MicroRNA-520a attenuates proliferation of Raji cells through inhibition of AKT1/NF-κB and PERK/eIF2α signaling pathway

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Abstract. Burkitt’s lymphoma (BL) is a fast growing cancer of the human lymphatic system, and an extremely invasive B-cell non-Hodgkin’s lymphoma. We explored the mechanism of apoptosis in Raji cells associated with the post-transcriptional regulation factors. To confirm that the predicted microRNA-520a (miR-520a) is matched with AKT1, 3’ untranslated region (UTR) luciferase activity of AKT1 was used in the assessment. In the presence of the mimics or inhibitors of miR-520a, cell function of Raji, such as proliferation, growth and apoptosis were analyzed. The expression of endoplasmic reticulum (ER) stress-related proteins were examined. Luciferase reporter analysis showed that miR-520a leads to decreased activity of luciferase gene fused with AKT1 3’UTR. Therefore, AKT1 is a direct target of miR-520a. Our data indicated that the mimics of miR-520a inhibited growth, proliferation of Raji cells and promoted its apoptosis, which was related to downregulation of AKT1/NF-κB and ER stress response mediated by PERK/eIF2α pathway. On the contrary, the inhibitors of miR-520a promoted growth, proliferation of Raji cells and inhibited its apoptosis, which was related to AKT1/NF-κB and PERK/eIF2α pathway. We identified miR-520a, which specifically binds to AKT1 mRNA 3’UTR. miR-520a is a crucial mediator for proliferation and ER stress in Raji cells through regulating the AKT1/NF-κB or PERK/eIF2α signaling pathway. Our findings suggest that targeting mir-520a is a promising therapeutic strategy in BL.

Introduction

Burkitt’s lymphoma (BL) is one of the greatly proliferative and invasive lymphomas world-wide, despite its low morbidity (1). BL can be usually healed with rigorous iatrochemistry, whereas its strong side-effects block the usage of the chemotherapy for children, the elderly or patients in developing countries (2). In Raji cells, the downregulation of HSP70 could block the pathway of PI3K/AKT and accentuated its sensitivity to iatrochemistry (3). Targeting the pathway of PI3K/AKT may be advantageous to treat patients with BL (3).

The microRNAs (miRNAs) are a group of tiny molecules and non-coding RNA, 22-25 nucleotides in length that function on regulation of gene expression at post-transcriptional level (4). miRNAs control gene expression by pairing with incompletely matching target sites of the 3’-untranslated regions (UTRs) of mRNA, and cause translational repression and/or mRNA destabilization, thereby downregulating the expression of the targeted proteins (4,5). The growing number of reports hold up the vital function of miRNAs on expression regulation at post-transcriptional level. Moreover, the regulated expression of genes involve numerous biological progressions, especially for the different pathogenetic disorders (including cancer) (5,6). A variety of miRNAs are regarded to symbolize a new category of diagnostic and therapeutic opportunities in cancer (6-8).

Raji cells are stable hematopoietic human cells (9) widely used, but, the mechanisms of miRNA-dependent regulation of Raji cell functions are unclear. Keklikoglou et al found that miR-520/373 family had tumor-suppressive effect against breast cancer. It serves as contact between TGF-β and NF-κB pathways, and may contribute to the interaction effect of inflammation and tumor metastasis or progression (10). In the current study, we investigated the effects of microRNA-520a (miR-520a), for mediating the function of Raji cells.

To assess the role of miR-520a in Raji cells, we first identified miR-520a sequences in the AKT1 mRNA, and then evaluated the levels of miR-520a expression in Raji cells. Our study demonstrated that AKT1 is one of the targets of miR-520a in Raji cells. AKT1 has been activated associated with NF-κB. AKT1 has also been revealed to control survival of cells by regulating activation of NF-κB and mediating
the expression of endoplasmic reticulum (ER) stress-related proteins, as well as to inhibit cell apoptosis by activating the NF-κB subunit, RelA/p65. We, therefore, studied the regulation effect of miR-520a on regulating activation of NF-κB in Raji cells. In particular, the cell behavior effects of miR-520a were associated with the regulation of AKT1 and NF-κB signaling pathways.

