ly3 cell line, which initially showed poor response to rituximab (1.54% ± 1.39% vs. 21.13% ± 4.39%, P < 0.01; Fig. 4A).

Because rituximab-mediated calcium influx is a sensitive indicator of rituximab action, we measured the calcium influx with fluo-4-AM using flow cytometry after rituximab (50 μg/mL) treatment. We observed that the addition of rituximab resulted in significant increase in intracellular calcium ions in OCI-ly3 and OCI-ly7 cell lines. Expectedly, rituximab-mediated intracellular calcium ions were enhanced in cells treated with Bay K8644 for 2 hours, whereas it was reduced in cells treated with nimodipine for 2 hours. Ionomycin (2 μg/mL) was used as a positive control for calcium influx (Supplementary Fig. S5A). Neither nimodipine nor Bay K8644 treatment alone led to a significant alteration in

Figure 2.

The effect of CACNA1C downregulation on rituximab-induced apoptosis and tumor inhibition. A and B, The interference with shRNA to CACNA1C reduced the percentage of rituximab-induced cell apoptosis (P < 0.05) in OCI-ly7 and OCI-Ly8 cell line. C, In xenograft mice models of OCI-ly7 cell line, downregulation of CACNA1C by its shRNA obviously suppressed rituximab-induced tumor shrinkage in tumor volume and weight (P < 0.05).
intracellular calcium ions. Concurrently, we did not find the influence of nimodipine and Bay K8644 on CD20 expression after treatment of 24 and 48 hours in the study (Supplementary Fig. S5B).

We tested the action of these compounds in \textit{in vivo} DLBCL PDX mice (Fig. 4B) and observed that rituximab combined with agonist (Bay K8644) markedly enhanced rituximab action, as estimated by tumor volume ($P = 0.024$) and weight ($P = 0.012$). Neither rituximab combination with antagonist (nimodipine) nor modulators of L-type calcium channel alone showed obvious effect on tumor growth.

\textbf{MiR-363 epigenetically regulating the expression of CACNA1C}

Target Scan, a computational algorithm, predicted \textit{CACNA1C} to be the direct target of \textit{miR-363}. We demonstrated the direct regulation of \textit{miR-363} on \textit{CACNA1C} expression using luciferase reporter assay in 293T cells, where we found that luciferase
expression was significantly inhibited by miR-363 with wild-type 3'-untranslated region (UTR) of CACNA1C, but not with mutant 3'-UTR of CACNA1C (Fig. 5A). Furthermore, ectopic expression of miR-363 in OCI-ly7 and OCI-ly8 cell lines led to significant loss of CACNA1C and as anticipated loss of CD20 protein expression in these cell lines (Fig. 5B). The overexpression of miR-363 significantly reduced rituximab-induced apoptosis in OCI-ly7 (27.07% ± 2.54% vs. 1.22% ± 1.06%, *P = 0.018) and OCI-ly8 cell lines (17.23% ± 2.21% vs. 2.94% ± 1.11%, *P = 0.014) after rituximab treatment of 48 hours (Fig. 5C). To explore direct regulation, we further knocked out miR-363 locus in the OCI-ly3 cell line using the CRISPR/Cas9 system, and as expected resulted in the upregulation of CACNA1C (Fig. 5D). These edited cells showed significantly increased apoptosis (0.80% ± 0.69% vs. 10.25% ± 1.80%, *P = 0.001; Fig. 5E), when treated with rituximab.

**Discussion**

We investigated the role of L-type calcium channel in rituximab-induced apoptosis and demonstrated that loss of CACNA1C expression correlates with rituximab-mediated immun chemotherapy resistance in DLBCL. The role of CACNA1C in normal B-cell activation is not known, but several studies showed that L-type calcium channels play a role in B-cell calcium influx and proliferation (22), and increased intracellular calcium correlates with apoptosis (29). Calcium influx can affect the depolarization of mitochondria, which is fundamental in the activation of executioner caspases (30). The increased levels of cytosolic calcium may also activate calcium-dependent proteases, such as calpains, which have been implicated in caspase-independent death (31). The elevated intracellular calcium has been shown...
to be critical in rituximab-induced apoptosis. Thus, enhancing calcium influx could be an approach to increase rituximab-induced direct cell apoptosis in DLBCL. We attempted to knock out CACNA1C using CRISPR/Cas9 system in DLBCL cell lines, but these knockout cells lost the ability of persistent growth (Supplementary Fig. S6), which indicated a critical role of CACNA1C in the long-term survival of DLBCL cells.

