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LINK-A lncRNA Promotes Proliferation and Inhibits Apoptosis of Mantle Cell Lymphoma Cell by Upregulating Survivin

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: LINK-A lncRNA acts as an oncogene in triple-negative breast cancer, but its involvement in other diseases is unknown. The present study was performed to investigate the involvement of LINK-A lncRNA in mantle cell lymphoma.

Material/Methods: Expressions of LINK-A lncRNA and survivin in plasma of patients with mantle cell lymphoma and healthy controls were detected by qRT-PCR and ELISA, respectively. ROC curve analysis was performed to investigate the diagnostic value of LINK-A lncRNA for mantle cell lymphoma. Correlations between plasma level of LINK-A lncRNA and survivin were analyzed by Pearson correlation coefficient. LINK-A lncRNA shRNA and expression vector were transfected into cells of human mantle cell lymphoma cell lines, followed by detection of cell proliferation, cell apoptosis, and survivin expression by cell proliferation assay, cell apoptosis assay, and Western blot analysis, respectively.

Results: We found that, compared with healthy controls, plasma levels of LINK-A lncRNA and survivin were significantly increased in patients with mantle cell lymphoma. Upregulation of LINK-A lncRNA sensitively distinguished patients with mantle cell lymphoma from healthy controls. Plasma levels of LINK-A lncRNA and survivin were positively correlated in mantle cell lymphoma patients but not in healthy controls.

Conclusions: LINK-A lncRNA overexpression promoted cell proliferation, inhibited cell apoptosis, and upregulated survivin expression, while LINK-A lncRNA knockdown had the opposite effect.

MeSH Keywords: Apoptosis • Cell Proliferation • Lymphoma, Mantle-Cell • RNA, Long Noncoding

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Background

Mantle cell lymphoma is a well-studied lymphoid malignant disease considered to be an aggressive subtype of non-Hodgkin's lymphoma, and it accounts for 6% of all cases of this disease [1]. The annual incidence rate of mantle cell lymphoma is around 1 per 200 000 people [2]. In spite of the low incidence rate, mantle cell lymphoma is considered to be a major cause of cancer-related death due to the high recurrence rate after initial treatment [3]. Moreover, development of drugs for long-term application of chemotherapy is also a serious challenge in the treatment of mantle cell lymphoma [4]. Therefore, identification of novel therapeutic targets is indispensable to improve the survival of patients with mantle cell lymphoma.

Survivin, also known as baculoviral inhibitor of apoptosis repeat-containing 5, is a member of the inhibitor of apoptosis (IAP) family [5]. Increased expression levels of survivin are frequently observed during the development of different types of cancers [6]. Overexpression of survivin not only increases the risk of cancer but also leads to failure in cancer treatment [7]. Inhibition of survivin is considered to be a promising target for cancer treatment [8]. The crosstalk between survivin and lncRNAs has been widely reported [9]. LINK-A is a recently identified lncRNA with an oncogenic role in triple-negative breast cancer [10]. In the present study we found that LINK-A also participates in mantle cell lymphoma through interactions with survivin.

Material and Methods

Patients and plasma

A total of 108 patients with mantle cell lymphoma were diagnosed and treated in Sir Run Run Shaw Hospital from March 2015 to March 2018. Among those patients, our study included 36 to serve as research subjects according to inclusion and exclusion criteria. Inclusion criteria were: 1) patients diagnosed with mantle cell lymphoma through pathological examinations, 2) patients diagnosed and treated for the first time, and 3) patients who could fully understand the experimental procedure and were willing to participate. Exclusion criteria were: 1) patients complicated with other malignancies, 2) patients who were treated before admission, and 3) patients with education level below high school. Those patients included 20 males and 16 females, and age ranged from 28 to 69 years, with a mean age of 50.2±7.1 years. We also included 32 healthy volunteers who received physiological examinations during the same period to serve as a control group. The control group included 18 males and 14 females, and age ranged from 27 to 66 years, with a mean age of 49.1±6.3 years. No significant differences in age or sex were found between the 2 groups. A plasma sample from each participant was obtained from the specimen library of Sir Run Run Shaw Hospital. This study was approved by the Ethics Committee of Sir Run Run Shaw Hospital. All participants were informed about the experimental protocol and signed the informed consent form.