Materials and methods

Cell culture. The cell line Raji was obtained from ATCC, USA. Cells were cultured in DMEM medium accompanied with 10% FBS (both from Gibco, Carlsbad, CA, USA) in incubator with 5% CO₂ humidified atmosphere at 37°C.

The luciferase reporter gene assays with dual reporter system. The 3'UTR of the AKT1 gene ~300 nt, which contain the binding site for predicted miR-520a. Then the cDNAs encoding the entire 3'UTR of AKT1 mRNAs were amplified from total RNA of Raji cells using XhoI and NorI linker/primers, and then cloned into the vector pGL4 (the luciferase reporter vector) including the gene expressing firefly and Renilla luciferase. AKT1 3'UTRs were cloned in reverse orientation as controls lacking the miRNA target sequence. Additionally, the complementary region of miR-520a sequence in position 768-774 of human AKT1 3'UTR, AGCCACUU, was mixed up with UCCUGAA of the hsa-mir-520a-3p sequence. These constructs were all identified with COS-7 cells transfected with the reporter construct and the indicated miRIDIAN microRNA mimics or its negative control (NC) sequences by Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA). The mutant construct of the AKT1 3'UTR was created with Site-Directed Mutagenesis kit (Promega, Madison, WI, USA). The activity of Renilla luciferase was normalized with the corresponding control of Dual-Glo Luciferase Assay System. The analysis of luciferase activity of cells was detected with the CCK-8 assay kit for detection of cell viability and then total proteins were extracted with RIPA lysis buffer. Total proteins were analyzed with electrophoresis method using SDS-PAGE gel, and transferred to polyvinylidene fluoride (PVDF) 0.45 μm membrane. At room temperature, 5% skim-milk was used to incubate the membrane. The membranes were probed with primary antibodies: anti-GRP78, anti-GADD (1:500); anti-AKT1 (1:1,000 dilution) (both from Abcam, UK), anti-NF-κB, anti-p-PERK, anti-eIF2α (1: 3,000 dilution; Cell Signaling Technology, Inc., USA), or anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:3,000 dilution; Santa Cruz Biotechnology, Inc., USA), at 4°C, overnight. Then they were incubated at room temperature with the secondary antibodies (1:8,000 dilution; Cell Signaling Technology, Inc.).

Cell viability or proliferation assay, and determination of apoptosis. The CCK-8 assay kit for detection of cell viability was purchased from Dojindo Laboratories. The absorbance of viability was analyzed in pre-treated cells in a 96-well plate for 16 h. The multiwell plate reader was used to measure the absorbance value of incubated cells with CCK-8 solution for 1 h at 37°C. The assay for proliferation was analyzed with XTT in Raji cells. The microtiter plate reader was used to determine the 450 nm XTT absorbance value of pre-treated cells. The apoptotic cells were determined with Annexin V/7-AAD staining and FACS technique or caspase-3/7 activity based on the manufacturer's instructions.

Statistical analyses. Statistical differences between groups were analyzed with two-tailed paired Student t-test. Data of qRT-PCR and luciferase reporter assays were expressed relative to the control in each experiment, and 95% confidence intervals were calculated. The normally distributed continuous variables are shown as the means ± standard deviation (SD). Abnormally distributed data between groups were analysed using Kruskal-Wallis ANOVA. SPSS software (version 18.0) was used for the statistical analyses. A difference with P<0.05 was considered statistically significant.

