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Evaluation of Relationship between Bone Marrow Flow Cytometric Typing and Pathological Immunohistochemical Analysis of Lymphoma Cell Leukemia

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ABSTRACT

The objective of the study was to observe the results of bone marrow flow cytometric (BMFC) typing and pathological immunohistochemical (PI) of lymphoma cell leukemia (LCL), and then provide scientific data for clinical typing of lymph node cell leukemia. The BMFC typing and PI data of 86 patients with LCL admitted to our hospital from January 2018 to January 2022 were retrospectively analyzed to compare the BMFC typing and PI expression, and investigate the differences in PI results among patients with different BMFC typing. There were significant differences in bone marrow flow cytometry immunogen level between T lymphocyte type and B lymphocyte type (P<0.05). CD3, BCL-2, CD7, TDT and CD34 were the dominant PI typing of T cell type. The expression rate of BCL-2 was 100.00% and that of CD3 was 91.67% (33/36), followed by TDT, CD7 and CD34. CD20, CD79a, BCL-6 and BCL-2 were the dominant PI typing of B-cell type. CD20 had the highest expression rate (100.00%), followed by CD79a and BCL-6. There were significant differences in PI typing antigen level between T lymphocyte type and B lymphocyte type (P<0.05). Regarding the correlation between the same antigen of BMFC typing and PI typing, only CD3, CD7 and CD20 were correlated (r>0, P<0.05). CD5 exhibited no correlation (P>0.05). BMFC typing and PI antigen typing of LCL are only correlated in terms of CD3, CD7 and CD20 antigens, with complementary effects shown in other antigens. It was concluded that clinical selection should be made by comprehensive consideration of patients' conditions.

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Authors' Contribution
YZ and BG conceived the design of the study, collected and reviewed the data, and coordinated the project. LF, BL and JX reviewed the literature, collected and analysed the data, and prepared the manuscript. YZ and JX helped in critical revision and finalizing the manuscript. All authors read, revised, and approved the final manuscript.

Key words
Lymphoma cell leukemia, Bone marrow flow cytometric typing, Pathology, Immunohistochemistry

INTRODUCTION

Lymphoma is a malignant tumor with abnormal lymphocyte malignant hyperplasia that develops in the lymph nodes and can invade the tissues outside the lymph nodes (Zhang *et al*., 2022). Some highly aggressive lymphomas will invade the bone marrow, leading to lymphoma cell leukemia (LCL) (Center, 2022). According to the data of GLOBOCAN 2018, malignant lymphoma ranks 9th among the malignant tumors and 10th among the death rates globally (Parkin *et al*., 2022). At present, histopathological examination is used as the gold standard for lymphoma, but with the progression of lymphoma, bone marrow cells of some patients will be invaded by lymphoma cells. As a result, bone marrow flow cytometric (BMFC) typing carries important significance in differentiating disease progression of lymphoma patients (He *et al*., 2022). Meanwhile, flow cytometry based on immunohistochemical results can be used to detect small residual lesions of LCL before bone marrow invasion (Liu *et al*., 2021). Thus, both bone marrow flow cytometry and pathological immunohistochemical (PI) are techniques for early identification of bone marrow cell invasion by lymphoma cells. However, there is insufficient accurate data on the differences and correlations between BMFC typing and PI results. Whether there is consistency between bone marrow and lymphoid tissue immunophenotyping demands further study. To this end, our hospital conducted a retrospective analysis to investigate the results of bone marrow flow cytometry and PI of patients with LCL, aiming to provide scientific data for clinical typing of lymph node cell leukemia and facilitate the selection of early immune typing strategies.

MATERIALS AND METHODS

A retrospective analysis was performed on 86 patients with LCL admitted to our hospital from January 2018 to

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January 2022, including 58 males and 28 females. The patients were aged 17~70 years, with a median age of 41.50 (18.20,68.50) years. Inclusion criteria: WHO diagnostic criteria for LCL in hematopoietic and lymphoid tissue tumor classification are met (Stock *et al*., 2013), complete clinical data; with informed consent and signed informed consent for this study. Exclusion criteria: Patients with other malignant tumors, patients with severe abnormalities in liver and kidney function.