Real-time quantitative reverse transcription PCR (qRT-PCR)

All total RNA extractions were performed using Trizol reagent (Invitrogen, USA) with all steps being performed in strict accordance with the manufacturer's instructions. The SuperScript III Reverse Transcriptase kit (Thermo Fisher Scientific) was used in reverse transcription to synthesize cDNA, followed by PCR reaction using SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, USA). Thermal conditions for PCR reactions were: 1 min at 95°C, followed by 40 cycles of 25 s at 95°C and 30 s at 59°C. Primers used in PCR reactions were: 5'-TTCCCCCATTTTTCCTTTTC-3' (forward) and 5'-CTCTGGTTGGGTGACTGGTT-3' (reverse) for human LINK-A lncRNA; 5'-GACCTCTATGCCAACACAGT-3' (forward) and 5'-AGTACTTGCGCTCAGGAGGA-3' (reverse) for β-actin. All qRT-PCR data were processed using $2^{-\Delta\Delta CT}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Plasma levels of survivin were measured using a human survivin quantikine ELISA kit (DSV00, R&D Systems). All operations were performed in strict accordance with the manufacturer's instructions.

Cell lines, cell culture, and transfection

Human mantle cell lymphoma cell lines JVM-2 (ATCC® CRL-3002™) and Z-138 (ATCC® CRL-3001™) were purchased from ATCC. Cells were cultured with ATCC-formulated RPMI-1640 Medium (ATCC 30-2001) containing 10% fetal bovine serum (FBS) (ATCC 30-2020) at 37°C in a 5% CO_2 incubator. pIRSE2 vector (Clontech, Palo Alto, CA, USA) was used to construct LINK-A lncRNA overexpression and shRNA vectors. The LINK-A shRNA sequence was: TTCCCCCATTTTTCCTTTTC. Cell transfection was performed using Lipofectamine 2000 reagent with vector concentration of 10 nM. Untransfected cells were used as control cells and cells transfected with empty vectors were used as negative control cells. LINK-A expression as detected by qRT-PCR at 12 h after transfection. Subsequent experiments were only performed if overexpression rate above 200% (compared with control cells) and knockdown rate below 50% was reached.

In vitro cell proliferation assay

After transfection, CCK-8 assay was performed to detect cell proliferation. Cells were collected and cell suspensions with a cell density of 6×10^4 cells per ml were prepared. Each well of

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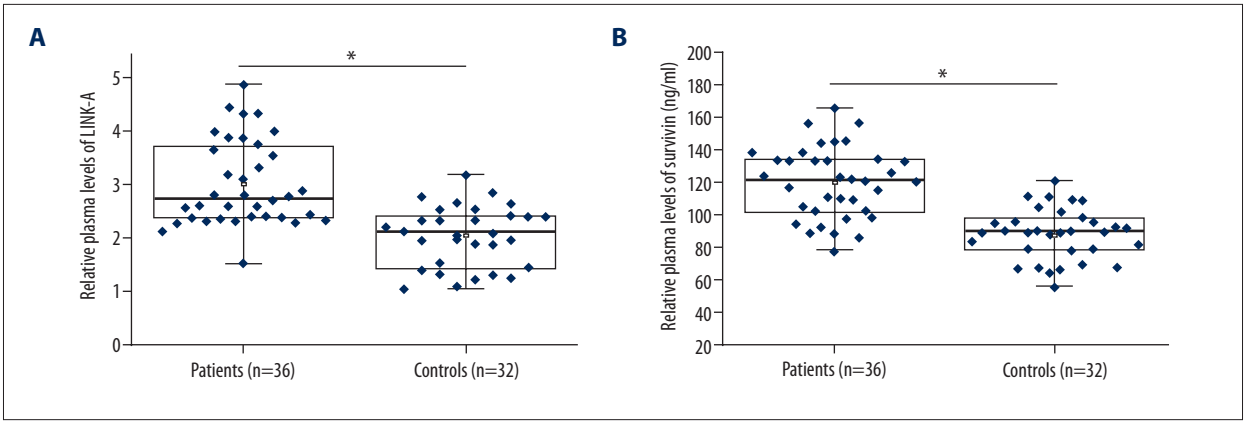

Figure 1. Plasma LINK-A lncRNA and survivin levels were significantly higher in mantle cell lymphoma patients than in healthy controls. Compared with controls, significantly higher plasma levels of LINK-A lncRNA (**A**) and survivin (**B**) were found in mantle cell lymphoma patients (* p<0.05).

a 96-well plate was filled with a 0.1-ml cell suspension containing 6×10^3 cells and cells were cultured in an incubator (37°C, 5% CO_2). CCK-8 solution (10 ul, Sigma-Aldrich) was added 24, 48, 72, and 96 h later. After that, cells were cultured for an additional 4 h and OD values at 450 nM were measured using a Fisherbrand™ accuSkan™ GO UV/Vis Microplate Spectrophotometer (Fisher Scientific).

