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Original Article

MLL2 protein is a prognostic marker for gastrointestinal diffuse large B-cell lymphoma

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Abstract: Mixed linage leukemia gene 2 (MLL2) is identified as a novel mutation gene in diffuse large B cell lymphoma (DLBCL). However, the significance of MLL2 protein expression for the prognosis of DLBCL is unclear. In this study, we detected MLL2 protein expression in primary gastrointestinal diffuse large B cell lymphoma (PGI-DLBCL) samples by using tissue microarray immunohistochemistry, and analyzed the correlation between MLL2 protein expression and tumor proliferation activity. In addition, we investigated clinical significance of MLL2 protein expression for PGI-DLBCL prognosis. We found that there was significant difference in MLL2 protein expression between PGI-DLBCL and reactive hyperplasia of lymph node. High expression of MLL2 protein indicated higher clinical stage. In older patients (>60 years) with PGI-DLBCL, MLL2 protein expression was positively correlated with Ki-67 expression and negatively correlated with patient survival. Our data suggest that MLL2 protein is overexpressed in PGI-DLBCL and appears as a prognostic factor for patients of PGI-DLBCL, especially for those older than 60 years old.

Keywords: Primary gastrointestinal lymphoma, MLL2 protein, tissue microarray, immunohistochemistry, prognosis

Introduction

Mixed lineage leukemia (MLL) indicates an evolutionarily conserved trithorax family of human genes that play critical roles in gene regulation and embryonic development. MLL2 is a member of MLL that possesses histone H3 lysine 4 (H3K4)-specific methyltransferase activity and plays important role in epigenetic regulation of transcription [1, 2]. Natarajan et al. reported that the level of MLL2 protein was increased in the nucleus and cytoplasm of breast and colorectal cancer cells [1]. Furthermore, abnormal MLL2 expression could be found in prostate cancer and gastric carcinoma compared with normal tissues [2, 3]. On the other hand, MLL2 mutations have been detected in small cell lung cancer and renal carcinoma [4, 5]. Recurrent mutation of MLL2 gene could be found in 16% of medulloblastoma by exon sequencing [6]. In addition, MLL2 mutations and abnormal expression have been detected in diffuse large B cell lymphoma (DLBCL) [7, 8].

The gastrointestinal (GI) tract is the most frequent site of primary extranodal non-Hodgkin's lymphoma. The main pathology type of GI lymphoma is DLBCL [9]. Primary gastrointestinal diffuse large B cell lymphoma (PGI-DLBCL) is a highly heterogeneous disease with different clinical characteristics and prognosis. PGI-DLBCL is highly aggressive and not sensitive to chemotherapy agents in some patients, leading to poor prognosis. However, some patients with PGI-DLBCL respond well to chemotherapy, such as CHOP-like regimen. Although molecular typing that divides DLBCL into germinal center (GC) and non-germinal center (non-GC) can predict the response to therapy and prognosis, the prognosis of PGI-DLBCL remains difficult.

Considering MLL2 mutations and abnormal expression in DLBCL, we wondered whether MLL2 protein could be a new prognostic factor for PGI-DLBCL. In this study, we detected MLL2 protein expression in PGI-DLBCL samples by using tissue microarray immunohistochemistry, and analyzed the correlation between MLL2 protein expression and tumor proliferation activity. In addition, we investigated the clinical significance of MLL2 protein expression for PGI-DLBCL prognosis.

Material and methods

Subjects

Total 52 cases of PGI-DLBCL and 12 control cases of reactive hyperplasia of lymph node were randomly selected from the patients who visited the First Affiliated Hospital of Wenzhou Medical University. The study protocols were approved by Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University, and signed consent form was obtained from each patient. Follow-up data were obtained from the outpatient reexamination and telephone. The survival of dead patients was defined as the time from pathological diagnosis to the date of death. The survival of alive patients was defined as the time from pathological diagnosis to the last follow-up day. Clinical data were collected from the patients' medical records, including the age, lactate dehydrogenase (LDH) level, B symptoms, gastric or intestinal involvement, bulky mass size, infiltration depth, lymph node metastasis, Ann Arbor stage, international prognostic index (IPI) and Eastern Cooperative Oncology Group performance status (ECOG). All cases were de novo DLBCL, without histological evidence of mucosa-associated lymphoid tissue (MALT) lymphoma. No one had hepatitis C virus (HCV) and helicobacter pylori (H pylori) infection.

