JBUON 2019; 24(2): 763-769
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Long non-coding RNA H19 promotes proliferation of Hodgkin's lymphoma via AKT pathway

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Summary

Purpose: *To explore whether lncRNA (Long non-coding RNA) H19 could promote the development of Hodgkin's lymphoma (HL) by regulating cell proliferation via AKT pathway.*

Methods: *H19 expressions in 60 HL tissues, 40 RH (reactive hyperplasia of lymph nodes) tissues, L428, A20 and Ly1 cell lines were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). H19 siRNA and pcDNA-H19 were constructed. Cell viability after altering H19 expression was detected by EdU and cell counting kit-8 (CCK-8) assay. The mRNA level of AKT in HL tissues and RH tissues was detected by qRT-PCR. The relationship between AKT and H19 was further detected by Western blot.*

Results: *H19 was overexpressed in HL tissues and cell lines compared with those of controls. HL patients with huge lump and in Ann Arbor stage III-IV presented higher expression of H19. Besides, H19 expression was negatively correlated to overall survival (OS) of HL patients. In vitro experiments suggested that H19 downregulation decreased proliferation and viability of HL cells. AKT expression was upregulated in HL tissues compared with RH tissues, and was positively regulated by H19. Western blot results also indicated that H19 overexpression upregulated protein expression of AKT in HL cells.*

Conclusions: *Overexpressed lncRNA H19 promotes HL development by stimulating proliferation of HL cells via AKT pathway.*

Key words: *AKT pathway, cell proliferation, HL, LncRNA H19*

Introduction

Lymphomas are malignant tumors that originate from the lymphoid hematopoietic system. Based on the pathological features, lymphomas are classified into Hodgkin's lymphoma (HL) and non-Hodgkin's lymphomas (NHLs). The incidence of most malignant tumors gradually increase with age. However, the incidence of HL presents a bimodal curve, manifesting as younger people (15-35 years old) and older people (older than 55 years old) as frequently affected population [1,2]. In recent years, the incidence of HL has remarkably increased in China [3]. HL could be divided into nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) and classical Hodgkin's lymphoma (CHL) according to the phenotypes of infiltrating cells. CHL accounts for about 95% of HL cases [4,5]. CHL is a special tumor belonging to B cell-derived lymphomas in or beneath the germinal center with clonal immunoglobulin gene recombination [6]. Unlike the majority of B cell lymphomas, Hodgkin-Reed-Sternberg (HRS) cells - the malignant tumor cells of CHL - only account for about 1% of tumor masses. Mesenchymal cells and infiltrating cells are the majority components of the

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Received: 13/07/2018; Accepted: 02/08/2018

tumor masses [7]. Most HL patients achieve a good long-term prognosis by current chemotherapy and radiotherapy. We still need to further explore the potential mechanism of HL, so as to find new targets for better predicting and treating HL patients.

AKT, also known as protein kinase B (PKB), is a serine/threonine protein kinase. It is a downstream molecule of phosphatidylinositol 3-kinase (PI3K) [8]. AKT exerts an essential role in cell proliferation, migration, and inhibition of apoptosis [9,10]. Apoptosis is a normal cellular function that controls excessive proliferation. Cancer cells possess multiple anti-apoptosis mechanisms that can be regulated by AKT pathway [11]. Continuous activation of AKT can prevent PTEN-induced apoptosis [12]. Functionally, AKT exerts its anti-apoptotic effect by phosphorylating the downstream target protein Bcl-2 and Caspase-9 [13]. Studies have shown that abnormal activation of AKT pathway can promote cell invasion of breast cancer [14]. HER-2/neu promotes the survival and growth of prostate cancer cells via AKT pathway [15]. Increased survival of HRS cells regulated by AKT pathway could be inhibited via targeting OLT39 [16]. Activation of AKT stimulates aerobic glycolysis of cancer cells, thus providing energy for the continuous growth and survival of cancer cells [17].

