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ORIGINAL ARTICLE

The role of flow cytometry in the diagnosis of non-Hodgkin's lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node specimens

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Summary

Purpose: *In this study we aimed to compare the flow cytometry (FC) results of patients with B cell lymphoma, T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node and investigate the role of FC in malignant or non malignant conditions.*

Methods: *Ninety patients were divided into 5 groups according to histopathology results. Patients were compared according to cytokeratin and positivity percentage of the following surface markers: CD45, CD19, CD5, CD19-CD5, CD4, CD8, CD3,CD16-CD56, CD10, CD10-CD19, CD23, CD20, CD4-CD8, CD3-CD16-56, CD30, CD38, kappa and lambda light chains, CD20-CD23. Patients were also compared according to the intensity of the expression (exp) of same markers. ROC curve analysis was performed for CD19+ cell percentage, CD38 exp, kappa/lambda and lambda/kappa ratios.*

Results: *1) Kappa/lambda and lambda/kappa ratios can distinguish B cell lymphoma from T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node; 2) CD19+ cell percentage can distinguish T cell lymphoma from Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node; 3) CD38 exp can partly distinguish B cell lymphoma from T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node and T cell lymphoma from granulomatous inflammation, T cell lymphoma from reactive lymph node, Hodgkin's lymphoma from reactive lymph node.*

Conclusion: *Flow cytometry has a role in distinguishing lymphomas from non malignant lesions.*

Key words: *flow cytometry, granulomatous inflammation, kappa/lambda ratio, lymphoma, reactive lymph node*

Introduction

Lymphadenopathy can be reactive or secondary to infections, lymphomas, leukemias, metastases from carcinoma, autoimmune diseases, reactions to drugs etc. [1]. Traditionally, the technique of choice for diagnosis of lymphadenopathy was based on the histopathologic study of paraffin-embedded tissue. Currently, the use of immunohistochemistry (IC) for detecting cell spesific antigens is essential in classifying tumors, identifying prognostic factors and identifying targets for therapy [2]. However, IC in routine clinical practice has limitations: analysis subjectivity, limited reproducibility and time investment [3,4].

FC immunophenotyping requires only a small sample. It is able to detect aberrant cells at a frequency of 1/10000 cells [5]. The large number of specific monoclonal antibodies and the possibility of combining 4 or more fluorochromes to precisely define the cell profile and the neoplastic nature of lymphoid proliferations make FC a more sensitive and quicker tool than is IC [6-9]. Evaluating several antigens on 1 cell, including cytoplasmic antigens, gives quantitative results and can detect small abnormal cell populations against a reactive background. It can also assess coexpression of antigens on subsets of cells and the relative

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density of surface antigens. It has additional advantage of being able to identify a small population of abnormal cells that may not be apparent on morphology alone [10,11].

Although Fromm et al. [12] reported that 8 or more color FC can diagnose classical Hodgkin's lymphoma in lymph nodes with high sensitivity and specifity, the usefulness of FC in contributing to the diagnosis of classical Hodgkin's lymphoma involving lymph nodes has been limited [13]. There is limited data about FC in granulomatous inflammation and reactive lymph nodes.

The aim of this retrospective study was to report our experience in assessing the contribution of FC immunophenotyping in the diagnosis of B cell lymphoma, T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph nodes.

Methods

We retrospectively analysed the medical records of 90 consecutive patients who had persistent enlargement of lymph nodes and underwent biopsy for both FC and histopathology analysis. Patients were divided into 5 groups according to histopathology diagnosis: B cell lymphoma (N=18;Group 1), T cell lymphoma (N=14;Group 2), Hodgkin's lymphoma (N=20;Group 3), granulomatous inflammation (N=15;Group 4) and reactive lymph node (N=13;Group 5).

Lymph node specimens were obtained in the operation room with intact capsule and sent complete to laboratory in RPMI culture medium. Lymph node specimen without fat and necrosis in 10 mm^3 volume was placed in 50 mm medicon with DAKO medimachine system. 0.5-1.5 ml suspension buffer (RPMI or Phosphate buffered saline/PBS were added. Medicons were placed in medimachine and tissue was cut into pieces with microknives during 2-2.5 min. Medicon was taken out of medimachine and the suspension was filtered through 70 µm filcon. Cells were washed with PBS for 2-3 times and resuspended with cell pellet. A cell concentration between 5-40,000 was adjusted.