Cell apoptosis assay

After transfection, cell apoptosis assay was performed to detect cell apoptosis. Cells were mixed with serum-free medium to prepare cell suspensions at a cell density of 6×10^4 cells per ml. We added 10 ml of cell suspension into each well of a 6-well plate. Cells were cultured for 48 h, followed by digestion with 0.25% trypsin. Cells were then harvested and dissolved in DMEM medium. After centrifugation at 1000 g for 5 min, cells were stained with Annexin V-FITC (Dojindo, Japan) and propidium iodide (PI), followed by flow cytometry to detect apoptotic cells.

Western blot analysis

Total protein was extracted from *in vitro* cultured cells using RIPA solution (Thermo Fisher Scientific, USA). Protein concentrations were measured by BCA assay. After denaturing, protein samples were subjected to 10% SDS-PAGE gel electrophoresis with 30 μg protein per lane. Gel transfer was then performed, followed by blocking PVDF membranes in 5% skimmed milk at room temperature for 1 h. After that, membranes were incubated with rabbit anti-human primary antibodies of survivin (1: 1200, ab76424, Abcam) and GAPDH (1: 2000, ab8245, Abcam) at 4°C overnight. Then, membranes were further incubated with goat anti-rabbit IgG-HRP secondary antibody (1: 1000, MBS435036, MyBioSource) at 25°C for 2 h. ECL (Sigma-Aldrich, USA) was then used to develop signaling. Signals were processed and normalized using Image J software.

Statistical analysis

GraphPad prism 6 was used for all data analyses. Data are expressed as mean ± standard deviation. Comparisons between 2 groups were performed by *t* test and comparisons among multiple groups were performed by one-way analysis of variance followed by LSD test. Correlation analyses were performed by Pearson correlation coefficient. p<0.05 represented a statistically significant difference.

Results

Plasma LINK-A lncRNA and survivin levels were significantly higher in mantle cell lymphoma patients than in healthy controls

Plasma levels of LINK-A lncRNA and survivin in mantle cell lymphoma patients and healthy controls were measured by qRT-PCR and ELISA, respectively. As shown in Figure 1, plasma levels of LINK-A lncRNA (Figure 1A) and survivin (Figure 1B) were significantly higher in mantle cell lymphoma patients than in healthy controls (p<0.05).

Upregulation of plasma LINK-A lncRNA distinguished mantle cell lymphoma patients from healthy controls

ROC curve analysis was performed to evaluate the diagnostic value of plasma LINK-A lncRNA for mantle cell lymphoma. As shown in Figure 2, the area under the curve (AUC) was 0.8338. The standard error was 0.04800 and the 95% confidence interval was 0.7397–0.9279.

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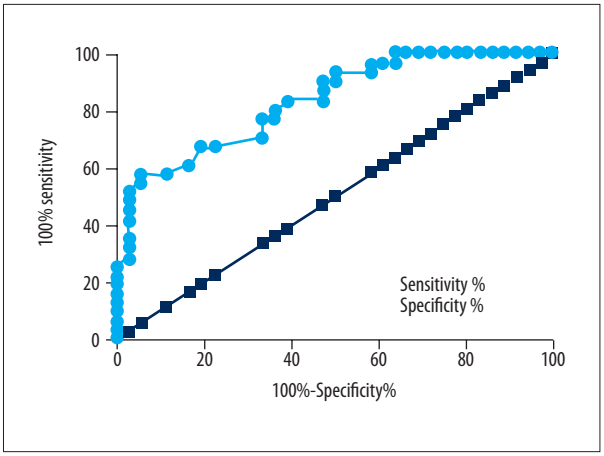

Figure 2. ROC curve analysis of the diagnostic value of plasma LINK-A lncRNA for mantle cell lymphoma.

Plasma LINK-A lncRNA and survivin were positively correlated in mantle cell lymphoma patients

Correlations between plasma LINK-A lncRNA and survivin were analyzed by Pearson correlation coefficient. As shown in Figure 3, a significant positive correlation between plasma LINK-A lncRNA and survivin was found in mantle cell lymphoma patients (Figure 3A) but not in healthy controls (Figure 3B).