Long non-coding RNA (lncRNA) is a type of RNA with a transcription length of over 200 nucleotides presented in the nucleus or cytoplasm. LncRNA can regulate gene expression at multiple levels. Relative studies have shown that lncRNA could regulate tumor development [18]. LncRNA H19 was the first tumor-associated lncRNA found on the 11p15.5 region of human chromosomes. H19 is overexpressed in embryonic development, mainly in mesoderm and endoderm derived tissues [19]. It is reported that H19 is upregulated in colon cancer, liver cancer, bladder cancer, breast cancer, gastric cancer, prostate cancer and ovarian cancer [20-24]. H19 is capable of promoting tumor formation and metastasis. Researchers have found that H19 expression is positively correlated to TNM stage of gastric cancer, which can be served as an independent predictor of OS in gastric cancer patients [25]. Upregulation of H19 can promote IDS-induced proliferation of bladder cancer cells [26]. H19 promotes cell proliferation by inhibiting β-catenin expression through competitive binding to microRNA-200a in colorectal cancer [27]. Meanwhile, studies have shown that H19 is closely related to AKT pathway in cancer cells. For example, H19 can promote the proliferation of gallbladder cancer cells through regulation of miR-194-5p

Table 1. Relationship between lncRNA H19 expression and clinical data of HL patients

Clinicopathologic features	*Number of cases*	*LncRNA H19 expression*		*p value*
		Low (n=30)	*High (n=30)*	
Age (years)				0.4383
<45	31	14	17	
≥45	29	16	13	
Gender				0.4257
Male	23	13	10	
Female	37	17	20	
Huge lump				
Yes	38	15	23	0.0321
No	22	15	7	
ESR				0.1965
Normal	29	17	12	
Rise	31	13	18	
Ann Arbor stage				0.0045
I-II	29	21	10	
III-IV	31	9	20	
LDH				0.5921
Normal	38	20	18	
Rise	22	10	12	
B Symptoms				0.5839
Yes	40	21	19	
No	20	9	11	

via targeting AKT2 [28]. Overexpression of H19 promotes trophoblast invasion and autophagy via activating PI3K/AKT/mTOR pathway [29]. Downregulation of H19 inhibits melanoma migration and invasion through inhibiting NF-κb and PI3K/AKT pathways [30].

Taken together, H19 is closely related to the occurrence and progression of tumors via AKT pathway. However, studies on the relationship between H19 and HL prognosis are rarely reported. In this study we aimed to explore the effect of H19 on regulating the proliferative ability of HL cells.

Methods

General data

60 HL tissues and 40 reactive hyperplasia of lymph nodes (RH) tissues were surgically resected in the Affiliated Yantai Yuhuangding Hospital of Qingdao University from July 2014 to September 2017. Tissue samples were immediately preserved in liquid nitrogen. This study was approved by the Affiliated Yantai Yuhuangding Hospital of Qingdao University Ethics Committee and all subjects signed informed consent. Clinical data of the enrolled HL patients are listed in Table 1.

Cell culture

Classical Hodgkin's lymphoma (CHL), diffuse large B-cell lymphoma cell line Ly1, B-cell derived cell line L428 and mouse B-cell derived cell line A20 were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in RPMI-1640 (Roswell Park Memorial Institute-1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and maintained in a 5% CO_2 incubator at 37°C.

Cell transfection

H19 complementary deoxyribose nucleic acid (cDNA) was constructed at the polyclonal sites of pcDNA3.1 vector. Ly428 and Ly1 cells were seeded in 24-well plates at a density of 2×10^5/ml. H19 downregulation and overexpression were achieved by transfection of H19 siRNA and pcDNA-H19, respectively, according to the instructions of Lipofectamine 2000 manufacturer (Invitrogen, Carlsbad, CA, USA).

RNA extraction and qRT-PCR (quantitative real-time polymerase chain reaction)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan). QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Tokyo, Japan), with 3 replicates in each group. Primers used in the study were as follows: GAPDH, forward: 5'-CGGAGTCAACGGATTTGGTCGTAT-3', reverse: 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; H19, forward: 5'-TTCAAAGCCTCCACGACTCT-3', reverse: 5'-GCTCACACTCA CGCACACTC-3'; AKT, forward: 5'-CCGTCGACACCATCGGTTACCCATACGACGTG-3', reverse: 5'-CCATCGATTCCACCGCCGCCTCAGGCCGTGCC-3'.

Western blot

Total protein was extracted from treated cells by radioimmunoprecipitation assay (RIPA) solution (Beyotime, Shanghai, China). Protein sample was separated by electrophoresis on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After membranes were blocked with skimmed milk, they incubated with primary antibodies (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. The membranes were then washed with TBST (Tris-buffered Saline with Tween 20) and followed by incubation of secondary antibody at room temperature for 1 hr. The protein blot on the membrane was exposed to chemiluminescence.