Results

Identification of miRNA sequences in AKT1 mRNA. In this study, miR-520a was validated in Raji cells. The length of
pre-miR-520a is ~85 nt (hsa-mir-520a: MI 0003149, CUCA GGCUGUGACCCUCAGGGAAGUACUUUCUGUUCUGAGAGAAAAGAAAGUGCUUCCCUUUGGACUGUUUCGGUUUGAG). In an intergenic region of chromosome 19, miR-520a is transcribed, and generates a 22-nt mature sequence, and it shows a predicted secondary folding structure (Fig. 1A and B).

For identifying the target gene of miR-520a, it was predicted within the 3'UTR of the presumed miR-520a binding sites. A member of serine/threonine protein kinase subfamily, AKT1 was predicted as the potential target gene regulated by miR-520a. To investigate whether miR-520a regulates AKT1 in Raji cells, we first predicted the miRNAs target AKT1 using the online software at http://targetscan.org/ (Table I). Furthermore, to identify whether miR-520a directly combines with AKT1 3' UTR, the dual-luciferase reporter assay was performed. The results demonstrated that the relative luciferase activities of AKT1 3' UTR decreased to 60% in Raji cells treated with miR-520a compared to control (Fig. 2A) (P<0.01). The results of dual-luciferase reporter assay in Raji cells indicated that microRNA-520a could directly combine with the 3' UTR of AKT1. For determining the presumed binding site of AKT1, we created the mutant construct of AKT1 (mutant-AKT1) with Site-Directed Mutagenesis kit in its 3' UTR. The luciferase activity of the AKT1 was significantly regulated by miR-520a, but there was no luciferase activity produced from the mutant-AKT1 construct (Fig. 2A). It indicated that, by targeting the 3' UTR of AKT1, miR-520a could not regulate the expression of NF-κB by targeting its 3'UTR (Fig. 2B).

To evaluate if miR-520a regulates the expression of AKT1 in Raji cells, western blotting was used for quantitative analysis at protein level. As showed in Fig. 2C, the protein expression levels of AKT1 were downregulated by miR-520a.

miR-520a inhibits expression levels of AKT1 and NF-κB in Raji cells. The transcription factor NF-κB has an essential role in regulation of cell proliferation (11,12). It has been reported that NF-κB could be activated through exhibiting constitutive PI3K/AKT activity (13,14). The present study was performed to illustrate upstream mechanisms of AKT and NF-κB activation related to miRNA regulation. We further studied the specificity effects of miR-520a on PI3K/AKT and NF-κB signaling pathway, respectively.

We found that the NF-κB pathway was activated by over-expression of AKT1, and suppressed by si-AKT1 (Fig. 3A).

After transfected with mimics of miR-520a for 48 h, the results demonstrated that miR-520a suppressed the expression of AKT1 and NF-κB in Raji cells. On the contrary, transfected with the inhibitors of miR-520a, both the expression levels of AKT1 and NF-κB increased significantly (Fig. 3B). Moreover, the mimics or inhibitors of miR-520a and si-AKT1 were co-transfected into Raji cells, the effect of mimics or inhibitors of miR‑520a on the AKT1/NF-κB pathway could be diminished by si-AKT1 (Fig. 3B).

miR-520a represses proliferation and viability of Raji cells. Functional research on potential biological consequences induced by miR-520a were performed in Raji cells. The role of miR-520a on cell viability in Raji cells were analyzed with CCK-8 assay. Then Raji cells were transfected with mimics of miR-520a for 48 h, the absorbance value of Raji cells was significantly reduced compared with the control. Moreover, the inhibitors of miR-520a significantly enhanced the absorbance

Table I. Prediction of miRNA targets with TargetScan.

