

Original Article

Effects of microRNA-203 on Farage cell apoptosis in diffuse large B cell lymphoma

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Abstract: Survivin is a most potent member in apoptotic inhibitor protein family, and exerts anti-apoptotic effect via inhibiting caspase-3 activity. Study has found significantly elevated Survivin expression in diffuse large B cell lymphoma (DLBCL) patients. Meanwhile, remarkably decreased microRNA (miR)-203 expression was observed in B lymphocytes of DLBCL patients, indicating its tumor suppressing role. Bioinformatics analysis showed satisfactory targeting relationship between miR-203 and 3'-UTR of Survivin mRNA. This study thus investigated whether miR-203 played a role in regulating Survivin expression and affecting apoptosis of DLBCL cell line Farage. A total of 38 DLBCL patients in our hospital were recruited to collect tumor samples, in parallel with 46 lymph tissues samples with reactive hyperplasia as the control group. MiR-203 and Survivin expression were tested, and their targeted relationship was assessed by dual luciferase reporter gene assay. In vitro cultured Farage cells were divided into mimic NC group, miR-203 mimic group, si-NC group, si-Survivin group and miR-203 mimic + si-Survivin group. Spectrometry was used to test caspase-3 activity, while flow cytometry was utilized for analysis of cell apoptosis, followed by Western blot analysis of protein expressions. Compared with control group, DLBCL tissues had significantly lowered miR-203 expression, plus higher Survivin expression. MiR-203 targeted 3'-UTR of Survivin mRNA and inhibited its expression. Elevation of miR-203 and/or silencing of Survivin expression significantly potentiated Caspase-3 activity and facilitated Farage cell apoptosis. MiR-203 inhibited Survivin expression via targeted inhibition, thus depressing its inhibitor effect on Caspase-3 activity, leading to accelerated Farage cell apoptosis.

Keywords: microRNA-203, survivin, DLBCL, farage cell, apoptosis

Introduction

Non-Hodgkin Lymphoma (NHL) is one group of malignant hematological disease originated from lymphatic organs such as lymph node, spleen and thymus and/or extra-nodal lymphatic tissues [1, 2]. Although 40% NHL patients obtained long-term remission by routine treatments, there were still 60% patients who were ineffective for normal treatment or had recurrence after remission [3]. NHL can be derived from B cells, T cells and NK/T cells, with majority (70%~85%) cases belonging to B cell NHL [4]. Diffuse large B cell lymphoma (DLBCL) is one of the most common B cell lymphoma, and occupies about 54% of all B cell NHL (B-NHL), and 30%~40% of all NHL patients [5]. Inhibitor of apoptosis proteins (IAPs) is one group of proteins with homologous structure and cell apoptosis inhibition function. Survivin, also named

as human baculoviral inhibitor of apoptosis repeat-containing 5 (BIRC5) is a member of IAPs family, and is the most potent apoptosis antagonizing molecule ever been found [6]. Survivin protein can impede cell apoptosis via inhibiting caspase-3 and caspase-7 activity, thus impeding cell apoptosis [7]. As an oncogene, Survivin has abnormally elevated expression in multiple tumor tissues including colorectal carcinoma [8], prostate cancer [6], esophageal carcinoma [9] and gastric cancer [10], whilst in normal tissues it has minimal expression level. Previous study has indicated significantly elevated Survivin expression in DLBCL patients [11] and its expression was correlated with patient's survival or prognosis [12].

MicroRNA (miR) is a type of non-coding small single stranded RNA with 18~22 nucleotide at length in eukaryotes, and can bind to 3'-UTR of

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target gene mRNA via completely or incompletely complementary pairing, thus participating in various biological processes including proliferation, cell cycle, differentiation and apoptosis via degrading target gene mRNA or inhibiting protein translation [13, 14]. The role of miR expression and abnormal function in NHL pathogenesis has drawn lots of research interests. Bioinformatics analysis revealed the existence of complementary binding sites between miR-203 and 3'-UTR of Survivin. Farage cell line originates from lymphatic tissues of DLBCL patients, and belongs to B lymphocytes. It is widely used as an *in vitro* model for studying DLBCL pathogenesis [15]. This study thus investigated whether miR-203 played a role in regulating Survivin expression, and affecting DLBCL cell line Farage apoptosis or DLBCL pathogenesis.