EdU assay

After cell transfection for 24 hrs, cells were labeled with 100 μL of medium containing 50 μmol/L EdU for 2 hrs. Cells were then fixed with 4% paraformaldehyde for 30 min and incubated with 0.5% TritonX-100. Ten min later, 100 μL of Apollo solution was added and the nucleus was stained with Hoechst in the dark. Image acquisition and analysis were performed using Image Pro software (Version X; Media Cybernetics, Silver Springs, MD, USA).

CCK-8 (cell counting kit-8) assay

Transfected Ly42 and Ly1 cells were seeded into 96-well plates at a density of 2×10^5/mL. Ten μL of CCK-8 solution (cell counting kit-8, Dojindo, Kumamoto, Japan) were added in each well after cell culture for 6, 24, 48, 72 and 96 hrs, respectively. The absorbance at 450 nm of each sample was measured by a microplate reader (Bio-Rad, Hercules, CA, USA). Each group had 5 replicates.

Statistics

SPSS 20.0 statistical software package (IBM, Armonk, NY, USA) was used for data analysis. Measurement data was expressed as mean ± standard deviation and compared using the *t*-test. $P < 0.05$ showed statistically significant difference.

Results

LncRNA H19 was overexpressed in HL

H19 was overexpressed in HL tissues compared to RH tissues detected by qRT-PCR (Figure 1A). HL patients with higher expression of H19 presented shorter OS than those with lower expression (Figure 1B). Clinical data of enrolled HL patients were recorded, including age, gender, huge lump, Ann Arbor stage, lactate dehydrogenase (LDH) level and B symptoms (Table 1). H19 expression was remarkably correlated to huge lump ($p=0.0321$) and Ann

Arbor stage I-II (p=0.0045) of HL patients (Figures 1C and 1D).

LncRNA H19 promoted proliferation of HL cells

The mRNA level of H19 in HL cell lines was detected. Among A20, Ly1 and L428 cell lines, Ly1 cells expressed the highest level of H19, whereas Ly48 cells expressed the lowest level (Figure 2A). Subsequently, we transfected si-H19-1, si-H19-2 and si-NC in Ly1 cells. Transfection efficacy was verified by qRT-PCR. The data demonstrated that si-H19-1 presented the highest transfection efficacy, which was used for the following experiments (Figure 2B). Similar, transfection efficacies of pcDNA3.1 vector and pcDNA-H19 in L428 cells were also verified (Figure 2C).

To explore the effect of H19 on regulating proliferation of HL cells, EdU assay was carried out. The results indicated that H19 downregulation resulted in decreased proliferative abilities of Ly1 cells (Figure 2D). Opposite results were obtained after H19 overexpression in L428 cells (Figure 2E). CCK-8 assay also demonstrated that H19 promoted the proliferative ability of HL cells in a time-dependent manner (Figures 2F and 2G).

LncRNA H19 regulated AKT expression

In order to verify whether H19 participates in HL development via AKT pathway, AKT expression in HL tissues and RH tissues was detected by qRT-PCR. The data elucidated that AKT was remarkably overexpressed in HL tissues compared with that of RH tissues (Figure 3A). Cox regression analysis also revealed that H19 expression was positively correlated to AKT expression (Figure 3B). *In vitro* experiments showed that protein expression of AKT was reduced after H19 knockdown in Ly1 cells (Figure 3C). On the contrary, H19 overexpression in L428 cells increased AKT expression (Figure 3D).

