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Original Article

Significance of microRNA-17, microRNA-19, c-myc, and E2F1 expression in T lymphoblastic lymphoma/leukemia and their relation to prognosis

Jing Li[1*], Peng Li[2*], Zhuanzhuan Yu[3], Peng Bu[1], Yanfeng Xi[1]

Departments of [1]Pathology, [2]Breast Surgery, Shanxi Cancer Hospital, Taiyuan, Shanxi Province, P. R. China; [3]Department of Pathology, Shanxi Medical University, Taiyuan, Shanxi Province, P. R. China. [*]Equal contributors.

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Abstract: Objective: To study the significance of microRNA-17, microRNA-19, c-myc, and E2F1 in T lymphoblastic lymphoma/leukemia (T-LBL/ALL) and their relationship to prognosis. Methods: Sixty cases of T-LBL/ALL with follow-up data were studied by using the immunohistochemical EnVision method for Ki-67, E2F1, and c-myc. Thirty cases of reactive lymph nodes were selected as the normal control group. Fluorescence in-situ hybridization (FISH) for the c-myc gene (located on chromosome 8q24) was performed to detect its breakage and gain/amplification. In addition, by way of the Quantitative real-time reverse transcription-polymerase chain reaction (PCR) method, we measured the microRNA-17 and microRNA-19 expression status in 60 cases of T-LBL/ALL. Results: Among the 60 cases of T-LBL/ALL, immunohistochemistry results showed that the percentages of tumor cell expression of E2F1 and c-myc were 51.7% and 66.7%. Ki-67 expression of ≤ 80% was found in 36 cases and > 80% was found in 24 cases. The positive rate of E2F1 protein was 51.7% (31/60) in 60 cases of T-LBL/ALL, but only 16.7% (5/30) in 30 cases of reactivated lymphoid tissue (χ^2 = 15.72, P < 0.05). C-myc protein expression was correlated with mediastinal width and Ki-67 index (P < 0.05). FISH results showed that among the 60 cases, the c-myc gene with breakage of 8q24 was detected in six cases (10.0%) and gains in 11 cases (18.3%). Thirty cases of reactive lymph nodes did not incur breakage or gains of c-myc gene, which was not significant between the c-myc gene and protein expression (P > 0.05). The expression of miR-17, 19 in T-LBL/ALL was higher than in the reactivated lymphoid tissues (P < 0.05). The significance of miR-17, 19 was related to c-myc protein (P < 0.05), but the relationship of miR-17, 19, E2F1, and c-myc was not significant (P < 0.05). Log-rank analysis results: The prognosis of E2F1 and the c-myc protein positive group was worse than that of the negative group (P < 0.05). The expression of miR-17 and miR-19 (> M) was related to the prognosis (P < 0.05). Multivariable Cox regression analysis results: miR-17, 19 (> M) and c-myc protein are risk factors that affect the prognosis of T-LBL/ALL (P < 0.05). Positive E2F1 protein in the mediastinal width was higher than that in the normal mediastinum (P < 0.05). Conclusions: miR-17, 19, E2F1, and c-myc may play an important role on the development of T-LBL/ALL. There may be independent prognosis factors. Their links are very significant. Under the c-myc, molecular regulation between miR-17, 19 and E2F1 promotes excessive proliferation of T cells, playing a role in tumor formation and development.

Keywords: Lymphoma, microRNA-17, microRNA-19, c-myc, E2F1, prognosis

Introduction

Lymphoblastic lymphomas and leukemias are malignancies of precursor cells committed to the lymphoid lineage [1]. Lymphoblastic lymphomas/LBL and Lymphoblastic leukemias/ALL are defined as a set of clinical and laboratory features similar to that of the same tumor entity in the World Health Organization (WHO) hematopoietic and lymphoid tissue tumor classification. T-LBL accounts for 85%-90% of all lymphoblastic lymphomas. Its prognosis is poorer than that of B-LBL, which occurs in children and adolescents. The classification of B-LBL/ALL is more clear, but T-LBL/ALL for molecular subtypes and pathogenesis, the prognosis is not [2].