Treatment

Total 46 cases had radical or palliative operation, while the other 6 cases were treated with rituximab plus CHOP chemotherapy (Rituximab 375 mg/m^2 d0, Cyclophosphamide 750 mg/m^2 d1, Epirubicin 50 mg/m^2 d1, Vincristine 1.4 mg/m^2 d1, Prednison 100 mg d1-5) after biopsy. 46 cases received chemotherapy after operation, including CHOP or CHOP-like regimen for 27 cases, R-CHOP for 19 cases.

Construction of tissue microarrays (TMAs)

Two 12×8 TMAs were constructed from 52 cases of PGI-DLBCL and 12 control cases of reactive hyperplasia of lymph node. In each case, 3 cases with a diameter of 1.2 mm were obtained from 3 different areas of the tissue blocks and embedded into a single microarray paraffin block. TMAs were kept at 4°C until they were ready for analysis.

Immunohistochemistry

TMAs were cut into 4 μm sections, deparaffinized and rehydrated through graded alcohol series. Slides were pretreated in the microwave with citrate buffer (pH 6.0) at 90°C for 10 min to retrieve antigen, immersed in 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity, and incubated overnight at 4°C with normal bovine serum to reduce nonspecific binding. Subsequently, each TMA block was incubated with antibodies against MLL2 (1:30, clone I-18, Santa Cruz Biotechology, CA, USA) or Ki-67 (1:100, clone SP6, Maixin Biotechology, Fuzhou, China), followed by incubation with secondary antibody. The stained sections of the TMA blocks were independently evaluated by 3 hematopathologists. The cases were divided into germinal center B-cell-like (GCB) or non-GCB DLBC as described previously [10]. Dark brown stain granules in nucleus were defined as positive. Stains were scored as low expression for the <80% positive tumor cells, high expression for ≥80% positive tumor cells [11]. Ki-67 located in nucleus. Claybank or yellow brown stain granules in nucleus were defined as positive. Stains were scored as low expression for the <60% positive tumor cells, high expression for ≥60% positive tumor cells [12].

Statistical analysis

Statistical analyses were performed using SPSS19.0 statistical software. χ^2 test was used to compare different clinical features between high and low MLL2 protein expression groups. The relationship between binary variable of high and low expression of MLL2 and Ki-67 were analyzed by using binary Logistic regression. Univariate analyses of prognostic factors by using Kaplan-Meier and Log-rank test. A multivariate survival analysis was performed by Cox's proportional hazards regression model. A *P* value of less than 0.05 was considered statistically significant.

Results

Survival analysis

Of 52 cases of PGI-DLBCL, 8 cases were positive for CD10 expression, 33 cases were positive for B cell lymphoma-6 (Bcl-6) expression

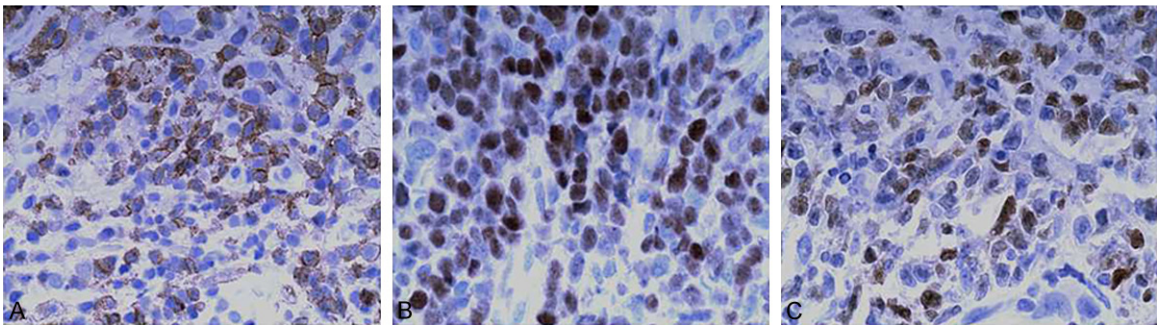

Figure 1. Immunohistochemical staining of PGI-DLBCL tissues. A. Positive staining of CD10. B. Positive staining of Bcl-6. C. Positive staining of MUM1. Magnification: 400 ×.