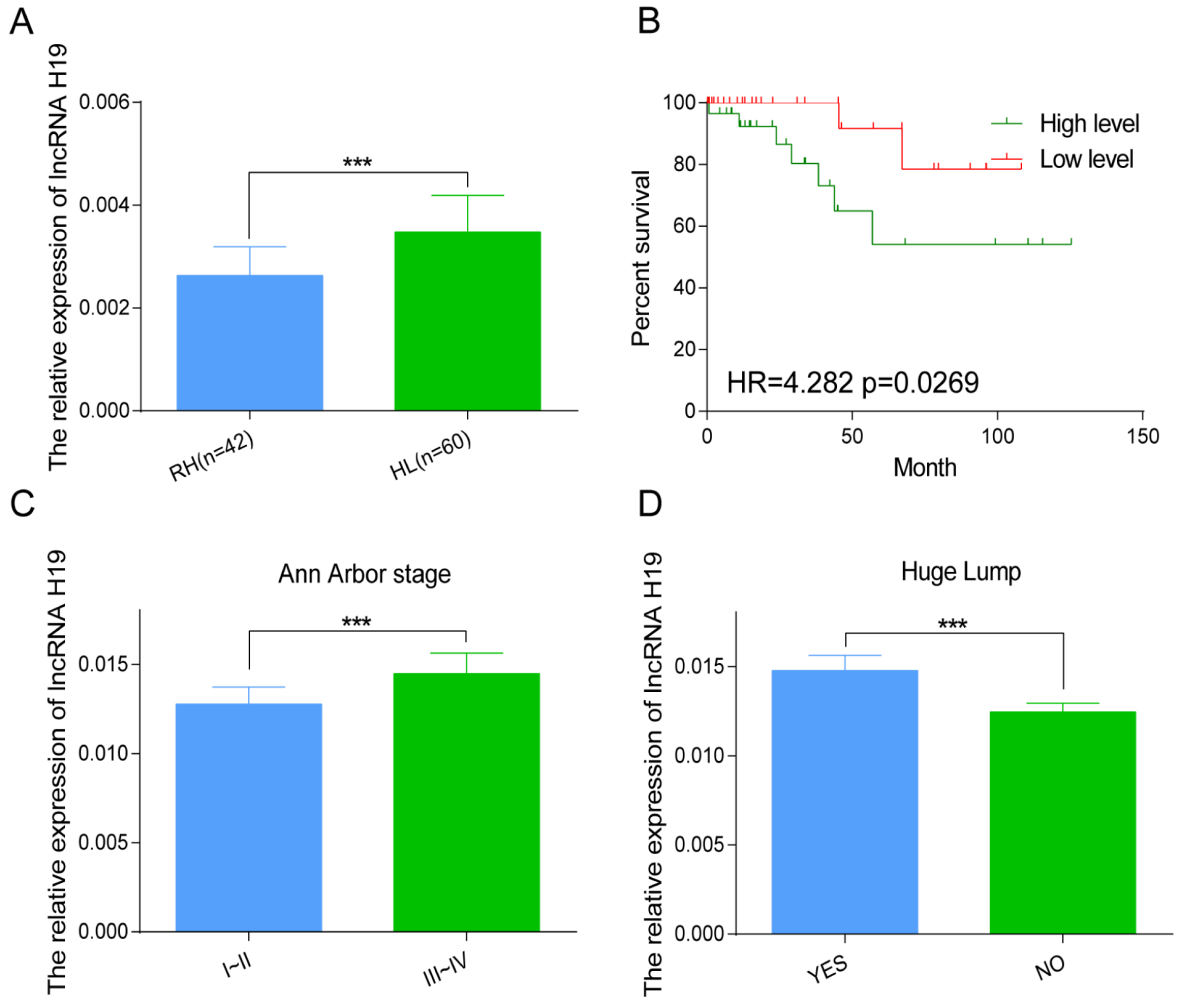

Figure 1. LncRNA H19 was overexpressed in HL. **A:** H19 was overexpressed in HL tissues than in RH tissues detected by qRT-PCR. **B:** HL patients with higher expression of H19 presented shorter OS than those with lower expression (Kaplan-Meier). **C,D:** H19 expression was remarkably correlated to huge lump (C, p=0.0321) and Ann Arbor stage I-II (D, p=0.0045) of HL patients. ***p<0.001.