MicroRNAs (miRNAs) are a relatively new class of naturally occurring, short, noncoding RNA molecules of approximately 22 nucleotides in length [3]. Through adjusting its target genes

mRNA 3'-Untranslated Regions/UTR, they are widely involved in cell proliferation, differentiation, apoptosis, and so on [4]. At present, 10%-30% of human genes are thought as to be microRNAs' regulation of target genes. In biological individual growth, cell apoptosis, differentiation, and the physiological and pathological process, they play an important role [5].

MicroRNA (miR)-17, 19 located in 13q31, plays a key role in cell cycle regulation. Its excessive expression occurs in many solid tumors. It can promote cell proliferation, gene transformation, and at the same time inhibit cell apoptosis. High expression of this cluster has been reported in lymphomas, which can promote the growth of T and B cells, and especially, has a promoting effect on TH1 cells. MiR-17, 19 is considered a key factor in TH1 adjustment [6, 7]. MiR-17, 19 with E2F1, may be involved in cell cycle regulation. It causes T cell proliferation; meanwhile, E2F1 and c-myc (located in 8q24) are involved in cell cycle regulation. There is a positive feedback effect. They play a role in inhibition, further inducing lymphoma formation [8].

Recent more studies have shown that c-myc gene translocation and the change of E2F1 exist in T-LBL/ALL. They are related to T-LBL/ALL's pathogenesis [9]. For this reason, we carried out this retrospective study to evaluate the prognostic value of miR-17, 19 in T-LBL/ALL, which is more common in Asia than in Western countries. In the past several years, we have collected T-LBL/ALL diagnostic specimens from 60 patients in our hospital in China, and we have immunophenotype data and clinical follow-up information for these patients. Such materials and clinical data have enabled us to investigate the expression of miR-17, 19, E2F1, and c-myc in archived formalin-fixed, paraffin-embedded (FFPE) lymph node specimens from these patients, with FFPE reactive lymph nodes as controls. Statistical analysis revealed a correlation of miR-17, 19, E2F1, and a c-myc expression level with overall survival of our patients.

Materials and methods

Tissue samples

A total of 60 FFPE lymph node specimens were included in this study. They were from patients diagnosed with T-LBL/ALL between 2001 and 2013 in Shanxi Cancer Hospital in Taiyuan, China and for which clinical follow-up information was available. T-LBL/ALL diagnosis was made by evaluating morphology and immunophenotype according to the WHO classification. An additional 30 reactive lymph node specimens were used as controls. Histopathological parameters, immunophenotypical findings, and patient outcome data were obtained from pathology records and hospital records.

Immunohistochemistry

Immunohistochemistry (IHC) study of FFPE specimens was performed using the EnVision System (Dako, Glostrup, Denmark). This is a two-step method in which application of the primary antibody is followed by a polymeric conjugate consisting of a large number of secondary antibodies bound directly to a dextran backbone, including E2F1, c-myc, and Ki-67. All the antibodies were from Dako, and the IHC procedures were completed on a Roche automated IHC instrument (USA). The antibodies and IHC procedures used in this study were part of a standard IHC panel for pathology laboratories to characterize lymphomas. Procedures were provided by the manufacturers. Positive localization of c-myc and Ki-67 is in the nucleus, but E2FI protein positive is mainly in the cytoplasm, with some visible expression in the nuclei [10].

Fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) was performed on FFPE specimens using a c-myc gene probe (CymoGen Dx, New Windsor, NY, USA), with 100 nuclei scored for each tissue specimen. The slides containing tissue specimens were first treated using a pretreatment kit (CymoGen Dx). The c-myc gene probe was a dual-color, break-apart rearrangement probe targeting the c-myc gene on chromosome 8q24. Hybridization and posthybridization washes were performed following standard protocol provided by the manufacturer. Rearrangement was defined as having a red and green fused signal separated into individual red and green signals. When such a break-apart signal pattern was seen in more than 5% of the tumor cells, results were recorded to be positive for c-myc rearrangement. When three or more intact/fused signals were seen in a nucleus,