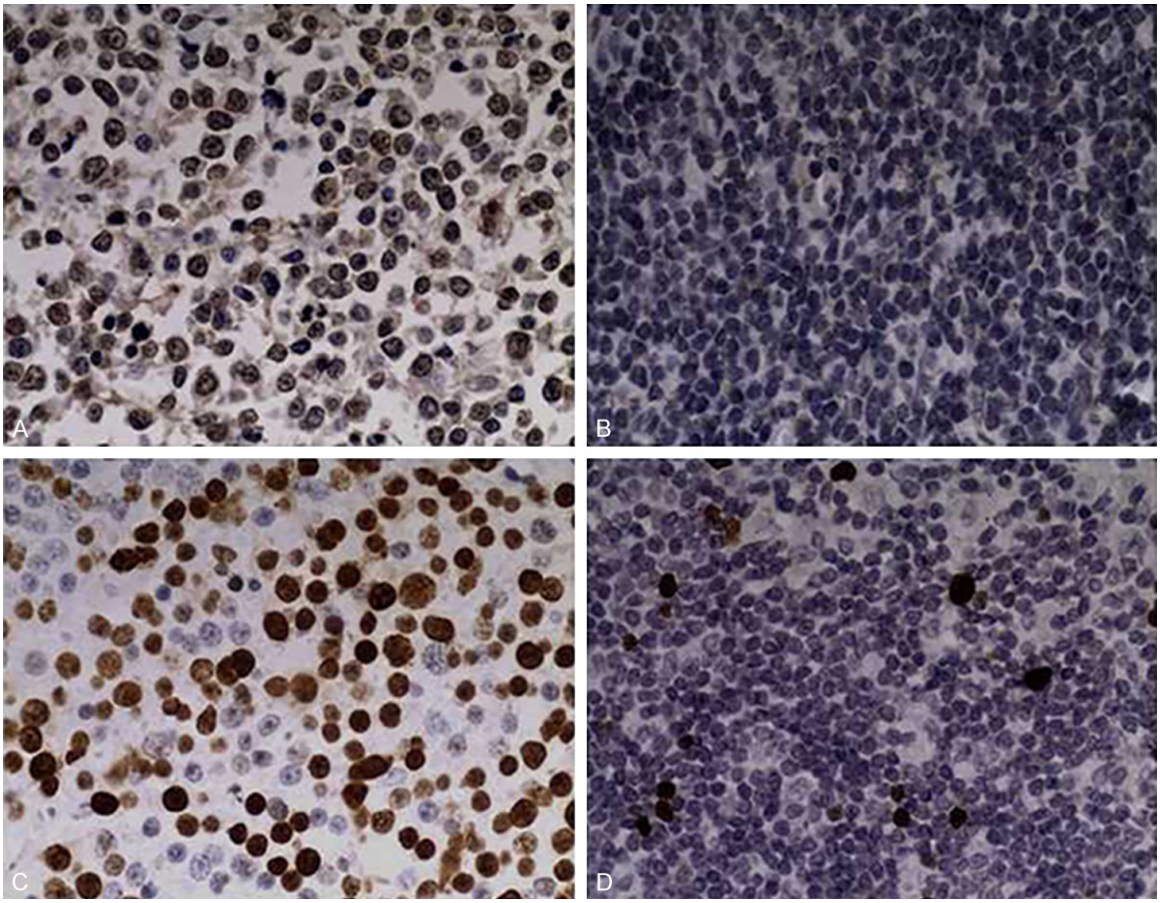

Figure 2. Immunohistochemical staining of MLL2 in PGI-DLBCL and reactive hyperplasia of lymph node tissues. A. Positive staining of MLL2 in PGI-DLBCL. B. Positive staining of MLL2 expression in reactive hyperplasia of lymph node. C. Positive staining of Ki-67 in PGI-DLBCL. D. Positive staining of Ki-67 expression in reactive hyperplasia of lymph node. Positive staining of MUM1. Magnification: 400 ×.

and 43 cases were positive for multiple myeloma oncogene 1 (MUM1) expression (**Figure 1A-C**). According to immunohistochemistry analysis, PGI-DLBCL was divided into 11 cases of GCB and 41 cases of non-GCB.

The median length of follow-up was 28 months (range 2-102 months). Median survival was (67±5) months (95% confidence interval: 58-77), including 20 cases of death and 32 cases of survival. In patients >60 years old, 25

Table 1. Comparison of clinical features between patients with high and low MLL2 protein expression in all patients

	MLL2 expression		
Characteristics	<80% (n=12)	≥80% (n=40)	*P*-value
Age >60 years	7 (58.3%)	25 (62.5%)	0.795
Performance, ECOG >2	3 (25.0%)	12 (30.0%)	0.650
Ann-Arbor stage III/IV	4 (33.3%)	29 (72.5%)	0.013*
B symptoms	4 (33.3%)	15 (37.5%)	0.793
Site involvement			0.838
Gastric	7 (58.3%)	22 (55.0%)	
Intestinal	5 (41.7%)	18 (45.0%)	
Bulky tumor (>5 cm)	8 (66.7%)	30 (75.0%)	0.568
Lymph node involvement	7 (58.3%)	31 (77.5%)	0.189
LDH elevation	2 (16.7%)	11 (27.5%)	0.447
IPI			0.128
0-2	11 (91.7%)	28 (70.0%)	
3-5	1 (8.33%)	12 (30.0%)	
GCB type	4 (33.3%)	7 (17.5%)	0.838