Materials and methods

Reagents and materials

Human DLBCL cell line Farage was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA); RPMI 1640 culture medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (US). Lipofectamine 2000 liposome transfection reagent was purchased from Invitrogen (US). ReverTra Ace qPCR RT Kit and SYBR Green dye were purchased from Toyobo (Japan). Mouse anti-human Survivin, anti-mouse IgG H&L and anti-rabbit IgG H&L with HRP labelling was purchased from Abcam (US). Rabbit anti-cleaved caspase-3 was purchased from CST (US). Annexin V/PI apoptotic kit was purchased from Yusheng (China). Caspase-3 activity kit was purchased from Beyotime (China). Dual-Luciferase Reporter assay system and pGL3-promoter were purchased from Promega (US).

Clinical information

A total of 38 DLBCL patients admitted in Huzhou Central Hospital from May 2015 to June 2016 were recruited. Tumor tissues were removed during the surgery, and were confirmed by the pathology examination. There were 21 males and 17 females, aged between 23 and 67 years (average age =44.5 years). Another cohort of 46 lymph node reactive hyperplasia patients was included in the control group. There were 26 males and 20

females, aged between 26 and 71 years (average age =46.3 years). No significant differences existed regarding sex or age between two groups. Fresh tissue samples were frozen in liquid nitrogen within 10 min after separation, and were kept at -80°C. All samples collections have informed consents of patients, and this study was reviewed and approved by the ethical committee of Huzhou Central Hospital.

Cell culture

Human DLBCL cell line Farage was kept in RPMI1640 medium containing 10% FBS and 1% penicillin-streptomycin, and were incubated in 37°C chamber with 5% CO₂, with medium changing every 2 days. Cells in log-phase growth were collected for experiments.

Construction of luciferase reporter gene

Using HEK293 genome as the template, full length fragment of 3'-UTR of Survivin gene was amplified. PCR products were purified from agarose gel, and were ligated into pGL-3M luciferase reporter plasmid after XbaI/NotI dual digestion. Recombinant plasmid was then used to transform DH5 α competent cells. Positive clones with primary screening were selected for further cell transfection and following experiments.

Luciferase reporter gene assay

Lipofectamine 2000 was used to transfect HEK293 cells with 500 ng pGL3-Survivin-3'UTR plasmid, 30 nmol/l miR-203 oligonucleotide fragments (or negative control), and 30 ng pRL-TK. After 6 h transfection, normal DMEM medium containing 10% FBS and 1% streptomycin-penicillin was used. After 48 h continuous incubation, dual-luciferase assay was performed. In brief, cells were washed twice in PBS, with the addition of 100 μ L PLB lysis buffer. With vortex at room temperature for 30 min, the mixture was centrifuged at 1000 rpm for 10 min. 20 μ L cell lysate was mixed with 100 μ L LAR II. Fluorescent value I was measured in a microplate reader. The enzymatic reaction was stopped in 100 μ L Stop & Glo, followed by quantification of fluorescent value II. The relative expression level of reporter gene was calculated as the ratio of fluorescent value I/ fluorescent value II.

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Cell grouping and culture

Cultured Human DLBCL cell line Farage cells were divided into 5 groups: non-treated group, mimic NC control, miR-203 mimic group, si-NC group, si-Survivin group, and miR-203 mimic + si-Survivin group. Opti-MEM was used to dilute oligonucleotide fragments and Lipofectamine 2000 for 5 min incubation at room temperature. Lipofectamine 2000 was then mixed gently with oligonucleotide fragment. After 30 min room temperature incubation, the mixture was added in serum-free medium. After 6 h, DMEM medium containing 10% FBS and 1% streptomycin-penicillin was added for 48 h continuous culture in further experiments. Oligonucleotide sequences were: mimic NC, 5'-UUCUC CGAAC GUGUC ACGUU U-3'; miR-203 mimic, 5'-GUGAA AUGUU UAGGA CCACU AG-3'; si-Survivin sense, 5'-GCAUC UCUAC AUUCA AGAAdT dT-3'; si-Survivin anti-sense, 5'-UUCUU GAAUG UAGAG AUGCdT dT-3'; si-NC sense, 5'-UUCUC CGAAC GUGUC ACGUdT dT-3'; si-NC anti-sense, 5'-ACGUG ACACG UUCGG AGAAAdT dT-3'.