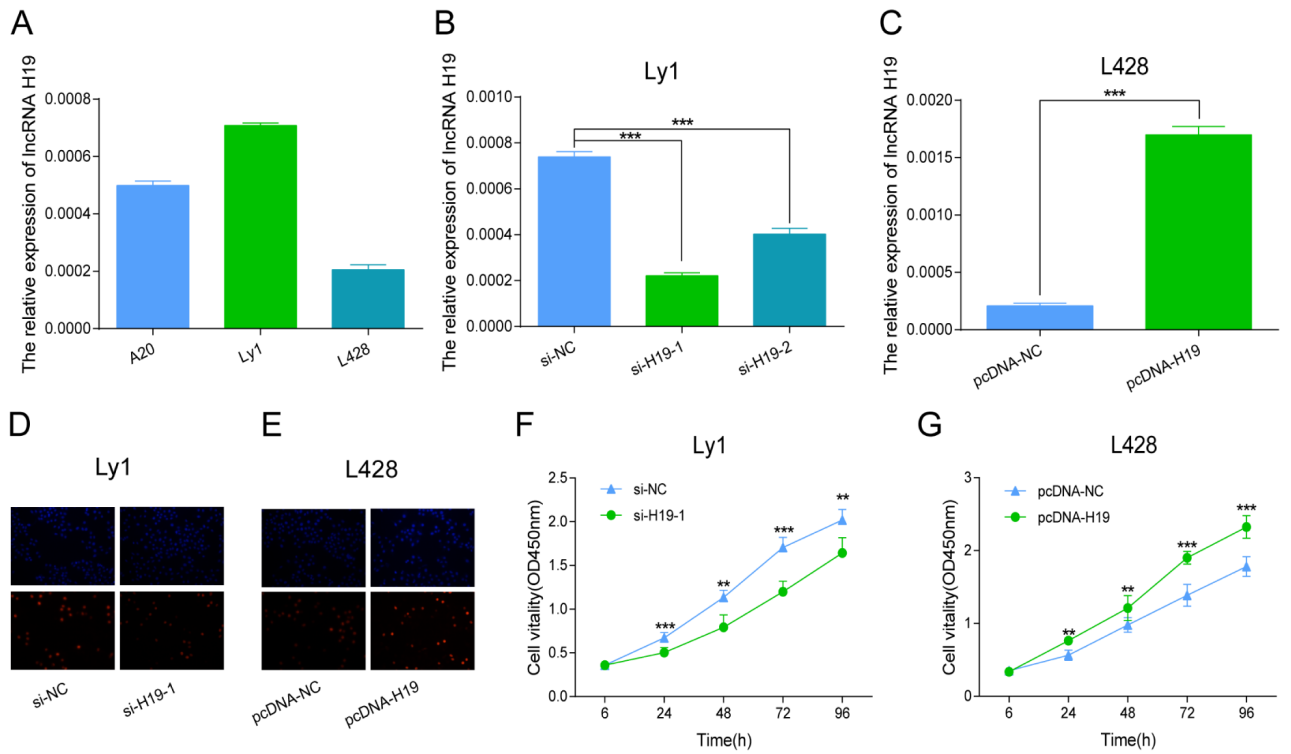

Figure 2. LncRNA H19 promoted proliferation of HL cells. **A:** The mRNA level of H19 in A20, Ly1 and L428 cell line. **B:** Transfection efficiency of si-H19-1, si-H19-2 and si-NC in Ly1 cells. **C:** Transfection efficiency of pcDNA3.1 vector and pcDNA-H19 in L428 cells. **D:** H19 downregulation resulted in decreased proliferative abilities of Ly1 cells. **E:** H19 upregulation resulted in increased proliferative capabilities of L428 cells. **F:** CCK-8 assay demonstrated that H19 downregulation resulted in decreased viability of Ly1 cells (Kaplan-Meier). **G:** CCK-8 assay demonstrated that H19 upregulation resulted in increased viability of L428 cells (Kaplan-Meier). **p <0.01, ***p<0.001.