Instruments and reagents

FC500 flow cytometer was used, and the immunophenotyping kit was purchased from Beckman, USA. PI instrument was Autostainer360/480S/720 automatic immunohistochemistry auto Stainer. PI instrument (benchMark-ULTRA) and kits were purchased from Ventana, USA. Constant temperature oven was manufactured by Dongguan Tengjie Machinery Technology Co., LTD.

Heparin was produced by Shanghai Hengyuan Biotechnology Co., LTD. Alcohol, anhydrous ethanol, methanol-hydrogen peroxide was purchased from Guangzhou Yongqi Chemical Trading Co., LTD. Xylene was purchased from ExxonMobil Chemical; PBS was produced by Baianmei Innovation Technology (Guangzhou) Co., LTD. Distilled water was made by our laboratory. The primary antibody and secondary antibody were provided by Abace Biotechnology. The color developing agent was produced by Wuhan Boster Biological Technology Co., LTD. Hematoxylin was produced by Shanghai Maokang Biotechnology Co., LTD. Neutral gum was produced by Hubei Anlixin Medical Industry Co., LTD. Phosphate buffer was produced by Wuhan Procell Life Science andTechnology Co., LTD

Specimen collection and preparation

1-2ml bone marrow specimens were selected as bone marrow flow cytometry specimens and detected by flow cytometry (FCM) within 24 h after anticoagulation with heparin. At the same time, lymph node or lymphoid tissue biopsy specimens were taken as PI specimens. Lymph node or lymphoid tissue specimens were treated with 75%, 80%, 95%×2, 100%×3 gradient alcohol and xylene for specimen preparation.

BMFC typing

The collected bone marrow specimens were separated as per mononuclear cells by lymphocyte fluid, and the cell number was adjusted to (0.5~1.0) ×106 /ml. Then, the bone marrow specimens were dissolved by phosphate buffer, and CD45 /SSC was classified into mature cell population and naive cell population. The expression levels of CD2, CD3, CD5, CD7, CD11c, CD19, CD20, CD22, CD23, CD79a, CD38, FMC-7, HLA-DR and sKappa antigens in bone marrow specimens were determined by flow cytometry. The experiment was conducted by two professional inspectors in strict accordance with the FC500 flow cytometer and related immunophenotyping kit instructions, and the experimental specimens were tested within 24 h.

PI

The prepared lymph node or lymphoid tissue specimens were placed in a constant temperature oven at 60℃ overnight. The endogenous catalase in lymph nodes or lymphatic tissues was treated with methanol and hydrogen peroxide, then cleaned with PBS and distilled water, and then the antigen in tissues was repaired. Primary and secondary antibodies were added to the treated tissues and incubated at room temperature. The incubation time was set at 30min. Color developing agent was added and hematoxylin was used for restaining. The specimens were treated with 70%, 95%×2, 100%×4 gradient alcohol and xylene, and then sealed with neutral gum for use. The expression levels of CD3, TDT, CD5, CD7, CD10, CD20, CD21, CD34, CD43, CD79a, LCA, BCL-2, BCL-6 and PAX-5 antigens were analyzed by PI instrument and corresponding kits.

Observation indexes

The same antigens in BMFC typing and PI typing were used as consistency observation indexes, and the positive expressions of the same antigens CD3, CD5, CD7 and CD20 in the two examination methods were used as the main observation indexes.

Statistical analysis

SPSS 22.0 statistical analysis software was used, the measurement data were expressed as $\bar{x}\pm s$ and tested by t test. The count data were expressed as %, and tested by χ^2 test. $P < 0.05$ indicated statistically significant difference. The correlation was analyzed by Pearson correlation, and the correction level was $\alpha=0.05$.