qRT-PCR for gene expression

cDNA was synthesized in a 10 μ L system including 1 μ g total RNA, 2 μ L RT buffer (5X), 0.5 μ L oligo dT + random primer mix, 0.5 μ L RT enzyme mix, 0.5 μ L RNase inhibitor, and ddH₂O. The reaction conditions were: 37°C for 15 min, followed by 98°C 5 min. cDNA products were kept at -20°C fridge. Using cDNA as the template, PCR amplification was performed under the direction of TaqDNA polymerase using primers (miR-203P_F: 5'-GTCGT TACCA GTGCA GGGTC CGAGG TATTC GCACT GGATA CGACC TAGT-3'; miR-203P_R: 5'-GCCCC TGAAA TGTTT AGGAC CAC-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; SurvivinP_F: 5'-AGGAC CACCG CATCTC TACAT-3'; SurvivinP_R: 5'-AAGTC TGGCT CGTTC TCAGT G-3'; β -actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β -actinP_R: 5'-TGTCA CGCAC GATTT CC-3'); In a PCR system with 10 μ L total volume, we added 4.5 μ L 2XSYBR Green Mixture, 1.0 μ L of forward/reverse primer (at 2.5 μ M/L), 1 μ L cDNA, and 3.0 μ L ddH₂O. PCR conditions were: 95°C for 15 s, 60°C for 30 s and 74°C for 30 s. The reaction was performed on Bio-Rad CFX96 fluorescent quantitative PCR cyler for 40 cycles to collect fluorescent data. The acquired qPCR data was quantitatively analyzed by the

2- $\Delta\Delta$ CT method which was represented as the fold change relative the internal control.

Western blot assay

RIPA buffer was used to lyse cells. Supernatant was saved for protein quantification. 50 μ g protein samples were separated by 10% SDS-PAGE for 3 h, and were transferred to PVDF membrane for 1.5 h. The membrane was blocked with 5% defatted milk powder for 60 min, followed by primary antibody (anti-Survivin at 1:300, anti-cleaved caspase-3 at 1:100 or anti- β -actin at 1:500) incubation at 4°C overnight. By PBST washing (5 min \times 3 times), HRP-labelled secondary antibody (1:10,000 dilution) was added for 60min incubation. After PBST rinsing for three times (5 min each), ECL reagent was added for 2~3 min dark incubation. The membrane was then exposure in dark and scanned for data analysis.

Spectrometry for caspase-3 activity

Standard dilutions of 0, 10, 20, 50, 100 and 200 μ M pNA were prepared from 10 mM stock. Absorbance values at 405 nm wavelength were measured by a microplate reader to plot a standard curve with pNA concentration against A405 value. Attached cells were digested in trypsin, and were collected into culture medium for 4°C centrifugation for 5 min at 600 \times g. Supernatant was carefully removed and washed out by PBS. 100 μ L lysis buffer was added for every 2 \times 10⁶ cells. Cells were lysed at 4°C for 15 min, and were centrifuged at 18000 \times g with 4°C for 10 min. Supernatants were saved for further use. Ac-DEVD-pNA was placed on ice, mixed with buffer and test samples, with 10 μ L Ac-DEVD-pNA. The mixture was incubated at 37°C for 120 min. A405 value was measured when color changed significantly.

Flow cytometry for cell apoptosis

Cells were collected by centrifugation, and were then washed in PBS twice. 100 μ L Binding Buffer was used to re-suspend cells. The mixture was added with 5 μ L Annexin V-FITC and 5 μ L PI staining solution. After gentle mixture, the mixture was incubated in dark for 10 min, with the addition of 400 μ L 1 \times Binidng Buffer, and was immediately loaded for online testing in