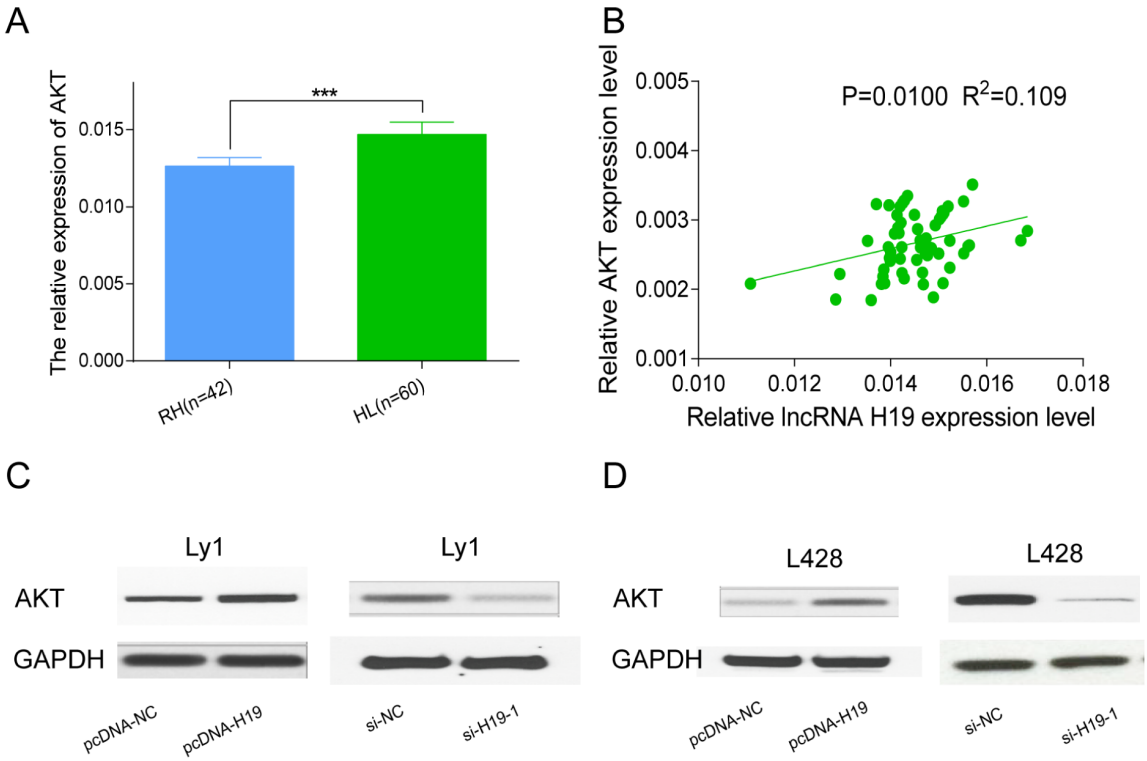

Figure 3. LncRNA H19 regulated AKT expression. **A:** AKT was remarkably overexpressed in HL tissues compared with that of RH tissues. **B:** Cox regression analysis also revealed that H19 expression was positively correlated to AKT expression. **C:** Protein expression of AKT was reduced after H19 knockdown in Ly1 cells. **D:** H19 overexpression in L428 cells increased AKT expression (***p<0.001).

Discussion

Lymphomas belong to the most common types of hematological malignancies. Based on the origin of lymphoma cells, lymphomas are divided into B-cell and T-cell lymphomas. Most lymphomas are derived from B cells, accounting for approximately 95%. B-cell lymphomas usually originate from the germ center, such as in HL. Clinically, HL patients have a good prognosis. The majority of HL patients could be cured after early diagnosis and receiving standardized treatment. However, 20% of HL patients eventually die of HL recurrence or progression. Therefore, it is of significance to explore the occurrence and development mechanisms of HL.

AKT is a downstream protein kinase of PI3K. Activated PI3K stimulates AKT, thereafter causing a series of cascade reactions that mediate cell growth, proliferation, survival, and death. Relative studies have found that AKT1 is amplified in gastric cancer. AKT2 amplification was found in ovarian cancer, pancreatic cancer, and breast cancer. Overexpression of AKT3 is confirmed in breast cancer and prostate cancer [31]. Meanwhile, studies have shown that AKT is closely related to the development of B cells [32]. During the stages of B cell maturation, activation, and differentiation, AKT pathway participates in the central immune tolerance of B cells, clearance of autoreactive B cells, transformation of B cell antibodies and mutation of somatic cells [33,34]. The specific role of B cells in HL, however, is rarely reported.

In the present study, we speculated whether H19 could regulate the proliferation of HL via AKT pathway. We found that H19 was overexpressed in HL tissues and cell lines, indicating the potential role of H19 in HL. H19 expression was positively correlated to huge lump and Ann Arbor stage of HL patients. Subsequently, *in vitro* effects of H19 on HL cells were investigated. Both EdU and CCK-8 results demonstrated that H19 overexpression promoted proliferation and viability of HL cells. To further verify whether AKT pathway is involved in the H19 regulation of HL, mRNA level of AKT in HL and RH tissues were detected by qRT-PCR. AKT expression was positively regulated by H19. Similar results were also obtained after detecting protein expression of AKT in HL cells. To sum up, we consider that H19 overexpression promotes the proliferative activity of HL cells via AKT pathway, which provided a novel idea for HL treatment.

Conclusions

Overexpressed lncRNA H19 promotes HL development by stimulating the proliferation of HL cells via AKT pathway.

Conflict of interests

The authors declare no conflict of interests.

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