Table 1. Correlation of E2F1 expression with clinicopathological characteristics of patients with T-cell lymphoblastic lymphoma

Parameters	Postive	Negative	X^2	P
Age (y)				
≤ 18	16	15	0.001	1
> 18	15	14		
Sex				
Male	23	20	0.202	0.777
Female	8	9		
Arbor				
I-II	6	5	0.045	1
III-IV	25	24		
Marrow involvement				
Yes	17	18	0.322	0.609
No	14	11		
Widened mediastinum				
Yes	23	13	5.384	**0.034**
No	8	16		
LDH				
≤ 250 U/L	15	14	0.000	1
> 250 U/L	16	15		
KPS				
< 80	6	11	2.546	0.154
≥ 80	25	18		
IPI score				
1-2	18	17	0.002	1
3-4	13	12		
Ki-67 index				
< 80%	15	21	0.000	0.07
≥ 80%	16	8		
C-MYC protien				
Postive	22	18	0.534	0.586
Negative	9	11		
C-MYC rearrangement				
Yes	3	3	0.007	1
No	28	26		
C-MYC amplification				
Yes	6	5	0.045	1
No	25	24		
MicroRNA17				
< 0.739	9	12	1.004	0.418
≥ 0.739	22	17		
MicroRNA19				
< 0.223	12	15	1.025	0.437
≥ 0.223	19	14		

Statistically significant values are shown in bold.

the results were recorded as copy number gain/amplification of c-myc.

MiRNA analysis

Total RNA was extracted from the FFPE lymph nodes of each patient using the Recover All Total Nucleic Acid Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Quantitative real-time reverse transcription-polymerase chain reaction (PCR) was carried out using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems [ABI], Foster City, CA, USA). The expression levels of miR-17 (ABI 4395419), miR-19 (ABI 4373098), and control U6 small nuclear RNA (ABI 4395470) were quantified by real-time PCR. Each reaction was performed in a final volume of 10 μL containing 2 μL of the cDNAtemplate, 2 μL of nuclease-free water, 1 μL of 20× primer/probe mix from the TaqMan microRNA assay, and 5 μL of 2× TaqMan universal PCR master mix (ABI). All reactions were run in triplicate on a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). The reaction mixture was incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Expression values were normalized to endogenous control U6. Relative quantification (RQ) of miRNA expression was calculated using the $\Delta\Delta C_q$ method described by Schmittgen and Livak [12]. RQ = $2^{-\Delta\Delta Cq}$, the $\Delta\Delta C_q$ value being calculated using ΔC_q values for lymphoma tissues and the mean value of ΔC_q for reactive lymph node controls for each miRNA ($\Delta\Delta C_q = \Delta C_{qtumor} - \Delta C_{qcontrol}$, where $\Delta Cq = C_{qmiR\text{-}17 \text{ or } 19} - C_{qU6}$) [13].

Statistical analysis

Statistical comparison of differences in miRNA levels in tumor vs control and comparison of the relationship between miRNA expression levels and each pair of clinicopathological parameters listed in **Table 1** were performed using the X^2 test and Fisher probabilities test. Differences between survival curves were analyzed using the Kaplan-Meier analysis and log-rank test in **Figure 2**. Multivariate Cox proportional hazard regression analysis with stepwise selection was used to evaluate independent prognostic factors associated with patient survival in **Table 2**. Results were considered statistically significant if the *P* value was < 0.05.