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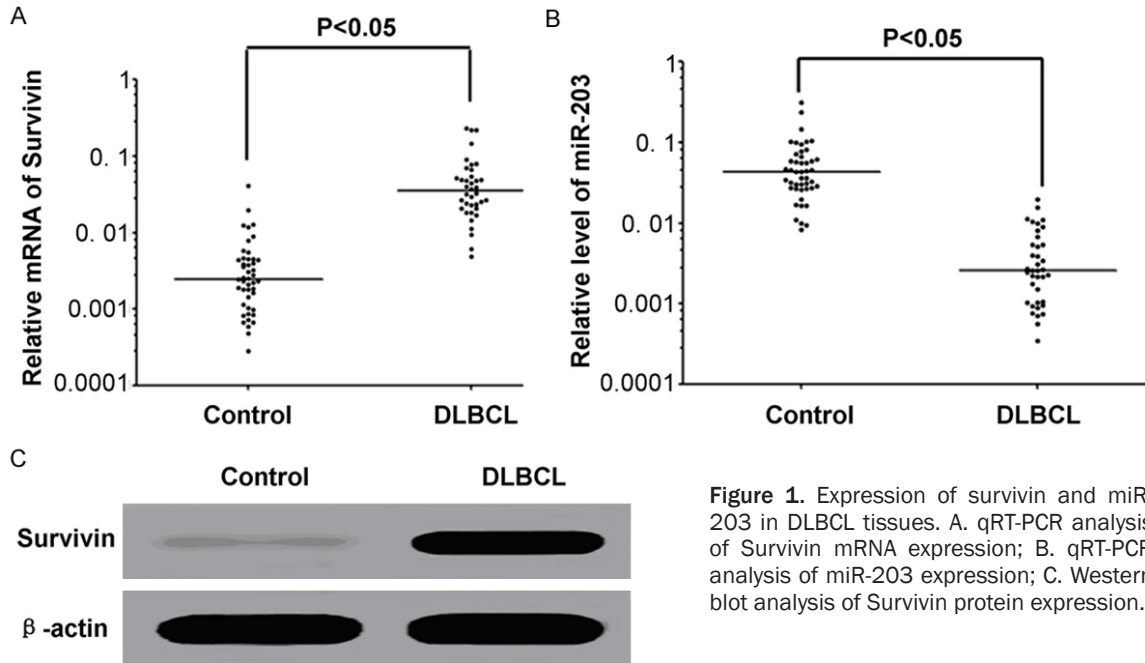


Figure 1. Expression of survivin and miR-203 in DLBCL tissues. A. qRT-PCR analysis of Survivin mRNA expression; B. qRT-PCR analysis of miR-203 expression; C. Western blot analysis of Survivin protein expression.

Beckman FC500MCL flow cytometry apparatus.

Statistical analysis

At least three independent experiments with triplicates each time were performed for each assay. SPSS18.0 software was used for data analysis. Measurement data were presented as mean \pm standard deviation (SD). Student's t-test was used to compare measurement data between groups. A statistical significance was defined when $P < 0.05$.

Results

Lower miR-203 and higher survivin expression in DLBCL tissues

qRT-PCR results showed significantly higher Survivin mRNA expression level in DLBCL patient tumor tissues compared with control group (**Figure 1A**). MiR-203 expression was significantly lower (**Figure 1B**). Western blotting results also showed remarkably higher Survivin protein expression in DLBCL tissues (**Figure 1C**). These results showed the possible role of miR-203 down-regulation in elevating survivin and facilitating DLBCL pathogenesis.

Survivin expression in Farage cells was regulated by miR-203

An online prediction using microRNA.org showed complementary binding sites between

miR-203 and 3'-UTR of Survivin mRNA (**Figure 2A**). Dual luciferase reporter gene assay showed that the transfection of miR-203 mimics or inhibitor significantly decreased/increased relative luciferase activity in HEK293 cells, proving that Survivin was a target gene of miR-203 (**Figure 2B**). Transfection of miR-203 mimic or inhibitor also remarkably reduced or elevated Survivin expression level in Farage cells (**Figure 2C** and **2D**), further demonstrating that miR-203 could regulate Survivin expression.

MiR-203 inhibited survivin expression and facilitated Farage cell apoptosis

Transfection of miR-203 mimic elevated miR-203 expression. Whilst transfection of Survivin siRNA significantly reduced Survivin expression in Farage cells (**Figure 3A** and **3B**), making its inhibitor effects on Caspase-3 enzymatic activity significantly decreased (**Figure 3B** and **3C**), with more apoptotic cell numbers (**Figure 3D**).