RESULTS

A total of 36 cases of T lymphocyte type and 50 cases of B lymphocyte type were collected in this study. Seen from the cell morphology, T lymphocyte type cells exhibited diffuse growth, medium size, with a small amount of cytoplasm, showing starry sky phenomenon. B lymphocyte type exhibited round or oval nuclei with indentation and sometimes curved nuclei, fine chromatin, usually indistinct nucleoli, and focal starry sky

phenomenon in some cases. Using cell morphology alone cannot differentiate the immunophenotype.

The expression levels of CD2, CD3, CD5, CD7, CD11c, CD19, CD20, CD22, CD23, CD79a, CD38, FMC-7, HLA-DR and sKappa antigens in bone marrow specimens were determined by flow cytometry. Immunophenotype analysis confirmed 36 cases of T lymphocyte type and 50 cases of B lymphocyte type. CD7, CD38, CD3, CD11c and HLA-DR were the dominant bone marrow flow cytometry of T cell type. The expression rate of CD7 was 100% and that of CD3 was 58.33% (21/36). CD3 deletion was found in 9 cases. CD19, CD38 and CD20 were the dominant bone marrow flow cytometry of B cell type. CD19 had the highest expression rate (96.00%, 48 cases), followed by the positive rate of CD20 (64.00%, 30/50). There were significant differences in bone marrow flow cytometry immunogen level between T lymphocyte type and B lymphocyte type patients ($P < 0.05$). The BMFC typing results of T lymphocyte type and B lymphocyte type patients are shown in Table I.

Table I. Bone marrow flow cytometric typing distribution in T lymphocyte type and B lymphocyte type patients.

Immunophe-notyping	T lymphocyte (n=36)	B lymphocyte (n=50)	χ^2_c	P
CD2	12(33.33)	2(4.00)	11.149	0.001
CD3	21(58.33)*	3(6.00)	25.950	0.000
CD38	33(91.67)	21(42.00)	20.024	0.000
CD7	36(100.00)	2(4.00)	74.371	0.000
CD11c	18(50.00)	11(22.00)	133.080	0.000
HLA-DR	18(50.00)	11(22.00)	133.080	0.000
CD19	2(5.56)	47(94.00)	63.231	0.000
CD20	3(8.33)	32(64.00)	24.616	0.000
CD22	2(5.56)	16(32.00)	7.318	0.007
CD23	2(5.56)	16(32.00)	7.318	0.007
CD5	14(38.89)	1(2.00)	17.301	0.000
FMC-7	3(8.33)	18(36.00)	7.246	0.007
sKappa	0	18(36.00)	/	0.000

/, Fisher's exact probability method; *, indicates deletion.

The expression levels of CD3, TDT, CD5, CD7, CD10, CD20, CD21, CD34, CD43, CD79a, LCA, BCL-2, BCL-6 and PAX-5 antigens in lymph node or lymphoid tissue specimens were determined by PI. PI typing had the same results as BMFC typing, with T lymphocyte type in 36 cases and B lymphocyte type in 50 cases. Where, CD3, BCL-2, CD7, TDT and CD34 were the dominant PI typing of T cell type. The expression rate of BCL-2 was 100.00% and that of CD3 was 91.67% (33/36), followed by TDT, CD7 and CD34. CD20, CD79a, BCL-6 and BCL-2 were the dominant PI typing of B cell type. CD20 had the highest expression rate (100.00%), followed by CD79a and BCL-6. There were significant differences in PI typing antigen level between T lymphocyte type and B lymphocyte type patients ($P < 0.05$). The PI typing results of T lymphocyte type and B lymphocyte type patients is shown in Table II.

Table II. Pathological immunohistochemical typing distribution of T lymphocyte type and B lymphocyte type patients.