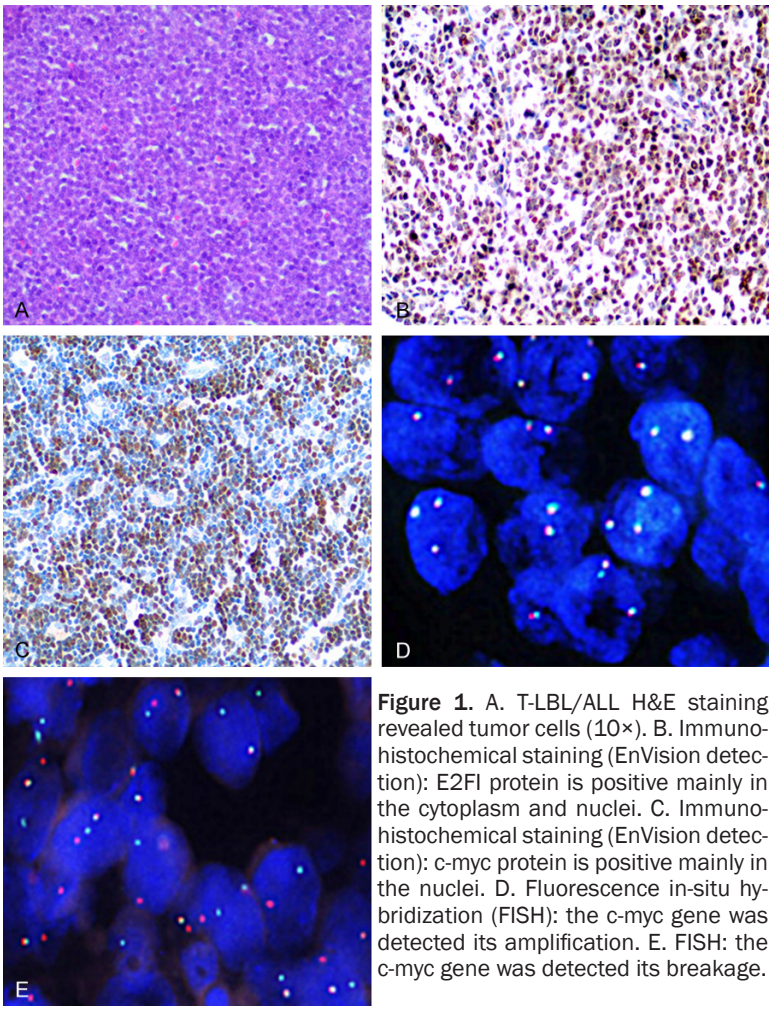

Figure 1. A. T-LBL/ALL H&E staining revealed tumor cells (10×). B. Immunohistochemical staining (EnVision detection): E2FI protein is positive mainly in the cytoplasm and nuclei. C. Immunohistochemical staining (EnVision detection): c-myc protein is positive mainly in the nuclei. D. Fluorescence in-situ hybridization (FISH): the c-myc gene was detected its amplification. E. FISH: the c-myc gene was detected its breakage.

Results

Clinical characteristics

Sixty patients (43 male, 17 female) had follow-up data of T-LBL/ALL, with a male-to-female ratio of 2.5:1. Ages ranged from 3 to 73 years, with a median age of 18. Fifty-six cases occurred primarily within the lymph nodes; 4 cases were extranodal. According to Ann Arbor staging, there were 11 cases in the I-II period and 49 in the III-IV period. Twenty-three patients presented B symptoms. Increased serum lactate dehydrogenase (> 250 IU/L) was detect-ed in 31 of the 60 patients. Our 60-case follow-up time ranged from 1 to 13 years. Five patients survived.

Pathological morphology and immunohistochemical features

Most tumor cells were of medium size had had a high nucleoplasm ratio. These nuclei assumed a circular or irregular, curvy shape, and the nuclear membrane was very thin. The chromatin was like fine dust, and the nucleolus was quite obvious. Nuclear division was seen more frequently. Much of the lymph node structure had disappeared, and there was diffuse infiltration of the tumor cell capsule (**Figure 1A**).

Among the 60 patients, 31 were positive for E2F1 (51.7%) (**Figure 1B**), and 40 for c-myc protein (66.7%) (**Figure 1C**). More than 80% of Ki-67 positive cells were observed in 24 of the 60 patients.

FISH analysis was subsequently performed on the FFPE tissues. The FISH probe detected c-myc gene copy number gain/amplification in 11 patients (18.3%) (**Figure 1D**) and c-myc rearrangements in six patients (10.0%) (**Figure 1E**).