Monoclonal antibody panel included CD45 FITC (Fluorescein isothiocyanate) / CD14 PE (Phycoerythrin), Mouse IgG2a FITC / Mouse IgG1 PE, CD20 FITC / CD5 PE, CD4 FITC / CD8 PE, CD3 FITC / CD56 PE, CD3 FITC / HLA-DR PE, CD10 FITC / CD19 PE, CD23 FITC / CD19 PE, kappa FITC / CD19 PE, lambda FITC / CD19 PE, CD30 PE, cytokeratin FITC and CD38 PE (Becton Dickinson Bidscienes, CA, USA).

Test procedure

Thirteen 12x75 mm tubes were used. Patients' name and the antibodies used were written on the tubes. Ten µl vortexed monoclonal antibody was put in each tube. A hundred µl of cell suspension was added, vortexed and incubated in the dark at room temperature for 20 min. Two ml of red blood cell lysing were added and rested at room temperature for 10 min, then centrifuged at 1800 rpm for 5 min. and the supernatant was removed. This washing process was repeated once more. Five hundred µl PBS were added into the tubes and acquisition of data was made. Cell group was gated and positive antigens were analyzed. Mononuclear cells were separated for kappa and lambda and washed twice with PBS.

Patients were compared according to cytokeratin and positivity (+) percentage of the following surface markers: CD45, CD19, CD5, CD19-CD5, CD4, CD8, CD3,CD16-56, CD10, CD10-CD19, CD23, CD20, CD4-CD8, CD3-CD16-56, CD30, CD38, kappa, lambda, CD20-CD23. Patients were also compared according to the intensity of the expression (exp) of the following antigens: CD45, CD19, CD5, CD4, CD4-CD8, CD8, CD8-CD4, CD3, CD16-CD56, kappa, lambda, CD10, CD10-CD19, CD 19-CD10, CD20, CD23, CD20-CD23, CD23-CD20, CD30, CD38.

Statistics

Analysis of variance (Tamhane, Tucey), and Least Significant Difference (LSD), Kruskal-Wallis and x2 tests were used to analyze the results. The sensitivity and specifity of positivity of surface markers and cytokeratin for the diagnosis were evaluated with the positive (PPV) and negative predictive value (NPV). A receiver-operating characteristic (ROC) curve was used to determine the diagnostic accuracy and cut-off value. Data were expressed as median or mean ± standard deviation (SD). A p-value<0.05 was considered as statistically significant.

Results

After FC analysis of lymph node specimens, cytokeratin and CD45+, CD19+,CD5+, CD19+-CD5+, CD4+, CD8+, CD3+, CD16,56+, CD10+, CD10+-CD19+, CD23+, CD20+, CD4+-CD8+, CD3+-CD16,56+, CD30+, CD38+,kappa+, lambda +, CD20+-CD23+ cell percentages of each group were compared (Table 1). There was no statistically significant difference between groups according to CD19+-CD5+, CD16-56+, CD10+-CD19+, CD4+-CD8+, CD3+-CD16-CD56+, CD30+, CD38+, CD20+-CD23+ cell percentages. Groups were also analyzed according to the intensity of the expression of antigens (Table 2). Cytokeratin was negative in all groups.

According to ROC curve analysis obtained for patients in each group, 11% cut-off for CD19 distinguished T cell lymphoma from Hodgkin's lymphoma with 87.5% sensitivity and 92.5%