Discussion

NHL is a group of malignant hematological disease originated from lymphatic organs such as lymph node, spleen and thymus and/or extranodal lymphatic tissues [1, 2]. The incidence and mortality of NHL are ranked as 7th and 10th among all cancers, with gradually increased trends in recent years [16]. NHL has features of invasiveness, dispersion, worse prognosis and

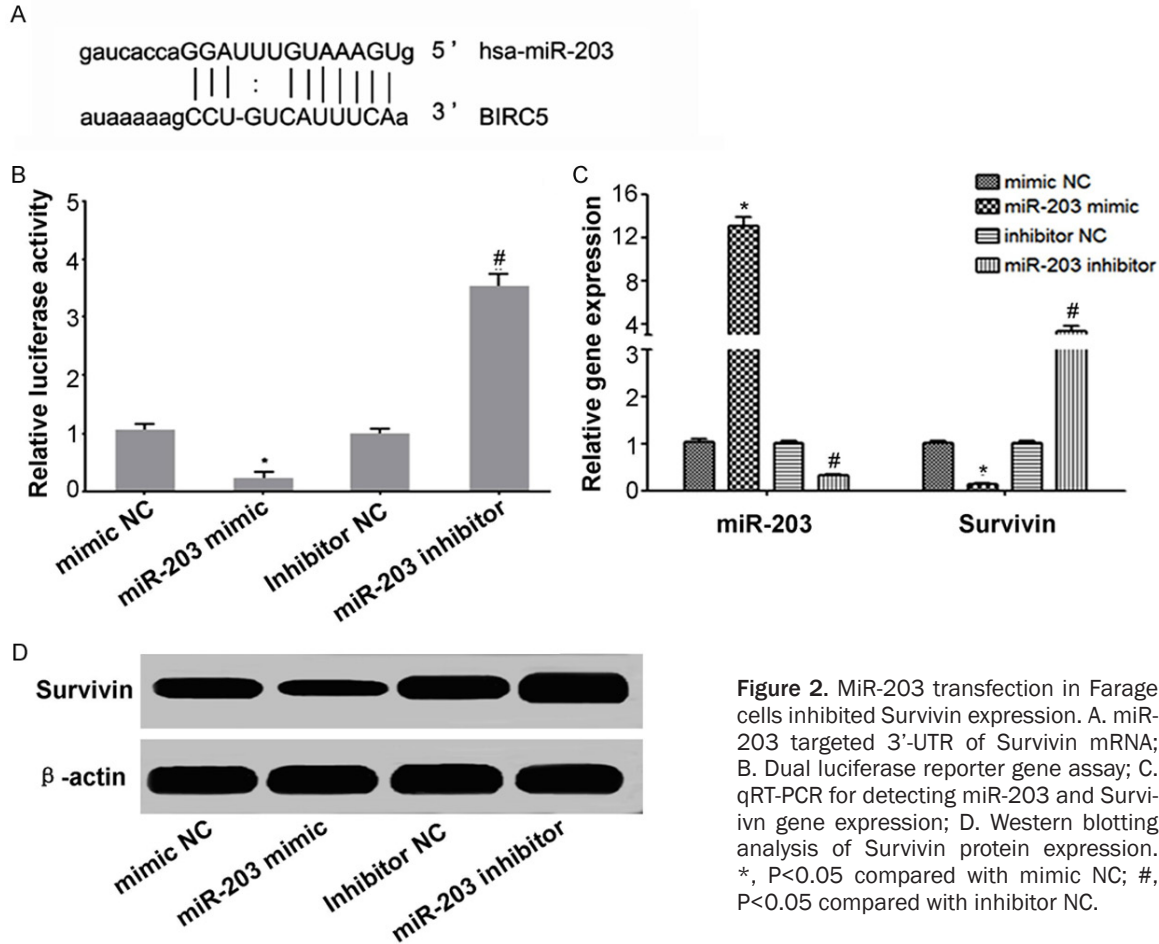


Figure 2. MiR-203 transfection in Farage cells inhibited Survivin expression. A. miR-203 targeted 3'-UTR of Survivin mRNA; B. Dual luciferase reporter gene assay; C. qRT-PCR for detecting miR-203 and Survivin gene expression; D. Western blotting analysis of Survivin protein expression. *, P<0.05 compared with mimic NC; #, P<0.05 compared with inhibitor NC.

higher mortality. DLBCL is one of the most common B cell lymphoma, and occupies about 54% of all B cell NHL (B-NHL), and 30%~40% of all NHL patients [5]. It is estimated that the incidence of DLBCL is 4~7 per 100,000 people, with increasing at 1.4% by years [17]. Although major progresses have been obtained including surgery, radio-/chemo-therapy and immune therapy, no significant improvements occur in survival and prognosis of DLBCL patients, whose 5-year survival rate maintains at 70% [18].