Correlation of miR-17, 19, c-myc, and E2F1 expression with clinicopathological characteristics and overall survival

MiR-17, 19 had high expression of T-LBL/ALL, which was higher than the lymph node reactive hyperplasia ($P < 0.05$). In the miR-17, 19 high expression group, c-myc protein expression, the positive rate, was higher than that of the low expression group ($P < 0.05$). C-myc protein expression correlated with mediastinal width and Ki-67 ($P < 0.05$). No statistical significance appeared between the c-myc gene and protein expression, c-myc gene, or immune indexes. E2F1 protein expression correlated with mediastinal width ($P < 0.05$). The miR-17, 19 gene had no statistical significance with E2F1 and c-myc protein expression (**Table 1**). The association between miR-17, 19 expression, E2F1 protein, c-myc, and overall patient survival was investigated by the Kaplan-Meier analysis and log-rank test. MiR-17, 19 (> M), E2F1 protein, and c-myc protein were associated with prognosis ($P < 0.05$). Multivariate analysis using the

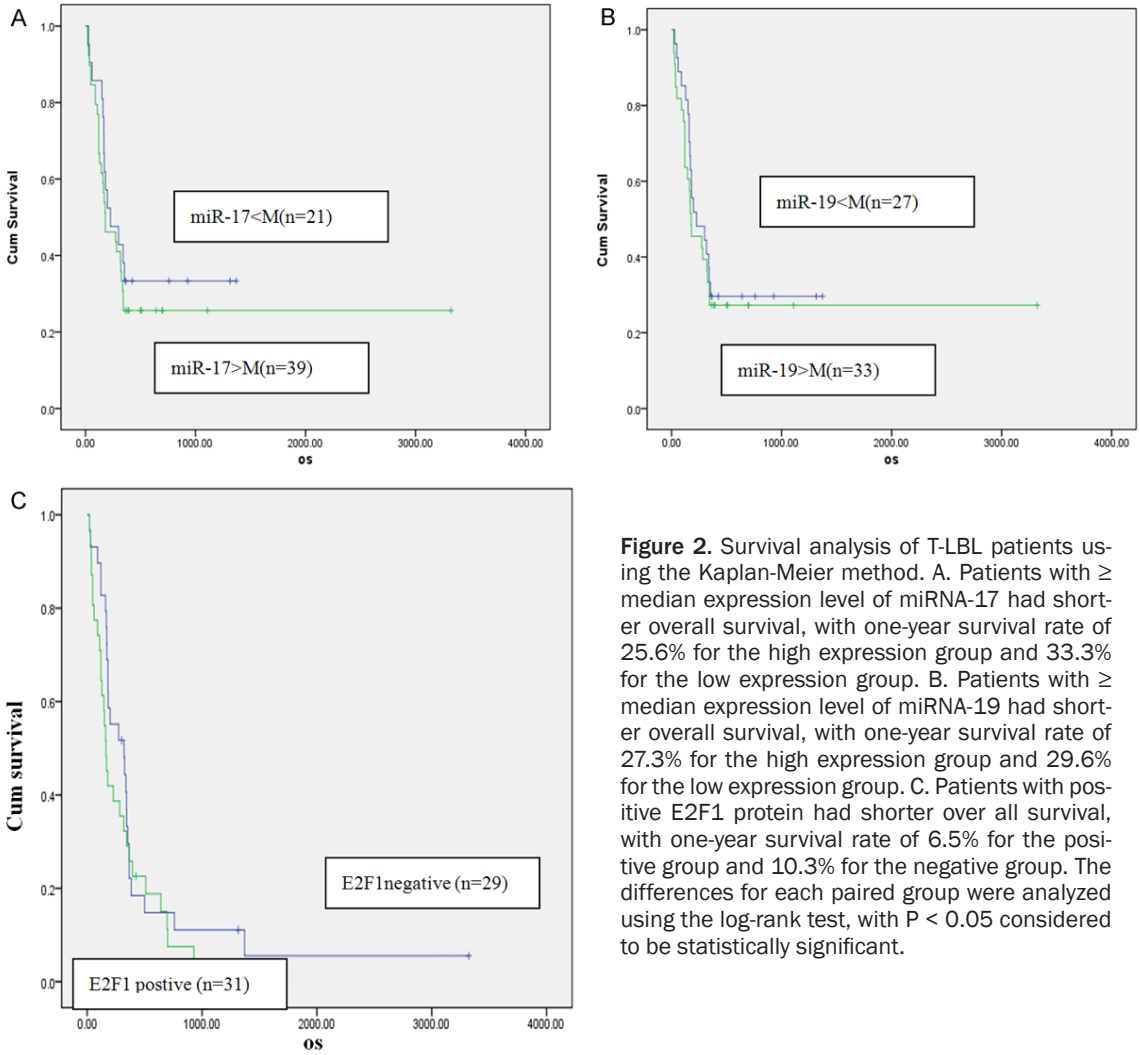

Figure 2. Survival analysis of T-LBL patients using the Kaplan-Meier method. A. Patients with ≥ median expression level of miRNA-17 had shorter overall survival, with one-year survival rate of 25.6% for the high expression group and 33.3% for the low expression group. B. Patients with ≥ median expression level of miRNA-19 had shorter overall survival, with one-year survival rate of 27.3% for the high expression group and 29.6% for the low expression group. C. Patients with positive E2F1 protein had shorter over all survival, with one-year survival rate of 6.5% for the positive group and 10.3% for the negative group. The differences for each paired group were analyzed using the log-rank test, with $P < 0.05$ considered to be statistically significant.