The independent association of CACNA1C expression with refractory disease and inferior survival was proven in DLBCL patients. We did not find distinctive expression of CACNA1C between GCB and ABC DLBCL subtypes. Of the normal B-cell subsets, centrocytes and plasmablast cells showed comparatively lower CACNA1C expression. Our study further revealed the function of the accessory L-type calcium channels CACNA1C in rituximab-induced apoptosis, and the interaction between CACNA1C and CD20 in cell membrane is critical for this process. We observed direct interaction between CACNA1C and CD20 molecule using confocal immunofluorescent and pull-down assays and demonstrated that loss of CACNA1C expression resulted in lower CD20 stability and also reduced rituximab-induced cell death. This direct relationship between CACNA1C expression and rituximab sensitivity was also demonstrated in DLBCL cell lines and xenograft mouse models.

Rituximab resistance remains to be a clinical challenge. Many studies have explored the regulatory mechanisms of CD20 expression, including transcription factors SPI1/PU.1 (32), Oct2 (33), and Pax5 (34). Other factors, such as epigenetic changes, somatic mutation, and noncoding RNA, could also affect CD20 expression (35). A recent study using RNAi screening method identified CREM (cAMP Responsive Element Modulator) as a top candidate for CD20 suppression, CHD4 (Chromodomain Helicase DNA Binding Protein 4) and MBD2 (Methyl-CpG Binding Domain Protein 2) as positive regulators of CD20 expression (36). In addition, CD20 regulation at the posttranscriptional level was also suggested. Bortezomib can sensitize CD20-positive lymphoma cells to rituximab-induced CDC after the incubation of 24 hours (37), and rituximab-resistant lymphoma cells were observed to exhibit upregulation of components of the ubiquitin-proteasome system (38, 39). In this study, we found that CACNA1C may perform a critical function in CD20 protein
stabilization through proteasome system, because lowered CD20 protein level was restored in cells with CACNA1C shRNAs after bortezomib incubation for 6 hours.

Furthermore, we investigated the effect of modulators of L-type calcium channel on rituximab-induced apoptosis and explored the potential approach to raise therapeutic efficacy for DLBCL in the future clinical trials. This study validated that inhibiting L-type calcium channel with nimodipine reduced rituximab action, whereas Bay K8644, an agonist of L-type calcium channel, increased it. These observations provide a rationale that modulating L-type Cav1.2 calcium channel may increase the clinical efficacy of rituximab. Actually, more attention has now been paid on calcium ion channels due to their involvement in the proliferation and invasion of cancer cells and their potential modulation by pharmaceuticals (40, 41). This is the first study showing that modulating Cav1.2 subfamily of L-type calcium channel can affect rituximab-induced cell apoptosis on DLBCL. Blocking L-type calcium channels in human B cells has been demonstrated to reduce B-cell receptor (BCR)-induced calcium entry (42). Some knowledge gaps on L-type calcium channels remain to be addressed in B lymphocytes and malignancy.

The miR-363 is a member of miR-106-363 cluster which is also a paralog of miR-17-92 superfamily (43–45). Although the miRNA-17–92 cluster is a paradigm of oncomirs and cooperates with c-Myc in the formation of mouse B-cell lymphoma (46), limited data are available on miR-106-363 cluster members. The miR-106-363 cluster is mapped to chromosome X (47), and high expression of the miR-106-363 cluster has been found in 46% of human T-cell leukemias (48) and colon and prostate cancers (49) suggesting its oncogenic potential. In our previous miRNAs profiling studies (24, 50), miR-363 was found to have a higher expression in mantle cell lymphoma and was associated with poor response to RCHOP regimen in DLBCL patients (24). However, the mechanism by which miR-363 mediates the poor response to RCHOP remains elusive. This study demonstrated that miR-363 can directly downregulate the expression of CACNA1C and may thus reduce rituximab-induced apoptosis. The relevance of miR-363 to RCHOP resistance will be further addressed in our future studies.

In summary, the alteration of CACNA1C expression and modulators of L-type channel modulates rituximab-induced apoptosis. Notably, the agonist of L-type calcium channel, Bay K8644, has been shown to synergize in rituximab-induced apoptosis and tumor suppression in DLBCL. The optimal dose and safety of Bay K8644 or its analogues are worth evaluating in the future. Reduction of CACNA1C appeared to be also associated with reduced CD20 expression that may reduce target binding by rituximab. Further studies on the interaction between CACNA1C and CD20 in calcium flux in lymphoma may lead to discovery of novel mechanisms of calcium ions transport in DLBCL that may have therapeutic implications. The effect of miR-363 on CACNA1C and rituximab efficacy further extended our insight into the mechanisms of refractory disease, and it may serve as a prognostic marker or novel therapeutic target in the future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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