Effects of LINK-A lncRNA overexpression and knockdown on cell proliferation and apoptosis

After transfection, cell proliferation and apoptosis were detected by CCK-8 assay and cell apoptosis assay, respectively. Compared with control cells (C) and negative control cells (NC), LINK-A lncRNA overexpression significantly promoted cell proliferation and LINK-A lncRNA knockdown significantly inhibited cell proliferation (Figure 4A, $p<0.05$). In contrast, LINK-A lncRNA knockdown had the opposite effect (Figure 4B, $p<0.05$).

Effects of LINK-A lncRNA overexpression and knockdown on survivin expression

After transfection, survivin expression was detected by Western blot analysis. As shown in Figure 5, compared with control cells (C) and negative control cells (NC), LINK-A lncRNA overexpression significantly promoted (Figure 5A, $p<0.05$) survivin expression, while LINK-A lncRNA knockdown significantly inhibited survivin expression in cells of JVM-2 and Z-138 cell lines (Figure 5A, $p<0.05$).

Discussion

The key finding of the present study is that LINK-A lncRNA, which is a characterized lncRNA in triple-negative breast cancer, also participates in mantle cell lymphoma. We provided experimental evidence that LINK-A lncRNA plays an oncogenic role in mantle cell lymphoma through interactions with survivin and its involvement in the regulation of cancer cell proliferation and apoptosis.

lncRNAs are key regulators in physiological processes and pathological changes [11]. It has been reported that the development of mantle cell lymphoma is also accompanied by changes in a large set of lncRNAs [12], indicating the involvement of lncRNAs in this disease. Those lncRNAs show upregulated or downregulated expression patterns to promote rather than inhibit cancer development. In a recent study, Wang et al. reported that lncRNA MALAT1 was significantly upregulated in patients with mantle cell lymphoma compared with healthy controls [13], indicating its role as an oncogenic lncRNA in this disease. LINK-A lncRNA, as a characterized oncogenic lncRNA in triple-negative lncRNA, is overexpressed in patients with this disease. In the present study, we observed that plasma levels of LINK-A lncRNA were significantly higher in patients

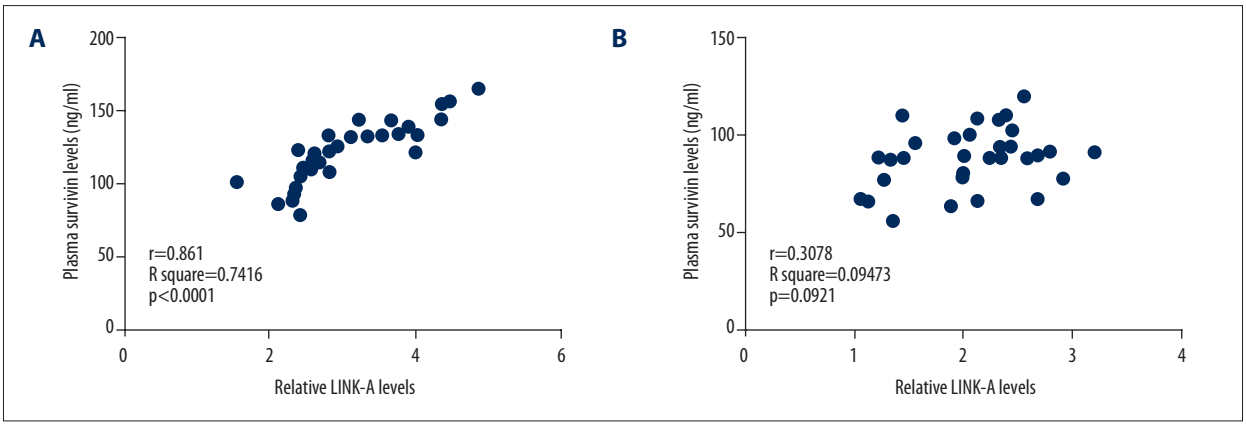

Figure 3. Plasma LINK-A lncRNA and survivin were positively correlated in mantle cell lymphoma patients. Pearson correlation analysis revealed a significant positive correlation between plasma LINK-A lncRNA and survivin in mantle cell lymphoma patients (**A**) but not in healthy controls (**B**).