Pathological immunotype	T lymphocyte (n=36)	B lymphocyte (n=50)	χ^2_c	P
CD3	33(91.67)	4(8.00)	56.405	0.000
TDT	27(75.00)	2(4.00)	44.085	0.000
CD5	12(33.33)	5(10.00)	7.185	0.007
CD7	15(41.67)	3(6.00)	14.005	0.000
BCL-2	36(100.00)	28(56.00)	19.036	0.000
CD34	18(50.00)	4(8.00)	17.251	0.000
CD43	12(33.33)	5(10.00)	7.185	0.007
CD79a	3(8.33)	37(74.00)	33.686	0.000
LCA	4(11.11)	21(42.00)	9.685	0.002
CD20	2(5.56)	50(100.00)	74.197	0.000
BCL-6	3(8.33)	32(64.00)	24.616	0.000
PAX-5	3(8.33)	11(22.00)	1.953	0.162

Table III. Correlation between bone marrow flow cytometric typing and pathological immunohistochemical typing.

Bone marrow flow cytometric typing	Pathological immunohistochemical typing			
	CD3	CD5	CD7	CD20
CD3	0.612*			
CD5		0.011		
CD7			0.521*	
CD20				0.301*

(*) $P < 0.05$.

Similarity between BMFC typing and PI typing Regarding the correlation between the same antigen of BMFC typing and PI typing, only CD3, CD7 and CD20 were correlated ($r > 0$, $P < 0.05$). CD5 exhibited no correlation ($P > 0.05$), as shown in Table III.

DISCUSSION

As a common malignant tumor, LCL has an increasing clinical incidence year by year (Ashoub *et al*., 2022). Due to its high heterogeneity, there are many missed and misdiagnosed patients, which easily delays the early treatment, leading to poor patient prognosis. With the increasing application of various leukocyte differentiation antigens, the biological evolution of lymphocytes has been widely identified (Miyoshi and Ohshima, 2018). Pathological diagnosis and PI are the primary diagnostic techniques for LCL. Where, PI uses the reaction of antigen and antibody to correlate the antigen with cell morphology and tissue structure. However, when LCL is highly suspected, it is susceptible to the influence of abnormally reactive lymph node cells, resulting in lower accuracy of detection results (Chen *et al*., 2019). Bone marrow flow cytometry has been mainly used for the diagnosis of hematological diseases in the past, which has multiple parameters, simple sampling and rapid examination. At the same time, flow cytometry can make up for the deficiency of PI examination results, with a higher sensitivity for the immune typing of lymphoma and the proportion of multiple cells, but it still cannot avoid false positive results in the examination process.

Non-hodgkin's lymphoma is mainly divided into T lymphocyte type and B lymphocyte type. T lymphocyte type is more common in nose and face, as well as skin and gastrointestinal tract, and the T cell markers are mainly positive in CD2, CD56 and CD30 (Yu *et al*., 2020). In addition, immunohistochemical evaluation of CD3, CD8, CD5, CD20, and CD30 has also been reported to confirm the diagnosis of T cell proliferation pedigree and monomorphic epithelial intestinal T cell lymphoma (Boșoteanu *et al*., 2022). A study found in statistics that T lymphocytes in 31 patients with non-Hodgkin's LCL expressed relatively consistent antigen in terms of PI and flow cytometric typing (Zhao *et al*., 2021). Studies have shown that T cell lymphoma is more prone to bone marrow invasion than B cell lymphoma (Kumar *et al*., 2009). According to bone marrow flow cytometry and PI antigen typing, bone marrow flow cytometry can lead to CD3 deletion, but PI does not cause antigen deletion. PI based on immunology only uses chemical reactions in its testing process to identify the position, content and specific properties of antigens in the body (Guan and Zhou, 2011).