Cell proliferation and apoptosis are two aspects for cell fate determination, as imbalance between these two factors affects tumor occurrence and progression. Apoptosis is a programmed cell death route and is important for maintaining homeostasis. Barrier of apoptotic regulation is one important reason for dysregulated growth of tumor cells with genetic mutation, eventually leading to tumor pathogenesis [19]. Under endogenous or exogenous stimuli

of apoptotic signal, cysteine proteinase caspase molecules are activated in a cascade reaction manner, leading to activation of downstream effector molecules and initiation of cell apoptotic signaling pathway [20]. Caspase-3 is one of the most important members in Caspase family. In both mitochondrial or death receptor dependent exogenous factor induced apoptosis process, caspase-3 induced signal transduction pathway is required as an executing molecule, as it can directly degrade intracellular structural proteins and functional molecules, eventually leading to cell apoptosis [21, 22]. Survivin is a member of IAPs family, and is the most potent apoptosis antagonizing factor ever been found [6]. Survivin can impede cell apoptosis via inhibiting caspase-3 and caspase-7 at the end stage of caspase cascade reaction, thus facilitating tumor occurrence and progression, enhancing malignant biological activity of tumors, and leading to worse prognosis [7]. Survivin can directly bind to active caspase-3 and caspase-7 specifically to inactivate

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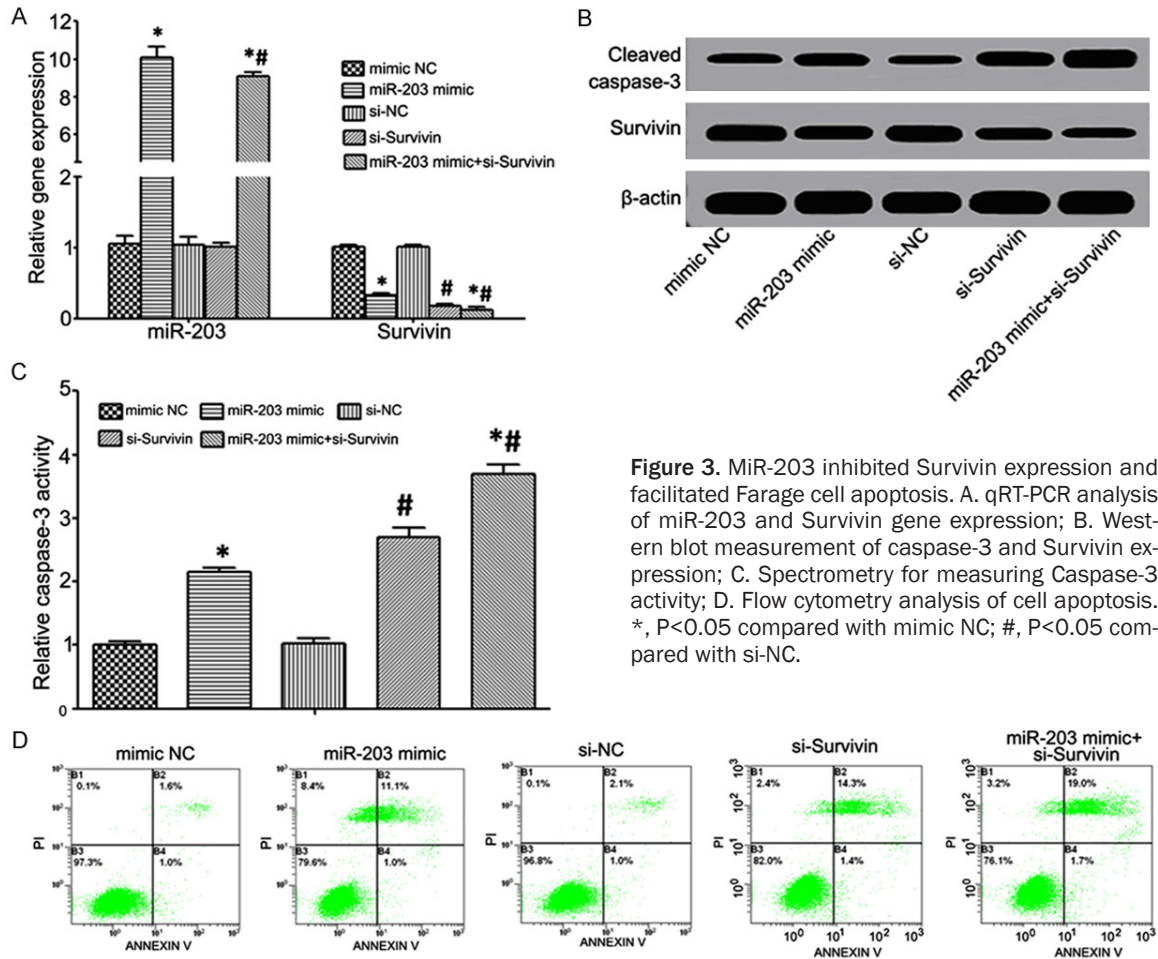