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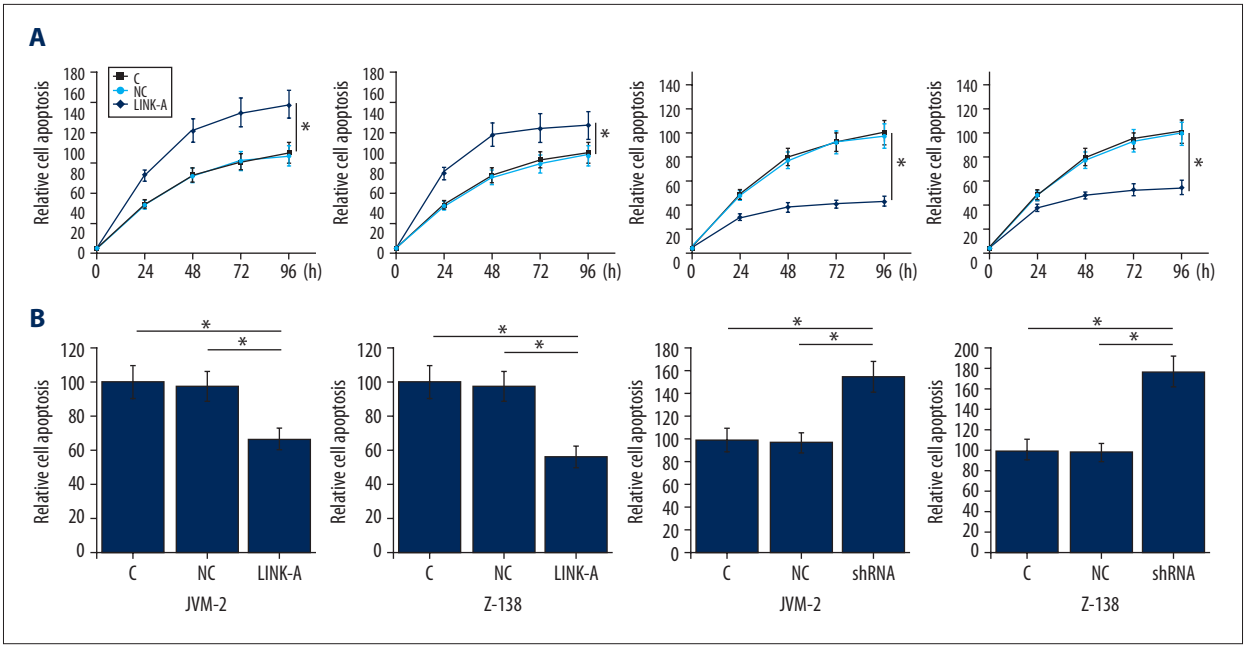

Figure 4. (**A, B**) Effects of LINK-A lncRNA overexpression and knockdown on cell proliferation and apoptosis. Data here show the effects of LINK-A lncRNA overexpression and knockdown on cell proliferation and apoptosis of cells of mantle cell lymphoma cell lines JVM-2 and Z-138 (* p<0.05).