In this study, 36 cases of T lymphocyte type and 50 cases of B lymphocyte type were collected. Accurate lymphocyte typing was made using BMFC typing and PI. Previous epidemiological investigation results showed that the proportion of T lymphocyte type and B lymphocyte type was about 15%/85% (Pan *et al*., 2019). However, cases collected in this study were still mainly B lymphocyte type, which conformed to the domestic clinical status, and the two examination methods showed consistency in the identification of lymphocyte type. Meanwhile, it was found in this study that the expression rate of CD7 and CD3 were 100% and 58.33% (21/36), respectively in BMFC typing of T cell type, and CD3 deletion was found in 9 cases. Pu *et al*. believed that CD3, CD5 and CD7 all belonged to the deletion-prone antigen phenotypes, which was similar to the finding in this study (Pu *et al*., 2022). CD19 had the highest expression rate (96.00%, 48 cases) in BMFC typing of B cell type, followed by CD20 (64.00%, 30/50). CD19 has been used as a therapeutic target for patients with diffuse B-lymphocyte lymphoma (De *et al*., 2022). During the development of lymphoma cells, partial antigen deletion or antigen enhancement may occur. This study found that the expression rate of CD20, the main antigen used for PI typing of B-cell lymphoma, was only 64.00%in BMFC typing. BMFC typing can be used for diagnosis, staging and prognosis testing of hematologic tumors, which belongs to the routine examination method of conventional lymphoma typing. In PI typing of T cell type, the expression rate of BCL-2 was 100.00% and that of CD3 was 91.67% (33/36). CD20 had the highest expression rate (100.00%) in PI typing of B cell type. Hence, the expression rate of CD20 antigen decreased in bone marrow compared with that in lymphoid tissue. PI results can provide a basis for targeted drugs, but for atypical patients whose lymph node pathology was difficult to determine and whose bone marrow involvement was unclear, bone marrow flow cytometry is still needed. However, seen from the correlation between the same antigen of BMFC typing and PI typing, CD3, CD7 and CD20 were correlated ($r > 0$, $P < 0.05$). Zhao *et al*. (2018) found that the immunophenotype of aggressive B-cell lymphomas was similar to that of mature B-cells, mainly CD20-negative, showing plasmablast characteristics in cells (Zhao *et al*., 2018; Montes-Moreno *et al*., 2010). The comparison of antigen positive rates of the two methods revealed statistically significant difference between T lymphocyte type and B lymphocyte type. This study further investigated the correlation between BMFC typing and PI typing in CD5 antigen, and the results showed no correlation between the two ($P > 0.05$). Yin *et al*. (2022) believed that CD5-positive (CD5) diffuse large B-cell lymphoma patients had clinical features different from CD5-negative patients. According to the distribution of CD5 antigen in this study, bone marrow flow cytometry was mostly detected in T lymphocyte type patients, while a small amount of pathological immune typing could be detected in T lymphocyte type and B lymphocyte type. Considering clinical conditions, the CD5 detection

results in bone marrow should prevail in terms of PI results. Studies have shown that the expression of some T cell antigens (CD2, CD3, CD5, CD7) decreased or disappeared in PI results (Hou *et al*., 2021). It can be speculated that in this study, CD5 antigen disappeared in T lymphocyte types, but could be detected by bone marrow flow cytometry. Therefore, for patients with LCL, the results of BMFC typing should be combined with the results of PI antigen typing, and the patient's immune antigen typing should be comprehensively considered to implement precision medicine programs using different targeted drugs, chemoradiotherapy drugs.

CONCLUSION

BMFC typing and PI antigen typing of LCL are only correlated in terms of CD3, CD7 and CD20 antigens, with complementary effects shown in other antigens. Clinical selection should be made by comprehensive consideration of patients' conditions.

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IRB approval

The research was carried out with the approval of Research Guidance Workshop Committee (Hainan Women and Children' Medical Center).

Ethical statement

The study was carried out in compliance with guidelines issued by ethical review board and institutional biosafety committee of Hainan Women and Children' Medical Center. The official letter would be available on fair request to corresponding author.

Statement of conflict of interest

The authors have declared no conflict of interest.

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