Table 1. Comparison of positive cell percentages in lymph node for each group

				1	2	3	4	5
CD19+	1	18	70 ± 30					
	2	13	6 ± 5	**		**		
	3	16	30 ± 16	**	**			
	4	13	33 ± 22	**	*			
	5	11	36 ± 14	**	**			
CD20+	1	18	51 ± 33					
	2	13	7 ± 6	**				
	3	16	11 ± 6	**				
	4	13	23 ± 15					
	5	11	23 ± 8					
CD19± Kappa+	1	28	40 ± 39					
	2	12	5 ± 3	**				
	3	19	13 ± 11	*				
	4	15	20 ± 12					
	5	13	18 ± 7					
CD19+ lambda+	1	28	28 ± 40					
	2	12	5 ±3					
	3	19	12 ± 8		*			
	4	15	16 ± 9		**			
	5	13	14 ± 7		**			
CD5+	1	28	22 ± 18					
	2	14	58 ± 25	**				
	3	20	56 ± 18	**				
	4	15	50 ± 18	**				
	5	13	52 ± 16	**				
CD4+	1	28	19 ± 18					
	2	14	32 ± 28					
	3	20	47 ± 18	**				
	4	15	38 ± 13	*				
	5	13	43 ± 17	**				
CD8+	1	28	19 ± 18					
	2	14	32 ± 28	*				
	3	20	47 ± 18					
	4	15	38 ± 13					
	5	13	43 ± 17					
CD3+	1	28	27 ± 21					
	2	14	54 ± 35	**				
	3	20	64 ± 18	**				
	4	15	51 ± 26	**				
	5	13	58 ± 17	**				
				Comparison (Probability)				
				1	2	3	4	5
CD4± CD8+	1	28	2.7 ± 3.1					
	2	14	3.8 ± 7.2					
	3	20	4.4 ± 4.4					
	4	15	3.1 ± 1.1					
	5	13	4 ± 2.7					
Kappa± lambda+	1	28	18.99 ± 28.5					
	2	12	1.26 ± 0.6	**				
	3	19	1.03 ± 0.6	**				
	4	15	1.2 ± 0.3	**				
	5	13	1.26 ± 0.22	**				
Lambda± kappa+	1	28	14.3 ± 29.6					
	2	12	0.9 ± 0.3	*				
	3	19	2.3 ± 3.7	*				
	4	15	0.8 ± 0.2	*				
	5	13	0.8 ± 0.1	*				

*p<0.05, **p<0.01. SD: standard deviation

Table 2. Comparison of positive cell percentages in lymph node for each group

	Group	*N*	*Median*	*Comparison (Probability)*				
				1	*2*	*3*	*4*	*5*
Kappa exp	1	27	193					
	2	12	505	**				
	3	19	264					
	4	15	212	**				
	5	12	242	**				
		x^2=13.9				SD=4		
Lambda exp	1	27	214					
	2	12	427	*				
	3	19	427					
	4	15	158	*				
	5	12	605	*				
		x^2=10.3				SD=4		
CD38 exp	1	21	469					
	2	7	636	**				
	3	8	892	**				
	4	11	274	**				
	5	7	304	**				
		x^2=16				SD=4		
CD45 exp	1	26	437					
	2	13	526					
	3	17	408					
	4	13	276					
	5	13	471					
		x^2=5.98				SD=4		

*p<0.05, **p<0.01
Exp: expression, SD: standard deviation

specificity, T cell lymphoma from granulomatous inflammation with 76.9% sensitivity and 92.3% specifity, T cell lymphoma from reactive lymph node with 100% sensitivity and 92.3% specificity.

ROC curve analysis was also generated for the intensity of the expression of the above mentioned antigens. A cut-off value 291 for CD38exp distinguished T cell lymphoma from granulomatous inflammation with 81.8% sensitivity and 100% specificity. Cut-off 304 for CD38exp distinguished T cell lymphoma from reactive lymph node with 57.1% sensitivity and 100% specificity. Cut-off 291 for CD38exp distinguished Hodgkin's lymphoma from granulomatous inflammation with 81.8% sensitivity and 87.5% specificity. Cut-off 638 for CD38exp distinguished Hodgkin's lymphoma from reactive lymph node with 100% sensitivity and 62.5% specificity.

Sensitivity and specifity of kappa/lambda and lambda/kappa light chain ratios for certain tresholds in B cell lymphoma patients were also evaluated (Tables 3 and 4). When the thresholds for kappa/lambda ratio were chosen as 1.5, 2, 2.5 and 3, the sensitivity and specificity were found highest for 3. When the threshold for lambda/kappa ratio were chosen as 1.5, 2, 2.5 and 3 the sensitivity were found highest for 2.

Discussion

In this study we evaluated the cell distribution in lymph nodes of patients with B cell lymphoma, T cell lymphoma, Hodgkin's disease, granulomatous inflammation and reactive lymph node by using FC and comparing the results for each group.