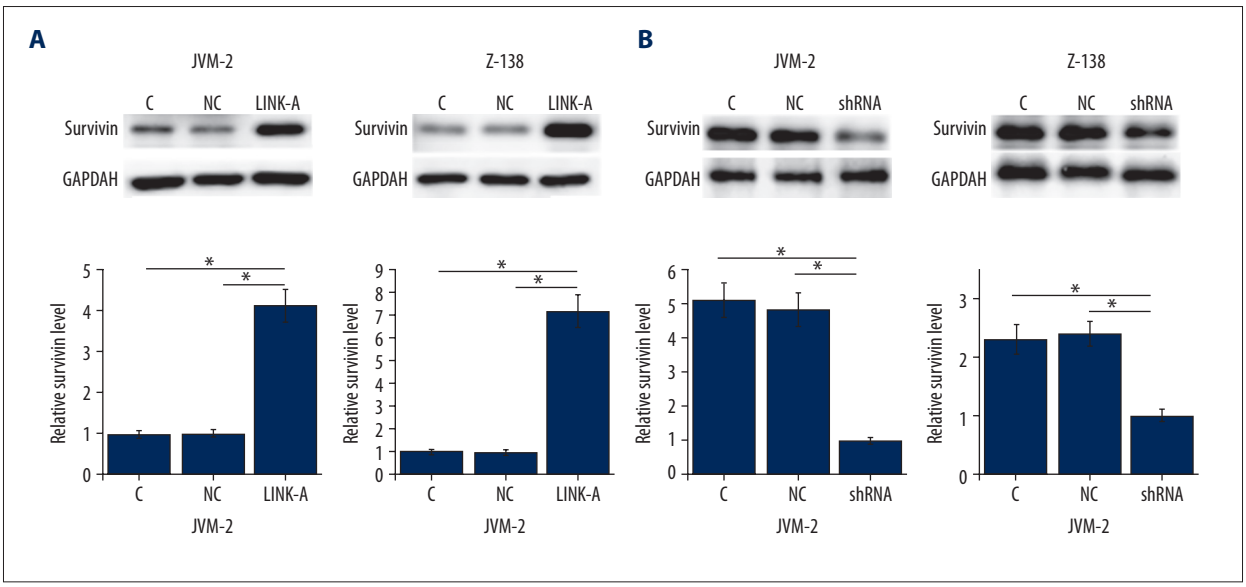

Figure 5. Effects of LINK-A lncRNA overexpression and knockdown on survivin expression. Data here show the effects of LINK-A lncRNA overexpression (**A**) and knockdown (**B**) on survivin expression in cells of JVM-2 and Z-138 cell lines (* p<0.05).

with mantle cell lymphoma than in healthy controls. In effect, upregulation of LINK-A lncRNA effectively distinguished mantle cell lymphoma patients from healthy controls. Those data indicate the role of LINK-A lncRNA as an oncogenic gene in mantle cell lymphoma and its potential application in the diagnosis of mantle cell lymphoma.

Survivin inhibits cancer cell apoptosis. Upregulation of survivin is observed at the very early stage of tumorigenesis and is continued during the entire process of tumor development [14,15]. Inhibition of survivin through multiple pathways has been proved to have potential in treatment of different types of cancers [16] and the inhibition of drug resistance [17]. Overexpression of survivin has also been observed in patients with mantle cell lymphoma [18]. Consistent with previous

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studies, our study also observed significantly upregulated plasma levels of survivin in patients with mantle cell lymphoma compared with healthy controls. A recent study reported that the lncRNA LINK-A interacts with Ptdlns(3,4,5)P_3 to hyperactivate AKT and confer resistance to AKT inhibitors [19]. Wu et al. reported that lncRNA LINK-A promotes glioma cell growth and invasion via lactate dehydrogenase A [20]. LINK-A lncRNA is reported to promote migration and invasion of ovarian carcinoma cells by activating the TGF-beta pathway [21]. However, there are no previous reports on LINK-A lncRNA and its possible relationships with mantle cell lymphoma. Interestingly, our study observed a close correlation between plasma levels of LINK-A and survivin in patients with mantle cell lymphoma. In addition, *in vitro* cell experiments also proved that LINK-A is a positive regulator of survivin in mantle cell lymphoma cells, and LINK-A overexpression promotes cancer cell proliferation but inhibits cancer cell apoptosis. However, due to the lack of significant correlations between plasma levels of LINK-A and survivin in healthy controls, the regulatory effect of LINK-A on survivin is unlikely to be direct.

Our study is not without limitations. To date, no studies have defined the mechanism by which survivin affects mantle cell lymphoma. More research is needed to determine if LINK-A lncRNA promotes survival of mantle cell lymphoma in light of the remarkable results of other relevant tumor entities [20,21]. In this study, we did not validate if the is such a potential causal relationship between LINK-A lncRNA and mantle cell lymphoma. Future studies will focus on identifying the disease-related mediators between LINK-A and survivin in mantle cell lymphoma. Further experiments and analyses are needed to elucidate the interaction between LINK-A lncRNA and AKT, which is probably involved upstream of survivin in mantle cell lymphoma. Further work is also needed to investigate the mode of action involved in the relationship between survivin and mantle cell lymphoma.

Conclusions

LINK-A is upregulated and may act as an oncogene in mantle cell lymphoma. The action of LINK-A in mantle cell lymphoma is at least partially mediated by the upregulation of survivin. LINK-A lncRNA overexpression promotes cell proliferation, inhibits cell apoptosis, and upregulates survivin expression, while LINK-A lncRNA knockdown has the opposite effect. Our future work will focus on investigations of the action mode that relates to survivin in patients with mantle cell lymphoma as well as potential causal relationships between targeted lncRNAs and mantle cell lymphoma.

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