Cox proportional hazards model showed that the upregulated expression of miR-17, 19 (> M) and c-myc protein were independently poor prognostic factors for patients with T-LBL/ALL (**Table 2**). Survival analysis shows that the 1-year overall survival rate was 24.3%. Median survival time was 182 days. In the lymph node reactive hyperplasia of miR-17, 19, the median-expression level of M was bounded. The T-LBL/ALL patients were divided into a high-expression group (> M) and a low-expression group (< M). In T-LBL/ALL, the overall survival rate of the miR-17, 19 high-expression in group was lower than that of the low-expression group ($P < 0.05$). Overall 1-year survival rates were 25.6% for the high- and 33.3% for the low-miR-17-expression group (**Figure 2A**); 27.3% for the high- and 29.6% for the low-miR-19-expression group (**Figure 2B**); 15.0% for the c-myc positive and 44.0% for the negative group; and 6.5% for the E2F1 positive and 10.3% for the negative group (**Figure 2C**), respectively.

Discussion

T-LBL/ALL is a type of lymphatic hematopoietic malignant tumor, which originates in the precursor of T lymphocytes and is affected by a variety of genetic mutations that influence cell survival, proliferation, and differentiation. Strong chemotherapy drugs were used in the clinic. Lately, remission and overall survival rates for T-LBL/ALL have improved, but there is still drug-treatment failure. About 30% of patients relapse in 2 years, with symptoms appearing mostly in the central nervous system [9]. Targeted treatment is not effective. To study the pathogenesis, especially for indi-

Table 2. Multivariate Cox regression analyses for prognostic factors

Parameters	Hazard ratio 95% CI	P
Arbor	0.826 (0.7159, 0.9175)	0.948
KPS	0.6732 (0.5993, 0.8341)	0.787
IPI score	0.3528 (0.2882, 0.5451)	0.216
Marrow involvement	0.5841 (0.4549, 0.7118)	0.227
Widened mediastinum	0.5984 (0.4724, 0.7276)	0.629
E2F1 protein	0.4312 (0.3865, 0.6469)	0.435
C-MYC protein	2.9321 (1.148, 4.758)	**0.019**
C-MYC rearrangement	0.05649 (0.0218, 0.1782)	0.065
C-MYC gain/amplification	0.1538 (0.0825, 0.2841)	0.359
Ki-67	0.3982 (0.2724, 0.5276)	0.361
miRNA17	0.6213 (0.5257, 0.7743)	**0.0412**
MiRNA19	0.5168 (0.4204, 0.6796)	**0.0460**

Statistically significant values are given in bold.

cators in relation to their prognosis and target therapy, was the focus of the present study.