ECOG, eastern cooperative oncology group; LDH, lactate dehydrogenase; IPI, international prognostic index; GCB, germinal center B-cell. *P<0.05.

cases had high expression of MLL2 protein, including 8 cases of survival and 11 cases of death, whereas 7 cases had low expression of MLL2 protein, including 5 cases of survival and 2 cases of death. Between the two groups of high and low expression of MLL2 protein in older patients, there are no meaningful difference in ECOG, Ann-Arbor stage, B symptoms, Site involvement, LDH level, IPI score, GCB type and treatment.

Immunohistochemical analysis of MLL2

MLL2 protein was stained both in the cytoplasm and in the nucleus of PGI-DLBCL tissues (**Figure 2A**). Twelve cases of reactive hyperplasia lymph node tissues had low expression of MLL2 protein (**Figure 2B**), without differences within the reactive follicles/germinal centers versus interfollicular areas. In 52 cases of PGI-DLBCL, 12 cases had low expression of MLL2 protein, while 40 cases had high expression of MLL2 protein. MLL2 protein expression in PGI-DLBCL and reactive hyperplasia lymph node tissues was statistically significant.

To characterize the proliferation ability of PGI-DLBCL tissues and reactive hyperplasia lymph node tissues, we performed Ki-67 staining. Positive expression of Ki-67 was located in the nucleus of PGI-DLBCL tissues, stained as dark brown or yellow brown granules (**Figure 2C**). Among 52 cases of PGI-DLBCL, 30 cases had high expression of Ki-67, which accounted for 58% (30/52), while 22 cases had low expression of Ki-67, which accounted for 42% (22/52). In reactive hyperplasia lymph node tissues, Ki-67 expression was located in lymphoid follicle germinal center of mature lymphocyte nucleus (**Figure 2D**).

Relationship between MLL2 protein expression and clinical features of PGI-DLBCL

MLL2 protein expression was correlated with clinical stages of PGI-DLBCL. The expression rate of MLL2 protein was 57.9% in 11 cases with I/II stage but was significantly higher in 29 cases with III/IV stages (87.8%) (P<0.05). MLL2 protein expression had no correlation with the age, B symptom, site involvement, the size of mass, infiltration depth, lymph node involvement, LDH, IPI and immunophenotyping of PGI-DLBCL (**Table 1**).

In 52 cases of PGI-DLBCL, the correlation between MLL2 and Ki-67 protein expression was not significant (P>0.05). Meanwhile, low expression of MLL2 protein in reactive tissue also had no significant correlation with Ki-67 expression (P>0.05). Additionally, the survival of the patients between those with high expression of MLL2 protein (>80%) and those with low expression of MLL2 protein (<80%) was not significantly different (P>0.05, **Figure 3A**). Cox multivariate analysis revealed that IPI, immunophenotyping and LDH were significantly related to the prognosis (P<0.05). However, in older patients (age >60 years), 25 cases had high expression of MLL2 protein and 7 cases had low expression of MLL2 protein, while 19 cases had high expression of Ki-67 (>60%) and 13 cases had low expression of MLL2 protein (<60%). In the older patient group, MLL2 protein expression was positively correlated with Ki-67 protein expression (P=0.03). In addition, patients with high expression of MLL2 protein had shorter survival (**Figure 3B**).

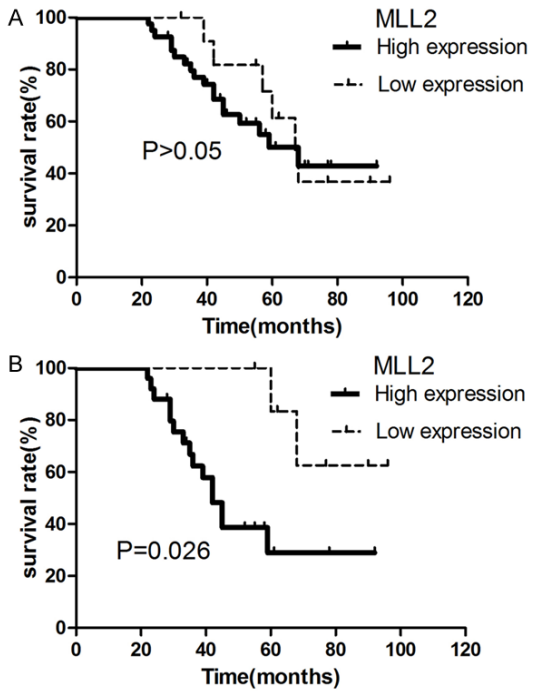

Figure 3. Survival curve analysis of PGI-DLBCL patients. A. The curve of all 52 PGI-DLBCL patients with different expression level of MLL2. B. The curve of older PGI-DLBCL patients (>60 years, n=32) with different expression level of MLL2.