Figure 3. MiR-203 inhibited Survivin expression and facilitated Farage cell apoptosis. A. qRT-PCR analysis of miR-203 and Survivin gene expression; B. Western blot measurement of caspase-3 and Survivin expression; C. Spectrometry for measuring Caspase-3 activity; D. Flow cytometry analysis of cell apoptosis. *, P<0.05 compared with mimic NC; #, P<0.05 compared with si-NC.

them [7]. Moreover, it can also exert cell apoptosis antagonizing effects via impeding self-activation of caspase-3 and caspase-7. Survivin can also interact with cell cycle regulatory protein CDK4 to form Survivin/CDK4 complex, which dissociates CDK4/p21 to release p21 and subsequent translocation into the mitochondrial to form caspase-3 complex for inhibiting its activity, thus suppressing apoptosis [23]. As an oncogene, Survivin is minimally expressed in normal human tissues except in embryos [24]. However, in tumor tissues it has abnormally elevated expression such as colorectal carcinoma [8], prostate cancer [6], esophageal carcinoma [9] and gastric cancer [10]. Previous study has indicated significantly elevated Survivin expression in DLBCL patients [11], with a correlation between expression level and patient's survival or prognosis [12], indicating potential oncogene role of Survivin in NHL pathogenesis. Previous study also revealed remarkably decreased miR-203 expression in B

lymphocytes of DLBCL patients, suggesting possible tumor suppressor role of miR-203 in DLBCL. Bioinformatics analysis revealed complementary binding sites between miR-203 and 3'-UTR of Survivin. Therefore this study investigated whether miR-203 played a role in regulating Survivin expression, affecting DLBCL cell line Farage apoptosis and DLBCL pathogenesis.

This study showed significantly elevated Survivin expression in lymphoma tissues of DLBCL patients compared with lymph node tissues from reactive lymph node hyperplasia patients, accompanied with significantly lower miR-203 expression. Zhang et al found higher Survivin expression in DLBCL patient's tumor tissues [12], with higher expression indicated advanced TNM stage, international prognostic index and serum lactate dehydrogenase level, plus shorter overall survival times [12]. Adida et al found significantly lower 5-year survival rate in Sur-

Survivin up-regulated DLBCL patients compared with those having lower Survivin levels. This study also detected higher Survivin expression in DLBCL patient's tumor tissues compared with control group, which is consistent with the result obtained by Adida et al [11] and Zhang et al [12]. Chim et al found significantly potentiated methylation of miR-203 gene promoter in NHL patient tumor tissues, plus lower miR-203 expression [25]. Ralfkiaer et al found suppressed miR-203 expression in T cell lymphoma patient tumor tissues [26]. Whilst Ralfkiaer et al also showed lower miR-203 expression in advanced T cell lymphoma patient's lymphatic tissue compared to those at non-progressed T cell lymphoma tissues [27]. Yamagishi et al found lower miR-203 level in B lymphocytes of DLBCL patients [28]. All these studies collectively suggested the possible tumor-suppressor role of miR-203 in NHL pathogenesis. Our study revealed lower miR-203 expression level in DLBCL patient's lymph node tissues, consistent with a previous study conducted by Yamagishi et al [28]. Dual luciferase reporter gene assay showed that transfection of miR-203 mimic or miR-203 inhibitor significantly elevated or decreased relative luciferase activity in HEK293 cells, and decreased/increased Survivin expression in Farage cells, demonstrating that miR-203 targeted and regulated Survivin expression. Transfection of miR-203 mimics and/or si-Survivin remarkably depressed Survivin expression in Farage cells, reducing its inhibitory role on caspase-3 enzyme activity, thus facilitating Farage cell apoptosis.

Conclusion

DLBCL tissue had lowered miR-203 expression and elevated Survivin expression. MiR-203 can inhibit Survivin expression via targeting, and reduce its inhibitory function on caspase-3 enzymatic activity, leading to facilitation of Farage cell apoptosis.

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Disclosure of conflict of interest

None.

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