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Figure 1. The predicted secondary folding structure of hsa-miR-520a. In this study, microRNA-520a (miR-520a) was validated in Raji cells. The length of pre-miR-520a is ~85 nt. In an intergenic region of chromosome 19, miR-520a is transcribed, and generates a 22-nt mature sequence, and it shows a predicted secondary folding structure. Figures were obtained using the (A) miRBase and (B) TargetScan online software.
miR-520a INHIBITS PROLIFERATION OF Raji CELLS RELATED TO AKT1/NF-κB AND PERK/eIF2α

The assay for proliferation was analyzed with XTT in Raji cells. Following transfection with mimics of miR-520a for 48 h were compared with control, the relative proliferation rate of Raji cells significantly decreased. Conversely, the inhibitors of miR-520a significantly increased the relative proliferation rate of Raji cells (Fig. 4B).

miR-520a promotes apoptosis of Raji cells. Because miR-520a inhibits the expression of AKT1, and AKT1 control cell survival and cell apoptosis, and we investigated whether miR-520a shows angiopreventive properties through inducing apoptosis. Annexin V-FITC/7-AAD was used for the measurement, flow cytometric analysis and caspase activity assay was performed. Our results demonstrated that miR-520a accentuated apoptosis of Raji cells (both early and late phase, P<0.01) compared to control. Conversely, miR-520a inhibitors suppressed apoptosis of Raji cells (Fig. 5A). Moreover, miR-520a in Raji cells also significantly increased the increase of caspase-3/7 activities. On the contrary, miR-520a inhibitors suppressed these activities (Fig. 5B). These results suggested that a caspase-dependent apoptotic mechanism is involved in miR-520a-induced cell death.

miR-520a associates with ER stress through PERK/eIF2α pathway in Raji cells. The expression of GRP78, GADD, p-PERK and eIF2α protein was determined by western blotting. In Raji cells, miR-520a significantly inhibited the expression of GRP78, GADD, p-PERK, and eIF2α. Furthermore, Raji cells treated with the inhibitors of miR-520a increased expression of GRP78, GADD, p-PERK, and eIF2α (Fig. 6).
Discussion

The great number of studies on these recently discovered molecules, miRNAs, indicated its vital value for prognostic, diagnostic and therapeutic diseases based on different styles of experimental models. It demonstrated that functions of these molecules had not only turned into the new instrument for clinic use, but also explored the novel mechanism of gene expression regulation (8). miRNAs-associated carcinogenesis was associated with many human cancers, and inhibition of carcinogenesis is vital for treatment of cancers. In the initiation of carcinogenesis, the basic cellular incident is related to activation of signaling pathway involved in cancer (15). The mechanism for transcriptional control of oncogene genes expression has been explored widely.

BL is a fastest growing cancer of the human lymphatic system, and one of the greatly proliferative and extremely invasive lymphomas despite its low morbidity in the world. The PI3K pathway in BL had been identified that was associated to its development (16). Chiu et al had found that miR-520a was associated with endoplasmic reticulum (ER) stress through PERK/eIF2α pathway in Raji cells. The expression of GRP78, GADD, p-PERK and eIF2α protein was determined by western blotting. In Raji cells, miR-520a significantly inhibited the expression of GRP78, GADD, p-PERK and eIF2α. Raji cells treated with the inhibitors of miR-520a obviously increased expression of GRP78, GADD, p-PERK and eIF2α.

Figure 4. MicroRNA-520a (miR-520a) represses proliferation and viability of Raji cells. Functional research on potential biological consequences induced by miR-520a were performed in Raji cells. The CCK-8 assay was used to analyze the effect of miR-520a on cell viability of Raji cells. Raji cells were transfected with mimics of miR-520a for 48 h, the absorbance value of Raji cells significantly reduced compared with the control. (A) Moreover, the inhibitors of miR-520a significantly enhanced the absorbance value of Raji cells. Besides, the assay for proliferation was analyzed with XTT in Raji cells. Transfection with mimics of miR-520a for 48 h compared with control, the relative proliferation rate of Raji cells significantly decreased. (B) Conversely, the inhibitors of miR-520a significantly increased the relative proliferation rate of Raji cells. The data are presented as means ± standard deviation (SD) from three independent experiments. *P<0.01; P-value was generated using Kruskal-Wallis ANOVA.