CD19+ cell percentage in B cell lymphoma patients was higher than in T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node. This was an expected finding since CD19 is a well known B cell associated marker. Lower CD19+ cell percentage was found significant in distinguishing T cell lym-

Table 3. Positive and negative predictive values of kappa/lambda ratio in B cell lymphoma

Treshold	*Sensitivity*	*Specifity*	*PPV*	*NPV*
1.5	94.1	96.6	88.9	98.3
2	100	96.7	88.9	100
2.5	100	96.6	88.2	100
3	100	100	100	100

PPV: positive predictive value, NPV: negative predictive value

Table 4. Positive and negative predictive values of lambda/kappa ratio in B cell lymphoma

Treshold	*Sensitivity*	*Specifity*	*PPV*	*NPV*
1.5	100	89.8	60	100
2	100	98.6	87.5	100
2.5	100	98.3	87.5	100
3	100	98.3	87.5	100

PPV: positive predictive value, NPV: negative predictive value

phoma from other disorders, but we could not find a similar comparison in the literature. CD20+ cell percentage in B cell lymphoma patients was found higher than in T cell lymphoma and Hodgkin's lymphoma but was not different from granulomatous inflammation and reactive lymph node.

In our study kappa+-CD19+ cell percentage in B cell lymphoma patients was higher than in T cell lymphoma and Hodgkin's lymphoma patients. This percentage was lower than in granulomatous inflammation and reactive lymph node in T cell lymphoma patients. Kappa/lambda and lambda/kappa ratios in B cell lymphoma were higher than in T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node. These findings were compatible with the literature which showed that lymphomas of B cell origin can be detected by their expression of B surface markers and light chain restriction [14-17].

It is more difficult to identify phenotypically abnormal T cells than abnormal B cells. FC plays only a part in the diagnosis of T cell lymphomas. The identification of populations of abnormal T cells may be achieved by demonstrating aberrant T cell antigen expression or by identifying restricted populations of T cells. Complete lack of staining for one or more pan T cell antigen(s) may prove that T cell population present is abnormal [18]. In our study CD5+ and CD3+ cell percentages in B cell lymphoma were lower than in T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph nodes. We also found that CD5 and CD3 increase in similar amounts in T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node and as a result can not be used in distinguishing the aforementioned diseases.

We found CD4+ cell percentage in B cell lymphoma was lower than in Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node. We concluded that CD4+ cell percentage is important in distinguishing B cell lymphoma from others but could not find similiar data in the literature.

Another finding in our study was lower kappa exp in B cell lymphoma from T cell lymphoma, granulomatous inflammation and reactive lymph node. Kappa exp in granulomatous inflamation and reactive lymph node was lower than in T cell lymphoma and Hodgkin's lymphoma patients. We could not find a report about the importance of kappa exp in distinguishing these diseases but our study showed that kappa exp is important in distinguishing T cell lymphoma, granulomatous inflammation and reactive lymph node.

Lambda exp was found lower in B cell lymphoma from T cell lymphoma and reactive lymph node but higher than in granulomatous inflammation. Lambda exp in granulomatous inflammation was lower than in Hodgkin's lymphoma. Lambda exp in reactive lymph nodes was higher than in Hodgkin's lymphoma and granulomatous inflammation. We concluded lambda exp helps in distinguishing reactive lymph nodes and granulomatous inflammation.

In our study CD38 exp in B cell lymphoma was lower than in T cell lymphoma and Hodgkin's lymphoma but higher than in granulomatous inflammation and reactive lymph nodes. CD38 exp in granulomatous inflammation and reactive lymph node was lower than in T cell lymphoma and Hodgkin's lymphoma. Although it was not reported before in the literature we found CD38exp was significant in distinguishing B cell lymphoma from Hodgkin's lymphoma and granulomatous inflammation.