Many studies have found that the expression of, miRNA16, miRNA17-92, miRNA148, miRNA-151, miRNA424, and miRNA125b is associated with tumor occurrence and prognosis [13]. MiR-17-92 and six different coded microRNAs play a role in lung cancer, Burkitt lymphoma, and others. Olive [14] and Tagawa [15] believe that miR-17-92 plays an important role in the pathogenesis of Burkitt lymphoma. Studies have found that miR-17, 19-related regulational genes include c-myc, NOTCH1, BIM1, FXWB7, PTEN, E2F1, and others [16]. The expression of miR-17, 19, located on 13q31, is excessive in a variety of malignant tumors, and so may be cancer genes. In many lymphomas, a high expression of miR17, 19 was found, which caused the genetic transformation of T and B cells. High expression of cells has a strong proliferative ability and has exhibited resistance to apoptosis cells. Especially, it has a role in promoting TH1 cells. MiR-17, 19 is regarded as the key factor in TH1 regulation, as the key of microRNA in T cell lymphoma [8, 17].

In some B cell lymphomas, excessive expression of miR-17, 19 directly affects cell cycle progression and promotes cell proliferation [18-20]. C-myc is a gene binding on a miR17-92 promoter. C-myc and E2F1 transcription factors, through activation of C13, rf25, allow it to promote miR-17, 19 expression, cell proliferation, cell apoptosis resistance [20], and metabolism. These factors affect epithelial and mesenchymal transformation and start tumor growth [21-24].

Molina et al. [25] reported that E2F1 can be a cancer gene that promotes the development of Burkitt lymphoma. Transcription factor E2F1 is one of a group of proteins that regulate the cell cycle from the G1 phase to the S phase. In a stationary phase, when E2F1 protein was bound with phosphorylation of the retinal mother cell, after the retina mother cell protein phosphorylation, E2F1 protein was released with biological activity and promoted the cell cycle. E2F1 belongs to one of the members of the family of the transcription factor, which includes 8 members such as E2F1-E2F8. E2F1-E2F3 had a transcription activation effect, while the rest of the members inhibited the transcription. E2F1 is considered one of the most important transcription factors, which converts cells from the G1 phase to the S phase. Abnormal E2F1 gene mutation or genetic modification, which are found in most tumors, causes E2F1 inactivation or activity. E2F1-3 activated transcription of c-myc, while c-myc also activated the transcription of E2F1-3. There was a positive feedback control loop between them [26]. At the same time, c-myc activated the transcription of gene cluster miR-17-92 [27] and the miR-17-92 gene cluster of target genes, including c-myc. There may be a negative feedback control loop between them [28].

Recent research demonstrates that c-myc plays a central role in T-LBL/ALL [29-32]. Although only < 5% of c-myc genes appeared to change in T-ALL cases, the increased c-myc existed directly in the signaling pathways involved in T-LBL/ALL. This made c-myc the most active cancer gene in T-LBL/ALL. This study found that c-myc protein expression was correlated with mediastinal width and Ki-67. We showed indirectly that c-myc protein was associated with some tumors' biological behavior. In about 50% of T-LBL/ALL cases, the NOTCH gene mutation led to increased c-myc transcription [29-31]. In the remaining cases, the c-myc gene was activated after translation, then it mediated FBXW7 or PTEN/PI3K/AKT, signaling a changed pathway, preventing the

ubiquitin or phosphorylation process, and promoting its degradation [33-36].

A report have confirmed that the HDAC inhibitor SAHA reduces the c-myc expression level. Although its mechanism is unclear [37, 38], it may interfere with c-myc expression through some mechanism. C-myc is now thought to be the main driving force for T-LBL/ALL canceration. Research on c-myc protein, gene levels, and miR17, 19 expression has caused great interest in T-LBL/ALL [32, 39]. The role of miR17 family members in lymphoma generation may be associated with c-myc.