Figure 5. MicroRNA-520a (miR-520a) promotes apoptosis of Raji cells. miR-520a inhibits the expression of AKT1, and AKT1 control cell survival and cell apoptosis. miR-520a shows angiopreventive properties through inducing apoptosis. Annexin V-FITC/7-AAD was used for the measurement, flow cytometric analysis and caspase activity assay were performed. Our results demonstrated that miR-520a accentuated apoptosis of Raji cells (both early and late phase, P<0.01) compared to control. (A) Conversely, miR-520a inhibitors suppressed apoptosis of Raji cells. Moreover, miR-520a in Raji cells also significantly induced the increase of caspase-3/7 activities. (B) On the contrary, miR-520a inhibitors suppressed these activities. These results suggested that a caspase-dependent apoptotic mechanism is involved in miR-520a-induced cell death. The data are presented as means ± standard deviation (SD) from three independent experiments. *P<0.01; P-value was generated using Kruskal-Wallis ANOVA.

Figure 6. MicroRNA-520a (miR-520a) associated with endoplasmic reticulum (ER) stress through PERK/eIF2α pathway in Raji cells. The expression of GRP78, GADD, p-PERK and eIF2α protein was determined by western blotting. In Raji cells, miR-520a significantly inhibited the expression of GRP78, GADD, p-PERK and eIF2α. Raji cells treated with the inhibitors of miR-520a obviously increased expression of GRP78, GADD, p-PERK and eIF2α.
suppressing cellular invasion in cancer cells could be induced by the inhibition of AKT/NF-κB pathway using HLE (17). In the present study, we ascertained the effects of miR-520a, and investigated the carcinogenesis process of BL. A number of factors such as miRNAs have crucial effect on gene expression regulation, which is involved in carcinogenesis, but the miRNA-mediated regulation of AKT/NF-κB pathway during carcinogenesis in BL is poorly understood. As shown in the current study, our bioinformatics analysis using online software (http://targetscan.org/) confirmed that 3′UTR site of AKT1 mRNA is complimentary to miR-520a. To further identify whether miR-520a directly combine with 3′UTRs of AKT1, the vector embracing the 3′UTR of the AKT1 gene was constructed, the sequences were from 768 to 774 nt. The results of luciferase reporter analysis indicated that the relative activity of AKT1 3′UTR significantly decreased in Raji cells transfected with miR-520a. It confirmed that the expression of AKT1 was modulated by miR-520a (Fig. 2A) (P<0.01). The miR-520a did not affect the mRNA expression levels of AKT1, but the results of western blotting verified that AKT1 expression in protein levels downregulated of miR-520a compared to control (Fig. 2B). It confirmed that miR-520a could directly bind with the 3′UTR of the AKT1 gene and negatively regulated its expression in Raji cells. Therefore, AKT1 mRNA is a direct target of miR-520a and this provides new therapy target for treatment of BL.

NF-κB is a key transcription factor and widely related to neoplasia by regulating the balance between cell apoptosis and proliferation. In addition, NF-κB signaling pathway was related to angiogenesis and metastasis in carcinoma (18,19). AKT acts as the basic point of signaling pathway, and controls the expression or activity of its downstream factors (20). AKT regulates cell functions, such as growth, proliferation, survival and apoptosis through taking advantage of its kinase activity. The key point of its function is the inhibition of cell apoptosis, especially in pro-oncogenic process (20), as well as the crucial anti-apoptotic roles of NF-κB (18). It is known that the activation of NF-κB could promote cell survival based on the functions of AKT. Dan et al found that AKT could facilitate the NF-κB activation dependent on IKK, and be associated with Raptor and mTOR by regulating anti-apoptotic gene expression (21). We found that the NF-κB pathway was activated by overexpression of AKT1 in Raji cells, and suppressed by si-AKT1 (Fig. 3A). The mimics of miR-520a suppressed the expression of AKT1 and NF-κB. On the contrary, the inhibitors of miR-520a upregulated the expression levels of AKT1 and NF-κB increased significantly. Moreover, the effect of mimics or inhibitors of miR-520a on the AKT1/NF-κB pathway could be diminished by si-AKT1. It indicated that miR-520a is a crucial mediator of AKT1/NF-κB signaling pathway in Raji.