Discussion

Histone methyltransferases regulate a variety of cellular processes such as the formation of heterochromatin, transcription, cell proliferation and adhesion, apoptosis and DNA damage repair, and are crucially involved in cancer progression [13-15]. MLL2 as a histone methyltransferase has been shown to be mutated or overexpressed in tumors such as breast cancer and colorectal cancer [16]. Previous data showed that MLL2 is a common target for chromosomal translocations associated with human acute leukemias [16].

In this study we examined MLL2 protein expression in PGI-DLBCL by using tissue microarray immunohistochemistry. Tissue microarray combines the protein expression level of gene and morphology of tissue, which different from gene chip and protein array. In addition, tissue microarray can save tissue specimen, reagent, time, and reduce systematic error.

Our data demonstrated that MLL2 proteins expression in reactive hyperplasia of lymph node tissue is significantly different from that in PGI-DLBCL. MLL2 mutation in lymphoma indicate that chromatin dysfunction play an important role in lymphoma [8]. Recently, a study showed that t(14; 18)(q32; q21) gene rearrangement occurred from the early benign B cell clone to tumor precursor B cells clone. However, MLL2 gene mutation occurred in the late stage of malignant B cell clone. Therefore, MLL2 gene mutation was considered as a secondary event in follicular lymphoma, and MLL2 gene mutation work as a helper or a passerby in the pathogenesis of lymphoma, but not as a caustic pathogenic factor [17].

Our study showed that MLL2 protein expression was positively correlated with Ki-67 expression in old patients with PGI-DLBCL (age >60 years). Natarajan et al. found that MLL2 expression level was positively correlated with malignancy of breast cancer and colorectal cancer [1]. We also investigated the clinical outcome of the patients with high MLL2 protein expressions. All cases had no underlying conditions, such as H pylori or HCV infections. For the entity of PGI-DLBCL with H. pylori positive is biologically distinct from H. pylori negative cases and has a better clinical outcome [18]. HCV infection may influence the outcome of B cell lymphoma, notwithstanding there is no statistical evidence that the prognosis of HCV-positive patients is inferior to that of HCV-negative subjects [19]. We found that MLL2 protein expressions were correlated to prognosis in elderly patients (age >60 years), which indicated that abnormal methylation regulated by MLL2 may be involved in elderly patients with PGI-DLBCL.

Although genome abnormalities such as chromosome translocation are the prominent presentation in lymphoma, abnormal methylation in lymphoma has been reported. Choi et al. found that abnormal methylation was frequently detected in promoter region in lymphoma [20]. Methylation is involved in lymphoma through influence on gene transcription by regulation of chromosome structure [21]. Moreover, the degree of methylation is related to clinical stage of DLBCL [22]. Amara et al. found that p16 methylation in DLBCL was related to clinical stage and B symptom, and DLBCL patients with p16 methylation had shorter overall survival (OS) and disease free survival (DFS) [23].

However, DLBCL patients with *GSTPI* gene methylation had low clinical stage and high complete remission [24]. Therefore, the role of methylation in lymphoma needs further investigation, which will help develop novel demethylation therapy for elderly lymphoma patients.

Currently, immunohistochemistry test for CD10, BCL-6 and MUM1 is used to distinguish between GCB and activated B-cell (ABC) type of DLBCL. Our data showed that there was no significantly difference in MLL2 protein expression between GCB and non-GCB groups. However, Shaknovich et al. sorted GCB and non-GCB groups based on the methylation of 263 genes and the accurate rate was up to 91% [25]. Therefore, it is interesting to investigate whether MLL2 combined with methylation of other genes could be used to distinguish between GCB and non-GCB.

Several limitations of this study need to be pointed out. First, we still do not understand why MLL2 protein expression was positively correlated with Ki-67 expression and negatively correlated with patient survival only in older patients (>60 years) with PGI-DLBCL, but not in all PGI-DLBCL patients. Further mechanistic studies are necessary. Second, the sample size of this study is small. Studies that employ larger samples are needed to confirm our conclusion.

In conclusion, our data suggest that MLL2 protein is overexpressed in PGI-DLBCL and appears as a prognostic factor for patients of PGI-DLBCL, especially for those older than 60 years old.

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Disclosure of conflict of interest

None.

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