Studies have shown that a known target gene of miR17-5p and miR-20a is a cell cycle transcription factor gene that was encoded by E2F1. In a certain concentration, transcription factors are controlled by activation involved in DNA replication and cell cycle genes to promote cell transformation from the G1 to the S phase and cell proliferation, but beyond a certain threshold it can promote cell apoptosis by the ARF-P53 pathway. MiR-17 negatively regulates the transcription E2F1 expression, then c-myc can induce E2F1 expression; the expression of the latter products, in turn, promotes the expression of c-myc, which exists between positive feedbacks. However, c-myc-induced E2F1 expression is the result of increased E2F1 mRNA content, although the extent of E2F1 protein increase was far less than that of E2F1 mRNA. Thus, there are certain factors inhibiting the E2F1 mRNA translation process and prompting miR-17 and other factors to negatively regulate E2F1. Inhibiting E2F1 mRNA and keeping the E2F1 protein within the threshold value effectively adjusts c-myc's mediating cell proliferation and avoids apoptosis caused by transformation of excessive E2F1 protein. These studies reveal that miRNA may act as a key modulator in cancer-related gene function.

We used the RT-PCR TaqMan method for 60 cases of T-LBL/ALL and 30 cases of lymph node reactive hyperplasia of paraffin samples to test miR17, 19 expression and took advantage of the immunohistochemical and the FISH method for c-myc, E2F1 protein expression, and c-myc genetic abnormalities. The expression of miR-17, 19 in T-LBL/ALL is higher than in the normal reactive lymph node. The overall survival rate of miR-17, 19 high-expression group (> M) is lower than that of the low-expression group (< M). The overall survival rate in E2F1 positive protein expression was 6.5%; in the negative group, it was 10.3%. The E2F1 positive expression rate in c-myc positive protein expression is 22/40 (55%), which is higher than in the negative group, which was 9/20 (45%). The E2F1 positive rate in the miR-17 high-expression group was 22/38 (59%), which was considerably higher than that of the low-expression group (9/22 [41%]). This proved that there is a positive correlation between E2F1 protein expression and the expression of miR-17. The E2F1 protein positive rate in KPS scores was more than 80 (25/43 [58%]), significantly higher than the KPS score of ≤ 80 (6/17 [35%]). E2F1 protein was significantly associated with the KPS score. The c-myc protein positive rate in miR-17, 19 high expression is higher than that of the low-expression group, for which there is a positive correlation [36-39]. Further evidence adjusting relations between them exists, which played the role of the T-LBL/ALL mechanism. The multivariable Cox analysis shows that the miR-17, 19 high-expression group (> M) and c-myc protein were independent prognostic factors. We demonstrated that the miR-17, 19 and c-myc changes are associated with a poor prognosis, as molecular regulation between the two pathways may promote development of disease.

The overall survival rate in the miR-17, 19 low-expression group was higher than that of high expression. The overall survival rate of c-myc and E2F1 protein positive was significantly lower than that of the negative group. The miR-17, 19 high-expression group, c-myc, and E2F1 protein positive group were correlated with a poor prognosis. The c-myc protein positive expression rate in miR-17, 19 (high-gene expression group) was higher than the rate of the low-expression group, which prompts us to speculate that it is possible to adjust the relationship between them. The c-myc protein-expression rate in the E2F1 positive protein group was higher than that of the E2F1 negative group. There was a molecular regulation between E2F1 and miR-17, 19 genes under the c-myc. Together, they promoted excessive proliferation of T cells and played a role in tumor formation and development.

The E2F1 and c-myc in T-LBL/ALL were believed to adjust miR-17, 19, express to apoptosis,

escape, and activate the apoptosis pathways. Studies [9, 10] showed that E2F1 and c-myc were the key adjustment factors in impacting miR-17, 19 levels, and they were of vital importance in the disease development mechanism. This hints that they can inhibit downstream molecular pathways of the disease and influence disease development by blocking E2F1 and c-myc expression. They will be important in guiding targeted therapy in the future [37-39].

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yanfeng Xi, Department of Pathology, Shanxi Cancer Hospital, No. 3 of Zhigongxin Street, Xinghualing Strict, Taiyuan 030013, Shanxi Province, P. R. China. Tel: +86-13934211344; Fax: +86-351-4651656; E-mail: xyf609@sohu.com

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