When we compared the CD19+ cell percents

of patients with T cell lymphoma and Hodgkin's lymphoma the diagnosis was in favor of Hodgkin's lymphoma if the CD19+ cell percent was >11% (sensitivity: 87.5% ; specificity: 92.5%). When we compared CD19+ cell percents of patients with T cell lymphoma and granuloma inflammation on the diagnosis was in favor of inflammation if the CD19+ cell percent was >11(sensitivity: 76.9% ; specificity: 92.3%). When we compared the CD19+ cell percents of patients with T cell lymphoma and reactive lymph node the diagnosis was in favor of reactive lymph nodeif the CD19+ cell percent was >11% (sensitivity: 100% ; specificity: 92.3%). We could not find similar data in the literature but concluded that CD19+ cell percentage is important in distinguishing T cell lymphoma from Hodgkin's lymphoma, granulomatous inflammation and reactive lymph node.

In our study a cut-off <291for CD38exp was in favor of granulomatous inflammation (sensitivity: 81.8% ; specificity: 100%). When we compared ROC curve analysis obtained for patients with T cell lymphoma and reactive lymph node a cut-off <304 for CD38exp was in favor of reactive lymph node (sensitivity:57.1% ; specificity:100%). When ROC curve analysis obtained for patients with Hodgkin's lymphoma and granulomatous inflammation a cut-off <291 for CD38exp was in favor of granulomatous inflammation (sensitivity: 81.8% ; specificity: 87.5%). When we compared ROC curve analysis obtained for patients with Hodgkin's lymphoma and reactive lymph node a cut-off <638 for CD38exp was in favor of reactive lymph node (sensitivity: 100%; specificity: 62.5%).

FC is one of the most convenient and powerful means for establishing B cell clonality. The kappa/lambda ratio in nonneoplastic specimens is usually in the range of 1/1 to 2/1. B cell lymphomas express a single clonal light chain so this ratio is generally increased or decreased. Jorgensen et al. [5] suggest investigating the case more closely if kappa/lambda ratio is >3/1 or if there is a significant lambda excess. Mature B cell neoplasms usually express only one class of immunglobulin light chain. The normal ratio of kappa/lambda is 1/1 to 2/1. Monoclonality is diagnosed when this ratio is >4/1 or >1/2 [19].

Chizuka et al. [20] analysed the predictive value of kappa/lambda light chain ratio in 105 B cell lymphoma patients. They found highest sensitivity (92.5%) and specificity (73.1%) when kappa/lambda ratio was equal to 2. PPV was 90% and NPV 72% for this ratio. Ten percent of patients with a kappa/lambda ratio >2 did not have B cell lymphoma and more than 20% of patients did not have B cell lymphoma when this ratio was <2. In the same study 38 of 53 (72%) B cell lymphoma patients had a kappa/lambda ratio higher than 2 and kappa light chain was dominant in 22 of them. Kappa/ lambda ratio was lower than 2 in 15 patients. Only 2 of the patients without B cell lymphoma had kappa/lambda ratio higher than 2. Diagnoses were chronic lymphadenitis and tuberculosis in these patients.

We also evaluated the specificity and sensitivity for certain kappa/lambda and lambda/kappa light chain ratio thresholds in B cell lymphoma patients. The highest specificity (100%) and sensitivity (100%) for kappa/lambda ratio was found for threshold 3 (PPV 100%, NPV 100%). Specificity was decreased for values lower than 3. Kappa/lambda ratio was higher than 3 in 14 of 28 (50%) B cell lymphoma patients. Kappa light chain was dominant in 17 of them. The highest specifity (100%) and sensitivity (100%) for kappa/lambda ratio in B cell lymphoma was found for threshold 2 (PPV 87.5%, NPV 100%). Lambda/kappa ratio was higher than 2 in 6 of 28 (21%) B cell lymphoma patients and lambda light chain was dominant in 11 of them. Kappa/lambda ratio higher than 3 or lambda/kappa ratio higher than 2 by FC analysis of lymph node suggests B cell lymphoma.

To our kowledge this is the first study in the literature comparing the FC results of B cell lymphoma, T cell lymphoma, Hodgkin's lymphoma, granulomatous inflammation and reactive lymph nodes together. Our results show that CD 19, CD 20, CD 3, CD4, CD5+ cell percentages, kappa, lambda, CD 38 exp and kappa/lambda, lambda/kappa ratios are important in distinguishing between these diseases. Kappa/lambda ratio higher than 3 or lambda/kappa ratio higher than 2 by FC analysis of lymph node suggest B cell lymphoma.

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