miR-520a-3p inhibits proliferation, apoptosis and metastasis in NSCLC by targeting MAP3K2, and miR-520a-3p may be used as a prognosis marker for NSCLC in clinical research (22). Mazan-Mamczarz et al demonstrated that overexpression of miR-520c-3p suppresses cell proliferation, overall gene expression, and initiates premature senescence progress of DLBCL cells. They found that overexpression of miR-520c-3p suppressed the growth of human xenograft tumors in a mouse model (23). In the present study, functional research on potential biological consequences induced by miR-520a were performed in Raji cells. Raji cells were transfected with mimics of miR-520a for 48 h, the viability and proliferation of Raji cells significantly reduced compared with control. Moreover, the inhibitors of miR-520a significantly enhanced viability and proliferation of Raji cells. Besides, the angiopreventive effect of miR-520a through inducing apoptosis was investigated. Our results demonstrated that miR-520a accentuated apoptosis of Raji cells compared to control. Conversely, miR-520a inhibitors suppressed TNF-induced apoptosis of Raji cells (Fig. 5A). Moreover, miR-520a in Raji cells also significantly induced the increase of caspase-3/7 activities. On the contrary, miR-520a inhibitors suppressed these activities (Fig. 5B). These results suggested that a caspase-dependent apoptotic mechanism is present, and involved in miR-520a-induced cell death. It demonstrated that miR-520a is an important mediator for proliferation in Raji cells by regulating the AKT1/NF-κB signaling pathway, and exerts effects to control Raji cell viability and accentuate caspase-dependent apoptosis.

ER stress is involved in pathogenic mechanism and pathogenesis of many disease processes. It was verified that in primary glial cells, ER stress dually regulated the activation of AKT. ER stress promoted the activation of AKT with short-term exposure, but long-term exposure induced inhibition of AKT activation. In stress conditions, AKT serves a vital role and hurts the function of ER (15,24). A series of exogenous and endogenous factors caused disorder of folding capacity in ER, and then induced ER stress. Initially, the purpose of ER stress was to rebuild the homeostasis of ER; the process is dependent on activation of unfolded protein response (UPR) signaling pathway (25). Moreover, the prolonged and severe ER stress induced function shifts of UPR in pro-survival, predominantly manifested toxic signal and executed mitochondrial apoptosis (26). The signaling pathways of ER stress are involved in certain anticancer modalities, the mechanism was related to enhancing immunogenicity on dying cells (27). According to the vital role of ER stress on potential therapeutics, it is beneficial to target its signaling pathway in anticancer therapy (15,25-28).

Our study determined the expression of GRP78, GADD, p-PERK and eIF2α protein. In Raji cells, miR-520a significantly inhibited the expression of GRP78, GADD, p-PERK and eIF2α. Furthermore, Raji cells treated with the inhibitors of miR-520a increased expression of GRP78, GADD, p-PERK and eIF2α. These results identified that miR-520a was associated with ER stress through PERK/eIF2α pathway in Raji cells.

In summary, we illustrated miR-520a, which specifically bind to AKT1 mRNA 3′UTR in Raji cells. miR-520a is a vital mediator of for proliferation and ER stress of Raji cells by regulating the AKT1/NF-κB or PERK/eIF2α signaling pathway, and exerts effects to control Raji cell viability and accentuate caspase-dependent apoptosis. Our findings suggest that targeting miR-520a, being involved in ER stress, is a promising strategy for the prevention and treatment of cancer, including BL.
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References


